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The PARP promoter of *Trypanosoma brucei* is developmentally regulated in a chromosomal context

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ABSTRACT

African trypanosomes are extracellular protozoan parasites that are transmitted from one mammalian host to the next by tsetse flies. Bloodstream forms express variant surface glycoprotein (VSG); the tsetse fly (procyclic) forms express instead the procyclic acidic repetitive protein (PARP). PARP mRNA is abundant in procyclic forms and almost undetectable in bloodstream forms. Post-transcriptional mechanisms are mainly responsible for PARP mRNA regulation but results of nuclear run-on experiments suggested that transcription might also be regulated. We measured the activity of genomically-integrated PARP, VSG and rRNA promoters in permanently-transformed bloodstream and procyclic form trypanosomes, using reporter gene constructs that showed no post-transcriptional regulation. When the constructs were integrated in the rRNA non-transcribed spacer, the ribosomal RNA and VSG promoters were not developmentally regulated, but integration at the PARP locus reduced rRNA promoter activity in bloodstream forms. PARP promoter activity was 5-fold down-regulated in bloodstream forms when integrated at either site. Regulation was probably at the level of transcriptional initiation, but elongation through plasmid vector sequences was also reduced.

INTRODUCTION

The kinetoplastid protozoa are unicellular parasites that branched very early in eukaryotic evolution (1) and exhibit many unusual characteristics. For example, nearly all genes are transcribed as part of polycistronic transcription units. Individual mRNAs are excised co-transcriptionally by 5′ *trans* splicing and 3′ polyadenylation. The position and timing of 3′ polyadenylation is determined mainly by the 5′ *trans* splicing of the next gene downstream (2–6). Genes showing different regulation are often found on the same transcription unit (e.g. 7,8), so that in general regulation of mRNA level is determined post-transcriptionally (9–13).

Trypanosoma brucei is a kinetoplastid parasite of mammals that is transmitted from one host to the next by tsetse flies. The parasite multiplies extracellularly in the blood and tissue fluids of the mammal, evading the immune response by frequently changing its surface coat of variant surface glycoprotein (VSG) (14). When the parasite is taken up by a tsetse fly, it transforms into the ‘procyclic’ form, a process that can be mimicked *in vitro* by a change in culture medium and by decreasing the growth temperature from 37 to 27°C. VSG gene transcription ceases; the VSG coat is shed and within a few hours replaced by a coat of procyclic acidic repetitive protein (PARP or procyclin; 15,16).

Several lines of pharmacological and genetic evidence imply that both classes of major surface protein are transcribed by RNA polymerase I (17–19): this is possible because in kinetoplastids the cap on the mRNA is provided by the 5′ *trans* splicing of a short, capped, mini-exon or spliced leader. The expressed VSG gene is situated at a telomere, up to 80 kb away from the VSG promoter, in one of twenty or so ‘expression sites’. Between the VSG gene and its promoter there are up to 10 moderately well-conserved genes that are co-transcribed (20). Measurements of transcription in crude nuclear preparations revealed that, although transcription of the VSG had ceased in procyclic trypanosomes, transcription of the promoter-proximal sequences had not (21,22). At first it seemed likely that the VSG promoter that had been active in the bloodstream form remained equally active in the procyclic form, but that transcription was attenuated within a few hundred base pairs of the initiation site (21,22). Recent experiments indicate, however, that in procyclic trypanosomes many (or all) VSG promoters are weakly active but subject to attenuation, whereas in bloodstream trypanosomes the activity of a single promoter is up to 20-fold up-regulated (23). By integrating reporter constructs bearing VSG or rRNA promoters into the VSG and rRNA loci, it was possible to demonstrate that the low activity of a VSG promoter in procyclic forms was both locus- and promoter-specific (23). It seems likely that the regulation of VSG promoters in bloodsteam forms during antigenic-type switching is achieved by an independent mechanism that is dependent on the telomeric location of the VSG expression sites (24,25).

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Control of PARP transcription has also been controversial. The PARP genes are arranged in four short transcription units on different chromosomes; each unit contains two or three PARP genes followed by one or two unrelated genes (26,27). Measurements of transcription in crude nuclear preparations or permeabilized cells show that transcription from the PARP promoter occurs in bloodstream forms as well as procyclic forms. There is no doubt that this transcription is driven by the PARP promoter (rather than by read-through from an upstream polymerase II transcription unit) because it is resistant to α-amanitin (22,28). Transcription of the PARP genes is undoubtedly less active in bloodstream forms than in procyclic forms but the estimates of the extent of regulation vary: for example, in one paper the level of regulation was estimated as 6-fold (22) whereas in others it appeared considerably more (28). Very recently, Vanhamme *et al*. (29) measured transcription of a chloramphenicol acyltransferase (CAT) gene integrated in the PARP locus: for their one cell population estimates of regulation varied from 25- to 6-fold. One possible source of inaccuracy is the known temperature-sensitivity of PARP- and VSG-promoter-mediated transcription; another possibility is specific transcript degradation. PARP expression is also subject to extremely strong post-transcriptional regulation (13,30,31); which is at least partially mediated through RNA degradation (31,32; Hotz *et al*., manuscript submitted).

In transient transfection assays of bloodstream and procyclic trypanosomes, the PARP, VSG and rRNA promoters all showed similar activities (9,33). We have therefore tried another method to measure the regulation of the PARP promoter in a chromosomal context. By integrating constructs showing no post-transcriptional regulation, and transcribed by different promoters, into different genomic positions we could confirm that PARP transcription is developmentally regulated 5-fold and that regulatory sequences are present within 250 bp of the transcriptional start site.

MATERIALS AND METHODS

Trypanosomes and transfection

Two *T.brucei brucei* strains were used. Most experiments were done with strain MiTat 1.2 trypanosomes, cultured *in vitro* as both bloodstream forms and procyclic forms. For cyclic transmission, trypanosomes of strain 247 were kindly provided by Dr Jenni (Schweizer Tropeninstitut, Basel) and were cultured as procyclic forms. Passage through tsetse flies and growth as bloodstream forms in mice were as previously described (34).

Trypanosomes were transfected as described (35,36). For pHD 330, 383, 129 and 301, both procyclic and bloodstream trypanosomes were diluted into microtitre dishes immediately after electroporation and hygromycin selection at 12.5 or 25 μg/ml applied the next day. Procyclic trypanosomes were diluted into medium conditioned by the growth of up to 5×10^6 procyclic trypanosomes/ml. Bloodstream clones with pHD 301 were re-cloned on plates (37). For plasmids targeted into the rRNA locus of bloodstream trypanosomes, the transfected parasites were diluted into several 5 ml flasks such that only about half the flasks yielded drug-resistant parasites, and resistant populations re-cloned by plating (37). Chloramphenicol acyltransferase (CAT) activity was measured by the two-phase kinetic assay (38), using extracts from actively-growing cells (procyclic: $<6 \times 10^6$ cells/ml; bloodstream: ~1 x 10^6/ml). Immunofluorescent detection of CAT was performed as previously described (39).

Plasmids

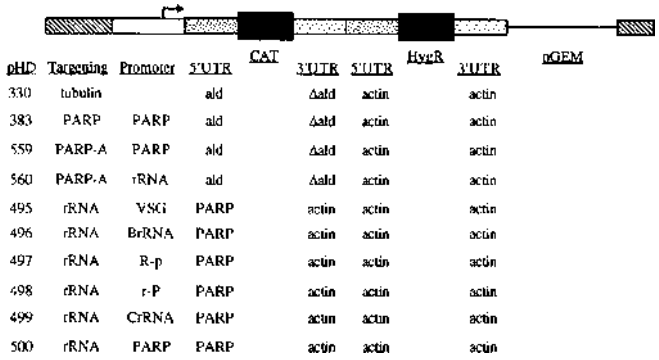
Plasmid pHD 301 was built by inserting the hygromycin resistance marker (hygR) into pHD 30 (9) and placing the PARP locus intergenic region (6) downstream. The intergenic region fragment was synthesized by PCR using as primers CZ 163a (ATTGAGGCCTGGGTTCGAATAATAGTTCCT) and CZ164 (ATTG*CCCGGGCGGCCGC*GCGCACATCAACTGGGATAAC) (*Stu*I, *Not*I and *Sma*I sites underlined or italicized) and a cloned PARP A locus plasmid as template (40). Plasmid pHD 301 was cleaved with *Kpn*I and *Not*I before transfection. All plasmids with CAT and hygR genes are diagrammed in Figure 1. Plasmids pHD 330 (41) and pHD 383 (42) contain CAT genes positioned upstream of a hygromycin resistance marker and are designed for targeting into the tubulin and PARP loci, respectively. Plasmid pHD 383 has a PARP promoter fragment of 640 bp, consisting of a proximal region of 282 bp separated from a distal region of 316 bp by a synthetic *Not*I site. Plasmid pHD 559 is the same but the PARP targeting region is extended to include an additional 440 bp. This was synthesized with oligonucleotides CZ 447 (CTAGAGC*TCTAGA*TCGTTCGCTACGAAACAA) (*Sac*I underlined, *Xba*I italicized) and CZ 448 (CTAGAGC*TCAGA*TCTAG-AGTCGGGGGCATGCA) (*Sac*I underlined, *Bgl*II italicized) using a cloned PARP A locus as template. In pHD 560 the proximal PARP promoter fragment was replaced by the BrRNA promoter (see below). Plasmids pHD 559 and pHD 560 were linearized at the synthetic *Bgl*II site before transfection.

Plasmids pHD 495–500 were derived from pHD 430 (42). First, pHD 459 was constructed: this has an rRNA targeting region, a PARP promoter with *tet* operators, and a CAT gene flanked by the PARP splice acceptor and actin 3′-UTR; downstream is a phleomycin resistance cassette with an actin 5′ and 3′-UTR. The construct is targeted into the non-transcribed spacer of the rRNA locus such that transcription is in the opposite direction to transcription of the neighbouring rRNA genes (42). From this was derived pHD 483, in which the hygR cassette replaces the phleomycin resistance cassette. Various promoters then replaced the inducible PARP promoter. The promoters used were PARP (43) (pHD 500), VSG (9) (pHD 495), rRNA promoters cloned by us (CrRNA, pHD 499) (19) and by Zomerdijk *et al*. (BrRNA, pHD 496) (19,44) and hybrid promoters with rRNA upstream activating regions and a PARP core, with the junction either upstream of (pHD 498 from pHD 263) or within (pHD 497 from pHD 348) the core promoter region (19).

For integration, all plasmid DNAs were linearized with *Not*I unless otherwise noted above.

DNA and RNA analysis

Poly(A)$^+$ RNA was isolated with the QuickPrep *Micro* mRNA Purification Kit (Pharmacia Biotech, Freiburg); total RNA was purified using TRIzol reagent (Gibco, Life Technologies Inc., Eggenstein). RNA was analysed by transfer hybridization with ^{32}P-labelled probes followed by phosphorimager quantitation. Trypanosome DNA was analysed by transfer hybridization using an enhanced chemiluminescence (ECL) kit (Amersham, Braunschweig) according to the manufacturer's instructions. PCR reactions on ~0.1 μg genomic DNA were performed with the following oligonucleotides: VIB13 (CAT antisense, CGATGCGATTGGGATATATCAACG); CZ68 (PARP promoter, 3′ region, antisense, ATTTTCAATGCACGCC); CZ160 (rRNA promoter sense, hybridizing to upstream part of core promoter, CAATAGGATAAT-



pHD	Targeting	Promoter	5′UTR	3′UTR	5′UTR	3′UTR
330	tubulin		ald	Δald	actin	actin
383	PARP	PARP	ald	Δald	actin	actin
559	PARP-A	PARP	ald	Δald	actin	actin
560	PARP-A	rRNA	ald	Δald	actin	actin
495	rRNA	VSG	PARP	actin	actin	actin
496	rRNA	BrRNA	PARP	actin	actin	actin
497	rRNA	R-p	PARP	actin	actin	actin
498	rRNA	r-P	PARP	actin	actin	actin
499	rRNA	CrRNA	PARP	actin	actin	actin
500	rRNA	PARP	PARP	actin	actin	actin

Figure 1. Schematic drawing of bicistronic constructs used in this study; not to scale. The arrow represents the transcription start site. BrRNA and CrRNA: rRNA promoters isolated in Borst and Clayton laboratories respectively. ald: aldolase 5′-UTR and splice signal; Δald: truncated portion of the aldolase 3′-UTR. r-P: rRNA–PARP promoter hybrid with junction upstream of the core promoter (position –80); R-p: rRNA–PARP promoter hybrid with junction within the core promoter (position –50)

AATGATAAAGT); CZ119 (upstream extremity of rRNA promoter fragment, CGCAGGTACCTTTCCACCCAGCGCGGGTGCA); CZ12 (PARP promoter 'core' centering on –30, sense, TTTTGC-TATTCCGTGTCTCTGGGTGGGCGTGCAT); CZ334 (rRNA intergenic targeting region, TGGCCCTGATGGCATGC); and CZ 148 (VSG promoter sense, ggggTTCTAAAAGAATCATATCCC-TATTACCACACCAGTTTATAT). To confirm the identity of the rRNA/PARP hybrid promoter in pHD 497, PCR reactions were performed with the following primer pairs: VIB 13 + CZ160 (positive), CZ68 + CZ334 (positive); and for pHD 498, CZ68 + CZ334 (positive), VIB 13 + CZ119 (positive) and CZ160 + VIB13 (negative).

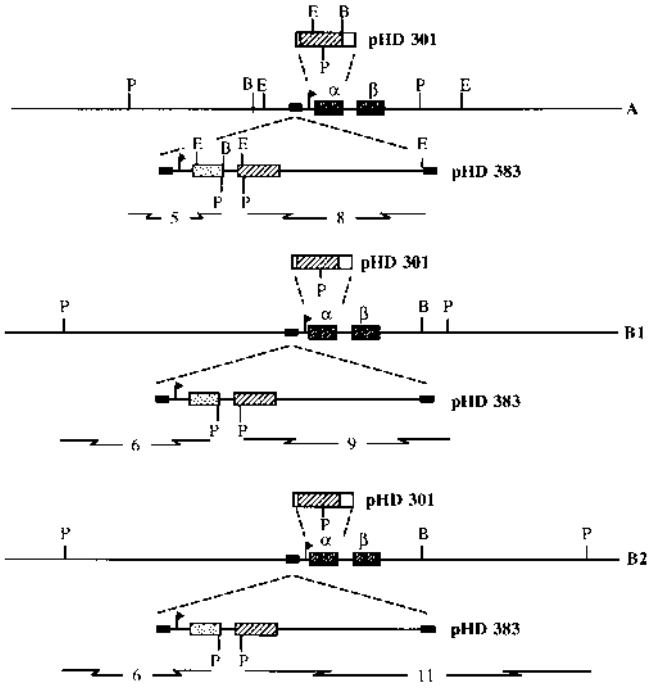


Figure 2. Restriction maps of the PARP loci. Plasmid pHD 301 contains a hygromycin resistance (hygR) gene (diagonal striped) that replaces a PARP gene (black stippled) after integration. The presence of a *Pst*I site (P) in the hygR gene resulted in the formation of restriction fragments diagnostic for each locus; integration could be confirmed using *Eco*RI and *Bam*HI. Plasmid pHD 383 integrates upstream of the PARP promoter in the region indicated by a thin black box. Restriction sites in the plasmids are shown in full only in the uppermost (A locus) panel. The sizes of *Pst*I fragments obtained after insertion of pHD 383 into each locus are indicated below the maps. Closed arrow, PARP promoter; E, *Eco*RI; B, *Bam*HI; P, *Pst*I.

RESULTS

Replacement of a PARP gene by a hygromycin resistance cassette

If the PARP promoter is active in its genomic location in bloodstream trypanosomes, it should be possible to use it to drive an integrated drug resistance marker in this form. We built a plasmid (pHD 301) designed to replace an entire PARP gene with a hygromycin resistance mRNA bearing the 3′- and 5′-UTRs of the actin genes (see Materials and Methods and Fig. 1). The actin genes are constitutively expressed throughout the life cycle (45) and their UTRs support similar levels of gene expression in both replicating trypanosome forms (9; Hotz, manuscript submitted). The excised replacement cassette was electroporated into cultured monomorphic bloodstream and procyclic trypanosomes (strain MiTat 1), and strain 247 procyclic trypanosomes. Hygromycin-resistant parasites were obtained easily from the monomorphic strain. (Efficiencies were not measured accurately but were at least 10^{-6} using 10 µg DNA/cuvette.) With strain 247 only one PARP-targeted line (247–301 P1 in Fig. 3) survived the cloning procedure; this was passaged (without selection) through tsetse, to produce bloodstream forms (247–301-B) which were then differentiated back into procyclics *in vitro* in the absence of selection (247–301-P2).

Independent cloned lines were checked for integration of the hygromycin resistance marker into the PARP locus using digestion with *Pst*I, *Pvu*II, *Sal*I, *Bam*HI and *Eco*RI (Fig. 2; data not shown). In the MiTat 1 strain, there are two copies of the PARP A locus and one copy each of the PARP B1 and B2 loci (40). These can be distinguished by *Pst*I digests (Figs 2 and 4). In the 247 strain, the results suggested that this strain may have two copies of the B1 locus and no B2 locus. In all cell lines examined, one hygR cassette had replaced a PARP Aα gene; the 301-B4 line (RNA shown in Fig. 3) had a replacement in the PARP B2 locus as well (data not shown).

The amounts of hygR RNA in the stably transformed lines were measured by blot hybridization (Fig. 3). The amounts of hygR mRNA indicated below the individual lanes are already standardized relative to the amount of actin transcript on the same filter (bottom row). The monomorphic bloodstream form lines (Fig. 3A) had on average 8.6-fold less hygR mRNA than the procyclic forms. The B4 lane had the same amount of HygR RNA as the other bloodstream lines although it had an additional copy of the gene.

The pleomorphic-bloodstream-form RNA had half as much actin mRNA as the procyclic forms although twice as much RNA was loaded (see legend). The cells used for the mRNA preparation were grown in mice for 5 days, long enough for some differentiation to intermediate forms to occur. The concomitant reduction in growth rate was probably responsible for the reduced levels of actin mRNA (46). Although the pleomorphic cells showed ~2-fold regulation of hygR RNA relative to actin, regulation was ~8-fold relative to the total amount of RNA loaded. As the parasites

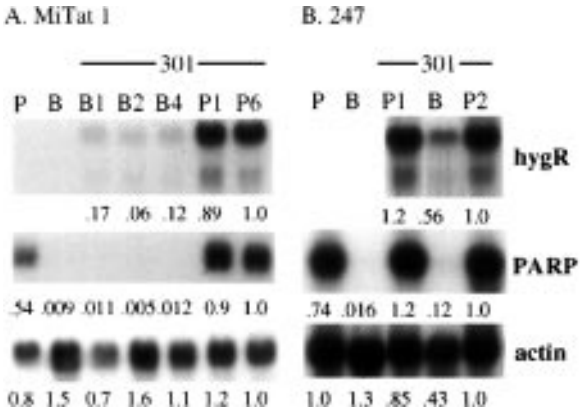


Figure 3. Northern blot of poly A^+ RNA from hygromycin-resistant trypanosomes containing integrated pHD 301. The RNA was purified by a single passage over oligo-d(T) so retained significant rRNA contamination. All lanes were loaded with 5 µg except the 301B lane which contained 10 µg. Blots were hybridized sequentially with HygR, actin and PARP probes. The relative amounts of RNA, quantitated using a phosphoimager, are indicated beneath the lanes. First, actin mRNA was quantitated and used as a standard to determine the relative amounts of polyA^+ RNA present on each lane. Next, other RNAs were quantitated and the measured levels corrected relative to the amount of actin RNA. (**A**) Mitat 1.2 monomorphic cell lines. RNAs from procyclic lines P1 and P6 (one copy of HygR gene, A locus) are compared with RNAs from bloodstream lines B1, B2 (each one copy of HygR gene, A locus) and B4 (two copies of HygR gene, A locus and B2 locus). RNAs from trypanosomes with no integrated pHD 301 are in the first two lanes. B, bloodstream; P, procyclic. (**B**) Strain 247 transformed cells (one copy of HygR gene, A locus). P1 are cells before tsetse passage; B are tsetse-transmitted bloodstream forms; P2 are procyclic trypanosomes derived from those. P and B are control RNAs from non-transformed monomorphic MiTat bloodstream and procyclic forms, respectively.

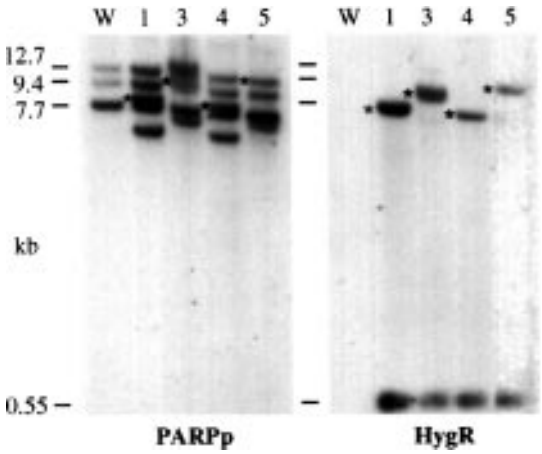


Figure 4. Blot hybridization of *Pst*I-digested DNA from procyclic trypanosome lines with integrated pHD 383. W, wild type trypanosomes; 1, 3, 4 and 5 are four independent cell lines. The blot was first hybridized with a PARP promoter probe (PARPp, the upstream conserved fragment of 282 bp, see Methods), then with a HygR probe (the complete HygR cassette). Novel fragments that hybridized with both probes are denoted to the left by an asterisk. Size markers in kb are on the left.

had passed through tsetse and mice in the absence of selective drug it is clear that the transcription of hygR in the PARP locus in bloodstream forms was not induced by selection. The estimates of PARP mRNA regulation were at least 100-fold for the monomorphic parasites. For the 247 line the PARP signal intensity was regulated 9-fold relative to actin and 36-fold relative to total RNA loaded. These estimates are very inaccurate: with signals as low as those from the bloodstream-form lanes, the measurements become exquisitively and unacceptably sensitive to the value (area of the blot) chosen to represent the background.

Targeting of reporter constructs into the PARP and tubulin loci

We now transfected parasites with constructs encoding both hygR and CAT (Fig. 1). In these constructs (pHD 330 and pHD 383) the CAT gene bears a 5′-UTR and splice acceptor signal from the aldolase locus, and a truncated version of the aldolase 3′-end (Fig. 1); we do not know precisely where polyadenylation occurs. The constructs were transfected into monomorphic bloodstream and procyclic trypanosomes. The genes were targeted either into the PARP locus, with recombination expected within the PARP promoter (pHD 383) or, as a control, into the tubulin array (pHD 330). Cells were cloned by limiting dilution immediately after transfection. If, in bloodstream forms, the PARP genes are normally in a closed chromatin conformation, one would expect insertion into the PARP locus (four copies per diploid genome) to be less efficient than insertion into the tubulin array, which is transcribed in both forms and present in ~30 copies per diploid genome (47). In procyclic forms the efficiencies were 6×10^{-5} for pHD 330 and 9×10^{-5} for pHD 383, measured relative to the number of cells surviving the electroporation. Fewer bloodstream forms survived the electroporation, but of the survivors the transformation efficiency was 1.25×10^{-5} for pHD 330 and 0.83×10^{-5} for pHD 383. Thus there was no marked difference between the PARP and tubulin loci in overall transformation efficiency.

Five independent cloned cell lines were selected from each transfection. All 330 lines had integration in the tubulin locus (Table 1). All 383 lines had integration immediately upstream of PARP genes, usually at the A locus (Table 1). Blot hybridization results for four procyclic lines are illustrated in Figure 4 (see maps in Fig. 2). The PARP loci can be distinguished by *Pst*I digestion, which yields fragments of 7–8 kb from the A locus (two copies per diploid genome), 9 kb from the B1 locus and 13 kb from the B2 locus (Figs 2 and 4, lane W). Integration of pHD 383 at the A locus (lanes 383–1 and 383–4) yields two novel *Pst*I fragments (~5.1 and 7.8 kb) that hybridize with a PARP promoter probe; the larger of these also reacts with the hygR probe (Figs 2 and 4). Integration in the B loci yields novel fragments of ~6 kb that lack hygR sequences; the hygR positive bands are of similar lengths to the original bands from the integration sites: 9 kb for the B1 locus (line 3) and 11–12 kb for the B2 locus (line 5). A 0.55 kb fragment internal to the pHD 383 plasmid is visible on the hygR blot in all 383 lanes.

Developmental regulation of CAT expression

In order to use CAT enzyme activity to measure transcription, there must be no post-transcriptional regulation. This was confirmed by measuring CAT activity from clones in which the construct was integrated into the tubulin locus (plasmid pHD 330), grown with or without selection (Table 2). There was no difference in CAT expression between bloodstream forms and procyclic forms whether or not hygromycin selection was applied; mean values for CAT in the procyclic forms were marginally higher only because of 2-fold greater expression in one line carrying three copies of the gene.

Table 1. Integration patterns of CAT-hygR plasmids into the PARP and tubulin loci of bloodstream (BF) and procyclic (Pro) trypanosomes

pHD 383			pHD 330		
	Clone no.	Integration		Clone no.	Copy no.
Bloodstream	1–5	A locus 1×	Bloodstream	1–5	1
Procyclic	1,2,4	A locus 1×	Procyclic	1,3,5	1
	3	B1 locus 1×		2	~3
	5	B2 locus 1×		4	~2

Plasmid pHD 383 is targeted to the PARP locus; the integration sites (locus A, B1 or B2) are indicated. Each of these clones contains a single integrated copy of the plasmid. pHD 330 targeted to the tubulin locus; integrated copy numbers are shown.

We now investigated the clones in which CAT was integrated at the PARP locus (Table 2). Procyclic forms with the CAT gene at the PARP locus showed ~11-fold more CAT activity than equivalent parasites with the gene in the tubulin locus and expression was not influenced by the presence of hygromycin. Bloodstream forms with the CAT gene at the PARP locus expressed only 2–3 times more CAT activity than equivalent parasites expressing CAT from the tubulin locus. Expression from the PARP locus was therefore ~5-fold developmentally regulated. The inclusion of hygromycin (12.5 μg/ml) in the medium of bloodstream forms reduced the growth rate by ~15% independent of the integration site and, in the experiment illustrated in Table 2, somewhat increased the level of CAT expression in the 383 lines. We therefore cultivated two cell lines of each type for 17 days, with or without drug, intermittently measuring CAT activity. No reproducible effect of hygromycin on CAT activity was found. Immunofluorescent staining showed that no intact trypanosomes were negative for CAT expression, but procyclic trypanosomes expressing CAT from the PARP locus had brighter fluorescence than the others. Bloodstream trypanosomes with CAT integrated at the PARP locus had similar fluorescence intensities to those with CAT integrated at the tubulin locus. Thus the low level of expression from the PARP locus in bloodstream forms was due to a uniformly low level of transcription in all cells.

Integration of an rRNA promoter in the PARP locus

To find out whether regulation was specific to the PARP promoter, we inserted a construct containing the rRNA promoter into the PARP A locus (pHD 560, Fig. 1). Only one bloodstream-form line was obtained in five experiments, each involving transfection of 10^7 cells with 10 or 25 μg of plasmid. The one cell line obtained had integration of two copies in tandem at the PARP A locus and expressed similar CAT levels to bloodstream cells containing one copy of the CAT gene with a PARP promoter (control plasmid pHD559) (not shown). Four equivalent procyclic cell lines containing the rRNA promoter construct (pHD 560) in the PARP A locus showed 2–3 times more CAT activity than the bloodstream clone. These results suggest that the rRNA promoter is 3–6-fold down-regulated in bloodstream forms when integrated at the PARP locus.

The activity of different promoters in the ribosomal RNA locus

To find out whether the control of CAT expression from the PARP promoter was dependent on integration specifically at the PARP locus, we designed new constructs for integration into the non-transcribed spacer of the rRNA locus, in inverse orientation relative to endogenous rRNA transcription. This spacer region is transcriptionally silent (23); transcriptional initiation is driven by the integrated test promoter (42); transcription is α-amanitin resistant (23) and initiates at the expected position (19). PARP, rRNA, VSG and rRNA–PARP hybrid promoters were inserted upstream of a CAT gene with a PARP 5′-UTR and actin 3′-UTR. Downstream was a hygR cassette (Fig. 1). Two rRNA promoters were tested that differ slightly in sequence: CrRNA, (19) and BrRNA (44). The rRNA–PARP hybrid promoter Rp had rRNA promoter sequence from position –240 to –50 and PARP promoter sequence from –50 to +25; promoter rP had distal rRNA promoter sequence up to –80 and proximal PARP promoter sequence. The relative activities of all the constructs in transient transfection assays are shown in Figure 6A. The PARP promoter was ~50% more active than the VSG promoter in procyclic trypanosomes and in bloodstream trypanosomes the situation was reversed. The hybrid promoters were poorly active in transient assays in bloodstream forms. Previous results of transient transfection assays (19) had suggested that the rP hybrid was more active in procyclic cells than the Rp hybrid. This was not confirmed with these constructs; we have no explanation for this discrepancy. Upon transfection into bloodstream and procyclic trypanosomes, the efficiency with which drug-resistant clones were obtained reflected the transient activities to some extent. Clones with intact VSG, rRNA and PARP promoters were easily obtained in both cell types, with efficiencies of $1–4\times 10^{-6}$ in procyclic cells. Efficiencies were ~4-fold lower in bloodstream forms. In contrast, cell lines were obtained only with difficulty with the hybrid promoter rP construct (efficiencies of $<10^{-7}$).

Table 2. Expression of CAT in the PARP and tubulin loci of bloodstream (BF) and procyclic (Pro) trypanosomes

Cell lines	Hygro	Div. time (h)	No. divs ± hyg.	c.p.m./h/10^5 cells	CAT: 383/330
330 BF	–	8.2 ± 1.0	17.9 ± 2.3	4400 ± 1188	
	+	10.5 ± 1.7	15.8 ± 3.1	4257 ± 1037	
383 BF	–	7.5 ± 0.4	19.4 ± 1.1	8661 ± 1458	2.0
	+	12.3 ± 5.4	14.4 ± 3.2	14 205 ± 3296	3.3
330 Pro	–	10.1 ± 0.5	4.8 ± 0.2	5433 ± 2082	
	+	10.4 ± 0.4	4.7 ± 0.6	4529 ± 1825	
383 Pro	–	9.1 ± 0.5	5.3 ± 0.3	65 312 ± 20 365	12.0
	+	9.4 ± 1.1	5.1 ± 0.5	47 806 ± 18 448	10.6

Activity is expressed as c.p.m./h/10^5 trypanosomes, all lines assayed in duplicate, values as mean ± standard deviation. Hygro, presence or absence of hygromycin; div time, mean division time (h); 383/330, ratio of CAT activities of 330 and 383 cell lines grown under corresponding conditions. The number of cell divisions was calculated from regular cell counts; that for the procyclic cells is an underestimate because the cells were not counted over the first 2 days of the experiment.

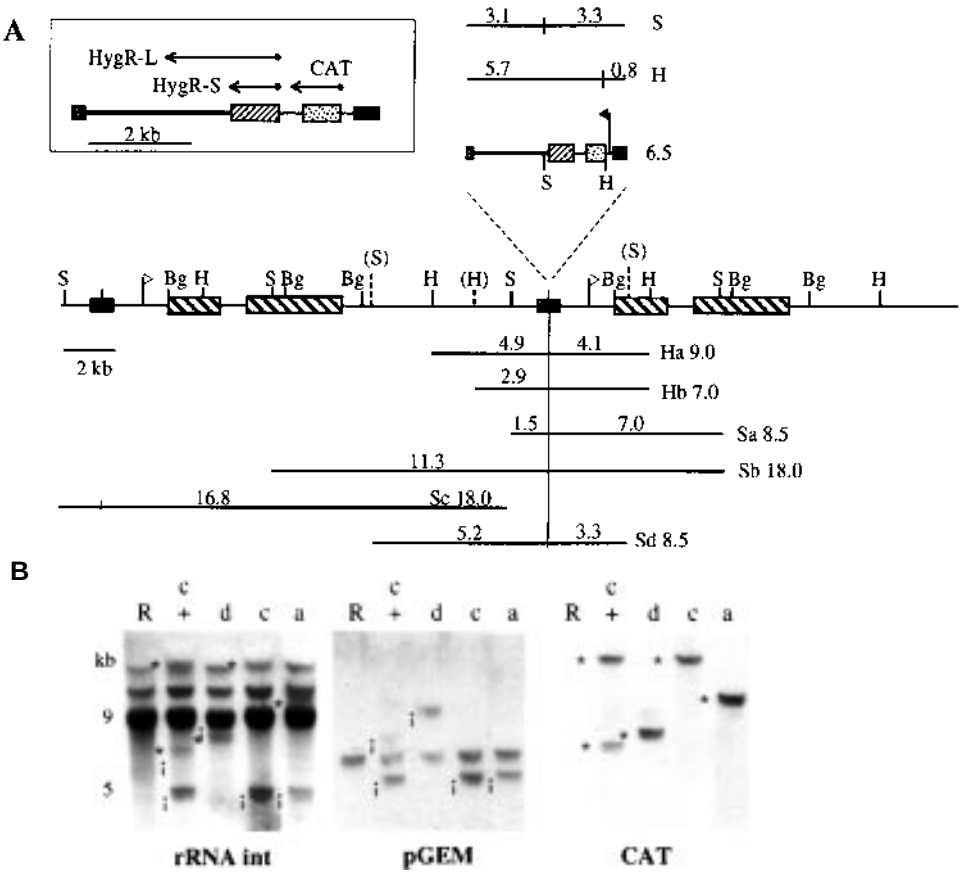


Figure 5. Integration of constructs into the rRNA non-transcribed spacer. (**A**) Restriction map of the rRNA locus. Open arrow, rRNA promoter; closed arrow, test promoter; thick striped blocks, rRNA genes; white stippled box, CAT gene; diagonal striped box, hygR gene; dark grey, targeting fragment. Sites shown in parentheses with dashed lines are not in the original published map but are inferred from the present experiments. Symbols as in Figure 2. Bg, *Bgl*II. Various types of cleavage pattern (a–d) with *Stu*I (S) or *Hin*dIII (H) are shown together with fragment sizes in kb. The boxed insert shows the mRNAs that are transcribed from integrated plasmid. (**B**) Blot hybridization of *Stu*I digested DNA from bloodstream trypanosome lines with constructs integrated in the rRNA locus non-transcribed spacer. Probes are the rRNA intergenic region targeting fragment (rRNAint), pGEM and CAT. Sizes of selected bands, read using markers, are indicated on the left. Fragments hybridizing with CAT and rRNAint are denoted with an asterisk, and fragments hybridizing with rRNAint and pGEM with a small circle. c+ is a line with integration pattern c, where a portion of the cells have an additional entire copy of the plasmid. The original cell line used (lane R) in these experiments has a plasmid integrated in the tubulin locus; hence the hybridization with the pGEM probe.

The integration sites of the plasmids were determined by blot hybridization. The presence of restriction enzyme polymorphisms among the rRNA repeats complicated the analysis; representative examples are illustrated in Figure 5B. *Stu*I digestion yields three main bands and several minor bands when wild-type DNA is hybridized with an rRNA-spacer (targeting-segment) probe. A type (a) insertion position (Fig. 5A) has two *Stu*I sites 8.5 kb apart, as shown in the original published map (48) (Fig. 5B, lanes R). Insertion of the plasmid DNA yields new fragments of 4.6 kb (hybridising to the targeting fragment and pGEM probes) and 10.3 kb (hybridizing to targeting fragment and CAT) (Fig. 5B, lanes a). (The starting trypanosome line, lanes R, has a plasmid encoding a bacterial repressor integrated at the tubulin locus: hence the band of ~4 kb that hybridized to the pGEM probe in all lanes.) We interpret the larger bands (Fig. 5B, rRNA int probe) in cells without the integrated plasmid (lanes R) as representing loss of one or more *Stu*I sites. A locus of type (b) has lost a *Stu*I site just upstream of our targeting sequence. Insertion of the construct yields a blot hybridization pattern in which the pGEM and targeting fragment probes hybridize to a band of 14.4 kb and the CAT probe [as for (a)] lights up a 10.3 kb band (Fig. 5A, data not shown). A locus of type (c) has lost the *Stu*I site within the rRNA gene downstream of our targeting sequence. Insertion of the construct yields a blot hybridization pattern in which the pGEM and targeting fragment probes hybridize to a band of 4.6 kb [as for (a)] and the CAT probe labels a very large (~20 kb) band (Fig. 5B, lanes c). If a second copy of the intact vector sequence is present it appears as an additional band hybridising to all probes. In the Figure a cell line (labelled c+) that has a c-type insertion and an additional copy is illustrated; only a subpopulation of the cells has the additional copy as the band is under-represented after hybridising to each probe. Insertion patterns of type (d) imply the presence of additional *Stu*I sites; the correct insertion in such clones could be deduced from the *Hin*dIII digestion pattern. The CAT-hybridizing fragment is ~7 kb and the pGEM-hybridizing band ~8.5 kb. *Hin*dIII digestion can yield hygR-containing fragments of 10.6 (a) or 8.6 kb (b) depending on the position of the upstream *Hin*dIII site (Fig. 5A, data not shown). Judging from the *Stu*I and/or *Hin*dIII digestion patterns, all cell lines had insertions in the rRNA spacer. To confirm that the promoters had remained intact, the integrated promoter–CAT fragments were amplified by PCR, using a reverse (antisense) CAT primer and primers specific

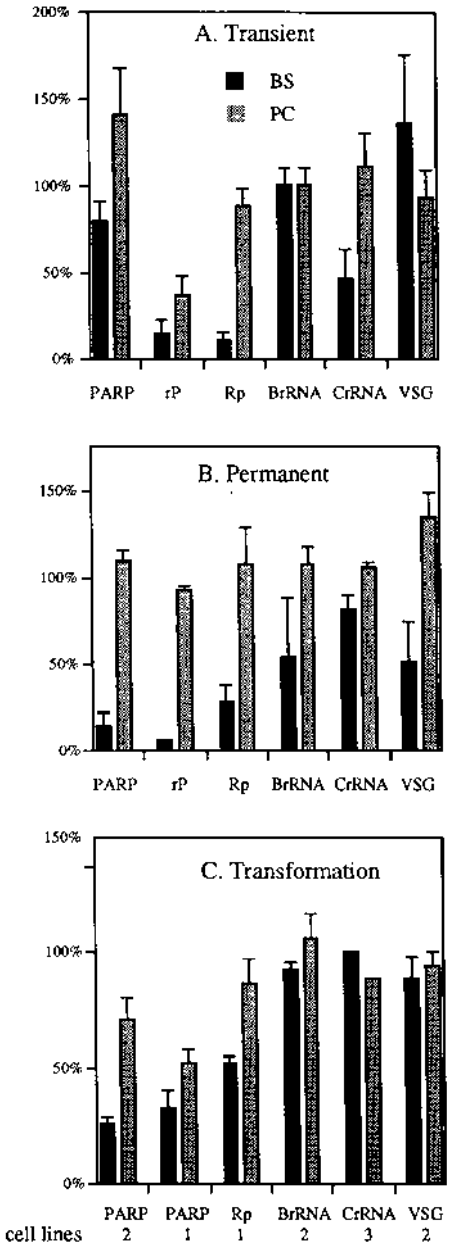


Figure 6. Activities of PARP, VSG, rRNA and hybrid promoters in driving CAT expression from the rRNA non-transcribed spacer. (**A**) The activities of the plasmids in transient assays; measured in triplicate for procyclic forms (PC) and twice in triplicate for bloodstream forms (BS). Results (mean and standard deviation) are expressed independently for bloodstream and procyclic forms, relative to the mean levels for the BrRNA promoter set at 100%. (**B**) Permanent cell lines. CAT activities of cell lines containing a single copy of the appropriate plasmid are expressed as mean and standard deviation. Data for 3–5 lines are included except for rP bloodstream (one line) and rP procyclic (two lines). All results are expressed relative to the average value for one rRNA promoter line (100%) that was included in all assays as a reference. (**C**) Transformation of bloodstream forms into procyclic forms. CAT was assayed from exponentially growing cultures of bloodstream forms or established (2 months) procyclic forms. Details of the lines used are in Figure 8. All lines were assayed at least twice in duplicate except for the CrRNA 3 line, which was assayed only once because the culture then died.

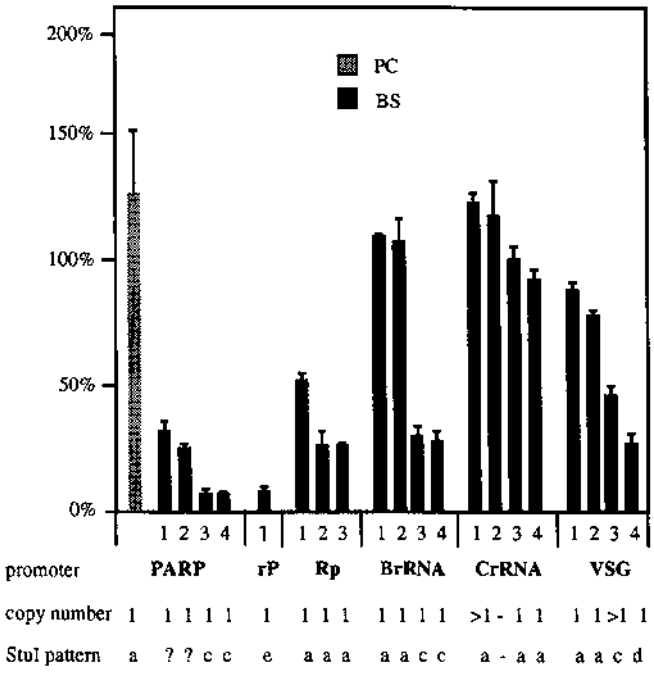


Figure 7. Variations in CAT expression between bloodstream trypanosomes with CAT cassettes integrated in the rRNA spacer. CAT assays were performed at least twice in duplicate. One of the CrRNA lines (CrRNA3) is set at 100%. All lines had integration in the rRNA spacer as judged by *Hin*dIII or *Stu*I pattern (Fig. 5) except that line CrRNA 2 was not examined. The *Stu*I pattern type is indicated. PC, procyclic line with a PARP promoter integrant.

to the 5′-extremity of each promoter. The status of the hybrid promoters was confirmed using primers from both PARP and rRNA promoters, to ensure that the hybrids had not been gene-converted to 'pure' forms (see Materials and Methods). In all cases, we obtained the expected PCR products, and failed to obtain products using negative control pairs of primers (not shown).

The CAT activities of different clones were measured at least twice. The results for 3 or 4 clones with a single integrated copy of the relevant plasmid [apart from rP where only one (bloodstream) or two (procyclic) lines were examined] are shown in Figure 6B. All promoters yielded similar CAT activities in procyclic trypanosomes and there was little variation between clones. In bloodstream trypanosomes, the PARP promoter was 5–8-fold less active than the rRNA promoters and the Rp promoter was in between. Expression from the integrated rP promoter in bloodstream forms was intermediate between that from the PARP and Rp promoters; the activity from the VSG promoter was similar to that from the rRNA promoters; and the average results for all promoters were somewhat less than the corresponding results for procyclic forms. Although bloodstream-form cell lines containing the fusion promoters showed a lower CAT activity than those with a VSG or rRNA promoter driving the CAT gene, no conclusions can be drawn about the location of regulatory sequences because the fusion promoters had low activities in transient assays.

Variations between bloodstream form clones were very considerable (see below). We therefore induced two clones of each type to differentiate into procyclic forms (in the absence of drug). The cell lines were derived from a monomorphic culture and differentiated very poorly: most cells died. Only half the cultures survived to produce growing procyclic cultures; these tolerated only 2-fold dilutions, growing slowly to maximal densities that were about four times lower than for normal procyclic cultures. CAT activities are shown in Figure 6C. Lines bearing rRNA and VSG

promoter integrants showed no regulation of CAT expression. One PARP line showed 2–3-fold up-regulation of CAT and the other rather less; Rp gave an intermediate result. The strange behaviour of these lines leads us to suspect that some components required for healthy procyclic growth were poorly functional or absent.

Table 3. Quantitation of HygR and CAT mRNA from data as illustrated in Figure 8

Stage	Promoter	CAT/actin	CAT/hygR	hygR-S/hygR-L
Bloodstream	rRNA	1.00	1.29 ± 0.77	1.22 ± 1.00
	rRNA	1.20		
	PARP	0.09	0.36 ± 0.24	2.52 ± 1.86
	PARP	0.07		
Procyclic	rRNA	1.34	0.66 ± 0.33	1.11 ± 0.72
	rRNA	1.97		
	PARP	1.07	0.78 ± 0.56	0.92 ± 0.53
	PARP	0.80		

Northern blots of total or poly(A)$^+$ RNA from bloodstream and procyclic trypanosome lines containing pHD 500 (PARP promoter) or pHD 499 (CrRNA promoter) integrated in the rRNA spacer were probed with hygR, CAT and actin probes.
CAT/actin: the levels of CAT mRNA were compared after standardising relative to the actin control (not shown), using one blot of poly(A)$^+$ mRNA. One bloodstream cell line was set arbitrarily at 1.0.
CAT/hygR: the relative amounts of CAT, and total hygR RNA were compared. Results are mean ± standard deviation, measured from three blots for each pair of cell lines. The result for one of the bloodstream (rRNA promoter) cell lines was set arbitrarily at 1.0 to control for differences in probe activity and for differences in the order of hybridization.
hygR-S/hygR-L: ratio of the long and short hygR transcripts (see Fig. 8). Results are mean ± standard deviation, measured from three blots for each pair of cell lines. Absolute ratios varied between experiments but the tendencies were always the same.

Different cloned bloodstream trypanosome lines with supposedly similar insertions in the rRNA spacer consistently had up to 3-fold differences in CAT activity (Fig. 7). There were no obvious differences between clones in growth characteristics. Most lines had only one integrated copy of the plasmid; the two lines containing more (BrRNA promoter line 1, with 2–3 copies, and VSG promoter line 3, with 1–2 copies) surprisingly had only slightly more activity than equivalent clones with only one copy. Clones with a 'c' pattern expressed less CAT than those with an 'a' pattern. So far no bloodstream lines have been obtained with a PARP promoter in an 'a'-type locus but insufficient clones have been examined to lend this observation significance. Overall the results hint at differences in transcription between different rRNA spacer sites and highlight the importance of analysing several clones.

Regulated transcription termination

If transcriptional attenuation is occurring in a locus, there should be a progressive decrease in transcript yield as the distance from the promoter increases. To find out if attenuation plays a role in regulation of PARP-promoter-driven transcription, RNA from duplicate bloodstream and procyclic cell lines was analysed. Each line contained a single integrated copy of the CAT-hygR construct bearing either the PARP or the CrRNA promoter. Transcription of the integrated constructs resulted in a single major CAT mRNA whose size indicated that it terminated within the shortened aldolase 3′-UTR (Figs 5A and 8). There were two hygR transcripts.

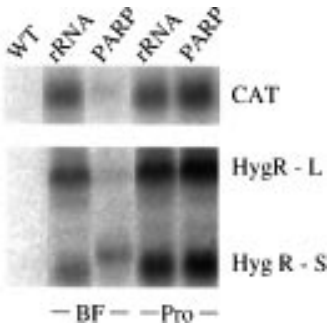


Figure 8. The effect of different promoters on transcript patterns. Approximately equal amounts of total RNA were analysed from duplicate bloodstream and procyclic cell lines with a single integrated copy of pHD 499 or pHD 500; phosphorimager results for one cell line of each type are shown but those for the others were similar. The pattern with polyA+ RNA was also similar. WT, wild-type procyclic trypanosomes; BF, bloodstream forms; Pro, procyclic forms. The nature of the promoter driving transcription is indicated above each lane. The labels against the bands correspond to those in Figure 5A (boxed insert). The phosphoimager data were imported into Adobe photoshop; the contrast of the hygR panel has been increased by ~10%. Quantitation for three such blots and including all eight cell lines is in Table 3.

The shorter hygR-S transcript (1.4 kb) presumably terminates within the actin 3′-UTR; the longer hygR-L transcript (2.5 kb) hybridized with a plasmid vector probe but not with a CAT probe (not shown) so must have 1.5 kb of plasmid sequence at its 3′ end. All cell lines that showed no developmental regulation of CAT levels had similar amounts of the longer and shorter hygR RNAs (see ratio of hygR-S to hygR-L in Table 3). Although the absolute ratios of the different RNA species varied somewhat between blots (Table 3), the overall picture (Fig. 8) was always the same and was not affected by the presence of hygromycin in the medium.

As expected, all lines had similar levels of CAT mRNA except the two bloodstream lines in which transcription was driven by the PARP promoter. These lines had 9 and 7% of the CAT activity expressed by the standard bloodstream rRNA promoter cell line. Corresponding CAT activities were 15 and 10% (lines PARP 3 and 4 in Fig. 7). The procyclic rRNA promoter lines had almost twice as much hygR RNA as the bloodstream rRNA promoter lines (Table 3), indicating a small degree of post-transcriptional regulation; the important point is that in procyclic forms the promoter type made no difference to either the ratio of CAT/hygR RNA, or the relative abundances of the two hygR transcripts. The comparison of the bloodstream lines was more intriguing. First, the lines with the integrated PARP promoter appeared to have rather higher levels of total hygR RNA relative to CAT than the rRNA promoter lines (Table 3). There was thus no evidence for preferential termination of PARP-promoter-driven transcription within the 1 kb encompassing the hygR gene. Secondly, the longer hygR-L mRNA was almost undetectable in these lines and the average length of the shorter hygR transcripts had increased (Fig. 8). This was very clearly a promoter-specific effect as the patterns in bloodstream and procyclic cell lines containing the integrated rRNA promoter were undistinguishable from each other. We think that the simplest explanation is that in bloodstream forms, PARP-promoter-driven transcription is preferentially terminating within the plasmid sequence, and that the consequent altered precursor structure affects the choice of polyadenylation site.

DISCUSSION

Most evidence so far suggests that the PARP promoter is recognized by RNA polymerase I. Developmental regulation of RNA polymerase I transcription is not unprecedented. In mammalian cells, transcription by RNA polymerase I is regulated according to growth rate (49), and various species of *Plasmodium* express different rRNA genes in the sexual and asexual stages of the life cycle (50). Our results show that regulation is dependent on chromosomal integration: the same is true for the recently-reported control of VSG promoter activity which is, however, also chromosome- and position-dependent (24,25). When our constructs were targeted to the rRNA spacer in bloodstream trypanosomes, considerable variations in the expression level were obtained. We do not know whether the various trypanosome rRNA loci are differentially transcribed, as appears to happen in yeast (51).

Relative levels of CAT expression from genes integrated in the PARP or tubulin loci were consistent with those previously observed using a luciferase reporter (42) and confirm that transcription of a typical RNA polymerase II-transcribed locus is about an order of magnitude less efficient than of a RNA polymerase I-transcribed locus. CAT activities obtained when the same cassette was integrated into the actin or aldolase transcription units were not significantly different from those observed from the tubulin locus (C. Hartmann, unpublished).

The control of PARP-promoter-mediated transcription from the PARP locus could be at the level of initiation or elongation (20,22). Some of the constructs that we integrated at the PARP locus contained only 25 bp of PARP-locus-derived sequence downstream of the initiation site. Thus if premature termination of transcription is involved in regulation, it must either occur within the first 25 residues (in which case regulation of initiation and of elongation become difficult to distinguish), or not be dependent on PARP locus sequence. In our constructs containing both a CAT gene and a hygR gene, the 3′ ends of the genes are respectively 0.9 and 2.2 kb distant from the transcription initiation site. (For comparison, the 3′-polyadenylation site of the endogenous PARP Aβ gene is 2.1 kb downstream of the initiation site.) Results of RNA analysis yielded no evidence for developmentally-regulated termination within the hygR gene, but clearly suggested that in bloodstream forms, the PARP-promoter-directed transcription of vector sequence was impaired. A similar effect was observed by Vanhamme *et al.* (29) who did not, however, detect the sequence-specificity. Several previous publications contain circumstantial (42,52) or direct (53,54) evidence that PARP promoter-driven transcription of plasmid vector sequence is rather inefficient, even in procyclic forms. It is not clear whether the regulation of plasmid transcription described here is relevant to transcription of the PARP genes *in vivo*. We do not know what sequences in the plasmid sequence are inhibiting elongation, or whether functionally-equivalent sequences are present in the PARP locus.

Regulation of PARP and VSG promoters occurs in a variety of chromosomal contexts. Berberof *et al.* (31) integrated constructs such that PARP promoter-directed transcription was in the same direction as RNA polymerase II transcription coming from upstream tubulin genes: the PARP promoter was only active in procyclic forms. When a VSG promoter was integrated in inverse orientation in the RNA polymerase II gene locus in procyclic trypanosomes, activity was very low but could be stimulated by the presence of a PARP promoter nearby (52). In contrast, a VSG promoter was highly active when integrated into the rRNA spacer in procyclic trypanosomes (23), and in bloodstream trypanosomes (this paper). This supports the conclusion that VSG promoter regulation is dependent on chromosomal location (23). Our cell populations bearing the integrated active VSG promoter in the rRNA spacer were expressing VSG (data not shown), suggesting that two VSG promoters can be active simultaneously in a bloodstream trypanosome.

Our analyses suggest that several elements are responsible for PARP promoter regulation. The results from the hybrid promoters were consistent with the presence of down-regulatory (for bloodstream forms) sequences in the PARP promoter upstream of position –50. The PARP promoter showed regulation whether it was inserted in the PARP or rRNA loci, implying that regulatory sequences are present downstream of position –247 relative to the transcription start. On the other hand, an rRNA promoter was also down-regulated in the PARP locus, indicating a further contribution from upstream sequences. The requirement for chromosomal integration implies that the regulation is dependent on chromatin structure, and it is quite possible that the sequences required are functionally degenerate and spread over the whole of the 640 bp conserved region upstream of the PARP genes.

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