Alpha-amanitin resistant transcription of protein coding genes in insect and bloodstream form *Trypanosoma brucei*

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Communicated by R.Nusse

The variant cell surface glycoprotein (VSG) gene expression sites of the protozoan *Trypanosoma brucei* are transcribed by an unusual α -amanitin resistant RNA polymerase. All other protein coding genes of *T. brucei* examined to date are transcribed by an α -amanitin sensitive RNA polymerase, presumably RNA polymerase II. We now show that transcription of protein coding genes by α -amanitin resistant RNA polymerases is not unique to the bloodstream form expressed VSG gene expression sites, but also occurs in insect form trypanosomes, which do not express VSG genes. In insect form trypanosomes transcription of the procyclin or PARP genes is resistant to α -amanitin to a degree comparable with that of VSG and ribosomal RNA (rRNA) genes. Comparison of the α -amanitin resistantly transcribed PARP and VSG gene families shows that they both produce one of the most abundant mRNAs [1–3% of poly(A)⁺] and they both encode the major cell surface proteins of their respective life cycle stages. Transcription of a subset of functionally comparable protein coding genes is thus mediated by an RNA polymerase which differs from the regular RNA polymerase II.

Key words: α -amanitin/procyclin genes/RNA polymerase/*Trypanosoma brucei*/variant cell surface glycoprotein gene

Introduction

The flagellated parasitic protozoan *Trypanosoma brucei* is transmitted between mammalian hosts by the tsetse fly. In the mammalian bloodstream *T. brucei* is covered by a dense, homogeneous variant cell surface glycoprotein (VSG) coat. *Trypanosoma brucei* escapes immune destruction in the bloodstream by undergoing antigenic variation as a result of the periodic generation of a few parasites which express an antigenically distinct VSG coat, made possible by a switch to the expression of a different VSG gene (Vickerman, 1969; Cross, 1975; Borst, 1986; Borst and Greaves, 1987; Van der Ploeg, 1987). The mechanism underlying antigenic variation of the bloodstream form trypanosomes has been widely studied showing that the active VSG gene and several expression site associated genes (ESAGs) are arranged in a tandem array in a polycistronically transcribed telomeric VSG gene expression site (Cully *et al.*, 1985; Kooter *et al.*, 1987; Shea and Van der Ploeg, 1988; Pays *et al.*, 1989). The transcription of these VSG gene expression sites occurs in a mutually exclusive manner and is mediated by an RNA

polymerase that is resistant to the drug α -amanitin at concentrations up to 1 mg/ml (Kooter and Borst, 1984). This level of resistance is characteristic of transcription by RNA polymerase I (RNA pol I), which in other eukaryotes exclusively transcribes ribosomal RNA (rRNA) genes. All other protein coding genes identified thus far in trypanosomes and other eukaryotes are transcribed by the α -amanitin sensitive (5 μ g/ml) RNA polymerase II (RNA pol II) (Evers *et al.*, 1989; Smith *et al.*, 1989). It is still unclear whether the α -amanitin resistant polycistronic transcription of the VSG gene expression sites is mediated by RNA pol I, or by a modified RNA pol II, or a transcription factor which may confer α -amanitin resistance (Evers *et al.*, 1989).

The differentiation of the bloodstream form trypanosome into the insect form (procyclic form) results in numerous biochemical changes. Insect form trypanosomes (i) lack a VSG coat, (ii) express an insect form specific, abundant procyclic acidic repetitive cell surface protein (PARP or procyclin) and (iii) have switched from glycolysis to mitochondrial respiration for ATP production (Roditi *et al.*, 1987; Mowatt and Clayton, 1987, 1988; Oppendoes, 1988).

To enhance our understanding of protein coding gene transcription by α -amanitin resistant RNA polymerases we searched for other, independently regulated, α -amanitin resistantly transcribed genes in order to compare their function, chromosomal location and control mechanisms with those of the VSG expression sites.

Results

Procyclic specific protein coding genes transcribed by α -amanitin resistant RNA polymerases

We had previously found that telomere repeats of *T. brucei* (clone TT6 in Figure 1A) are transcribed at similar levels by an α -amanitin resistant RNA polymerase in both insect and bloodstream form trypanosomes (Rudenko and Van der Ploeg, 1989). This transcription proceeded towards the chromosome end and we postulated that it resulted from read-through transcription downstream of telomeric genes. This finding indicated that transcription of non-rRNA genes by an α -amanitin resistant RNA polymerase might not be restricted to the VSG gene expression sites of the bloodstream form of *T. brucei* as was previously thought. We therefore screened uncharacterized procyclic cDNAs available in the laboratory for sensitivity to the drug α -amanitin in a run-on transcription assay. Nuclear run-on transcription analysis of four cDNA clones using procyclic *T. brucei* nuclei, showed that they were transcribed at a high rate in an α -amanitin resistant manner in insect form trypanosomes (clones CPT4-7, Figure 1A). Transcription of these cDNA clones was reduced to 50–70% of the original levels after the addition of 1 mg/ml α -amanitin (Table I). This level of resistance of the CPT4-7 clones is comparable with that of the rRNA genes. This is in contrast to the markedly reduced level of transcription

Fig. 1. Nuclear run-on analysis of the insect form specific cDNA clones with nascent RNA from insect form and bloodstream form nuclei. Nuclear run-on experiments were performed as described in Materials and methods. The slot blots contain 2 μ g of plasmid DNA: rDNA, an entire *T. brucei* ribosomal DNA repeat; pBR, plasmid pBR322; tub, an α - β tubulin genomic clone; 118, a VSG 118 cDNA; pUC, plasmid pUC18; 5S, a *T. brucei* 5S rRNA repeat; TT6, the telomere clone pTT6 containing a 300 nt GGGTTA repeat; a mini-exon repeat clone; and clone pR1, encoding an insect and bloodstream form expressed cell-surface protein (see Materials and methods for a detailed description of the different clones). The filters were hybridized with nuclear run-on RNA made in the presence of 1 mg/ml α -amanitin (1) or the absence of α -amanitin (0) (A) Nuclear run-on assay with insect form trypanosomes. The control panel is shown on the left. Plasmid clones CPT4-7 representing the different α -amanitin resistantly transcribed cDNA clones are shown on the right. Blue, is the Bluescript plasmid (Stratagene) negative control. (B) Nuclear run-on assay with bloodstream form trypanosomes. See (A) for description.

of the α -amanitin sensitive RNA polymerase II transcription units of the α and β tubulin and mini-exon genes and the RNA polymerase III transcribed 5S rRNA genes (Figure 1A, see Table I for quantitation). The transcription of clones CPT4-7 was significantly higher in the insect form of *T. brucei* than in the bloodstream form of the parasite where VSG gene transcription is high (Figure 1B, Table I). Transcription results in the generation of an abundant 800 nt RNA in insect form trypanosomes (Figure 2, lane P). Therefore, the expression of clones CPT4-7, as is the case for VSG genes, is predominantly transcriptionally controlled in a life cycle specific manner by α -amanitin resistant RNA polymerases. All hybridization to clone CPT4 was to the anti-sense strand of the cDNA clone and clones CPT4-7 did not show homology with rRNA genes (results not shown).

Sequence analysis of these cDNA clones identified them as members of a previously identified family of protein coding genes, the procyclic acidic repetitive proteins (Figure 3; PARPs or procyclins) (Roditi *et al.*, 1987; Mowatt and Clayton, 1987, 1988). The PARP cDNA clone CPT4, which we analyzed in detail (Figure 3), had a 127 amino acid open reading frame, characterized by 25 Glu-Pro repeats (underlined in Figure 3) and by non-repetitive 5' and 3' flanking sequences. The eight PARP genes in the *T. brucei* genome are organized at four separate loci with two PARP genes (α and β) arranged in tandem at each locus (Mowatt and Clayton, 1987, 1988). On the basis of restriction fragment length polymorphisms, the loci had previously been grouped into two A loci and two loci denoted B1 and B2 (Mowatt and Clayton, 1987). The sequence of the 3' end of clone CPT4 was identical to the partial 3' sequence of

Table I. Quantitation of *T. brucei* nuclear run-on analysis

Experiment no.	Procyclic			Bloodstream form	
	UTP	2	3	GTP	UTP
Pol I - rDNA	53%	71%	75%	56%	78%
Pol II - tub	7%	5%	9%	6%	7%
- mini-exon	5%	6%	12%	5%	10%
- PR-1	ND	11%	ND	4%	4%
Pol III - 5S	5%	8%	0%	9%	8%
Pol ? - VSG	*	*	*	*	56%
- TT6	53%	69%	68%	57%	ND
- CPT4	ND	55%	45%	44%	*
- CPT5	ND	71%	68%	45%	*

Experiments were performed with [32 P]UTP (three experiments with insect form nuclei and one with bloodstream form nuclei) and with [32 P]GTP (one experiment with insect form nuclei) as a radioactive marker. Experiments were performed as described in Materials and methods. The relative levels of transcription for each clone after addition of α -amanitin are presented as percentages of the incubations without α -amanitin. Abbreviations are as in Figure 1. PR-1 is an insect form and bloodstream form expressed pol II transcribed control gene. ND = not determined.

*Transcription levels were too low to allow quantitation.

the β PARP gene of a B locus (Mowatt and Clayton, 1988). Partial sequence analysis of the other cDNAs (CPT5–CPT7) showed that they represented other members of the PARP gene family (data not shown).

Fig. 2. Northern blot analysis of RNA from *T.brucei* hybridized with clone CPT4. RNA was purified as described in MacDonald *et al.* (1987) size-separated in an agarose gel, transferred to a nylon filter and hybridized with clone CPT4 as described in Maniatis *et al.* (1982). DNA probes were generated by random priming as described in Feinberg and Vogelstein (1983). Post-hybridizational washes were performed at 65°C and $0.1 \times \text{SSC}$. P, 20 μg total procyclic RNA; R, 20 μg of total RNA after RNase A treatment; B, 20 μg of total bloodstream form RNA. The same filter was rehybridized with an α - β tubulin control probe to show that equal amounts of RNA were loaded in the lanes.

Comparison of PARP chromosomal locations with VSG gene expression sites

In order to determine whether the PARP genes were expressed from a site at or near to the previously identified α -amanitin resistantly transcribed VSG gene expression sites, we compared the chromosomal locations of PARP and VSG genes. Using pulsed field gradient gel electrophoresis (PFGE), 80% of the genome of *T.brucei* has been separated into 19 different bands, ranging in size from 50–150 kb (band 1, the mini-chromosomes) to 5.7 megabasepairs (Mb; band 19) (Van der Ploeg *et al.*, 1984, 1989). Transcriptionally active VSG gene expression sites have thus far been located in band 5 (430 kb; VSG 1.8), band 10 (1.5 Mb; VSG 118) and band 15 (3 Mb; VSG 221) (Van der Ploeg *et al.*, 1984b, 1989). We determined that four chromosomes contain PARP genes the smallest being roughly 2.5 Mb and the largest 5.7 Mb in size. It is not possible to use chromosome separation conditions at which each of the individual bands over this broad size range is optimally separated in a single PFG gel. Figure 4 therefore shows a rough size separation, visualizing PARP genes at four distinct bands. More accurate mapping of the PARP genes to bands 14, 15, 18 and 19 was carried out in a series of PFG gels not shown here. As the PARP genes are present at four separate loci, each containing two genes (Mowatt and

Fig. 3. DNA sequence analysis of cDNA clone CPT4. The repeat region is underlined. The nucleotide sequence of the cDNA inserts was determined using the dideoxy chain termination method (Sanger *et al.*, 1977) with the Klenow fragment of the DNA polymerase at 37 or 48°C, or with the T7 DNA polymerase (Sequenase) according to the manufacturer's specifications (United States Biochemical Corporation).

Fig. 4. Determination of the PARP chromosomal location. Chromosome-sized DNA of *T.brucei* variant 118 clone 1 was size-separated by PFG. Chromosome sized DNA was prepared in low-melting point agarose (FMC Bioproducts-InCert) essentially as described in Van der Ploeg *et al.* (1984b, 1989). Agarose blocks with DNA from 5×10^7 trypanosomes were loaded in each lane. All separations were performed in 20 cm square, 1% low melting point agarose (BRL) gels at 15°C, in a Pharmacia LKB Pulsaphor inhomogeneous submarine electrophoresis unit with modified TBE buffer (89 mM Tris, 89 mM H3B03, 0.2 mM EDTA). DNA was electrophoresed at 3 V/cm with a pulse frequency of 6500 s for 7 days. After electrophoresis, gels were stained with ethidium bromide and blotted to nitrocellulose filters and hybridized with probe CPT4. Post-hybridizational washes were performed at 65°C and $0.1 \times \text{SSC}$. The accurate chromosomal location of the PARP genes was obtained from the comparison of different PFG gels run at different voltages and pulse frequencies for optimal separation of different bands (data not shown). This particular PFG gel demonstrates that four different chromosomes hybridize with the PARP cDNA probe.

Clayton, 1987, 1988), and since we detect hybridization with four bands in the PFG, the hybridization at the slot of the PFG gel must be due to trapping of some of the

chromosomes. Trapping has been found for other genes as well (Van der Ploeg *et al.*, 1984b, 1989; our unpublished data). The chromosomal location of the PARP genes does not coincide with that of known VSG gene expression sites, except for band 15 which contains the VSG 221 expression site. Band 15, however, seems to contain at least two chromosomes as judged from its non-stoichiometric intensity of ethidium staining. Thus most if not all of the PARP genes have a chromosomal location that is distinct from that of the known VSG gene expression sites. Unlike VSG genes, PARP genes do not seem to be located near (within tens of kilobasepairs) telomeres (data not shown). This indicates that a telomeric location may not be a prerequisite for transcription by α -amanitin resistant RNA polymerases.

Discussion

α -Amanitin resistant transcription of protein coding genes had previously been exclusively detected in the bloodstream form of *T.brucei*, and had been hypothesized to be unique to VSG gene expression sites. Our data shows that α -amanitin resistant transcription of protein coding genes also occurs in insect form trypanosomes. The insect form specific PARP gene transcription may be the counterpart of the bloodstream form specific VSG gene expression site transcription.

Comparing PARP and VSG transcription units will prove useful in elucidating their control mechanisms. PARP and VSG genes produce one of the most abundant mRNAs (1–3% of poly(A)⁺) (Mowatt and Clayton, 1987, 1988; Hoeijmakers *et al.*, 1980; this paper) and both PARP (Richardson *et al.*, 1988) and VSG genes encode the major membrane proteins of their respective life cycle stages. The life cycle specific expression of the PARP genes and VSG gene expression sites is predominantly transcriptionally controlled. This is in contrast to the α -amanitin sensitively transcribed protein coding genes of trypanosomes studied thus far (phospho-glycerate kinase and heat shock (70 kd genes) whose mRNA levels appear to be primarily post-transcriptionally regulated (Gibson *et al.*, 1988; M.G.-S.Lee and L.H.T.Van der Ploeg, unpublished). It is unclear whether the post-transcriptional control of mRNA abundance will be a discriminating feature of α -amanitin sensitive versus resistantly transcribed protein coding genes. Transcription initiation sites for the PARP genes, in contrast to VSG genes, are located within the first 300 bp upstream of the 3' splice acceptor site at which mini-exon addition for the α -PARP mRNAs occurs (G.Rudenko and L.H.T.Van der Ploeg, unpublished). The physical maps at the transcription initiation sites were unaltered when active and inactive PARP genes were compared. PARP genes also have fixed chromosomal locations and are not rearranged in bloodstream form versus insect form *T.brucei* (this paper and data not shown). It is therefore unlikely that DNA rearrangement events alone control PARP transcription. A subset of functionally analogous protein coding genes (PARP and VSG genes) thus appears to be controlled by an RNA polymerase which differs from the regular RNA pol II.

Materials and methods

Trypanosome strains and culture conditions

Trypanosoma brucei strain 427 bloodstream form variant 118d and variant 118 clone 1 were used for the experiments (Michels *et al.*, 1983; Lee and

Van der Ploeg, 1987). The *T.brucei* 427 procyclic trypanosomes were initially obtained from Dr R.Brun and have been maintained in SDM79 medium at 24°C as described by Brun and Schonenberger (1979). Procyclic *T.brucei*, strain TREU 667 BUT 55 was used for the construction of a cDNA library (kindly provided by C.Clayton) (described in Mowatt and Clayton, 1987, 1988).

Nuclear run-on analysis and hybridization

Slot blots were made by transferring 2 μ g of plasmid DNA or 3 μ g of single stranded (ssDNA) M13 phage onto nitrocellulose filters (pore diameter 0.45 μ m) using the Schleicher & Schuell Minifold II Slot-blotter according to Kafatos *et al.* (1979).

Nuclei were isolated from trypanosomes using a Stansted cell disrupter according to Kooter *et al.* (1984). Briefly, bloodstream form trypanosomes were grown to a high parasitemia in rats, harvested by cardiac puncture, and immediately passed through the cell disrupter. Variability in the level of α -amanitin resistant PARP gene transcription has been found with different bloodstream form nuclei preparations. Nuclei made from bloodstream form trypanosomes as rapidly as possible had consistently the lowest levels of PARP transcription relative to the control genes and presumably best reflect the *in vivo* situation. Procyclic trypanosomes were concentrated to 10⁹ cells/ml and passed through the cell disrupter. Nuclei were stored at -140°C in 100 μ l aliquots in nuclei storage buffer at a concentration of 5–10 $\times$ 10⁸ nuclei/ml.

Nascent RNA elongation was performed according to Kooter *et al.* (1987). Incubations were performed for 5 min at 37°C for nuclei from bloodstream form trypanosomes and at 22°C for nuclei preparations from insect form trypanosomes. Nuclear run-on reactions in the presence of α -amanitin (Serva Fine Biochem. Inc.) were performed as described in Rudenko and Van der Ploeg (1989).

Nascent RNA was hybridized to the filters for 48 h at 42°C and washed according to Rudenko and Van der Ploeg (1989). The end stringency was 0.1 $\times$ SSC and 0.1% $\times$ SDS at 65°C. Quantitation of the nuclear run-on signal was performed by dissolving the nitrocellulose filters in Econofluor (Dupont) and by counting the solution in a liquid scintillation counter. The background hybridization to the plasmid control was subtracted.

Description of recombinant plasmids

The *T.brucei* telomere clone pT4 and the TT6 *T.brucei* telomere subclone contain the 300 bp insert of pT6 (described previously by Van der Ploeg *et al.*, 1984a) cloned into the plasmid T3/T7lac (Boehringer Mannheim). The *T.brucei* ribosomal clone pR4 is as described in Kooter and Borst (1984); the 5S rRNA clone is as described in Lenardo *et al.*, (1985); the mini-exon repeat clone pCL103 is described in Laird *et al.* (1985); the VSG 118 cDNA clone TcV118-2 in Bernards *et al.* (1981); and the α - β tubulin genomic clone pTb $\alpha\beta$ T-1 in Thomashow *et al.* (1983); the CPT4-7 cDNAs were cloned as *EcoRI* inserts into the plasmid pUC18. The PR1 clone is a 3350 bp cDNA in pUC 18, encoding a *T.brucei* receptor-like membrane protein (PR1 is identical to clone CRAM; M.G.-S.Lee *et al.*, unpublished).

Acknowledgements

We thank Dr Gwo-Shu Mary Lee for sharing unpublished results and Dr Christine Clayton for the gift of a procyclic *T.brucei* cDNA library and for communicating unpublished results. We thank all colleagues from the laboratory for critical reading of the manuscript. This work was supported by a Grant from the National Institutes of Health no. A1 21784 to LHTVDP and by a grant from the John D. and Catherine T. MacArthur Foundation. LHTVDP is a Burroughs Wellcome scholar in molecular parasitology.

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Received on August 7, 1989; revised on September 15, 1989