Antigenic variation in *Trypanosoma brucei*: a telomeric expression site for variant-specific surface glycoprotein genes with novel features

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ABSTRACT

African trypanosomes evade the immune response of their host by periodically changing their variant surface glycoprotein (VSG) coat. Each coat is encoded by a separate VSG gene. Expressed genes are in a telomeric expression site (ES) and there are several sites in each trypanosome. To study the transcription control of VSG genes in *Trypanosoma brucei* we have analyzed an ES, called the dominant ES (DES), that readily switches off and on. The promoter area of the DES is very similar to that of the 221 ES (Zomerdijk et al., 1990). It can be switched off and on *in vivo* without detectable DNA alterations in the vicinity of the transcription start and it can drive high transient expression of a reporter gene in transfection experiments. However, there are also two major differences between the DES and the 221 ES. First, one version of the DES contains an additional upstream transcription unit overlapping the VSG gene ES promoter. The presence of this upstream transcription is dispensable, however, for the VSG gene ES promoter is active, even if transcription through this start from the upstream promoter is blocked using UV light. Moreover, a second version of the DES present in another trypanosome variant does not produce these upstream transcripts. Secondly, we find that the inactivation of DES transcription in one trypanosome variant is accompanied by DNA alterations in the DES upstream (>2 kb) of the transcription start; reactivation of DES transcription is accompanied by another alteration far upstream. Although we cannot exclude that these DNA rearrangements are incidental, our results raise the possibility that the activity of ES promoters is negatively controlled *in cis* by far upstream sequences not included in transfection constructs and that alterations in these sequences may lead to (in)activation of the promoter.

INTRODUCTION

Mammalian infective forms of African trypanosomes, such as *Trypanosoma brucei*, are covered by a dense surface coat (1). This coat consists of a single protein species, the variant-specific surface glycoprotein or VSG (2). Each VSG is encoded by a separate gene and there are some 1000 VSG genes (and pseudo genes) per trypanosome nucleus (3,4). By repeatedly switching the expressed VSG gene, *i.e.* antigenic variation, a small fraction of the trypanosomes continue to escape the antibody-mediated immune response of the mammalian host. Expressed VSG genes are invariably located in a telomeric expression site (ES) (5). Hybridization studies suggest that *T.brucei* 427 may contain as many as 20 different ESs (6,7). Usually only one ES is operational at any time. Hence, there is a tight differential transcriptional control of ESs.

The three ESs in *T.brucei* 427 characterized thusfar, the 221 ES, the 1.8 ES and the dominant ES, are similar: they consist of a single transcription unit of 40–60 kb (8,9,10) that includes several expression-site associated genes or ESAGs, besides the VSG gene (6,8,11,12). Analogous results have been obtained for an ES in another strain of *T.brucei* (13) Transcription runs towards the telomere (5) and is resistant to α-amanitin concentrations up to 1 mg/ml, unlike transcription of most other protein-coding genes in trypanosomes (8,14).

Antigenic switching can occur either by replacement of the VSG gene in the active ES by another VSG gene (5,15,16,17), or by activation of another ES and inactivation of the formerly active one (18,19,20,21). How a trypanosome switches from one ES to another is not yet known, but this *in situ* (in)activation occurs without detectable DNA rearrangements. The observation that two ESs can be simultaneously active (22,23) suggests that there is no cross-talk between ESs (i.e. not a single mobile promoter, enhancer or transcription terminator) and that each ES is activated and inactivated independently of other ESs (22). We have previously tested whether this stochastic (in)activation process involved DNA rearrangements near the promoter, as previously proposed (22,24), by cloning the promoter area of the VSG gene 221 ES in an active and in an inactive configuration. No DNA alterations were found within 1.4 kb of the promoter (7).

The 221 ES is not optimal for biochemical analysis. Its promoter is not easily (in)activated (25) and it is located in a rather large (4 Mb) chromosome that is difficult to isolate (7). We have

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therefore continued our ES analysis with the dominant ES (DES), the site used in *T.brucei* strain 427 for most of the VSG genes studied (25). The DES lies on a 2 Mb chromosome (26,27) and it is readily switched on and off (20).

We report here on the DES promoter area of three trypanosome variants, 118a, 1.8c and 118a', related to each other via single relapses (20), as illustrated in figure 1. All three variants carry the VSG 118 gene in the DES; in 118a and 118a' it is transcribed, in 1.8c it is not. No major DNA rearrangements were previously detected in the DES in the sequence 118a → 1.8c → 118a' (10,20), but this analysis only looked at regions downstream of the promoter. We have now cloned the DES promoter area and studied the region upstream of the transcription start. Although the DES promoter itself is very similar to the 221 ES promoter studied previously (7), we find remarkable alterations upstream of this promoter when it is inactivated (118a → 1.8c) and reactivated (1.8c → 118a'). Although the significance of these alterations for the control of VSG gene transcription remains to be established, our results suggest that there may be more to ES control than could be inferred from previous work on the 221 ES (7).

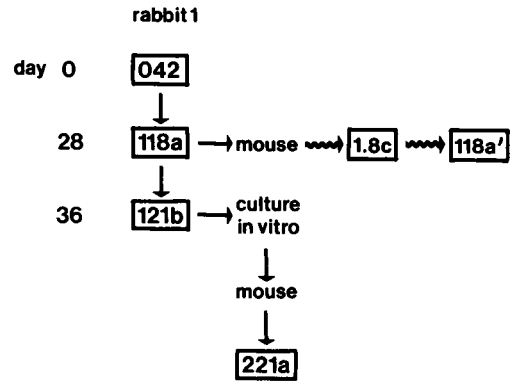

Figure 1. Derivation of trypanosome clones of *T.brucei*, strain 427 (2,20,28). The numbers within the boxes designate the variant antigen type of the trypanosomes.

MATERIALS AND METHODS

Trypanosomes

The trypanosomes used belong to strain 427 of *T.brucei brucei*. Trypanosome variants 118a (MiTat 1.5a), 1.8c and 118a' were described by Michels et al. (20,28). Trypanosomes were grown in Sprague Dawley rats and blood was collected from animals with a high parasitemia by cardiac puncture. Trypanosome populations were tested for antigenic homogeneity by indirect immunofluorescence using VSG polyclonal antibodies. Buffy coat was used for RNA isolations; for PFGE analysis trypanosomes were separated from blood cells by DEAE-cellulose chromatography (29). Procyclic culture form trypanosomes were grown in the semi-defined medium (30).

Recombinant DNA: DES chromosome-enriched DNA library

The DES resides in chromosomal DNA of approximately 2000 kb that migrates relatively free of other chromosomes in PFG gels (26,27). DNA of the DES chromosome in *T.brucei* variant 118a', isolated from 0.5% GTG-agarose (FMC Corp.) pulsed field gradient (PFG) gels in 1×TBE (31) run at 14°C for 24 h at a field strength of 10 V/cm (200 V, 170 mA) that was switched every 180 s, was partially digested with Sau3AI and the sticky ends were partially filled in with Klenow DNA polymerase in the presence of dATP and dGTP. DNA fragments in the 10–25 kb range were isolated and ligated in phage lambdaGEM-11 XhoI half-site arms (partially filled in with Klenow in the presence of dCTP and dTTP; ref. 32; Promega), *in vitro* packaged and plated on *E.coli* strain MB406 (recBC$^-$, recsbc$^-$ permissive). Phage DNA was purified as described (31). The DES promotor clone was identified following the same strategy as described for the 221 ES promoter region clones (7). Eleven recombinant phages were selected with a probe for the ScaI-EcoRV conserved region (figure 2 A, probe c) indicative of ES promoters. These eleven were then screened with a primer complementary to the hypervariable region shown in figure 2, with at five positions a base ambiguity. Under stringent conditions only one phage insert hybridized to this primer. Internal fragments of the phage insert encompassing promoter sequences and ESAG-X genes from the DES were checked for comigration with *T.brucei* 427 variant 118a' nuclear DNA digests on Southern blots. This was done for the following digests: BamHI, SalI-BamHI, HindIII-SalI, HindIII-SphI and HindIII and the probes ScaI-EcoRV (420 bp fragment from the 221 ES promoter area; ref. 7) and probe I (5' end of ESAG-X; ref. 8). An originally 3.2 kb SalI fragment (5' SalI from the phage arm and the 3' is the conserved SalI site of the VSG gene ES promoter region) was cloned into the SalI site of pGEM3Zf(−) (Promega) giving rise to pDS1. Upon amplification of this plasmid the 50 bp repeats at the 5' end were almost all lost without affecting other sequences in this construct, resulting in a SalI insert length of 1.6 kb. We have compared our sequence of part of the DES promoter area with that recently obtained by K. Gottesdiener and L.H.T. Van der Ploeg in New York on independent plasmid clones isolated by a chromosome walking procedure (personal communication). The two sequences between the SpeI site and the SalI site were identical (see figure 2).

The plasmids used in the procyclic transfection experiments were constructed as follows: into the SacI site of pJF6dScaI (7), encompassing the parpA gene splice acceptor region fused to the CAT gene, we cloned the 1.6 kb SalI DES promoter region fragment from pDS1 in its correct (pRK8(+)DES) and reverse (pRK8(−)DES) orientations.

We used the following constructs for the slotblot analysis presented in figure 4 B: the expression site associated clones pTg221.12, pTg221.11, pTg221.4 (fragment f) and Tg221.8 were isolated from genomic libraries as described (8,33). The subclones pTg221.12 sub 1 (fragment a), PTg221.11 sub 4 (fragment c), pTg221.11 sub 3 (fragment d), pTg221.11 sub 1 (fragment e) and Tg221.8 sub 1 (fragment g) are presented in Johnson et al. (9). ESAG-1D (fragment h), a downstream clone of the DES is described by Crozatier et al. (10). The 118 VSG cDNA clone pTcV118.3 (fragment i) is described by Bernards et al. (16). The ESAG-X clone (fragment b) is a cDNA of ESAG-X from variant 221a, lacking approximately 250 bp at its 5' end (unpublished results).

Two-dimensional Pulsed Field Gradient Electrophoresis

Genomic DNA was separated in the first dimension as described above for optimal separation of the 2 Mb chromosome-sized DNA molecules. We sliced out lanes down the gel and washed those slices in several changes of 10 mM Tris HCl pH 7.9, 1 mM EDTA for a period of 2 h at room temperature. The slices

were subsequently equilibrated in several changes of the restriction enzyme buffer recommended by the manufacturer over a period of 2 hr. Restriction enzyme (approximately 100 Units) was added to these slices in a small volume (approximately 3.5 ml) of fresh enzyme buffer and incubated for 16 h at the appropriate temperature recommended for the enzyme. The slice was then placed in a large slot spanning a whole 1% agarose gel, and the DNA fragments were resolved in the Beckman Gene Line System (Transverse Alternating Field Electrophoresis) in a buffer containing 10 mM Tris, 0.5 mM EDTA (free acid), pH 7.5 during 22 h at 10°C; the electric field (200V, 160 mA) switched every 25 seconds. Sizes of the DNA molecules were estimated from oligomerized lambda DNA molecules, ran in parallel.

cDNA synthesis and Polymerase Chain Reaction

A synthetic 17-mer deoxynucleotide AB416 (5'-CAATTCAT-CCACAGTAG) complementary to the conserved bases 913–929 (relative to the A in the ATG start codon; ref. 7, 13) downstream of the hypervariable region in ESAG-X from the 221 ES was used to prime cDNA synthesis on 10 μg total RNA of *T.brucei* variant 118a and 118a' (see figure 2 B). cDNA synthesis was performed in 20 μl essentially as described (28). The reaction was diluted to 1 ml in 10 mM Tris.HCl pH 8, 1 mM EDTA and 1 to 5 μl was used with Taq DNA polymerase (Perkin Elmer Cetus) in a polymerase chain reaction (34) according to the manufacturer's instructions. A 17-mer synthetic deoxynucleotide AB300 (5'-CGCTATTATTAGAACAG) identical to bases 7 to 23 of the mini-exon sequence of *T.brucei* 427 (35) and a synthetic 15-mer deoxynucleotide AB285 (5'-CGACTCTTTTACACG) complementary to the conserved bases 537–551 in ESAG-X from the 221 ES were used to amplify ESAG-X cDNAs (see figure 2 B).

DNA sequence analysis

Double stranded plasmid DNAs, derivatives of pGEM3Zf (Promega), were sequenced using the dideoxy chain termination method (36). In addition to the SP6 and T7 primers, oligonucleotides were synthesized using phosphoramidite chemistry and were used as internal primers to complete the sequence on both strands. Computer analysis were performed using the software of the University of Wisconsin Genetics Computer Group (37). PCR products were directly sequenced using 100×less of one of the two primers in the PCR amplification to produce single stranded DNA. The sequence for the DES promoter area in *T.brucei* variant 118a' can be found in the EMBL/GenBank/DDBJ nucleotide sequence databases under the accession number X56598.

DNA transfection of procyclic trypanosomes by means of electroporation

The method was essentially as described (38), adapted for insect form *T.brucei* by C.Clayton and coworkers (39) and is described in detail in Zomerdijk et al. (7). Typically, 2.5×10^7 trypanosomes were shocked twice at 7.5 kV/cm, 25 μF in the presence of 0.5 to 5.0 pmoles of circular plasmid DNAs. CAT activity was assayed as described (40), using 250 μM n-butyryl CoA (Sigma) and 88 μM D-thero[dichloroacetyl-1,2-^{14}C]--chloramphenicol (NEN, DuPont) at a final specific activity of 59.5 mCi per mmole. Assays were for 2 h at 37°C. After xylene and two aqueous phase extractions (40), the xylene fractions were mixed with 10 ml of Opti-fluor (Packard) and radioactivity was determined by liquid scintillation counting. CAT activities were correlated with a standard dilution series of *E.coli* chloramphenicol acetyl transferase (P-L Biochemicals, Inc. USA). One unit of this enzyme catalyzes the acetylation of one nanomole of chloramphenicol per minute at 37°C.

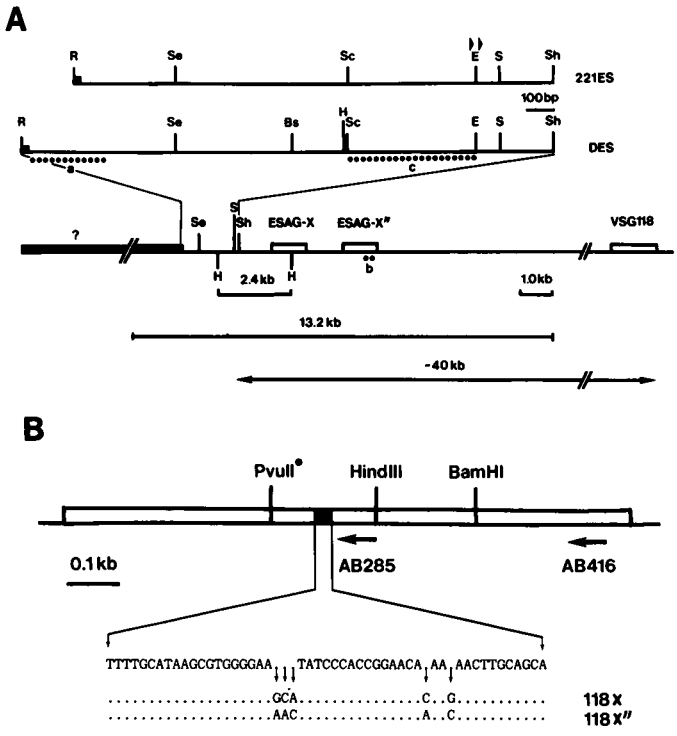

Figure 2. Physical map of the DES promoter area. **A.** The physical map was constructed with a phage isolated from a DES chromosome-enriched DNA library of *T.brucei* variant 118a'. The phage insert (13.2 kb) is shown underneath the map. Boxes indicate expression site associated genes (ESAGs) and the VSG gene. The promoter area is approximately 40 kb upstream of the expression-linked copy of the VSG 118 gene. The DES contains two ESAG-X genes that differ from each other in their hypervariable regions and from those of all other known ESAGs (7). Sequence comparison indicates that the ESAG-X gene closest to the promoter region of the DES is the homologue of ESAG-X from the 221 ES and ESAG-7 in the AnTat 1.3A ES; the second ESAG-X gene copy in the DES is related to ESAG-X" from the 221 ES and ESAG-6 in the AnTat 1.3A (7,13). The black bar symbolizes the 50 bp repeats; the 5' border of this region is unknown. The promoter area of the DES is enlarged above the map, and aligned with the corresponding region of the 221 ES. The arrow heads indicate the transcription start sites in the 221 ES (7). Relevant restriction sites are indicated and the probes of various parts of the promoter area are indicated with a dotted line (**a**, **b** and **c**). Restriction sites: Bs, BssHII; E, EcoRV; H, HindIII; R, RsaI (every 50 bp repeat contains two RsaI sites, but only one is indicated within the 50 bp repeat region); S, SalI; Sc, ScaI; Se, SpeI; Sh, SphI. **B.** The hypervariable region sequences of ESAG-X and ESAG-X' from the DES are identical to the corresponding sequences of the cDNAs of these genes in *T.brucei* variants 118a and 118a'. Both genes appear to be equally expressed in trypanosome variants 118a and 118a' used for the mRNA sequence analysis (see Materials and Methods). The hypervariable region is schematically indicated by a black bar in ESAG-X and an enlargement of this region is shown underneath. The sequences presented are those of the cDNA-PCR product of ESAG-X-like genes in *T.brucei* variants 118a and 118a' (top line; bases 433 to 482 relative to the adenine in the first translation initiation codon ATG in ESAG-X and in ESAG-6 and 7 (ref. 13)). The sequences of ESAG-X (118X) and ESAG-X" (118X") are from the phage insert derived from a DES chromosome enriched library of variant 118a' DNA. The location of the primers used for the cDNA synthesis (AB416) and the PCR amplification (AB285) are indicated. The asterisk on PvuII indicates that it is not a single PvuII site but a cluster of three sites.

Isolation and blotting analysis of RNA and DNA

Total RNA, DNA isolation and blotting were performed as described (7). The Southern and slot blots were hybridized in 5×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 10 mM EDTA. 0.5% SDS, 0.5% tetrasodium pyrophosphate, 5×Denhardt, 100 μg/ml denatured salmon sperm DNA and 100 μg/ml *E.coli* tRNA at 60°C for 40 h and subsequently washed in 0.3×SSC, 0.1% SDS at 60°C. The Northern blots were washed at a stringency of 0.1×SSC, 0.1% SDS at 65°C.

RNase protection and *in vitro* transcription

RNase protection and *in vitro* transcription were performed as described (41). 10 to 30 μg of total RNA was hybridized with RNA probes under standard conditions (80% formamide, 40 mM Pipes pH 6.4, 400 mM NaCl and 1mM EDTA) at 65°C, followed by an RNase A and T1 treatment (12 μg RNAse A and 3 U RNAse T1, 30 minutes at 30°C). Protected fragments were visualized by electrophoresis in a 6% acrylamide, 7 M urea gel, followed by autoradiography. The Schleicher and Schuell SCR 072/0 minifold II was used for slotblots. DNA binding was performed essentially as described (10,42).

UV irradiation

UV irradiation was performed under conditions defined by Johnson et al. (9). Trypanosomes of variant 118a in 10 ml of blood from a rat with high parasitemia were diluted in 500 ml Baltz medium at 37° C to approximately 2×10^8 trypanosomes per ml. Samples (70 ml) of Balz medium with trypanosomes were irradiated with UV light for 0 min.(2 samples), 2 min.(2 samples) or 5 min.(2 samples) while continuously shaken in an open tray of 652.5 cm^2. With the UV lamp used, 1 second of irradiation corresponds to 0.14 $erg.mm^{-2}$. After irradiation the trypanosomes were incubated at 37° C for 1 hour to allow run-off of RNA polymerases engaged prior to irradiation. Subsequently total RNA was isolated from one of two samples, irradiated for 0 min., 2 min. or 5 min. The remaining samples were used for the isolation of nuclei.

Preparation of nuclei from trypanosomes and elongation of nascent RNA in nuclear run-ons

Nuclei from bloodstream form trypanosomes were prepared by passing blood of highly infected rats, diluted 50 times in Baltz medium to 2×10^8 trypanosomes per ml., through a Stansted cell disrupter as described (8). Approximately 1.4×10^{10} nuclei were used per run-on transcription reaction as described (7).

RESULTS

Cloning the expression site promoter region

Previous work has shown that the sequences of trypanosome ESs are repetitive (6,11,43,44,45,46,47,48,49) and that this includes the promoter area (7,8,13,50). Sequence homology is so high that promoter fragments from different ESs cannot be distinguished by hybridization. To enrich the promoter fragments from a given ES, we made clone banks from chromosome-sized DNA, size-fractionated in pulsed field gradient (PFG) electrophoresis gels (7,51). To allow linkage of the putative promoter segment to semi-unique sequences at a distance, we cloned large DNA segments using phage lambda as vector. Clones containing sequences related to the ES promoter were identified with a highly conserved 420 bp ScaI-EcoRV probe derived from the 221 ES (7) and indicated in Figure 2 A (probe c). To identify the phages derived from the DES we exploited subtle sequence differences in the multiple ESs present in the trypanosome genome (7,51,52). A useful difference was previously found in the Expression Site Associated Gene called ESAG-X located immediately downstream of the promoter region (7). Although the overall conservation in the ESAG-X gene family is high, these genes contain a hypervariable region that can be used to distinguish between ESs. The sequences of the hypervariable regions of the ESAG-Xs present in the DES were obtained from the ESAG-X mRNAs present in trypanosome variants in which the DES was active. These sequences were then used to identify a phage DNA insert derived from the DES. The details of the procedure are described in the Materials and Methods, the outcome is presented in figure 2.

The DES putative promoter region is similarly organized as the 221 ES promoter region

Figure 2 A compares the promoter areas from the 221 ES and the DES. Most restriction sites are conserved between the two and both are flanked at their 5′ ends by imperfect direct repeats of 50 bp. These repeats are devoid of restriction enzyme recognition sites, except for RsaI. Mapping and partial sequence analysis of the phage lambda insert suggest that this repeat region is minimally 1.6 kb long. The upstream border of this repeat region in the DES is not known.

To determine precisely the differences between the 221 ES and the DES, the region between the 50 bp repeats and the conserved SalI site (figure 2 A) in the promoter region was sequenced. The nucleotide sequence database accession number for the sequence is X56598. Sequences downstream of the SpeI site (figure 2) are 93% identical in the DES and the 221 ES, but the two DNAs differ upstream of the SpeI site in length and sequence. The latter DES segment is only 59% identical with the corresponding region in the 221 ES, but is not single copy (not shown); it has a G+C content of only 22%. The homology rises again to 93% in the first 4 repeats at the 3′ end of the imperfect 50 bp repeat array.

Expression of DES promoter-CAT gene chimeric constructs are expressed in transiently transformed trypanosomes

The VSG gene 221 ES promoter region has been shown to promote CAT-gene expression from promoter-CAT gene chimeric constructs that were introduced by electroporation in

Table I. The DES and 221 ES promoter driving CAT-gene expression in transient transfections of *T.brucei*

DNA constructs[1]	promoter[2]	amount of DNA[3]	CAT activity[4] A	B
pRK8(+)	221ES(+)	0.5	8	7
		5.0	109	61
pRK8(+)DES	DES(+)	0.5	2	n.d.
		5.0	30	n.d.
pJF6	parpA(+)	0.5	n.d.	8
		5.0	n.d.	62
pRK8(−)	221ES(−)	5.0	0.3	0.3
no DNA			0.3	n.d.

[1]described in Materials and Methods (and ref. 7,39);
[2]correct (+) or reverse (−) orientation relative to the CAT gene;
[3]pmoles per transfection of 2.5×10^7 trypanosomes;
[4][CAT activity in units]$\times10^2$ (see Materials and Methods) per 1×10^7 trypanosomes in two independent transfection experiments, A and B (n.d.: not determined).

procyclic form trypanosomes (7). The highly homologous corresponding region in the DES is also able to drive CAT expression from a similar construct. The base differences between the 221 ES and DES promoter areas are thus apparently non-essential as both sequences have promoter activity as summarized in Table I.

An α-amanitin resistant transcription unit overlapping the promoter region of the DES in *T.brucei* variant 118a, but not in variant 118a'

As the promoter regions of the DES and the 221 ES are highly homologous and show comparable promoter activity in a transient expression system, one would expect that the transcription start sites for both promoters would also be the same *in vivo*. This was indeed observed in run-on transcription experiments with variant 118a' (not shown). In variant 118a, however, there are also transcripts coming from an upstream promoter, as schematically indicated in figure 3. The results on which this interpretation is based are shown in figures 4, 5 and 6.

Figure 4 A shows that labeled nascent RNA molecules synthesized in nuclei isolated from *T.brucei* variant 118a hybridized to all fragments of the DES promoter area subclone. Identical results were obtained with the RNA made in the presence or absence of 1 mg α-amanitin per ml. Fragments upstream of the EcoRV site did not hybridize with RNA synthesized after irradiation of trypanosomes with UV light (fragments 3,5,6,7, 9 and 10). The hybridization to these upstream fragments in non-irradiated trypanosomes is therefore due to promoter distal transcripts, since their synthesis is blocked after UV irradiation (*cf.* ref. 9). Fragments containing the EcoRV-SalI region remain detectable after UV irradiation (fragments 1,2,4 and 8), suggesting that there is a transcription start around the EcoRV site, as in the 221 ES of variant 221a, (figure 4; ref. 7) and in the DES of variant 118a' (data not shown). The transcripts in the upstream promoter region of the DES in variant 118a that disappear upon UV treatment, must be encoded minimally 3−10 kb away from the promoter. This was inferred from the UV-inactivation of transcription in the ES, where sequences 3−10 kb downstream of the promoter in the ES had disappeared (figure 4 B).

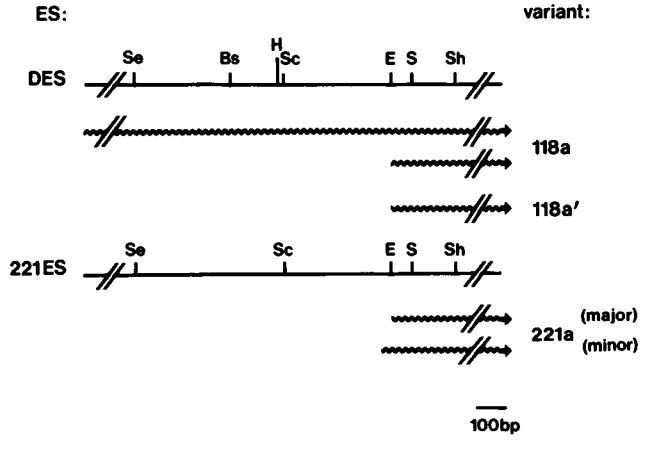

Figure 3. Schematic presentation of the transcription in the promoter regions of the DES in variants 118a and 118a' and the 221 ES promoter region in variant 221a. Wavy lines, below the restriction maps of the promoter regions, represent the regions transcribed and the direction of transcription. Restriction sites as in figure 2, which also presents details of the maps.

The transcripts from the DES promoter area in variants 118a and 118a' were mapped in detail using RNase protection assays. As expected, the pattern of protected fragments representing promoter region transcripts in *T.brucei* variant 118a was more complex than in variant 118a', due to transcription of the area

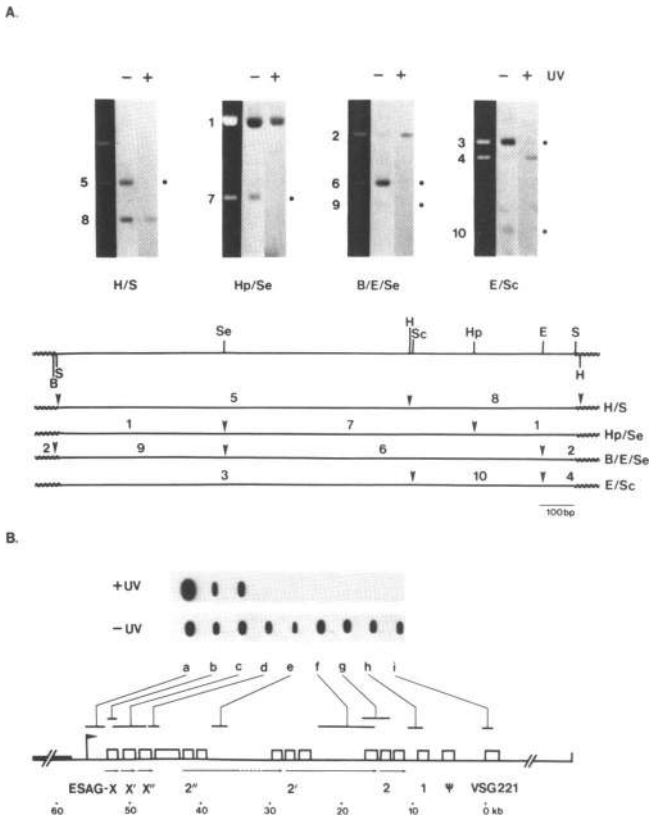

Figure 4. Transcription of the DES promoter area in nuclei isolated from *T.brucei* variant 118a. **A.** A restriction map of the DES promoter area clone pDS1. The 1.6 kb SalI DES promoter region insert is represented by a straight line. Wavy lines represent vector sequences. The sites above the line are insert derived, those below the line are from the vector. Depicted under the map are the numbered restriction fragments, for which the sites are indicated by an arrow head. The restriction fragments are indicated at the left of each ethidium bromide stained gel lane containing different digests of the pDS1 clone. The middle and the right-hand side of each panel are Southern blots of these digests hybridized with RNA synthesized in the presence of [α-^{32}P]UTP and 1mg/ml α-amanitin by nuclei isolated from *T.brucei* variant 118a, either irradiated for 2 min with UV light (+)or not irradiated (−). Hybridizing fragments that disappear upon a UV treatment are indicated by a dot at the right of these blots. Restriction fragment 1 contains the whole vector and promoter region sequences at both ends. Fragment 3 and 4 are separated by a ScaI site in vector sequences (not indicated). Restriction sites: B, BamHI; E, EcoRV; H, HindIII; Hp, HpaI; S, SalI; Sc, ScaI; Se, SpeI. **B.** Transcriptional inactivation. RNA synthesized in nuclei isolated from nonirradiated trypanosomes or irradiated trypanosomes (2' UV irradiation) was hybridized to a slotblot containing a set of DNA clones that represent ESs in general (see 8,10). The physical map of the 221 ES in variant 221a is shown. The first nucleotide of the VSG gene 221 found in the mature VSG 221 mRNA is set at 0 kb, the upstream positions are given in negative numbers. The position of the VSG gene 221, the pseudo-VSG gene (33), ESAG-1 (6), -X, -X', X" (ref. 7 and unpublished results), 2, 2' and 2" (8) are indicated underneath the boxes, among other putative ESAGs. The flag at position −57 kb indicates the 5' border of the transcribed region. The thick line indicates the 50 bp repeat region at the 5' end of the ES. Regions duplicated and triplicated in the ES are indicated by arrows just beneath the map. DNA segments cloned from this ES or representative of it and used in the slot blot hybridization with RNA synthesized in nuclei isolated from *T.brucei* variant 118a either for 2 min with UV light irradiated (+) or not irradiated (−) are indicated above the map and are described in detail in the Materials and Methods.

upstream of the EcoRV site (figure 5, probes A, B and C). The deduced 5′ ends of promoter region transcripts in total RNA of trypanosome variant 118a and 118a′ are depicted in figure 5. The transcription start in the DES promoter of variant 118a′ maps at an equivalent position to the 221 ES start in variant 221a (7). We find multiple 5′ ends of transcripts from variant 118a derived

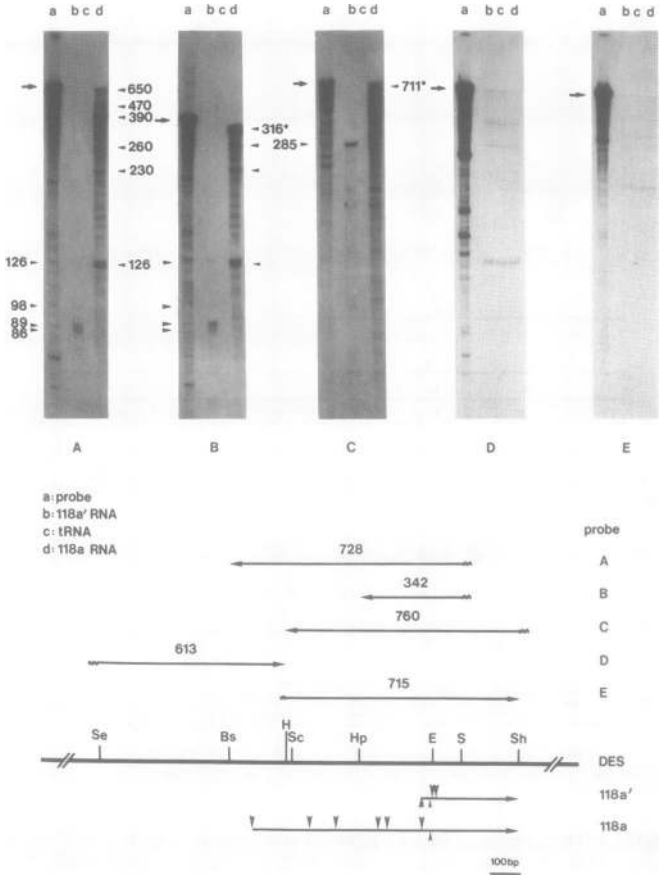

Figure 5. RNase protection to map the start of transcription in the DES of *T.brucei* variant 118a′ and 118a. Total trypanosome RNA was hybridized for 16 h in 80% formamide at 60°C (see Materials and Methods) with various run-off transcripts synthesized *in vitro*. The sense and antisense *in vitro* synthesized probes are depicted above the map of the DES promoter area, with their length in nucleotides (nt) of trypanosome (straight line) and pGEM3Zf(−) vector polylinker (wavy line) sequences. The antisense probes used are: SalI-BstEII run-off SP6 transcript of 728 nt (**A**); SalI-HpaI run-off SP6 transcript of 342 nt (**B**); SphI-HindIII run-off T7 transcript of 760 nt (**C**); sense probes are: SpeI-HindIII run-off T7 transcript of 613 nt (**D**); HindIII-SphI run-off SP6 transcript of 715 nt (**E**). 15 μg of total RNA of variant 118a′ (lanes marked **b**) and 30 μg of total RNA of variant 118a (lanes marked **d**) was hybridized with the uniformly labeled probes indicated below the gels. As a control 30 μg *E.coli* tRNA (lanes marked c) was hybridized to the probes. The hybrids were treated with RNase A and T1, size fractionated on a 6% polyacrylamide, 7 M urea gel and visualized by autoradiography. Probes not treated with RNase are shown in lanes marked **a** and an arrow at the left of each panel. The sizes of the protected fragments are indicated alongside the gel; at the left for total RNA from variant 118a′, at the right for total RNA from variant 118a. The protected fragments observed with probe **B** that are identical to those detected with probe **A** are indicated by an arrow head only. The black dot (for the protected fragments 316 and 711 bp) indicates that the probe is protected over its full length. The results for the various probes with total RNA from *T.brucei* variants 118a′ and 118a are summarized in a schematic presentation of the 5′ ends of DES promoter proximal transcripts below the map of the DES promoter region. Large arrow heads indicate the 5′ ends of abundant transcripts, the little arrow heads minor ones. The transcripts run towards the VSG 118 gene in the DES (arrows). Restriction sites : Bs, BstEII; E, EcoRV; H, HindIII; Hp, HpaI; S, SalI; Sc, ScaI; Se, SpeI; Sh, SphI.

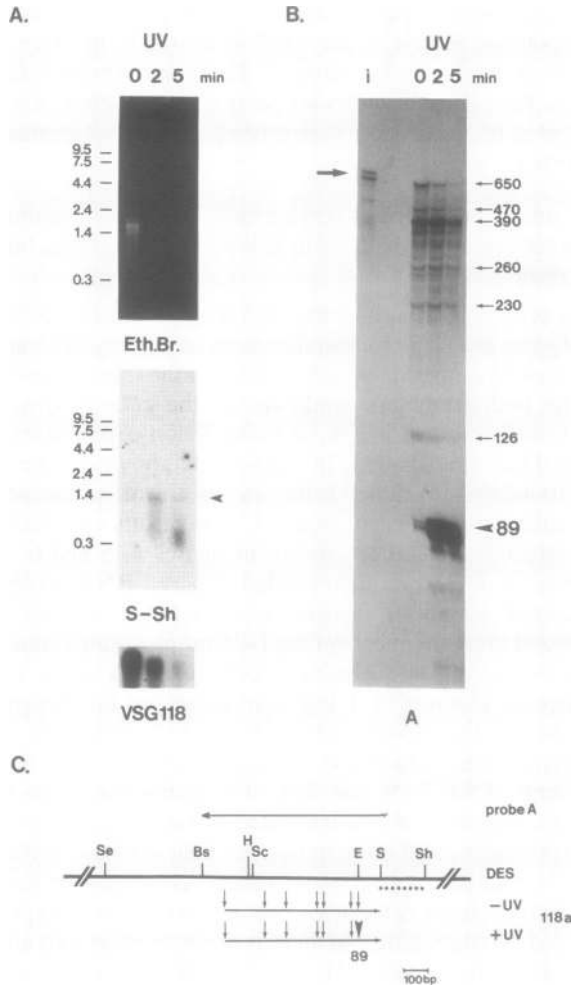

Figure 6. Accumulation of DES promoter proximal transcripts upon UV irradiation of trypanosomes. **A.** Northern blot of total RNA of variant 118a, isolated from nonirradiated and irradiated trypanosomes, hybridized with a ES promoter proximal and VSG gene 118 probe. Bloodstream form trypanosomes, variant 118a, were diluted in Baltz medium and incubated for 1 h under conditions described (9) without prior treatment (0 min. UV) or after 2 min. or 5 min. of UV irradiation. Subsequently total RNA was isolated from the different samples, that contained an equal number of trypanosomes. The separate isolations were performed simultanously and are presumed to have occurred with equal efficiency. A fixed fraction, i.e. not corrected for the amount of RNA, was size fractionated in a formaldehyde gel that was subsequently blotted. At the top the ethidium bromide (Eth. Br.) stained gels and underneath the hybridization with the SalI-SphI probe (see C; dotted line) are shown. The sizes of the fragments of the marker (BRL RNA ladder) are indicated alongside the gel. A transcript of 1.4 kb indicated by an arrow head has accumulated after UV irradiation. The hybridization to the 550 bp EcoRI-PstI fragment of pTcV 118.2 (16) is marked VSG118. **B.** RNase protection with total RNA of trypanosome variant 118a, isolated from irradiated and nonirradiated trypanosomes. A fixed fraction of the total RNA isolated from trypanosomes irradiated for 2 min. or 5 min., half of that fraction of RNA isolated from nonirradiated trypanosomes (0 min.) not corrected for the of the amount of RNA were hybridized with uniformly labeled probe A (i, input not RNase treated; see map in **C**). Hybridization conditions are as described in Materials and Methods. The hybrids were subsequently treated with RNase A and T1, size fractionated on a 6% polyacrylamide, 7 M urea gel and visualized by autoradiography. The sizes of the protected fragments are indicated alongside the gel. The arrow head points at the fragments of 86 and 89 bp that accumulate upon UV irradiation. The sizes of the protected fragments not affected by the UV treatment are indicated by the small arrows and the sizes are in basepairs. **C.** Map of the DES promoter area, with above it the probe **A** used in the RNase protection experiment presented in **B**, and below the map a summary of the 5′ ends mapped before and after a UV treatment (− and + UV, respectively). The arrow head indicates the accumulated 86 and 89 bp protected fragments. the dotted line indicates the probe used for the RNA blot hybridization (see **A**). Restriction sites: Bs, BstEII; E, EcoRV; H, HindIII; S, SalI; Sc, ScaI; Sh, SphI.

from the area upstream of the transcription starts in variant 118a'. We conclude that these are transcript processing sites rather than transcriptional starts, as the promoter for these transcripts is not located immediately 5' of these transcripts. We detect no transcripts running in the opposite direction in the DES promoter region of variants 118a and 118a' (Figure 5, probes D and E). Transcription in the DES promoter region thus occurs in the direction of the VSG gene 118 only.

UV-inactivation of the upstream transcription unit in the DES of variant 118a does not lead to loss of VSG gene ES promoter function

Promoter proximal precursor RNA molecules in trypanosome VSG gene ESs are rapidly processed and thus have a low steady state level in total RNA (8). UV irradiation stabilizes unstable RNA precursor molecules, probably by an inhibition of RNA processing (13). UV irradiation also inhibits RNA elongation (53), but this effect is minimal near the promoter. Hence, promoter proximal transcripts accumulate after UV irradiation (13). This effect is hardly noticeable in short-time run-on experiments (ref. 7 and figure 4), but it is substantial in steady state RNA (13). Figure 6 A shows that a 1.4-kb transcript hybridizing with a promoter region probe indeed accumulates in variant 118a trypanosomes after UV irradiation. The newly synthesized RNA becomes shorter when the irradiation period is increased to five minutes. This is mainly due to RNA degradation (*cf.* ref. 13) as the VSG mRNA is also largely degraded under these conditions. The transcripts that accumulate were mapped by RNase protection. Figure 6 B shows that the signal of the fragments of 86 and 89 bp is strongly increased when probe A (see figure 6 C) is hybridized to total RNA of irradiated trypanosomes (compare 0, 2 and 5 min UV). This suggests that the 'standard' VSG ES start may also be used in variant 118a and that this start is active if transcription through this start site from the upstream promoter is prevented by blocking RNA elongation with UV light. The signals of the protected fragments corresponding to transcripts that start upstream of the EcoRV site (figure 6 B and C) are hardly affected by prior UV

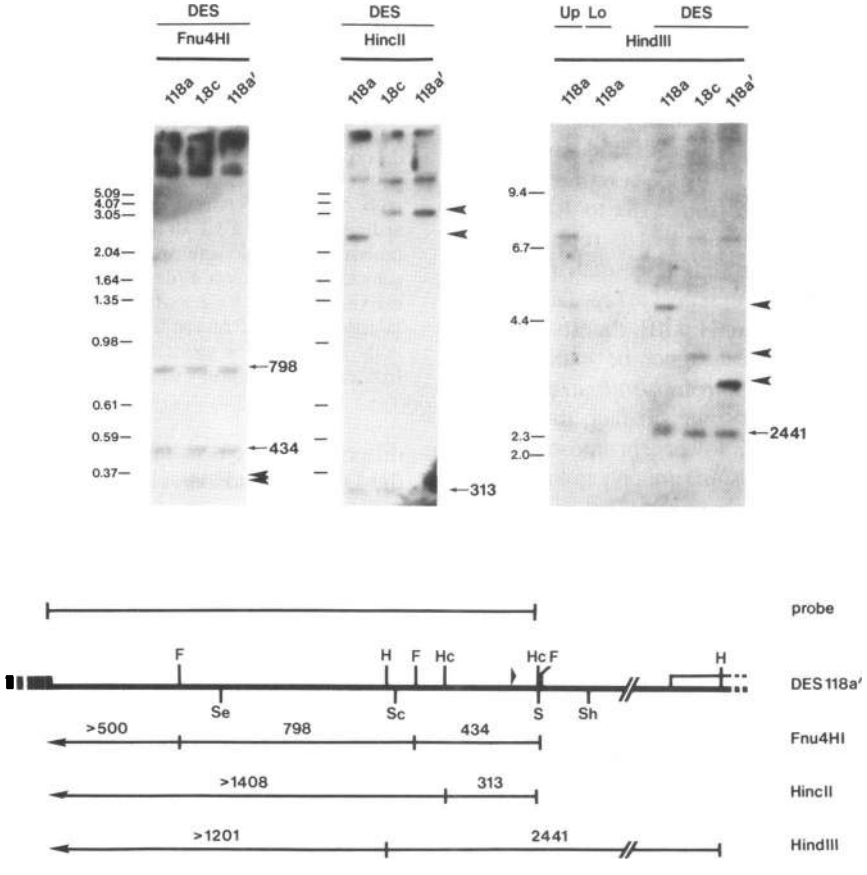

Figure 7. DNA blot of restriction fragments from the promoter area of the DES chromosome of variants 118a, 1.8c and 118a'. DES chromosome enriched DNA from the *T.brucei* variants 118a, 1.8c and 118a' was isolated from PFG gels, digested with the restriction enzymes Fnu4HI, HincII and HindIII, size-fractionated, blotted onto nitrocellulose and hybridized with a probe as indicated above the map of the DES promoter area from variant 118a' (from the 50 bp repeat, indicated at the 5' end by the thick black bar, up to the SalI site). The length in bp of the restriction fragments present in the three variants are indicated on the right side of each blot and refer to those depicted underneath the map. Fragments that differ in size between the different variants are indicated by the arrow heads at the right of each blot. On the left-hand side of each blot the sizes in kb of marker fragments are indicated. DNA enriched for the bands migrating just ahead (Lo, lower) and behind (Up, upper) the DES chromosome in PFG gels was also digested with HindIII as a control for cross-contamination. In the map of the DES promoter region only relevant restriction sites are indicated; those above the line are relevant for the digests, those below show the position of some of the characteristic and well conserved restriction sites present in ES promoter regions. The open box represents the ESAG-X 5' end. The arrow head pointing left to right indicates the positions of the 5' ends of promoter transcripts in variant 118a' (within the 434 bp Fnu4HI fragment). The sizes of the most upstream Fnu4HI, HincII and HindIII fragments are not known but must be larger than 500, 1408 and 1201 bp respectively in variant 118a'. Restriction sites: F, Fnu4HI; H, HindIII; Hc, HincII; S, SalI; Sc, ScaI; Se, SpeI; Sh, SphI.

irradiation (compare 0, 2 and 5 min UV), suggesting that these transcripts are normally relatively stable. There are no large open reading frames in this area, however.

Sequence alterations linked to the promoter region correlate with DES telomere transcription inactivation and reactivation

In previous comparisons of the downstream parts of the DES in variants 118a and 118a′ no differences were found (10,20). As 118a′ is directly derived from 118a via two single relapses (figure 1), it looked as if the DES had been inactivated (118a→1.8c) and reactivated (1.8c → 118a′) in these relapses without any DNA alterations. In fact, variants 118a and 118a′ seemed identical for all practical purposes (20). The differences we now find between the DES promoter region transcripts from these variants clearly show that this plausible interpretation is incorrect and they raise the possibility that the (in)activation of the DES is associated with DNA alterations (far) upstream of the promoter. This was tested by hybridization analysis of restriction digests of DNA isolated from the DES chromosome area of PFG gels. Figure 7 shows a Southern blot hybridized to a probe covering the whole promoter region of the DES in variant 118a′. The restriction fragments between the most upstream Fnu4HI site and the SalI site are conserved in the DES promoter regions of variants 118a, 1.8c and 118a′. Further upstream the physical maps differ, however. The most upstream Fnu4HI fragment is minimally 500 bp (see physical map of the DES promoter region in variant 118a′) and extends into the 50 bp repeat area. This large fragment runs in the compression zone of the gel in figure 7. In the switch from 118a to 1.8c a new Fnu4HI fragment appears (350 bp long) that is replaced by a doublet in the 1.8c to 118a′ transition. These small fragments must come somewhere from the 50 bp repeat array. Differences are also evident in the HincII and HindIII digests of DES chromosome DNA. The differences cannot be explained by contaminating fragments from chromosome-sized DNA molecules flanking the DES chromosome. In fact, the HindIII digest suggests that the upper and lower chromosome-sized molecules that flank the DES chromosome are contaminated with DES DNA.

The 50 bp repeat region is uniquely linked to the ES telomere of the DES chromosome

The DNA alterations that we detect are associated with the 50 bp repeat region. To ensure that there is not more than one 50 bp repeat region in the DES, we used two-dimensional pulsed field gel electrophoresis. Chromosome-sized DNA molecules were separated in PFG gels, digested in gel with an infrequently cutting restriction enzyme such as NotI, run in the second dimension, blotted and hybridized to several consecutive probes. Figure 8 shows DNA of variant 118a cut with NotI. There is a single NotI DES chromosome fragment of approximately 125 kb that hybridizes with the VSG 118 cDNA probe, a telomeric repeat probe, an ES promoter probe and a 50 bp repeat probe. Note that there is a second 50 kb NotI fragment coming from the DES chromosome that hybridizes with the telomeric repeat probe, but not with the 50 bp repeat probe or the other ES probes. These results demonstrate that the DES chromosome contains a single copy of the conserved ES promoter and a single array of 50 bp repeats, both linked to the telomeric VSG 118 gene present in the DES. The other end of this chromosome seems to lack an ES and 50 bp repeats. Analogous results were obtained with DNA from variants 1.8c and 118a′ (not shown). No size

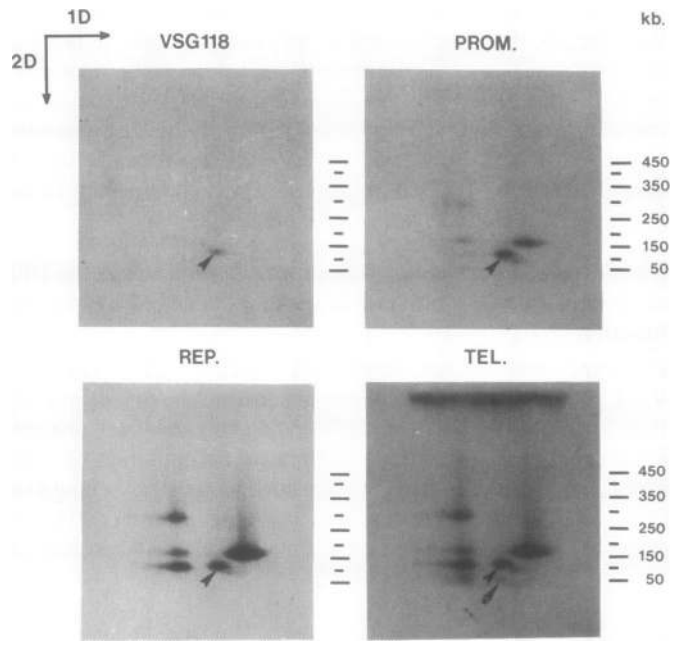

Figure 8. Linkage of the 50 bp repeat region to the DES telomere. *T.brucei* 427 variant 118a chromosome-sized DNA molecules were separated in the first dimension (1D) on PFG gels, digested in gel with the restiction enzyme NotI, run in the second dimension (2D) and blotted (see Materials and Methods for details). The same blot was hybridized with the 550 bp EcoRI-PstI fragment of pTcV118.2 (VSG118; ref. 16), stripped and reprobed with the 576 bp SpeI-HindIII probe from the DES promoter region (PROM.; see physical map figure 2), stripped and reprobed with a 1.1 kb fragment from the 50 bp repeat region (REP.) and probed with a telomere specific probe pT6 (61). The arrowhead on the autoradiographs points at the approximately 125 kb telomeric NotI fragment carrying the DES; the arrowhead with dot points at the other approximately 50 kb telomeric NotI fragment from the DES chromosome. The compression zone in the first dimension yields in the second dimension three telomeric fragments hybridizing with a promoter and a 50 bp repeat probe.

differences were detectable in the telomeric NotI fragments from the DES chromosomes of these three variants. The differences found between the variants in this chromosome in figure 7 are therefore too small to detect in a DNA fragment of approximately 125 kb.

DISCUSSION

DNA rearrangements linked to the 50 bp repeats are correlated with inactivation and reactivation of the DES

We have previously shown that the 221 ES can be switched off without changing a single bp within 1.4 kb of the transcription start (7). The results presented here confirm this. DNA rearrangements near the 'standard' promoter are clearly not responsible for the inactivation and reactivation of the DES. However, we have found alterations upstream of the major transcription start for the DES that accompany the activation and inactivation of this VSG gene ES in single relapse experiments. We attribute the alterations in restriction fragments to DNA rearrangements rather than to DNA modification as the alterations were also observed with restriction enzymes that are not affected by the DNA modification that results in partial digestion of inactive telomeric VSG gene copies (54,55). The precise molecular nature of the alterations that we observe remains unknown. They are localized in an area of 50 bp repeats that is resistant to conventional cloning procedures in *E. coli* (ref.7

and this paper) and the further analysis of this region may require isolation of trypanosome DNA clones in yeast.

Although we observe alterations both when the DES is switched off (118a to 1.8c) and when it is switched on (1.8c to 118a'), the latter switch is not a simple reversal of the former. The alterations may therefore be incidental. Nevertheless, the possibility that they cause (in)activation of the DES is an attractive one for theoretical reasons (*cf.* ref. 24) and on the basis of our transfection experiments that argue against a control of ESs by trans-activating factors. These experiments show that the ES promoter can promote high CAT activity under conditions where most endogenous ES promoters are shut off. In fact, the ES promoter works as well in these transient transfections as the promoters of the procyclin genes (Table I) and the rRNA genes, that are expected to be fully functional in the procyclic trypanosomes used in these experiments (unpublished results). Although we cannot exclude that the transfected plasmids circumnavigate endogenous nuclear controls, the simplest interpretation of our experiments is that ES promoters are negatively regulated *in cis* by sequences not included in the promoter constructs used thusfar. Rearrangements in these *cis* dominant control regions could regulate ES promoter activity. It will be difficult to verify this speculative scenario, as the putative control regions are highly repetitive and resistant to cloning. Fortunately the DES readily switches off and on (20) and it should be possible to extend the available switching series and test prospectively whether alterations in the 50 bp repeat array accompany each additional switch.

Loss of upstream transcription in the DES does not effect ES promoter activity

The observation that transcription upstream of the VSG gene ES promoter in the DES of variant 118a had vanished in variant 118a' was unexpected. This transcription is resistant to high concentrations of α-amanitin indicating that the additional promoter has the properties of a ribosomal promoter or an authentic VSG gene ES promoter. The function of this promoter is unclear, as the transcripts starting from this promoter are absent in variant 118a' and in variants using the 221 ES. In fact, L.H.T. van der Ploeg and co-workers, who have independently obtained results similar to ours, have cloned part of the upstream transcription unit from variant 118 clone 1 (a 118a variant) and shown that it has undergone a deletion somewhere in the transition from variant 118a to variant 1.8c (personal communication). What could such a discardable transcription unit provide? One possibility is that it is just a way of installing and removing an active ES promoter by a recombination or gene conversion event. The transcripts coming from this promoter may suppress initiation at the 'standard' promoter, as we could not detect a clear start at the 'standard' DES promoter of variant 118a in trypanosomes that had not been irradiated with UV light (*e.g.* figure 5, probe A). Moreover we found no clear gap in transcription in the promoter area of the DES in variant 118a (figure 4). Another possibility is that the upstream transcription unit contains genes that are only advantageous under certain conditions *in vivo*. We have speculated that ESAGs code for cell-surface components restricted to the flagellar pocket (8). A limited variability of such components could be advantageous because antibodies against an ESAG product do appear in chronic trypanosome infections (56) and vaccination of mice with purified flagellar pocket membranes can partially protect against subsequent trypanosome challenge (57). The switch from one ES to another bearing another set of ESAGs might therefore change the antigens exposed in the flagellar pocket. The selection for limited change in exposed epitopes of surface receptors/transporters might account for the presence of a hypervariable region in ESAGs-X and -X" (ref. 7,56 and this paper), where most nucleotide substitutions give rise to amino acid changes in different variants.

Our results confirm that an ES promoter fragment suffices to provide high promoter activity in transient expression in procyclic trypanosomes (7), as also recently reported by Jefferies et al. (58). The VSG gene 221 ES promoter yields CAT activities very similar to those obtained with an authentic procyclic stage (parp) promoter (Table I). The differences between the promoter regions of the DES and the 221 ES do not have a dramatic effect on promoter activity in the assay employed (Table I). Even with the more precise definition of a VSG gene ES promoter now available (ref. 7 and unpublished results) and an analogous dissection of the promoters of the parp or procyclin genes (39,59) and the rRNA genes of *T. brucei* (unpublished results), there is no homology detectable between these three promoters that generate α-amanitin resistant transcripts. Either these promoters are recognized by different polymerases (60), or they are transcribed by the same polymerase, *e.g.* RNA polymerase I (46), but promoter recognition is mediated by different factors recognizing different DNA sequences but the same polymerase. The further analysis of this problem awaits the development of a faithful *in vitro* transcription system.

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