Many trypanosome messenger RNAs share a common 5' terminal sequence

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ABSTRACT

The mRNAs for different variant surface antigens of Trypanosoma brucei start with the same 35 nucleotides. This sequence is encoded by a separate mini-exon, located in a 1.35-kb repetitive element. We have reported that trypanosomes contain many transcripts that hybridize to mini-exon probes, even if they do not make the surface antigens. We show here that these transcripts have the mini-exon sequence at their 5' end; they do not contain other sequences from the mini-exon repeat element and are polyadenylated. We have cloned DNA complementary to trypanosome mRNAs and randomly selected 17 clones containing mini-exon sequences. Thirteen of these are derived from different genes that do not code for surface antigens. We conclude that the mini-exon sequence is a common element at the 5' end of many trypanosome mRNAs. As the 200 genes for mini-exons are highly clustered, linkage of the mini-exon sequence to the remainder of most mRNAs may require discontinuous transcription.

INTRODUCTION

Trypanosomes can evade host antibodies by repeatedly changing their surface coat (1). This coat consists of a single protein species: the Variant Surface Glycoprotein or VSG (2). The progeny of a single trypanosome can produce at least one hundred antigenically different VSGs, encoded by a large repertoire of VSG genes (3, 4).

VSG gene activation frequently involves gene rearrangements (see (5) for review). A well defined rearrangement is the formation of an extra copy of one of the numerous silent VSG genes (designated Basic Copies or BCs) (6-10). In T.brucei, strain 427 this Expression-Linked extra Copy or ELC is transcribed in a dominant expression site located at the telomere of a 2-Mb chromosome (10-13). The duplicated VSG gene segment, which is thought to displace the preceding ELC by gene conversion, contains the complete protein coding region (9, 10), but lacks a mini-exon coding for the 5' 35 nucleotides of the mature VSG mRNA (14-16). Mini-exons are part of a 1.35-kb repeat element present in about

200 copies per nucleus (17). As most of these elements are tandemly arranged in arrays of more than ten units (17,18), we proposed that such a mini-exon repeat array is present in the dominant expression site. However, attempts to find mini-exon repeats upstream of expressed VSG genes have failed (De Lange, T., unpublished).

In addition to VSG mRNA, T.brucei contains many other transcripts that contain mini-exon sequences (17). Analogous transcripts are also present when VSGs are not synthesized and in trypanosome species that do not undergo antigenic variation (17; De Lange et al., manuscript in preparation), showing that the role of mini-exons is not confined to VSG gene expression.

In this paper we report a more detailed analysis of the transcripts in T.brucei that contain mini-exon sequences. The data suggest that mini-exons code for the 5' terminus of many mRNAs in trypanosomes. We discuss the possibility, raised by this observation, that many trypanosome mRNAs are the product of transcription of two coding regions that are not physically linked in the DNA: the repetitive mini-exon and the exon(s) coding for the remainder of the mRNA.

MATERIALS AND METHODS

Trypanosomes. The trypanosomes used belong to strain 427 of Trypanosoma brucei brucei. Trypanosome clones MITat 1 4a, 1 5a, 1.6b and 1.1a have been described by Cross (2). The origin of trypanosome clones 118c, 1.196 and 1.8b is described by Michels et al. (10) and Van der Ploeg et al. (13). Cultured (procyclic) trypanosomes were obtained from Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland). Trypanosomes were amplified in rats and purified from blood elements by published procedures (19).

Isolation and blotting of nucleic acids. Procedures for the isolation of trypanosome nuclear DNA and RNA have been described by Bernards et al. (9) and Van der Ploeg et al. (14). Poly(A)$^+$ RNA was purified by oligo(dT) cellulose chromatography as described by Hoeijmakers et al. (20). RNA blots were prepared using glyoxylated RNA, according to the procedure described by Thomas (21). DNA blots were prepared essentially as described by Southern (22). To facilitate transfer of large DNA fragments, DNA

gels were treated with 0.25 N HCl for 20 min at room temperature prior to the blotting procedure. Pulsed-field-gradient gel electrophoresis of intact chromosomes was carried out as described by Van der Ploeg et al. (13) in 1% agarose gels run at 20°C, alternating the electric fields (17.5 V/cm N-S; 6.25 V/cm W-E) every 35 seconds. Filter hybridizations with nicktranslated (23) restriction fragments isolated from low melting agarose gels and purified by DEAE-cellulose column chromatography (24) were performed as described (17). Post-hybridization washes were in 0.1 x SSC at 65°C.

cDNA cloning. A library of cDNA of mRNA from trypanosome clones 1.8b, 1.6b, 1.1a and 1.196 was constructed essentially as described by Maniatis et al. (25) using oligo(dT) as primer and (a mixture of) poly(A)$^+$ RNA as template. The cDNAs were annealed in the PstI site of the vector pBR322 by GC tailing and introduced into E.coli C600. The resulting library was screened with a synthetic 22 nucleotide probe (17 and see Fig. 4) using hybridization conditions described previously (17), and with a 1.35-kb XmnI fragment which contains the entire mini-exon repeat element, isolated from clone cPR1 (17) using hybridization conditions described in the preceding paragraph. Recombinant plasmids were isolated from clonal cultures using the procedure described by Birnboim and Doly (26).

Nucleotide sequence analysis. The DNA sequence of the 5' end of cDNA 8 was determined by the chemical degradation procedure (27) using five modification reactions (G, A+G, C+T, C, A+C) on 5'-^{32}P-labeled XmnI fragments. The 5' ends of T.brucei mRNAs were sequenced by reverse transcriptase catalysed cDNA synthesis with a synthetic 22-mer as primer (17 and see Fig. 4) using the reaction conditions described by Michels et al. (10).

RESULTS

Many different poly(A)$^+$ RNAs contain the mini-exon sequence.

All transcripts containing mini-exon sequences are recovered in the poly(A)$^+$ fraction after standard oligo(dT) cellulose chromatography (17). Figure 1 shows that these transcripts elute from oligo(dT) cellulose at the same NaCl concentration as the VSG mRNA indicating that they contain A-stretches as long or longer than the dT-oligomers on the column material (14-20 residues). As

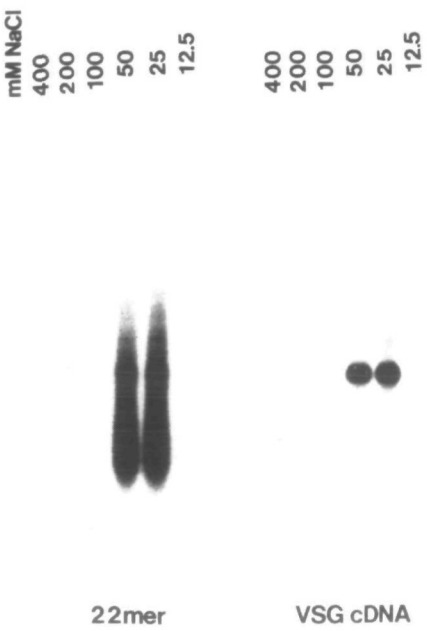

Figure 1. Mini-exon transcripts and VSG mRNA co-elute from oligo(dT) cellulose. RNA (1 mg) from 118c (10) trypanosomes was fractionated twice on oligo(dT) cellulose at room temperature using standard elution buffer (without NaCl) in the first selection round and elution buffer with a decreasing NaCl concentration (indicated in the figure) in the second selection round. One tenth of each fraction (50 ul) was ethanol precipitated, glyoxylated and size-fractionated on a 1.2% agarose gel. This RNA was transferred to nitrocellulose and hybridized to the 22-mer mini-exon probe shown in Fig.4 (left) and to a cDNA of VSG 118 mRNA (20) (right).

similar transcripts are present in T.cruzi (De Lange et al., manuscript in prep.) and in cultured T.brucei (17 and see below), both of which do not synthesize a surface coat, we can exclude that they represent only VSG mRNA precursors and/or processing products.

To further analyse these RNAs we cloned DNA complementary to a mixture of poly(A)$^+$ RNAs from four trypanosome variant antigen types in E.coli and screened this cDNA library with a synthetic mini-exon probe of 22 nucleotides. Approximately 5% of the colonies hybridize to this probe (Fig.2). None of the positive colonies hybridize to a probe for the entire 1.35-kb mini-exon repeat element under stringent conditions (0.1 x SSC; 65°C.); the single colony detected in Figure 2 does not hybridize to the 22-mer. This result is in line with our observation that a probe for the 3' flanking region of the mini-exon (a 0.64-kb PvuII fragment located 23 bp downstream of the mini-exon) fails to detect transcripts in RNA blots (not shown). We conclude that only a minor fraction of the transcripts have sequences from the mini-exon repeat element beyond the mini-exon and that therefore the majority

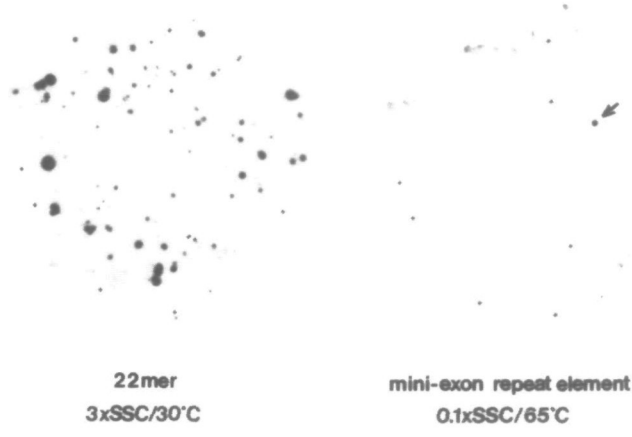

Figure 2. Many cloned cDNAs contain mini-exon sequences, but no other sequences from the mini-exon repeat element. Duplicate filters with ± 1000 lysed bacterial colonies containing cDNAs inserted in pBR322 were hybridized to the 22-mer mini-exon probe (left) and to a 1.35-kb XmnI fragment containing the entire mini-exon repeat element (right). After hybridization the filters were washed as indicated in the figure. The + signs on the autoradiographs are markers; the arrow indicates the single colony that hybridizes to the 1.35-kb XmnI fragment. The 22-mer probe is shown in Fig. 4.

of the transcripts that contain mini-exon sequences represent non-VSG mRNAs.

To obtain information on the fraction of mRNAs that contains the mini-exon sequence, we determined the relative hybridization of the 22-mer to VSG mRNA and to total poly(A)$^+$ RNA by densitometry. Comparison of the scans of poly(A)$^+$ RNA from bloodstream trypanosomes and cultured trypanosomes, shown in Figure 3, indicates that the VSG mRNA gives rise to a readily identifiable peak when present. The VSG mRNA peak accounts for about 10% (9% and 11% in two independent experiments) of the total 22-mer hybridization as shown in Figure 3, suggesting that the VSG mRNA makes up about 10% of the mini-exon containing mRNAs. In this calculation we assume that each mRNA contains only one mini-exon sequence (see below). As VSG mRNA is a major component of the poly(A)$^+$ fraction (28), our data suggest that many mRNAs in T.brucei contain a mini-exon sequence. The low percentage of cDNA clones that contain a mini-exon (5%) is probably due to the low efficiency with which 5' ends are cloned in the procedure used.

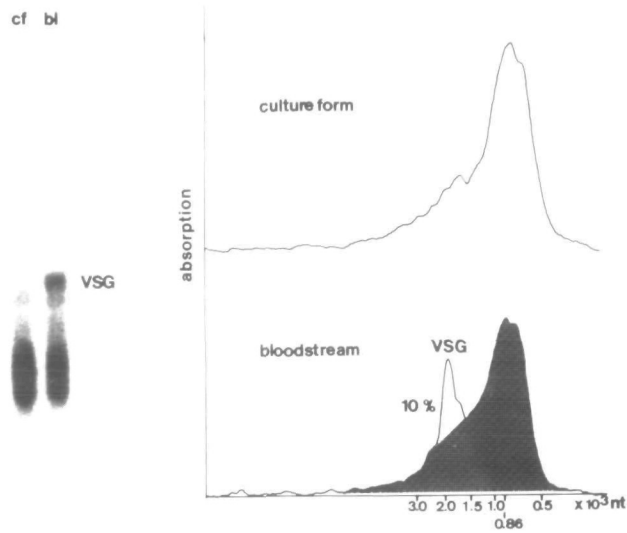

Figure 3. VSG mRNA makes up 10% of the mini-exon transcripts. A nitrocellulose strip containing 2 µg poly(A)$^+$ RNA from culture form trypanosomes (cf) and bloodstream trypanosomes (bl), which express VSG 1.8 (clone MITat 1.8b) was hybridized to the 22-mer mini-exon probe. The autoradiograph (left) was scanned (right) with a Beckman DU-8B densitometer. The 22-mer hybridization to VSG mRNA was calculated from the lower plot (non-shaded area) and compared to the total hybridization (VSG peak + shaded area). In two independent experiments the VSG peak was 9% and 11% of the total area.

The mini-exon sequence is at the 5'end.

Two lines of evidence indicate that the transcripts detected with the mini-exon probe contain one complete mini-exon at the 5'end. First we used the 22-mer as a primer for the synthesis of DNA complementary to poly(A)$^+$ RNA from bloodstream and cultured trypanosomes. Figure 4 shows that the major cDNA product is 35 nucleotides long, irrespective of the presence of VSG mRNA; minor products are visible at 36 and 38 nucleotides, but very few longer products are seen. Therefore this sequence must be located at the 5'end of mRNAs which contain it.

Secondly, we determined the nucleotide sequence of one of the cDNAs that hybridize to the 22-mer (cDNA 8, see below) and found this cDNA to contain 34 out of the 35 bp of the mini-exon at the edge of the cloned insert, directly flanked by the GC-tail added during cloning. The relevant nucleotide sequence is shown in Figure 5. Downstream, the mini-exon in cDNA 8 is flanked by a

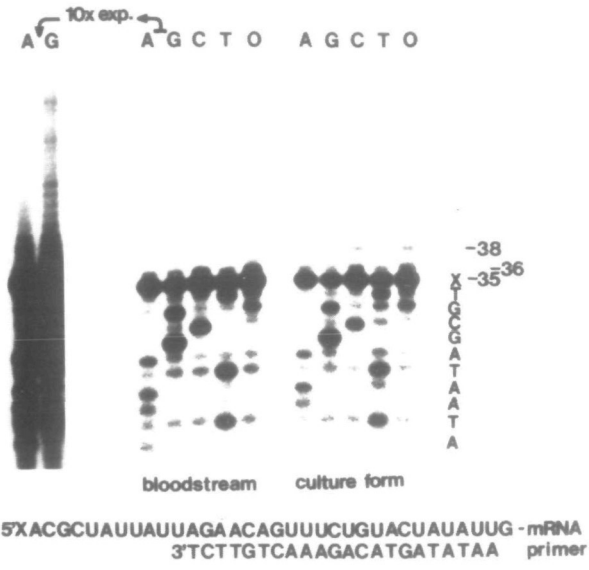

Figure 4. The mini-exon is predominantly located at the 5'end. DNA complementary to poly(A)$^+$ RNA from bloodstream trypanosomes expressing VSG 118 (MITat 1.5a) and culture form trypanosomes was synthesized by 22-mer primed reverse transcription of 1 µg template RNA. The reaction mixture contained either one (A, G, C or T) or no (O) dideoxynucleotide and α-^{32}P-dATP. The cDNA products, separated on a 8% sequencing gel, were detected by autoradiography for 4 hrs and for 40 hrs (A and G lanes on the left). The size of strong stop products is indicated in nucleotides. The presumed position of the primer - the 22-mer probe for the mini-exon - with respect to the 5'end of mRNAs is shown.

sequence that has no homology with the sequence flanking the mini-exon in T.brucei genomic DNA (17; De Lange et al., manuscript in prep.). At position 59 the cDNA sequence contains an ATG

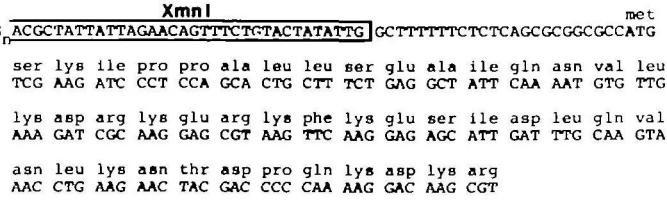

Figure 5. Nucleotide sequence of the 5'end of cDNA 8. The sequence was determined by the chemical degradation procedure from the XmnI site shown. The block at the 5'end contains 34 bp of the mini-exon. The A presumably present at the 5'end of mini-exon transcript (17) is missing. The mini-exon is direct-flanked by the G-tail added during cloning. The cDNA contains an open reading frame of at least 45 amino acids, as shown.

codon, followed by an open reading frame. This suggests that the mini-exon is at the 5' terminus of a non-VSG mRNA. We conclude that most mRNAs contain one copy of the mini-exon sequence at their 5' end.

Identification of thirteen non-VSG genes coding for transcripts, that contain the mini-exon sequence.

To characterize the nature of the non-VSG mRNAs that contain mini-exon sequences, 17 cloned cDNAs were randomly selected for further analysis. Each of these cDNAs contained (part of) the mini-exon sequence, but no other mini-exon repeat sequence. Hybridization of these cDNAs with restriction digests of trypanosome nuclear DNA showed that the 17 cDNAs were derived from 15 different loci. An example of this analysis is shown in Figure 6; the other data are summarized in Table I. The cDNAs in clones 10 and 16 probably originate from the same gene, as do cDNAs 2 and 12, which contain VSG 1.8 cDNA. Although cDNAs 21 and 23 recognize the same set of PvuII fragments in nuclear DNA (see Table I) they are not identical, because cDNA 23 lacks the PstI site pre-

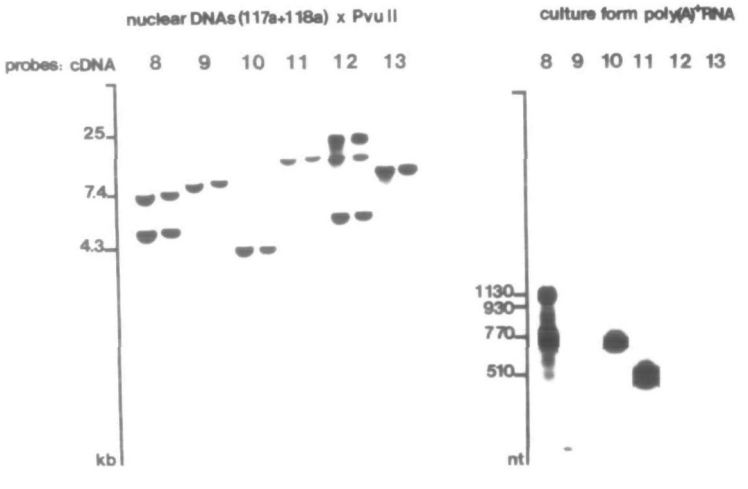

Figure 6. Analysis of cDNAs by hybridization to nuclear DNA and poly(A)$^+$ RNA. PstI (sub-) fragments (see Table I) from six mini-exon containing cDNAs were hybridized to nitrocellulose strips with size-fractionated, PvuII-digested DNA from MITat 1.4a (expresses VSG 117a) and MITat 1.5a (expresses VSG 118a) (left) and to nitrocellulose strips with 2 ug poly(A)$^+$ RNA from culture form trypanosomes (right). Filters were washed in 0.1 x SSC at 65°C after hybridization. The RNA blots hybridized with cDNAs 9, 12 and 13 were exposed 4-fold longer.

Table I. Structure and origin of seventeen cDNAs that contain (part of) the mini-exon. Plasmids were digested with PstI, size-fractionated on a 1% agarose gel, transferred to nitrocellulose and hybridized to the 22-mer mini-exon probe. The size of the PstI insert fragments is shown in the second column. PstI fragments that hybridize to the 22-mer (*), were isolated and used as hybridization probes (except for cDNA 19 where the whole plasmid was used as a probe). The PvuII fragments detected in the nuclear DNAs from two trypanosome clones (determined as shown in Figure 6) are listed. The patterns in the two DNAs were identical. The same probes were used on blots of poly(A)$^+$ RNA from culture form trypanosomes (as described in the legend of Figure 6) and an equal amount of poly (A)$^+$ RNA from a VSG 1.8 expressor (MITat 1.8b). The transcripts detected in culture form RNA are listed. The same transcripts are present in the VSG 1.8 expressor; cDNAs 2 and 12 detect the VSG mRNA in this RNA. The last column lists the chromosomal location of the loci determined as shown in Figure 7.

cDNA	PstI inserts(s) (bp)	PvuII fragments (kb)	transcript size(nt)	chromosomal location	
2	480 + 350*	>25 + 16 + 5.7	-	ori + 2 Mb	VSG 1.8
12	370*+ 225	>25 + 16 + 5.7	-	ori + 2 Mb	
9	350*	8.5	-	ori	- VSG?
10	490 + 320*	4.3	730	ori	identical
16	430 + 300*	4.3	730	ori	
21	440 + 160*	>25 + 5.0 + 2.7	720	ori + 2 Mb	related
23	650*	>25 + 5.0 + 2.7	900 + 720	ori + 2 Mb	
1	1350*+ 360	2.8 + 2.3	1600	ori	
8	760*	7.4 + 4.9	1130 + 770	ori	
11	450*	15.5	510	ori	
13	510 + 320*	11.0	930	ori	
14	365*	7.0	1000 + 870	ori	
15	405*	8.4 + 2.3	620	ori	
18	700*	23.5	840	ori	
19	230 + 170 + 90*	9.5	560	ori	
20	530*	13.5	1100	ori	
22	550*	>25	900	ori	

sent less than 160 bp downstream of the mini-exon in cDNA 21 (see Table I).

At least thirteen out of the fifteen loci do not contain a VSG gene. This follows from hybridization of the corresponding cDNA probes to poly(A)$^+$ RNA from cultured trypanosomes (lacking VSG mRNA synthesis) shown in Figure 6 and summarized in Table I. In addition to the cDNAs complementary to the mRNA for VSG 1.8, only the cDNA probe from clone 9 fails to detect a discrete transcript in this RNA. This clone may therefore also contain VSG cDNA.

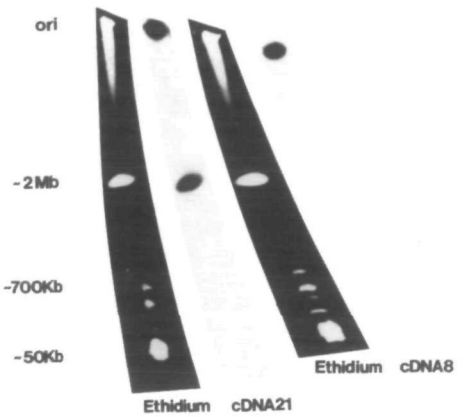

Figure 7. The chromosomal location of genes coding for mini-exon containing transcripts. Intact chromosomes of 118c trypanosomes were fractionated in a pulsed-field-gradient gel (see ethidium stained lanes), blotted onto nitrocellulose filter and hybridized to PstI(sub-) fragments from cDNAs 21 and 8; ori= origin.

Chromosomal location of transcribed non-VSG genes.

To study the chromosomal location of the thirteen non-VSG genes, the cDNAs were hybridized to nitrocellulose filters containing chromosome fractions, size-fractionated by pulsed-field-gradient gel electrophoresis (13). None of the cDNA probes recognizes the small chromosomes (Figure 7 and Table I). All probes hybridizes to the largest chromosome fraction in which the bulk of the mini-exons is located (13). In addition cDNAs 21 and 23 hybridize to a chromosomal fraction of ±2 Mb, which also contains some mini-exons (13). Hence, physical linkage between non-VSG genes and mini-exons is not disproven by this relatively coarse test.

DISCUSSION

Our data show that many mRNAs of T.brucei have a 5' terminus that is encoded by the 35-bp mini-exon. It is even possible that all trypanosomal mRNAs share this sequence; no other mRNAs apart from VSG mRNAs and those analysed by us have been studied.

Mini-exons are not present within 10 kb of VSG main exons (17); likewise the mini-exon sequence of the non-VSG mRNA cloned in cDNA 8 is not present within 10 kb upstream of the remainder of the gene (Kooter, J.M., De Lange, T. unpublished). This is as expected because the vast majority of mini-exons resides in repeat arrays of at least ten tandemly linked units (17). We have proposed that such arrays function as an efficient multiple pro-

moter for transcription of neighbouring genes (17). As there are about 200 mini-exons per nucleus there might be roughly twenty repeat arrays but not many more. However, the abundance of mRNAs with a mini-exon sequence at their 5' end suggests that many more than twenty genes need a mini-exon. To explain this, two alternatives can be considered. First many genes could be clustered downstream of a mini-exon repeat array. Expression of genes organized in this manner requires a differential splicing and/or termination mechanism, because the main exon of one gene will be in the intron of the next. Both differential splicing and differential termination have been described for viral- and cellular genes (29-31) and close linkage of genes is to be expected in trypanosomes, because their low complexity (only 6 x E.coli,(32)) leaves little room for spacers. Very large transcription units have also been found in other eukaryotes (cf. 33-35).

The second, rather outlandish (5), alternative invokes discontinuous transcription. After transcription of the mini-exon, RNA polymerase II might release the template, but not the transcript, and reinitiate near a (VSG) main exon to produce a precursor RNA, which is processed to yield a mature messenger (36). Two precedents for such a jumping polymerase-transcript complex are known: during retroviral replication reverse transcriptase moves from one template to another while holding on to the strong stop transcript (37); a similar jump has been postulated to occur during synthesis of some mRNAs by the RNA polymerase of coronaviruses (38). Mini-exon and main exon transcripts might also be synthesized independently, however, and become linked by bimolecular splicing.

The postulation of discontinuous transcription creates a conceptual problem concerning VSG gene expression, because reinitiation by the polymerase-mini-exon transcript complex (or independent transcription) involves the ELC gene rather than one of the numerous BC genes. Hence, some signal in the DNA or higher order structure of ELC genes should help RNA polymerase to find the correct VSG gene. Boothroyd and co-workers (36) have suggested that the $(ATT)_{90}$ stretch 2.3 kb upstream of the ELC gene for VSG 117a could fulfill this function. An $(ATT)_{90}$ stretch has also been found, however, within 1.5 kb upstream of a (non-tran-

scribed) chromosome-internal BC VSG gene in T.brucei strain 1125 (Eijgenraam, F., pers. communication).

Thusfar, trypanosomes are the only organisms known to produce mRNAs with uniform 5'ends. Could this terminus be advantageous for translation or mRNA stability? If so, it is unclear why this sequence is not simply encoded adjacent to each gene. Therefore, we favour the idea that the common 5' ends are the consequence of the unusual way in which protein coding genes are transcribed in trypanosomes. The unknown merits of this transcription system seem to be appreciated by trypanosomatids in general, since mini-exons and their transcripts are found in such diverse species as Trypanosoma vivax, Trypanosoma cruzi and Crithidia fasciculata (17; 18; De Lange et al., manuscript in prep.). The mini-exon sequence shared by many mRNAs may therefore be yet another unusual feature shared by the kinetoplastid flagellates (see (5)).

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Abbreviations: BC, basic copy; bp, base pair(s); cDNA, complementary DNA; ELC, Expression-Linked extra copy; kb, kilo-basepair(s); MITat, Molteno Institute Trypanozoon antigen type; Mb, Megabasepairs; mRNA, messenger RNA; nt, nucleotide(s); SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0); VSG, Variant Surface Glycoprotein.

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