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Transcriptional Regulation of Metacyclic Variant Surface Glycoprotein Gene Expression during the Life Cycle of *Trypanosoma brucei*

SHEILA V. GRAHAM AND J. DAVID BARRY*

Wellcome Unit of Molecular Parasitology, Anderson College, University of Glasgow, Glasgow G11 6NU, Scotland

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In antigenic variation in African trypanosomes, switching of the variant surface glycoprotein (VSG) allows evasion of the mammalian host immune response. Trypanosomes first express the VSG in the tsetse fly vector, at the metacyclic stage, in preparation for transfer into the mammal. In this life cycle stage, a small, specific subset (1 to 2%) of VSGs are activated, and we have shown previously that the system of activation and expression of metacyclic VSG (M-VSG) genes is very different from that used for bloodstream VSG genes (S. V. Graham, K. R. Matthews, P. G. Shiels, and J. D. Barry, Parasitology 101:361–367, 1990). Now we show that unlike other trypanosome genes including bloodstream VSG genes, M-VSG genes are expressed from promoters subject to exclusively transcriptional regulation in a life cycle stage-dependent manner. We have located an M-VSG gene promoter, and we demonstrate that it is specifically up-regulated at the metacyclic stage. This is the first demonstration of gene expression being regulated entirely at the level of transcription among the Kinetoplastida; all other protein-coding genes examined in these organisms are, at least partly, under posttranscriptional control. The distinctive mode of expression of M-VSG genes may be due to a stochastic mechanism for metacyclic VSG activation.

African trypanosomes, which are protozoan parasites that cause potentially fatal diseases in humans and domestic livestock (4), have a life cycle which alternates between the tsetse fly and the mammal (75). In the mammalian bloodstream, *Trypanosoma brucei* is densely coated with a single glycoprotein species, the variant surface glycoprotein (VSG). It is by antigenic variation, the process of switching between different VSGs and thus changing the variable antigen type of the parasite, that trypanosomes evade the specific host immune response (18). There are over 1,000 transcriptionally silent VSG genes, mostly located internally in chromosomes, with a minority located at telomeres (74). The sheer size of the VSG gene repertoire presents major problems for conventional mechanisms for differential gene expression. This is achieved by the use of programmed gene rearrangements in conjunction with specialized transcription units. Usually only one VSG gene is active at a time in bloodstream trypanosomes, and activation is achieved by two main mechanisms. In the first, which is the only mechanism available to the vast majority of VSG genes, duplicative transposition allows a transcriptionally silent VSG gene to be copied to 1 of up to 20 special bloodstream expression sites located at the telomeres of some trypanosome chromosomes (21, 41), whereas in the second, bloodstream VSG genes that already reside at telomeres which can act as expression sites become activated in situ (49). Bloodstream expression sites comprise long, complex polycistronic transcription units containing, from 3′ to 5′, the VSG gene, several kilobases of 70-bp repeats (thought to be involved in gene conversion [2]), at least eight expression site-associated genes (ESAGs) (53), and, located 40 to 60 kb upstream of the VSG gene, a promoter for transcription of the entire expression site (20, 26, 38, 52, 79).

There are major differences in gene expression between African trypanosomes and higher eukaryotes. One, which may result in most regulation of trypanosome gene expression being exerted posttranscriptionally, is that all trypanosome protein-coding genes analyzed thus far are organized in polycistronic transcription units. Genes within such transcription units are cotranscribed from a common 5′ promoter, but polycistronic primary transcripts are not formed since cleavage, *trans*-splicing, and polyadenylation occur rapidly to produce individual mRNAs as transcription proceeds through each gene (71); these RNA-processing mechanisms may be functionally linked in the Kinetoplastida (36, 42, 45, 60). Since many polycistronic transcription units contain genes which are transcribed from the same promoter but yield very different steady-state RNA levels in the cell and/or are differentially expressed during the life cycle of trypanosomatids, regulation of their expression must be mediated at least partly at a posttranscriptional level (14, 28). The best-studied polycistronic transcription units are the telomeric expression sites used by VSG genes during the bloodstream phase of the life cycle. During the trypanosome life cycle, these expression sites are under posttranscriptional regulation and contain genes which are differentially expressed. In the bloodstream, there is more than a 100-fold difference in the final steady-state levels of ESAG and VSG mRNAs from an active bloodstream expression site, despite their being transcribed from a common promoter at a constant level (41). Three promoter regions for bloodstream expression sites have been studied to date (26, 37, 51, 77–79), and they direct transcription of the expression site by an α-amanitin-insensitive RNA polymerase (20, 41, 52, 57). As far as is known, there is always a bloodstream expression site promoter active throughout the life cycle, even when VSG expression ceases as trypanosomes differentiate to the procyclic form in the tsetse fly. Although the promoter remains

* Corresponding author. Mailing address: Wellcome Unit of Molecular Parasitology, The Anderson College, 56 Dumbarton Rd., Glasgow G11 6NU, Scotland. Phone: 141 330 4875. Fax: 141 330 5422. Electronic mail address: gbga05@udcf.gla.ac.uk.

active, transcription elongation is aborted near the 5′ end of the polycistronic transcription unit, leaving the VSG gene unexpressed: life cycle stage-specific regulation of bloodstream VSG gene expression sites is accomplished downstream of transcription initiation (16, 50, 51, 55, 79).

Although this general picture of trypanosome gene expression is emerging, there is one set of genes which appears to conform to a different pattern: the VSG genes activated in the tsetse fly. When ingested into the midgut of the tsetse fly, bloodstream trypanosomes differentiate to the procyclic form and rapidly replace the VSG coat with a new, nonvariant surface coat composed of the glycoprotein procyclin/PARP (46, 54). Expression of the VSG is resumed only when differentiation to the nondividing metacyclic stage occurs in the fly salivary glands. Resynthesis of the VSG coat at this stage is thought to be essential to allow parasite survival and proliferation following transfer back to the mammalian host (75). At the metacyclic stage, only a small, specific subset of VSG genes (≤27; 1 to 2% of the repertoire) is expressed (19, 22, 69). Unlike the sequential activation of VSGs in the bloodstream, all metacyclic variable-antigen types are activated simultaneously, although as usual only one is expressed per cell, yielding an antigenically mixed metacyclic trypanosome population (66). Metacyclic VSG (M-VSG) genes have a specific chromosomal location; they are located at the telomeres of the longest trypanosome chromosomes (17). Furthermore, they have a life cycle stage-dependent activation mechanism, using in situ activation in the metacyclic stage but using duplicative gene conversion when activated de novo in bloodstream trypanosomes (31). Since M-VSG genes are always resident in their expression telomeres at the metacyclic stage (31), these telomeric loci must also contain the promoters for expression of the M-VSG genes. Metacyclic expression telomeres are structurally unique: they contain very few or no 70-bp repeats and at most one ESAG, and they comprise a low-copy-number sequence (30, 43, 44, 64), making them very different from bloodstream expression sites (53).

To investigate how M-VSG expression is regulated during parasite differentiation and in particular what role the unusual metacyclic expression telomeres might play, we have analyzed transcription of two M-VSG gene-containing telomeres. A previous study of M-VSG gene expression reported only Northern blot analyses of steady-state RNA, an approach which reveals nothing about transcription per se (43, 64). Our transcription studies of metacyclic stage-derived trypanosomes, in which the genes are normally expressed, and in bloodstream and procyclic trypanosomes, in which they are normally silent, reveal a number of surprising features: unlike other trypanosome genes, M-VSG genes are transcribed at the metacyclic stage as short, monocistronic transcription units flanked upstream by extensive, transcriptionally silent regions. Further, the putative metacyclic promoter regions are transcriptionally active at only the appropriate life cycle stage. These are the first trypanosome VSG genes, indeed the first trypanosome genes, shown to be exclusively transcriptionally controlled in a life cycle stage-dependent manner, and regulation of these metacyclic expression sites therefore appears to differ greatly from that of bloodstream expression sites.

MATERIALS AND METHODS

Trypanosomes. A virulent, cloned line of *T. brucei* EATRO 795 which retains fly transmissibility was used in these studies as described previously (31, 68). Tsetse fly transmissions, maintenance in mammalian hosts, and DNA preparations were carried out by standard procedures (32, 33). Procyclic culture form trypanosomes were established from EATRO 795 trypanosomes by standard methods and were maintained in SDM-79 medium (11). Metacyclic trypanosomes were cloned directly from dissected fly salivary glands or from salivary probes into guinea pig serum and grown in mice for up to 7 days. After this time, around 10^8 organisms were present with variable antigen type (VAT) purities in the range of 90 to 100%.

Recombinant clones. The clones derived from the basic copy locus of the metacyclic VSG gene ILTat 1.22 have been described previously (17, 44). These are λMT1.22B and pMG7.1-1 (Fig. 1A). The region of the 1.22 telomere containing the transcriptional start site of the 1.22 M-VSG gene was subcloned into pBluescript. We subcloned first the 1-kb 5′ *Bam*HI-*Eco*RI fragment from pMG7.1-1 to yield pMT1.22-BE, then the 400-bp *Bam*HI-*Pst*I fragment contained in this subclone to give pMT1.22-BPs, and finally the 2.0-kb *Hin*dIII-*Pst*I fragment, located at the 5′ end of pMG7.1-1, yielding pMT1.22-HPl (Fig. 1A). All subclones and deletion plasmids were checked by sequencing.

Because the nature of the 3′ untranslated region and, to a lesser extent, the 5′ splice acceptor site, has been found to be important in controlling reporter gene expression in transient-transfection assays in a life cycle stage-dependent manner (35), the recombinant plasmids for transient-transfection studies were of two types: the first were all derivatives of pJP44, which contains, in a 5′-to-3′ direction, the PARP B locus promoter (15), a PARP splice acceptor site, a chloramphenicol acetyltransferase (CAT) reporter gene, and the 3′ end of the PARP B α gene to provide polyadenylation signals (63), and the second were derivatives of pHD52, which contains, in a 5′-to-3′ direction, the 221 bloodstream expression site promoter region, an actin splice acceptor site, a luciferase gene, and an actin gene 3′ untranslated region (35). For our experiments, we replaced the luciferase gene in pHD52 with the CAT gene by cleaving pHD52 with *Hin*dIII and *Bam*HI to remove the luciferase gene and directly replacing this with the corresponding *Hin*dIII-*Bam*HI fragment containing the CAT gene from pJP44 to give pHD52CAT (63). Instead of using pJP44 directly for the PARP-based constructs, we used the construct p5′parpCAT3′parp, which contains essentially the same insert sequences as pJP44 but subcloned into *Sma*I-*Cla*I-cut pBluescript (KS−) to yield more flanking restriction sites (45). Plasmid p221CAT3′parp was constructed by replacing the PARP B promoter in p5′parpCAT3′parp, cut out as an *Sst*I-*Sma*I fragment with the 221 VSG gene promoter from pHD52 (35) also isolated as an *Sst*I-*Sma*I fragment. The promoterless construct (p-CAT3′parp) was made by cleaving p5′parpCAT3′parp with *Sma*I and *Not*I, blunt ending, and self ligation. This removes the 278-bp PARP promoter region found in pJP44 while retaining the PARP splice acceptor site. Putative promoter fragments from the metacyclic-expression telomeres were cloned upstream of the PARP splice acceptor site by *Not*I-*Sma*I digestion of the parent construct as above followed by substitution of the PARP promoter fragment with the appropriate metacyclic expression site fragment isolated from its pBluescript subclone by double digestion with *Not*I and a restriction enzyme at the 3′ end of the insert, yielding a blunt end. Short and long regions from the telomere encompassing the putative promoter were cloned into the CAT reporter construct to yield p1.22sCAT3′parp (400-bp fragment; equivalent to the insert in the plasmid pMT1.22BPs), and p1.221CAT3′parp (2.0-kb fragment; equivalent to the insert in the plasmid pMT1.22HPl). To be sure that orientation of insert sequences in the plasmid vector had no effect on expression in transient-transfection assays, all constructs were prepared and assayed in both pBluescript (KS−) and (SK−). The orientation of the inserted sequences with respect to the plasmid vector had no effect on CAT activity (data not shown). For the actin-based constructs, we modified pHD52CAT which we derived from pHD52, which contained a bloodstream expression site promoter (35). For the PARP promoter construct, we removed the VSG promoter from pHD52CAT by digestion of the plasmid with *Kpn*I and *Sma*I and replaced this fragment with the corresponding *Kpn*I-*Sma*I PARP promoter-containing fragment from pJP44 to give p5′parpHD52CAT (equivalent to the construct pHD30 but with the CAT gene instead of the luciferase gene [35]). For the promoterless control construct, we cleaved pHD52CAT with *Kpn*I and *Sma*I, then blunted it at the *Kpn*I site and religated it to yield p-HD52CAT (equivalent to the construct pHD54 [35] but with the CAT reporter gene instead of the luciferase gene). For the test construct, the 1.22 M-VSG putative short promoter region was removed from pMT1.22BPs as an *Sst*I-*Eco*RV fragment and was inserted into *Sst*I-*Sma*I-cut pHD52CAT upstream of the actin splice acceptor signal to replace the bloodstream expression site promoter, yielding p1.22sHD52CAT. The long version of the 1.22 M-VSG gene promoter was also cloned into pHD52CAT by replacing the *Kpn*I-*Sma*I promoter region with a *Kpn*I-*Sma*I fragment from pMT1.22-HPl. There is a *Kpn*I site 152 bp downstream of the *Hin*dIII site that marks the 5′ boundary of the insert in p122-HPl (Fig. 1A), and the *Sma*I site is located in the 3′ polylinker of the construct.

Other plasmid clones used were pPRO2001, a procyclin/PARP cDNA clone (54); pTbα,β-T1, a clone containing an α- and β-tubulin repeat unit (67); and pR4, containing a ribosomal DNA repeat unit (40).

DNA sequence analysis. Sequencing was carried out with single-stranded templates rescued from the plasmid pBluescript with the host bacterium *Escherichia coli* XL1-Blue (Stratagene) or on denatured double-stranded plasmid DNA by the dideoxy-chain termination method (Sequenase kit; Amersham International). The sequence of both strands of recombinant plasmids was obtained with the recommended primers for pBluescript or specific primers synthesized on an Applied Biosystems PCR-mate oligonucleotide synthesizer. Computer analysis was carried out with the Genetics Computer Group sequence analysis software package.

Nuclear run-on analysis. Preparation and storage of nuclei and run-on reactions were carried out exactly as described previously (40, 41), except that only up to 10^8 nuclei were used in reactions with metacyclic stage-derived cells since this was the maximum number obtainable from the early bloodstream trypanosome clones (31). Metacyclic stage-derived and bloodstream run-on incubations were carried out at 37°C, while procyclic reactions were carried out at 27°C. α-Amanitin was used at a concentration of 500 μg ml^{-1}. Hybridizations were carried out at 55°C in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 48 h, and washes were done in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C.

Purification of RNA, Northern and Southern blotting, and hybridization. RNA was prepared by lithium chloride-urea lysis of trypanosomes followed by phenol extraction (3). RNA was fractionated by electrophoresis on denaturing formaldehyde gels following denaturation of 5 μg of total RNA by incubation for 10 min in the presence of 90% dimethyl sulfoxide–10% lithium chloride (58). RNA was Northern (RNA) blotted directly onto a nylon membrane (Hybond-N; Amersham International plc) and immobilized on the filter by UV irradiation. DNA was Southern blotted onto a nylon membrane following denaturation in 0.5 N NaOH and neutralization (58). Radiolabelled probes were prepared by random hexanucleotide priming of restriction fragments separated by electrophoresis in low-melting-point gels (23). Hybridizations, washing of blots, and removal of hybridized probes were carried out as detailed in the Hybond protocol. Following removal of probes, filters were autoradiographed to check that no residual hybridization remained.

Transient transfection of trypanosomes. Five micrograms of supercoiled, CsCl-purified plasmid DNA was electroporated per transfection cuvette into procyclic culture cells exactly as described previously (7, 63, 79) with a single pulse of 1,500 V and 25-μF capacitance from a Bio-Rad Gene Pulser. For metacyclic stage-derived trypanosomes, blood was harvested from mice 5 days following fly feed by cardiac puncture with 0.2% sodium citrate as an anticoagulant. Erythrocytes were removed by centrifugation through preformed Percoll gradients (27) buffered with culture medium HMI-9 (12). Between 10^6 and 10^7 trypanosomes were obtained from each infected mouse depending on the time between infection and harvesting. Trypanosomes were washed in Zimmermann postfusion medium plus glucose (ZPFMG) as described previously (12). Following electroporation with 20 μg of DNA per cuvette, parasites were transferred to 5 ml of HMI-9 per cuvette and cultured overnight at 37°C. CAT reactions were carried out for 2 h at 37°C, and assays were done by xylene extraction (61, 79). Transfections were performed in replicate, and results presented are an average of those from six experiments, although values for CAT activity obtained with the constructs tested were remarkably reproducible.

PCR amplification. (i) 3′RT-PCR. First-strand cDNA was synthesized from total RNA isolated from transiently transfected, day 5 metacyclic stage-derived cells by using reverse transcriptase (RT) and the primer PWM5ANC (45). CAT-specific cDNAs were then PCR amplified with oligonucleotides PWMEco (CGAGAATTCGGTGGCAGCAGCCAACT) (45) and CATSG4 (GCCCGCC TGATGAATGCTCATCCGG), 470 nucleotides upstream from the CAT stop codon. A second round of amplification was carried out by nested PCR with oligonucleotide CATSG1 (TGGCAGGGCGGGGCGTAA), 18 nucleotides upstream from the CAT stop codon and PWM5Eco. PCR amplification was performed for 35 cycles of 30 s at 94°C, 1 min at 60°C, and 1 min at 70°C in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl, 100 μg of bovine serum albumin (BSA) per ml, and 100 pmol of each primer. PCR products were resolved by gel electrophoresis in 1.5% agarose.

(ii) 5′RT-PCR. 5′RT-PCR was carried out with a 5′RACE kit (Life Technologies) exactly as described in the manufacturer's protocol. The primer for first-strand synthesis was 1.22CATGSPSG1, which is homologous to sequences 215 to 190 bp downstream of the start codon for the CAT gene (5′CCGGATGAG CATTCATCAGGCGGGC3′). cDNAs were purified from primer and unincorporated nucleotides and then tailed with an oligo(dC) anchor. The first round of amplification was carried out with oligonucleotide GSP1 (1.22GSPSG2; 5′CAG GTCCGGGCCTCCGAGAGTTGG3′ [see Fig. 5B]) and the anchor primer supplied with the kit, which contains an oligo(dG) anchor region attached to a universal amplification primer region. A second round of PCR was carried out with oligonucleotide GSP2 (1.22GSPSG3; 5′CAATGCTTCCAGGGTTC3′ [see Fig. 5B]) and the universal amplification primer supplied with the kit. PCR amplification was performed for 35 cycles of 30 s at 94°C, 1 min at 60°C (first round of PCR) and 48°C (second round of PCR), and 1 min at 70°C in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl, 100 μg of BSA, and 100 pmol of each primer. PCR products were resolved by gel electrophoresis in 1.5% agarose. PCR products were cloned into a T-vector system (Promega), and recombinant plasmids were sequenced by the dideoxy-chain termination method (Sequenase kit).

RESULTS

Metacyclic stage-derived trypanosome clones. Study of VSG gene activation in the tsetse fly presents several technical difficulties. First, most trypanosome lines used for study of antigenic variation are adapted to growth in laboratory animals by rapid syringe passaging and display a number of abnormalities, including incapacity to progress through their life cycle and a greatly reduced rate of antigenic variation (68). The normal rate of switching, as displayed by normal tsetse fly-transmitted lines, is too high to permit direct molecular analysis. Second, VSG gene activation in the metacyclic population is polyclonal (66), necessitating study of trypanosome clones. Third, insufficient metacyclic cells are produced in the fly for direct molecular analysis. We have overcome these difficulties through development of a trypanosome line only partially adapted to continued animal growth. This line has reduced VSG switching and is still capable of fly transmission (31, 68). By injecting single metacyclic cells into mice, we are able to exploit the fact that the M-VSGs continue to be expressed for up to 6 to 7 days as the trypanosomes multiply, thus enabling direct molecular analysis of a limited number of clonal cells. In this study, we have derived four clones expressing ILTat 1.22 VSG and 16 clones expressing ILTat 1.61 VSG. All clones were found to be at least 99% homogeneous for VSG expression for the 2 days prior to experimentation.

Metacyclic expression telomeres are noncoding apart from the VSG gene. VSG genes in active expression sites lie immediately 5′ adjacent to short subtelomeric repeats flanked 3′ by longer GGGTAA telomeric repeats (10, 73). We have studied the metacyclic VSG genes encoding the ILTat 1.22 and ILTat 1.61 VSGs. We present results here only for the ILTat 1.22 gene; however, very similar data were obtained for the ILTat 1.61 gene. In the genome of trypanosomes of our cloned line of stock EATRO 795, there is one copy of the 1.22 M-VSG gene whose 5′ end is located typically 8 kb from the 3′ end of the telomere.

Steady-state transcription of the telomere harboring the ILTat 1.22 metacyclic VSG gene was analyzed by Northern blot analysis. We had previously characterized 18 kb of sequence upstream of the 1.22 gene, as shown in the map in Fig. 1A (44) and had detected no known ESAG sequence within the cloned region of the 1.22 metacyclic expression locus (λMT1.22B) by hybridization to ESAG-specific probes (44). Now 11 probes, most of approximately equal length and representing the entire cloned region of the telomere, were prepared and hybridized to Northern blots containing total RNA from procyclic culture cells, which do not express VSG genes (Fig. 1B to D, lanes 1) and from bloodstream (Fig. 1B to D, lanes 2) and metacyclic stage-derived (Fig. 1B to D, lanes 3) trypanosomes expressing the M-VSG gene. Metacyclic stage-derived organisms express the M-VSG gene in situ, while bloodstream parasites activate the gene by duplication, presumably to a bloodstream expression site (44). None of the probes (probes 2 to 10) from the 1.22 telomere hybridized except probe 1 (Fig. 1B), which contains part of the VSG gene, and probe 11, from the extreme 5′ end of the cloned region, which contains a partial *ingi* retroposon element (44) that is dispersed and repetitive in the trypanosome genome (39, 47) (Fig. 1C). Because transcripts from other copies of *ingi* in the trypanosome genome would hybridize to this probe, this region may not be transcribed on this telomere. Figure 1D shows the blot in Fig. 1B rehybridized to a PARP cDNA probe (54) following removal of the first probe to show that undegraded RNA is present in lane 1. These results indicated that over the region we had cloned, the 1.22 metacyclic telomere did not encode stable mRNAs other than that for the VSG gene itself.

M-VSG genes are transcribed as a short monocistronic transcription unit. To determine which regions of the 1.22 metacyclic telomere were transcribed and where transcription of the 1.22 M-VSG gene started, we prepared ^{32}P-labelled nascent transcripts, by nuclear run-on, from metacyclic stage-derived trypanosomes expressing the 1.22 VSG gene in situ

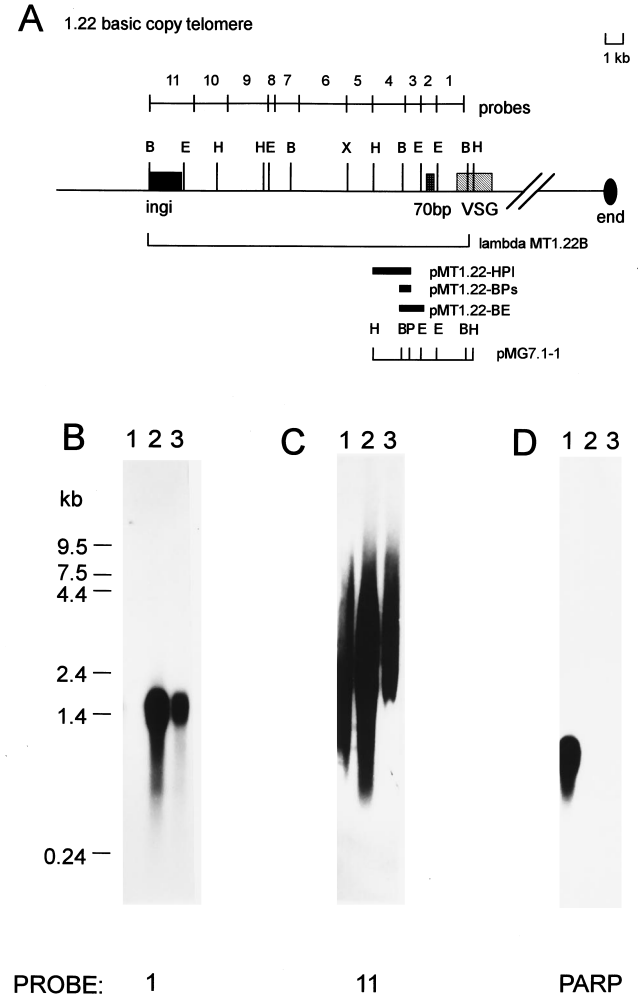


FIG. 1. Structure and steady-state analysis of transcription of the basic copy telomere harboring the ILTat 1.22 M-VSG gene. (A) Restriction maps of the genomic and plasmid clones (31, 44) derived from the telomere containing the ILTat 1.22 M-VSG gene. Abbreviations used for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I. Probes 1 to 11 refer to fragments of telomere-derived DNA used as probes in Northern blot analyses. Hatched box, VSG gene region (VSG); stippled box, 70-bp repeat region (70bp); black box, partial *ingi* retroposon sequence (ingi); end, end of the telomere. Horizontal black bars indicate fragments containing the putative promoter region subcloned into pBluescript. (B to D) Total RNA (5 μg) from (i) procyclic culture cells, which do not express the VSG gene (lanes 1); (ii) bloodstream trypanosomes expressing the 1.22 gene by duplicative transposition, probably to a bloodstream expression site (lanes 2); and (iii) metacyclic stage-derived trypanosome clones expressing the 1.22 gene in situ from the metacyclic expression telomere (lanes 3) (31) were separated by electrophoresis in denaturing formaldehyde gels. Northern blots containing the three RNAs were hybridized with the 11 telomere-derived probes or with a PARP cDNA fragment as a control (D), as shown below each panel. Only those blots to which probes hybridized are shown (B, probe 1; C, probe 11). D is a reprobing of the filter probed in panel B. Hybridizations were carried out in 50% formamide–5× SSC at 42°C. All probes were labelled by random priming to similar specific activities (10^9 dpm/μg). Blots were washed initially at 3× SSC and 65°C and then to 0.5× SSC and 65°C. Autoradiographs performed following the final wash are shown.

(88% expressors of 1.22) and hybridized them (Fig. 2C) to a Southern blot of *Eco*RI and *Bam*HI digests of λMT1.22B (Fig. 2A and B; *Eco*RI and *Bam*HI fragments are indicated by numbered letters). Figure 2C shows that apart from the *ingi*-containing fragment (fragment B3: shown as black bars on the map of the λMT1.22B clone) which hybridizes to *ingi*-related transcripts arising from elsewhere in the genome, only those regions of the telomere downstream of the *Bam*HI site 3.0 kb from the 5′ end of the VSG gene hybridized, i.e., *Bam*HI fragment B1 and *Eco*RI fragments E1, E2, and E3 (shown as wavy lines on the map of the λMT1.22B clone). The strength of each signal was measured by densitometry scanning, which showed that the hybridization signal on fragment B1 was very similar to the sum of the signals on fragments E1, E2, and E3 in Fig. 2C, even taking into account the small amount of hybridization which must be present to fragment E5, which overlaps fragment E1. In addition, hybridization to the 8-kb E3 band (underlined on the λ clone map [Fig. 2A]) was weak relative to its size, and quantification of the level of hybridization to this fragment by densitometry scanning indicated that only approximately 1 kb of sequence hybridized. Assuming that hybridization was to the 3′ end of E3 since fragment B2 did not hybridize, this places the initiation site for transcription of the 1.22 VSG gene close to the *Bam*HI site 3.0 kb 5′ of the VSG gene. To confirm this, we subcloned the *Bam*HI-*Eco*RI fragment, wherein transcription appeared to start, into pBluescript (pMT1.22-BE [Fig. 2A]) and hybridized nascent transcripts from a metacyclic stage-derived trypanosome clone (93% expressors of 1.22) (Fig. 2G) as above to a *Bam*HI-*Eco*RI-*Pst*I triple digest of this clone (Fig. 2F). Since both insert fragments hybridized (and fragment B2 in the first experiment did not hybridize), transcription of the 1.22 VSG gene must start upstream of the *Pst*I site, 2.6 to 3.0 kb upstream of the VSG gene. The region wherein transcription initiates is single copy in the genome of trypanosome stock EATRO 795 as shown previously (44).

In this experiment, lack of hybridization to *Bam*HI fragment 2 and to *Eco*RI fragment 4, taken together with the observed weak hybridization to *Eco*RI fragment 3 (which we assign to the 3′ end of this region), implies that there is a transcriptionally silent region of at least 13 kb 5′ of the VSG gene transcription unit on this telomere. The use of a run-on assay which limited nascent transcript processing (40, 41) and the size of the transcriptional gap observed preclude the possibility that the observed start is due to the presence of an attenuator sequence or subsiduary promoter downstream of the main promoter, as seen sometimes in bloodstream expression sites (62), or of an RNA-processing site (52). Since there is no transcription upstream of the 1.22 transcription unit for at least 13 kb, and by analogy with previously characterized trypanosome promoters, it is reasonable to assume that the promoter for the M-VSG gene is present adjacent to the transcription initiation site and that the M-VSG gene is transcribed as a monocistronic transcription unit located close to the end of the chromosome.

Our studies of transcription of the M-VSG telomeric expression locus containing the ILTat 1.61 gene show a transcriptional start site also located 2.6 to 3.0 kb upstream of the monocistronic VSG gene transcription unit and a large transcriptional gap (>15 kb) on this telomere upstream of the VSG gene transcription unit (data not shown). We have shown previously that the 1.22 M-VSG gene is transcribed by an α-amanitin-insensitive RNA polymerase (29). This is also true for the 1.61 M-VSG gene (data not shown), as it is for all VSG genes (53).

Life cycle stage-specific transcription. Next, we investigated whether the putative 1.22 metacyclic promoter region could direct transcription at other life cycle stages by hybridizing *Eco*RI and *Bam*HI digests of the λMT1.22B clone (Fig. 2B) with ^{32}P-labelled nascent transcripts from procyclic trypanosomes (Fig. 2E). Hybridization was observed only to the restriction fragments of the lambda clone containing *ingi*-related sequences (fragments B3 and E5). No hybridization to the

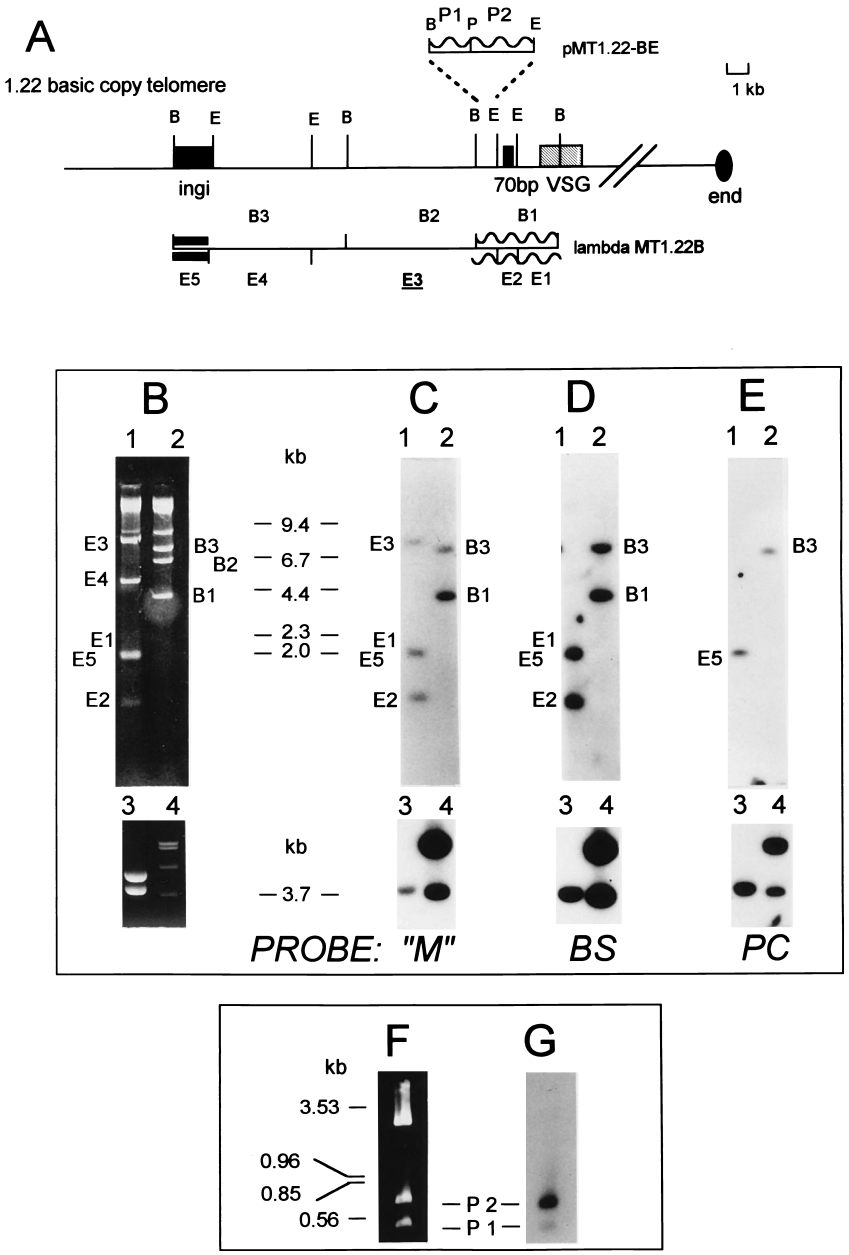


FIG. 2. Nuclear run-on analysis of transcription of the 1.22 M-VSG expression locus. (A) Restriction map of the 1.22 metacyclic expression telomere. The insert of the λ genomic clone derived from it, λMT1.22B, containing 18 kb of sequence homologous to the 1.22 VSG gene locus (44) is shown below, and the insert of the plasmid subclone pMT1.22-BE is shown above. *Eco*RI fragments of the λMT1.22B insert are numbered E1 to E5 and *Bam*HI insert fragments are numbered B1 to B3 in a 3′-to-5′ direction with respect to the telomere; plasmid subclone insert fragments are numbered P1 and P2. Wavy lines on the clone maps represent the M-VSG transcription unit fragments which hybridize in the experiments shown in panels C and G, and the heavy black bars represent hybridization to the partial *ingi* retroposon element in panels C, D, and E. Abbreviations: E, *Eco*RI; B, *Bam*HI; P, *Pst*I; 70bp, 70-bp repeat region; VSG, 1.22 M-VSG gene region; ingi, *ingi* retroposon sequence; end, end of the telomere. (B) Ethidium bromide-stained gel of *Eco*RI (lane 1) *Bam*HI (lane 2) restriction digests of λMT1.22B. Only the insert fragments are numbered. (C) Hybridization of ^{32}P-labelled nascent transcripts run on in vitro in nuclei isolated from metacyclic stage-derived trypanosomes expressing the 1.22 M-VSG gene in situ, to a Southern blot of the gel in panel B. (D) ^{32}P-labelled nascent transcripts run on in nuclei isolated from bloodstream trypanosomes expressing the 1.22 M-VSG gene by duplicative transposition to a bloodstream expression site (31, 44), hybridized to the same Southern blot following removal of the metacyclic stage-derived probe. (E) ^{32}P-labelled nascent transcripts run on in nuclei isolated from procyclic trypanosomes, which do not express the VSG gene, hybridized to the same blot following removal of the second, bloodstream-specific probe. The photograph at the bottom of panel B shows an ethidium bromide-stained gel containing *Hin*dIII digest of pTbαβ-T1, which contains an α- and β-tubulin repeat (67) (lane 3) and a *Pst*I digest of pR4, which contains a ribosomal DNA repeat unit (40) (lane 4). Tenfold less DNA was applied to lane 4 of the gel than to lane 3. The photographs beneath panels C, D, and E show the results of hybridizing a Southern blot of this gel with the same nascent RNA probes used in the blots above. (F) An ethidium bromide-stained gel of a *Bam*HI-*Pst*I/*Eco*RI triple digest of the plasmid subclone pMT1.22-BE. (G) ^{32}P-labelled nascent transcripts run on in nuclei isolated from a metacyclic stage-derived trypanosome clone expressing the 1.22 VSG gene in situ hybridized to a Southern blot of the gel in panel F. Hybridizations were in 3× SSC at 55°C, and blots were washed in 0.1× SSC at 65°C, except for the blot in panel E, which was washed at low stringency (3× SSC at 65°C).

transcriptional start region identified in metacyclic stage-derived trypanosomes (fragment E3) was detected nor was hybridization to the clone pMT1.22-BE, which contains the metacyclic transcriptional start site, detected (data not shown). Thus, the putative 1.22 M-VSG gene promoter is silent in the procyclic stage. The experiment was then repeated with nascent RNA from bloodstream trypanosomes. When 1.22 is activated in the bloodstream, the gene is duplicated into a bloodstream expression site (31, 44), with the copied segment stretching from the 70-bp repeat region at the 5′ side through the gene. When ^{32}P-labelled nascent transcripts from such a bloodstream trypanosome clone were hybridized to *Eco*RI and *Bam*HI fragments of the λMT1.22B clone (Fig. 2D), only the *ingi*-containing sequences (fragments B3 and E5) and those restriction fragments being transcribed from the duplicated copy in the bloodstream expression site, namely those (fragments E1, E2, and B1) containing the VSG gene and the 70-bp repeat region (Fig. 2A), hybridized. As before, fragments B2 and E4 did not hybridize but, most notably, fragment E3, which was transcribed in metacyclic stage-derived trypanosomes, did not hybridize in this experiment, showing that the putative metacyclic promoter region was transcriptionally silent in bloodstream trypanosomes. Again, no hybridization to the clone pMT1.22-BE, which contains the metacyclic stage-specific transcriptional start site, was detected (data not shown). In all run-on experiments, the efficiency of probe hybridization was monitored by simultaneous hybridization to blots of two plasmids, one containing an α- and β-tubulin gene repeat and the other containing a ribosomal DNA repeat unit (Fig. 2C to E, lanes 3 and 4). Tenfold fewer cells were used in the metacyclic stage-derived experiments than in the procyclic and bloodstream experiments. The level of hybridization to the ribosomal control plasmid in the procyclic experiment was reduced, as noted previously (41).

We have identified a transcriptional start and located the putative promoter for an M-VSG gene which operates specifically at the metacyclic stage: it is inactive in the procyclic and bloodstream stages. Corresponding results of a similar series of experiments examining transcription of the 1.61 M-VSG gene during the life cycle lead to very similar conclusions, i.e., that M-VSG genes are under transcriptional regulation during the trypanosome life cycle.

Metacyclic VSG gene promoters. To test for promoter function and because our finding of the apparent transcriptional silence of the M-VSG gene putative promoter in the procyclic form contrasted with the reported activity of bloodstream VSG gene promoters (37, 51, 55, 77–79), we assayed the ability of the telomere region around the putative start site for the 1.22 gene to direct transcription of the CAT reporter gene, by transient transfection (7) of procyclic and metacyclic stage-derived trypanosomes. For transient transfection of procyclic trypanosomes, we used CAT constructs with the trypanosome actin gene splice acceptor sequence and 3′ untranslated region and constructs with the PARP gene splice acceptor sequence and 3′ untranslated region in case different RNA-processing signals had a significant effect on expression of the reporter gene from the metacyclic promoter. For transient transfection of metacyclic stage-derived trypanosomes, we used only the constructs containing actin RNA-processing signals, since these yield greater efficiency of CAT expression in bloodstream trypanosomes than do those containing PARP RNA-processing elements (35) (the metacyclic stage-derived populations are morphologically bloodstream forms) and we obtained too few metacyclic stage-derived trypanosomes to allow us to compare the levels of CAT expression using both the actin and PARP-based constructs.

As positive controls for transient transfection of procyclic trypanosomes, we used two previously characterized trypanosome promoters: (i) the PARP promoter (63) either in the construct p5′parpHD52CAT, which contains an actin splice acceptor sequence and actin gene 3′ untranslated region or in the construct p5′parpCAT3′parp (45), which contains a PARP splice acceptor site and PARP 3′ untranslated region, and (ii) the 221 bloodstream VSG expression site promoter (79) either in the construct pHD52CAT which contains an actin splice acceptor signal and an actin 3′ untranslated region or in the construct p221CAT3′parp which contains PARP RNA-processing signals. As negative controls, we deleted the promoter regions from the constructs p5′parpCAT3′parp and pHD52 CAT but left the splice acceptor sites intact (p-CAT3′parp, p-HD52CAT). To assay the activity of the putative metacyclic VSG gene promoter, we replaced the promoter regions in p5′parpCAT3′parp and pHD52CAT with a 400-bp fragment (shown in Fig. 1A as the insert in plasmid pMT1.22-BPs) derived from the telomere region containing the transcriptional start site for the 1.22 M-VSG gene to create p1.22s CAT3′parp and p122sHD52CAT. Similar levels of CAT activity were obtained with each pair of constructs (data not shown); the nature of the RNA-processing signals used did not seem to affect CAT expression significantly in the procyclic transient-transfection experiments as previously reported (35). Only the results with the actin-based constructs are presented in Fig. 3B since it was these plasmids which we used subsequently to transiently transfect metacyclic stage-derived trypanosomes. Transient transfection of procyclic cells revealed that while the PARP (data not shown) or bloodstream expression site promoters in the positive controls yielded similar, high levels of CAT activity (86.9×10^3 dpm/3×10^6 cells), the construct containing the M-VSG gene putative promoter gave close to background levels (0.8×10^3 dpm/3×10^6 cells; $6\% \pm 1\%$ of the positive controls) (Fig. 3B). In case the 400-bp segment of the 1.22 putative promoter region used in these experiments did not include all the sequences directing transcription, we also assayed a longer fragment from the 1.22 metacyclic expression locus which encompassed the shorter fragment and included the next 1.6 kb of sequence upstream (the inserted DNA in the construct pMT1.22HPl [Fig. 1A]). Only background levels of CAT activity very similar to those obtained with the promoterless negative control were obtained (data not shown). Our results confirm that unlike bloodstream expression site promoters (37, 51, 55, 77–79), the putative metacyclic promoter does not function effectively in procyclic trypanosomes. These promoter-containing constructs are intact, as we have verified by sequence analysis.

For transient transfection of metacyclic stage-derived trypanosomes, we used only the actin-based constructs (pHD52 CAT, p-HD52CAT, and p122sHD52CAT [Fig. 3A]). As bloodstream VSG expression sites become activated in bloodstream trypanosomes between days 6 and 7 following the infecting fly feed (31), we assayed metacyclic promoter activity in a background of metacyclic stage-specific VSG gene transcription at day 5 following fly feed when no variants are detectable (31). Relatively few trypanosomes were available at this early stage of infection (3×10^6 maximum), and the efficiency of transient transfection of bloodstream trypanosomes and presumably of metacyclic stage-derived trypanosomes is much lower than that for procyclic culture forms (10- to 20-fold in our experiments) (12, 35). Despite this, we found that in six separate experiments with the day 5 metacyclic stage-derived cell populations, the 1.22 short-version putative promoter fragment construct, p1.22sHD52CAT, which gave low levels of CAT expression in procyclic transient-transfection experiments in comparison

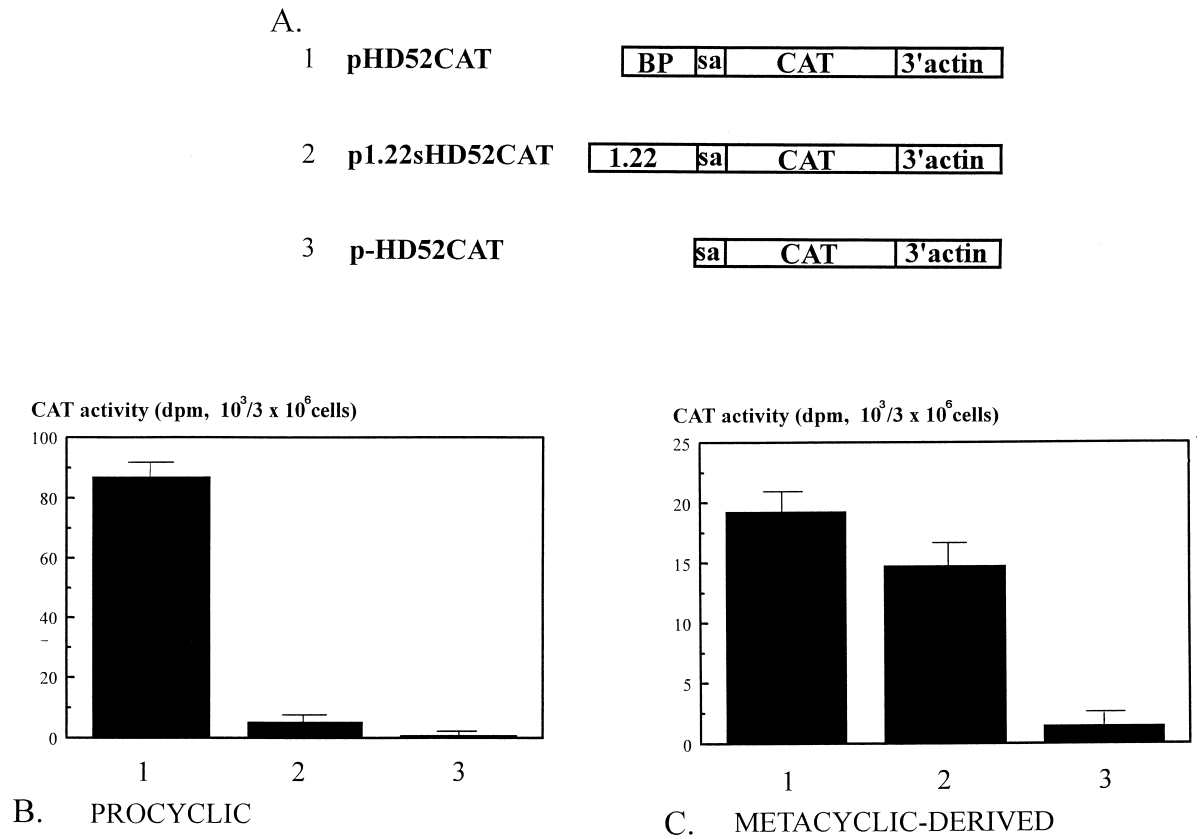


FIG. 3. Functional analysis of a putative metacyclic VSG gene promoter in procyclic and metacyclic stage-derived trypanosomes. A 400-bp fragment of the putative promoter region for the 1.22 M-VSG gene (the insert contained in the plasmid pMT1.22BPs) was assayed for the ability to direct transcription of the CAT gene in transient transfection of procyclic and metacyclic stage-derived trypanosomes. (A) Schematic illustrations of the plasmids assayed. pHD52CAT, bloodstream expression site promoter positive control; p1.22sHD52CAT, the test construct; p-HD52CAT, promoterless negative control. Construction of these plasmids is described in Materials and Methods. (B) Assay in procyclic cells. The bar chart shows CAT activity in 10^3 disintegrations per minute per 3×10^6 cells for each construct in procyclic transient transfections. (C) Assay in metacyclic stage-derived cells. The bar chart shows CAT activity in 10^3 disintegrations per minute per 3×10^6 cells for each construct in metacyclic stage-derived transient transfections. Values for each bar chart are the mean and standard deviation of six separate experiments. Abbreviations: sa, splice acceptor region; CAT, CAT coding sequence; BP, bloodstream VSG gene expression site promoter; 1.22, 1.22M-VSG gene putative promoter region.

with the bloodstream expression site promoter, gave a CAT activity of 14.7×10^3 dpm/3×10^6 cells, which was 76% ± 4% of that obtained with the bloodstream expression site promoter in pHD52CAT (19.2×10^3 dpm/3×10^6 cells) in the same experiment (Fig. 3C). The level of CAT activity obtained with the 221 bloodstream expression site promoter in metacyclic stage-derived trypanosomes was very similar to what we obtained with the same construct in transient-transfection experiments in true bloodstream trypanosomes (data not shown). In the same experiments, the 1.22 short-version putative promoter directed CAT expression more efficiently than did the bloodstream expression site promoter (1.9- ± 0.5-fold). However, when placed in a chromosomal context, the promoter was inactive (76). Transient transfection of uninfected mouse blood with the positive and negative test constructs (pHD52CAT, p-HD52CAT) gave background levels of CAT activity (data not shown). Our results show that a region of DNA encompassing the transcriptional start site for the 1.22 M-VSG gene can direct CAT expression to a level only slightly lower than that obtained with the well-characterized 221 bloodstream expression site promoter when metacyclic stage-derived trypanosomes were used but that the same sequence can drive expression of the CAT reporter gene in procyclic trypanosomes to a level less than 10% of that obtained with the bloodstream expression site promoter. These results support our conclusion that the 1.22 promoter region is activated during the trypanosome life cycle specifically at the metacyclic stage in the fly.

To verify promoter activity during transient transfections, we performed 3′RT-PCR to identify CAT RNA specifically. The PCR used a nested set of CAT gene-specific oligonucleotides (see Materials and Methods) to detect CAT RNA expressed from the putative 1.22 metacyclic promoter in transiently transfected day 5 metacyclic stage-derived trypanosomes. As the plasmids used to transiently transfect metacyclic stage-derived cells were the actin-based constructs (Fig. 3A), specific PCR products contained an actin 3′ untranslated region and could be detected by hybridizing a Southern blot of the gel-fractionated PCR products with an actin probe (pActine: 1.9-kb *Sal*I-*Bgl*II fragment [8]) to detect CAT-actin chimeric amplified cDNAs. Figure 4 shows that while no CAT-actin transcripts were detected by RT-PCR in cells transfected with the negative control (p-HD52CAT; lane 4) transient transfection with p1.22sHD52CAT specifically yielded a 315-bp RT-PCR product (lane 5) very similar to that obtained with the positive control (lane 3). RNA from mock-transfected cells (lane 1) and RNA from cells transfected with the positive control plasmid but not treated with RT (lane 2) each gave no detectable

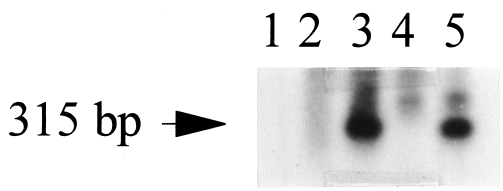


FIG. 4. Detection of CAT transcripts in transiently transfected, metacyclic stage-derived trypanosomes by PCR. Shown are the results of a Southern blot analysis of PCR-amplified cDNAs derived from total RNA from day 5 metacyclic stage-derived trypanosomes transiently transfected with the constructs shown in Fig. 3A. Lanes: 1, PCR-amplified cDNA synthesized from RNA from mock-transfected metacyclic stage-derived trypanosomes; 2, PCR-amplified cDNA from RNA from metacyclic stage-derived cells transiently transfected with pHD52CAT but not incubated with RT for first-strand cDNA synthesis; 3, as for 2 but in the presence of RT to give the positive control; 4, PCR-amplified cDNA from RNA from metacyclic stage-derived cells transiently transfected with the negative control (p-HD52CAT); 5, PCR-amplified cDNA from RNA from metacyclic stage-derived cells transiently transfected with the test construct (p1.22sHD52CAT). PCR products were separated by gel electrophoresis, and the gel was Southern blotted. The Southern blot was hybridized to an actin genomic probe, pActine (8), to detect the amplified 3′ ends of the mRNAs transcribed from the transiently transfected constructs. Hybridization was in 5× SSC at 65°C, and the blot was washed in 0.1× SSC at 65°C.

RT-PCR product. These results verify that the 400-bp fragment containing the transcriptional start site for the 1.22 M-VSG gene can drive expression of a CAT reporter gene in metacyclic stage-derived trypanosomes.

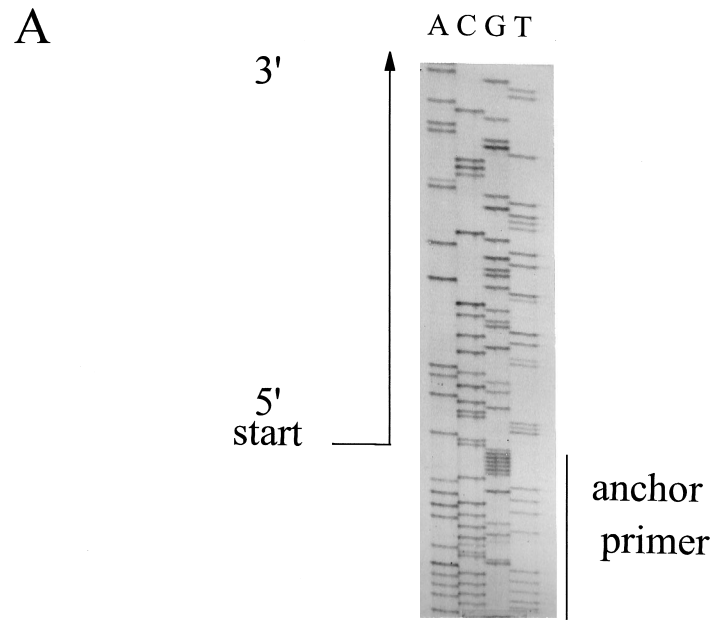
Initial attempts to map the transcriptional start site of the 1.22 M-VSG gene by using RNase protection were thwarted by the experimental difficulties with tsetse-transmitted trypanosomes: too few trypanosomes and too few clones were available to carry out sufficient experiments to obtain conclusive results. However, we were able to achieve this by applying 5′RT-PCR to total RNA isolated from day 5 metacyclic stage-derived trypanosomes transiently transfected with p1.22sHD 52CAT. The oligonucleotide used to direct first-strand cDNA synthesis was a 25-mer homologous to nucleotides 190 to 215 downstream of the start of the CAT coding region; then two further oligonucleotides were used to prime two subsequent rounds of PCR, and these are illustrated in Fig. 5B, which shows the sequence of the putative proximal promoter region for the 1.22 gene. A single major PCR product was obtained, and amplified DNAs were cloned and sequenced. The sequencing autoradiogram in Fig. 5A shows the sequence of the anchor primer and, immediately adjacent, the initiation site and downstream region in the primary transcript from one of the cloned PCR products. All eight clones sequenced gave the same initiation site corresponding to a G residue or the C residue next to it located at 2,828 or 2,829 bp 5′ of the VSG gene and indicated in Fig. 5B. It is not possible to be precise about the exact start site, since the 5′ anchor used in the 5′RT-PCR ends in a run of G residues, but there is a G residue in the sequence of the promoter region located exactly at the start site. It is possible that the observed start is specific to transcription from transiently transfected constructs; however, in a single RNase protection study with total RNA isolated from a metacyclic stage-derived trypanosome clone (93% expressors of 1.22), a start site located within 5 bp of the start site described above was obtained (data not shown).

DISCUSSION

Metacyclic stage-specific gene expression. As the metacyclic stage of *T. brucei* develops in the salivary glands of the tsetse fly, it assumes a number of preadaptations that enable it to survive transmission to the mammal and to initiate infection (75). The principal preadaptation is the acquisition of the VSG surface coat, which is necessary for the parasite to tackle both the nonspecific and specific immune mechanisms of the mammal (18). When synthesis of the surface coat initiates in the metacyclic stage, a specific subset of VSG genes is activated, giving rise to a mixture of variable antigen types, which we believe strongly enhances the probability of this population being transmitted in the field into reservoir animals already partially immune to the VSG repertoires of local trypanosomes (6). We have investigated activation and regulation of expression of M-VSG genes and have uncovered a system radically different from that used for VSG activation and expression in the bloodstream. Bloodstream VSG genes are activated (usually) by duplicative transposition and are expressed from long, complex polycistronic transcription units (20, 41, 52) whose expression is directed by promoters which do not appear to be transcriptionally regulated in a life cycle stage-dependent manner (37, 51, 55, 79). In contrast, at the metacyclic stage, M-VSG genes are activated in situ (i.e., without duplicative transposition) at the telomeres of the largest set of trypanosome chromosomes (17, 31, 43). These telomeric regions are transcriptionally silent apart from the M-VSG gene itself, and M-VSG genes are expressed as monocistronic transcription units which are under transcriptional regulation during the parasite life cycle. Moreover, these features of M-VSG gene expression are unusual in trypanosomes, in which all genes examined thus far are organized in polycistronic transcription units and are under the control of promoters which are constitutively active at least at some level during the life cycle (14, 28).

We have studied transcription of two telomeres carrying M-VSG genes that are activated in situ in the metacyclic stage. Both telomeres are unusual in that they contain long stretches devoid of coding sequences, whereas other telomeric and nontelomeric regions of the large and intermediate-size chromosomes studied in trypanosomes so far are densely packed with genes (14). Nuclear run-on analyses in the very small numbers of nuclei available from metacyclic stage-derived trypanosome clones have allowed us to determine that transcription initiates at the same location on both telomeres: 2.6 to 3.0 kb from the 5′ end of the VSG gene (for the 1.22 MSG gene, the exact initiation site determined by 5′RT-PCR is located 2,828 or 2,829 bp upstream of the VSG gene). Initiation within these regions is confirmed by our failure to detect transcription outside the M-VSG gene transcription units on the remainder of the cloned regions of the metacyclic expression telomeres (13 kb for the 1.22 M-VSG gene; 15 kb for the 1.61 M-VSG gene [28a]). Although the promoters are adjacent to the genes, the 2.6- to 3.0-kb gap between the transcription initiation site and the 5′ end of the VSG gene is reasonably large, but this may be due, in part, to the inclusion of two important sequences—a pyrimidine-rich sequence upstream of the gene (34), which may direct transcript *trans*-splicing, and a short region of 70-bp repeats (2) that may act as the 5′ limit of gene conversion when these genes are duplicated into the bloodstream expression site during activation in bloodstream trypanosomes (44). However, this region could include further sequences essential for directing correct regulation of M-VSG gene expression or as a spacer to ensure the correct distance between the promoter region and the end of the chromosome.

Early phenotypic studies led to the prediction that the expression systems for bloodstream and metacyclic VSG genes would differ from each other (70). Bloodstream VSG genes are transcribed from a promoter which is located 40 to 60 kb upstream and are cotranscribed with a series of at least eight different ESAGs (53) whose final steady-state mRNA levels



B

Bam HI

1 **GGATCC**CTATAGAGTATCAGAATATCTCAAGACGAGTTTCCTTGCTTAAAGCCGACATCC

61 GTACCCAAAAAGAATAAGTTGTAGTAAGTGTATTTCTCGCTGTTCTGACTCTTAGGGTTT

+1

121 TAGATTTCGTGCCGAGGACAGAATGGGAAACCATCGTCTATGGGCTCATCGCCATTTCCG

181 CAGCGACATTCGTCTGGCGCTTGAGGTGTAGCTTTGTGAACCCTGGAAGCATTGAAAGT

241 CTACCGTATTTTCCGCCAACTACTCGGAGGCCCGGACCTGGCAGGCAATTTTCGATGTGA

301 AACCTTGTCAATGCGCTCGCAATACGTGTCTTGTCAGCGTGCGACTCCGCAAGCCTGGCC

361 CATGCGACTCGACCGCGGGGGTAGTGGCATTGATAGAGGTGGCGGCGATGCTTTTCTCCG

Pst I

421 **CTGCAG**

FIG. 5. Identification of the transcription start site for the 1.22 M-VSG gene. (A) Sequence of the junction of the 5′RT-PCR anchor primer ending with the oligonucleotide G anchor, followed by the start of the primary transcript for the CAT gene driven by the 1.22 M-VSG gene promoter/transcription initiation region (the insert in the plasmid subclone pMT1.22-BPs), in RNA isolated from day 5 metacyclic stage-derived cells transiently transfected with p1.22sHD52CAT. "5′ start" indicates the G or C nucleotide with which the transcript initiates, and the direction of transcription runs upward as indicated by the arrow. (B) Sequence of the DNA insert in plasmid pMT1.22-BPs, showing the transcription initiation site (arrowhead, +1) and the sequences of the two primers GSP1 and GSP2 (arrows) used to amplify the 5′ end of the primary transcript produced from the transiently transfected construct, p1.22sHD52CAT. The grey boxed region indicates the extent of the sequence displayed in the autoradiograph above.

are 100-fold lower than those for the VSG (41). However, differentiation stage-specific regulation of the bloodstream expression site is mediated after transcription initiation: when the bloodstream stages develop to the procyclic stage, cessation of VSG synthesis is achieved not by bloodstream promoter inactivation but by disruption of RNA elongation and processing (16, 50, 51, 55, 79). Our nuclear run-on analyses have shown that metacyclic promoters direct transcription only in metacyclic stage-derived trypanosomes and not in procyclic or bloodstream forms. Moreover, the putative promoter region from the 1.22 M-VSG gene telomere failed to direct expression of a CAT reporter gene in transient-transfection assays in procyclic trypanosomes to the level obtained with either the PARP or 221 bloodstream expression site promoters, but the putative promoter was active to a level of 76% that of the bloodstream expression site promoter in metacyclic stage-derived trypanosomes. The levels of CAT activity which we observed in our metacyclic stage-derived transient-transfection experiments were very similar to those observed for bloodstream expression site promoter regions in similar transfection studies (37, 77, 78). It has been suggested that high levels of activity of bloodstream expression site promoters in the pro-

cyclic culture form may represent an escape from tight control in a chromosomal context (79). If this is so, our data suggest that metacyclic VSG gene promoters are under more stringent control than bloodstream VSG gene promoters.

Recently, it has been reported that another M-VSG gene, encoding the MVAT4 VSG, can be activated in situ in bloodstream trypanosomes and expressed as a monocistronic transcription unit from an adjacent promoter. Nothing is reported about its activation and expression in the metacyclic stage, but results imply that the MVAT4 promoter is not stage regulated and the gene must therefore be under posttranscriptional regulation during the trypanosome life cycle (1). This is different from what we have observed for the 1.22 M-VSG gene promoter and what we predict for metacyclic promoters in general (6, 70). It should be borne in mind that the MVAT4-expressing trypanosomes are rare, having required very extensive selection in the laboratory (1). Rudenko et al. (55) have shown that transcription of VSG genes in bloodstream expression sites proceeds from a promoter whose regulation depends on a combination of its own structure and its location. This suggests that the MVAT4 expression in bloodstream trypanosomes is fortuitous and is not related to the true, stage-regulated M-VSG expression we have studied. Whether the MVAT4 promoter is a genuine metacyclic promoter in the correct chromosomal context and correct life cycle stage remains to be demonstrated. The M-VSG genes we have studied represent another class of VSG genes; to our knowledge, they are the first trypanosome VSG genes, indeed the first trypanosome genes, found to be under true transcriptional regulation during the parasite life cycle.

Why transcriptional regulation? Why do the M-VSG gene expression loci display these two main deviations from what has been seen as the norm in trypanosomes, namely, monocistronic transcription units and transcriptional activation? We believe that there may be a common reason, driven by the selective pressure exerted on the parasite by its mammalian host population. In the bloodstream phase, antigenic variation operates as a function of time, the objective being to switch the VSG periodically, in concert with the ongoing waves of anti-VSG antibody. The predicament of the metacyclic population is fundamentally different. As a preadapted stage whose purpose is to infect the mammal, the critical factor is the moment of entry into the new host: time is not relevant. We have argued elsewhere (6) that metacyclic trypanosomes are likely to encounter anti-VSG antibody on entering their usual host, i.e., domestic and game animals, which are probably capable of overcoming trypanosome infection and self-curing by generating antibodies against the entire VSG repertoire of local trypanosomes (5, 48). Only as antibodies against individual VSGs wane can the metacyclic population gain entry (6). Transmission of the parasite would therefore be greatly enhanced by the metacyclic population expressing a mixture of VSGs rather than just one. This necessitates a stochastic activation mechanism for M-VSG genes, with one gene being activated at random in each cell, a situation which has indeed been observed directly in tsetse fly salivary glands (66). To achieve random activation, a simple transcriptional switch is likely to be easier to control than differential expression at the posttranscriptional level. The organization of metacyclic VSG genes in simple transcription units may facilitate this mechanism. In addition, if the bloodstream and metacyclic VSG expression systems are to remain functionally distinct, there is likely to be a restriction on the amount of recombination, and hence DNA sequence homology, between them. This may be why metacyclic expression telomeres lack most ESAGs and have very short, or no, 70-bp repeat regions (43, 44); it may also be one reason why the VSG gene is transcribed as a monocistronic transcription unit.

Monocistronic expression of M-VSG genes means that any ESAG expression must occur from elsewhere in the genome, as we have observed (29). This may happen because it would be more economical to maintain one or a few sets of expressible ESAGs outside the metacyclic expression site, rather than have up to 27 separate sets adjacent to the M-VSG genes to ensure expression in the metacyclic stage. The ESAGs may therefore be subject to coordinate expression by another control system at the metacyclic stage. An indication of this type of control comes from study of ESAGs 6 and 7, which together encode a transferrin-binding protein that is present in the bloodstream but not in the procyclic stage (57, 65). ESAG 6 and 7 mRNAs are absent in the procyclic stage because of termination of transcript elongation within the gene (51) or termination 5′ of the coding regions (55, 79). However, in metacyclic stage-derived organisms, transcripts homologous to ESAG 6 and 7 DNA probes are detected at a level similar to that seen in bloodstream parasites (29). This implies that if the ESAG 6 and 7 mRNAs are being transcribed from one or several bloodstream expression sites, their up-regulation does not occur at the level of transcription initiation at the metacyclic stage. If so, two gene control mechanisms come into operation as the metacyclic stage develops: transcriptional for the M-VSG genes and posttranscriptional for at least one other gene.

Despite the fundamental structural and functional differences between the metacyclic and bloodstream expression sites, there is one major similarity. In both bloodstream and metacyclic form trypanosomes, only one telomere is active at a time. Exclusive expression of bloodstream expression sites has been discussed at length elsewhere (53, 72), and a favored proposal is that it may arise from their (sub)telomeric location, via interaction with nuclear substructure, a belief afforded some evidence from cytological study (13). Inactive bloodstream expression sites in bloodstream stage trypanosomes include a modified nucleotide that is not detectable in the procyclic stage and is found only toward telomeres (24, 25). Thus, it may be that bloodstream expression sites compete for a single site in the nuclear skeleton and that outcompeted telomeres become modified. There is no reason why these ideas may not also generally apply, wholly or partly, to exclusive expression in the metacyclic stage. A single nuclear site could also be the source of specific *trans*-acting transcriptional initiation factors. Finally, our finding that two promoters subject to programmed transcriptional activation are located at telomeres is intriguing, because chromosome ends in other eukaryotes are associated with position effect variegation, the reversible transcriptional repression of genes (9, 59). As discussed previously, trypanosomes appear to use predominantly posttranscriptional regulation of gene expression, and in fact there is as yet no evidence for other genes being regulated solely by transcriptional activation during the life cycle (14, 28). What arises from these observations is the intriguing possibility that in a system committed to RNA-based control mechanisms, a special set of telomeres, with their position effect, have been commandeered to help provide differentiation stage-specific transcriptional control.

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REFERENCES

1. **Alarcon, C. M., H. J. Son, T. Hall, and J. E. Donelson.** 1994. A monocistronic transcript for a trypanosome variant surface glycoprotein. Mol. Cell. Biol. **14:**5579–5591.
2. **Aline, R., G. Macdonald, E. Brown, J. Allison, P. Myler, V. Rothwell, and K. Stuart.** 1985. (TAA)n within sequences flanking several intrachromosomal variant surface glycoprotein genes in *Trypanosoma brucei*. Nucleic Acids Res. **13:**3161–3177.
3. **Auffray, C., and F. Rougeon.** 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumour RNA. Eur. J. Biochem. **107:**303–314.
4. **Barry, J. D.** 1986. The molecular biology of African trypanosomes. Trop. Dis. Bull. **83:**R1–R25.
5. **Barry, J. D.** 1986. Antigenic variation during *Trypanosoma vivax* infections of different host species. Parasitology **92:**51–65.
6. **Barry, J. D., S. V. Graham, K. R. Matthews, P. G. Shiels, and O. A. Shonekan.** 1990. Stage-specific mechanisms for activation and expression of variant surface glycoprotein genes in *Trypanosoma brucei*. Biochem. Soc. Trans. **18:**708–710.
7. **Bellofatto, V., and G. A. M. Cross.** 1989. Expression of a bacterial gene in a trypanosomatid protozoan. Science **244:**1167–1169.
8. **Ben Amar, M. F., A. Pays, P. Tebabi, B. Dero, T. Seebeck, M. Steinert, and E. Pays.** 1988. Structure and transcription of the actin gene of *Trypanosoma brucei*. Mol. Cell. Biol. **8:**2166–2176.
9. **Blackburn, E. H.** 1991. Structure and function of telomeres. Nature (London) **350:**569–573.
10. **Blackburn, E. H., and P. B. Challoner.** 1984. Identification of a telomeric DNA sequence in *Trypanosoma brucei*. Cell **36:**447–457.
11. **Brun, R., and M. Schonenberger.** 1979. Cultivation and *in vitro* cloning of procyclic forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop. **36:**289–292.
12. **Carruthers, V. B., L. H. T. Van der Ploeg, and G. A. M. Cross.** 1993. DNA-mediated transformation of bloodstream-form *Trypanosoma brucei*. Nucleic Acids Res. **21:**2537–2538.
13. **Chung, H. M., C. Shea, S. Fields, R. N. Taub, and L. H. T. Van der Ploeg.** 1990. Architectural organisation in the interphase nucleus of the protozoan *Trypanosoma brucei*: location of telomeres and mini-chromosomes. EMBO J. **9:**2611–2619.
14. **Clayton, C.** 1992. Developmental regulation of nuclear gene expression in *Trypanosoma brucei*. Prog. Nucleic Acid Res. Mol. Biol. **43:**37–65.
15. **Clayton, C. E., J. P. Fueri, J. E. Itzhaki, V. Bellofatto, D. R. Sherman, G. S. Wisdom, S. Vijayasarathy, and M. R. Mowatt.** 1990. Transcription of the procyclic acidic repetitive protein genes of *Trypanosoma brucei*. Mol. Cell. Biol. **10:**3036–3047.
16. **Coquelet, H., M. Steinert, and E. Pays.** 1991. Ultraviolet-irradiation inhibits RNA decay and modifies ribosomal-RNA processing in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **44:**33–42.
17. **Cornelissen, A. W. C. A., G. A. M. Bakkeren, J. D. Barry, P. A. M. Michels, and P. Borst.** 1985. Characteristics of trypanosome variant antigen genes active in the tsetse fly. Nucleic Acids Res. **13:**4661–4676.
18. **Cross, G. A. M.** 1990. Cellular and genetic aspects of antigenic variation in trypanosomes. Annu. Rev. Immunol. **8:**83–110.
19. **Crowe, J. S., J. D. Barry, A. G. Luckins, C. A. Ross, and K. Vickerman.** 1983. All metacyclic variable antigen types of *Trypanosoma congolense* identified using monoclonal-antibodies. Nature (London) **306:**389–391.
20. **Crozatier, M., L. H. T. Van der Ploeg, P. J. Johnson, J. Gommers-Ampt, and P. Borst.** 1990. Structure of a telomeric expression site for variant specific surface antigens in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **42:**1–12.
21. **Cully, D. F., H. S. Ip, and G. A. M. Cross.** 1985. Coordinate transcription of variant surface glycoprotein genes and an expression site associated gene family in *Trypanosoma brucei*. Cell **42:**173–182.
22. **Esser, K. M., M. J. Schoenbechler, and J. B. Gingrich.** 1982. *Trypanosoma rhodesiense* blood forms express all antigen specificities relevant to protection against metacyclic (insect form) challenge. J. Immunol. **129:**1715–1718.
23. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
24. **Gommers-Ampt, J., J. Lutgerink, and P. Borst.** 1991. A novel DNA nucleotide in *Trypanosoma brucei* only present in the mammalian phase of the life-cycle. Nucleic Acids Res. **19:**1745–1751.
25. **Gommers-Ampt, J. H., A. J. R. Teixeira, G. Van der Werken, W. J. Van Dijk, and P. Borst.** 1993. The identification of hydroxymethyluracil in DNA of *Trypanosoma brucei*. Nucleic Acids Res. **21:**2039–2043.
26. **Gottesdiener, K., H. Chung, S. D. Brown, M. G.-S. Lee, and L. H. T. van der Ploeg.** 1991. Characterization of VSG gene expression site promoters and promoter-associated DNA rearrangement events. Mol. Cell. Biol. **11:**2467–2480.
27. **Grab, D. J., and J. J. Bwayo.** 1982. Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*. Acta Trop. **39:**363–366.
28. **Graham, S. V.** 1995. Mechanisms of stage-regulated gene expression in Kinetoplastida. Parasitol. Today **11:**217–223.

28a. **Graham, S. V.** Unpublished results.

29. **Graham, S. V., and J. D. Barry.** 1991. Expression site-associated genes transcribed independently of variant surface glycoprotein genes in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **47:**31–41.
30. **Graham, S. V., K. R. Matthews, and J. D. Barry.** 1993. *Trypanosoma brucei*: unusual expression-site-associated gene homologies in a metacyclic VSG gene expression site. Exp. Parasitol. **76:**96–99.
31. **Graham, S. V., K. R. Matthews, P. G. Shiels, and J. D. Barry.** 1990. Distinct, developmental stage-specific activation mechanisms of trypanosome VSG genes. Parasitology **101:**361–367.
32. **Hajduk, S. L., C. R. Cameron, J. D. Barry, and K. Vickerman.** 1981. Antigenic variation in cyclically transmitted *Trypanosoma brucei*. Variable antigen type composition of metacyclic trypanosome populations from the salivary glands of *Glossina morsitans*. Parasitology **83:**595–607.
33. **Hajduk, S. L., and K. Vickerman.** 1981. Antigenic variation in cyclically transmitted *Trypanosoma brucei*. Variable antigen type composition of the first parasitaemia in mice bitten by trypanosome-infected *Glossina morsitans*. Parasitology **83:**609–621.
34. **Huang, J., and L. H. T. Van der Ploeg.** 1991. Requirement of a polypyrimidine tract for *trans*-splicing in trypanosomes—discriminating the PARP promoter from the immediately adjacent 3′ splice acceptor site. EMBO J. **10:**3877–3885.
35. **Hug, M., V. B. Carruthers, C. Hartmann, D. S. Sherman, G. A. M. Cross, and C. Clayton.** 1993. A possible role for the 3′-untranslated region in developmental regulation in *Trypanosoma brucei*. Mol. Biochem. Parasitol. **61:**87–96.
36. **Hug, M., H.-R. Hotz, C. Hartmann, and C. Clayton.** 1994. Hierarchies of RNA-processing signals in a trypanosome surface antigen mRNA precursor. Mol. Cell. Biol. **14:**7428–7435.
37. **Jefferies, D., P. Tebabi, and E. Pays.** 1991. Transient activity assays of the *Trypanosoma brucei* variant surface glycoprotein gene promoter—control of gene expression at the posttranscriptional level. Mol. Cell. Biol. **11:**338–343.
38. **Johnson, P. J., J. M. Kooter, and P. Borst.** 1987. Inactivation of transcription by UV irradiation of *T. brucei* provides evidence for a multicistronic transcription unit including a VSG gene. Cell **51:**273–281.
39. **Kimmel, B. E., O. K. Olemoiyoi, and J. R. Young.** 1987. *Ingi*, a 5.2-kb dispersed sequence element from *Trypanosoma brucei* that carries half of a smaller mobile element at either end and has homology with mammalian LINEs. Mol. Cell. Biol. **7:**1465–1475.
40. **Kooter, J. M., and P. Borst.** 1984. Alpha-amanitin insensitive transcription of variant surface glycoprotein genes provides further evidence for discontinuous transcription in trypanosomes. Nucleic Acids Res. **12:**9457–9472.
41. **Kooter, J. M., H. J. van der Spek, R. Wagter, C. E. d'Oliveira, F. van der Hoeven, P. J. Johnson, and P. Borst.** 1987. The anatomy and transcription of a telomeric expression site for variant-specific surface antigens in *T. brucei*. Cell **51:**261–272.
42. **LeBowitz, J. H., H. Q. Smith, L. Rusche, and S. M. Beverley.** 1993. Coupling of poly(A) site selection and *trans*-splicing in *Leishmania*. Genes Dev. **7:**996–1007.
43. **Lenardo, M. J., K. M. Esser, A. M. Moon, L. H. T. Van der Ploeg, and J. E. Donelson.** 1986. Metacyclic variant surface glycoprotein genes of *Trypanosoma brucei* subsp. *rhodesiense* are activated in situ, and their expression is transcriptionally regulated. Mol. Cell. Biol. **6:**1991–1997.
44. **Matthews, K. R., P. G. Shiels, S. V. Graham, C. Cowan, and J. D. Barry.** 1990. Duplicative activation mechanisms of two trypanosome telomeric VSG genes with structurally simple 5′ flanks. Nucleic Acids Res. **18:**7219–7227.
45. **Matthews, K. R., C. Tschudi, and E. Ullu.** 1994. A common pyrimidine-rich motif governs *trans*-splicing and polyadenylation of tubulin polycistronic pre-mRNA in trypanosomes. Genes Dev. **8:**491–501.
46. **Mowatt, M. R., and C. E. Clayton.** 1987. Developmental regulation of a novel repetitive protein of *Trypanosoma brucei*. Mol. Cell. Biol. **7:**2838–2844.
47. **Murphy, N. B., A. Pays, P. Tebabi, H. Coquelet, M. Guyaux, M. Steinert, and E. Pays.** 1987. *Trypanosoma brucei* repeated element with unusual structural and transcriptional properties. J. Mol. Biol. **195:**855–871.
48. **Nantulya, V. M., A. J. Musoke, F. R. Rurangirwa, and S. K. Moloo.** 1984. Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei* and *Trypanosoma congolense* after spontaneous recovery from syringe-passaged infections. Infect. Immun. **43:**735–738.
49. **Pays, E.** 1989. Pseudogenes, chimaeric genes and the timing of antigenic variation in African trypanosomes. Trends Genet. **5:**389–391.
50. **Pays, E., H. Coquelet, A. Pays, P. Tebabi, and M. Steinert.** 1989. *Trypanosoma brucei*—posttranscriptional control of the variable surface glycoprotein gene expression site. Mol. Cell. Biol. **9:**4018–4021.
51. **Pays, E., H. Coquelet, P. Tebabi, A. Pays, D. Jefferies, M. Steinert, E. Koenig, R. O. Williams, and I. Roditi.** 1990. *Trypanosoma brucei*: constitutive activity of the VSG and procyclin gene promoters. EMBO J. **9:**3145–3151.
52. **Pays, E., P. Tebabi, A. Pays, H. Coquelet, P. Revelard, D. Salmon, and M. Steinert.** 1989. The genes and transcripts of an antigen gene expression site

from *T. brucei*. Cell **57:**835–845.
53. **Pays, E., L. Vanhamme, and M. Berberof.** 1994. Genetic controls for the expression of surface antigens in African trypanosomes. Annu. Rev. Microbiol. **48:**25–52.
54. **Roditi, I., M. Carrington, and M. Turner.** 1987. Expression of a polypeptide containing a dipeptide repeat is confined to the insect stage of *Trypanosoma brucei*. Nature (London) **325:**272–274.
55. **Rudenko, G., P. A. Blundell, M. C. Taylor, R. Kieft, and P. Borst.** 1994. VSG gene expression site control in insect form *Trypanosoma brucei*. EMBO J. **13:**5470–5482.
56. **Rudenko, G., M. G. S. Lee, and L. H. T. Van der Ploeg.** 1992. The PARP and VSG genes of *Trypanosoma brucei* do not resemble RNA polymerase II transcription units in sensitivity to sarkosyl in nuclear run-on assays. Nucleic Acids Res. **20:**303–306.
57. **Salmon, D., M. Geuskens, F. Hanocq-Quertier, D. Nolan, L. Ruben, and E. Pays.** 1994. A novel heterodimeric transferrin receptor encoded by a pair of VSG gene expression site-associated genes in *T. brucei*. Cell **78:**75–86.
58. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
59. **Sandell, L. L., and V. A. Zakian.** 1992. Telomere position effect in yeast. Trends Cell Biol. **2:**10–14.
60. **Schurch, N., A. Hehl, E. Vassella, R. Braun, and I. Roditi.** 1994. Accurate polyadenylation of procyclin mRNAs in *Trypanosoma brucei* is determined by pyrimidine-rich elements in the intergenic regions. Mol. Cell. Biol. **14:** 3668–3675.
61. **Seed, B., and J.-Y. Sheen.** 1988. A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene **67:**271–277.
62. **Shea, C., M. G.-S. Lee, and L. H. T. Van der Ploeg.** 1987. VSG gene 118 is transcribed from a cotransposed Pol I-like promoter. Cell **50:**603–612.
63. **Sherman, D. R., L. Janz, M. Hug, and C. Clayton.** 1991. Anatomy of the *parp* gene promoter of *Trypanosoma brucei*. EMBO J. **10:**3379–3386.
64. **Son, H. J., G. A. Cook, T. Hall, and J. E. Donelson.** 1989. Expression site associated genes of *Trypanosoma brucei rhodesiense*. Mol. Biochem. Parasitol. **33:**59–66.
65. **Steverding, D., Y.-D. Stierhof, M. Chaudhri, M. Ligtenberg, D. Schell, A. G. Beck-Sickinger, and P. Overath.** 1994. ESAG 6 and 7 products of *Trypanosoma brucei* form a transferrin binding protein complex. Eur. J. Biochem. **64:**78–87.
66. **Tetley, L., C. M. R. Turner, J. D. Barry, J. S. Crowe, and K. Vickerman.** 1987. Onset of expression of the variant surface glycoproteins of *Trypanosoma brucei* in the tsetse fly studied using immunoelectron microscopy. J. Cell Sci. **87:**363–372.
67. **Thomashow, L. S., M. Milhausen, W. J. Rutter, and N. Agabian.** 1983. Tubulin genes are tandemly linked and clustered in the genome of *Trypanosoma brucei*. Cell **32:**35–43.
68. **Turner, C. M. R., and J. D. Barry.** 1989. High frequency of antigenic variation in *Trypanosoma brucei rhodesiense* infections. Parasitology **99:**67–75.
69. **Turner, C. M. R., J. D. Barry, I. Maudlin, and K. Vickerman.** 1988. An estimate of the size of the metacyclic variable antigen repertoire of *Trypanosoma brucei rhodesiense*. Parasitology **97:**269–276.
70. **Turner, C. M. R., J. D. Barry, and K. Vickerman.** 1986. Independent expression of the metacyclic and bloodstream variable antigen repertoires of *Trypanosoma brucei rhodesiense*. Parasitology **92:**67–73.
71. **Ullu, E., K. R. Matthews, and C. Tschudi.** 1993. Temporal order of RNA-processing reactions in trypanosomes: rapid *trans* splicing precedes polyadenylation of newly synthesized tubulin transcripts. Mol. Cell. Biol. **13:**720–725.
72. **Van der Ploeg, L. H. T., K. Gottesdiener, and M. G.-S. Lee.** 1992. Antigenic variation in African trypanosomes. Trends Genet. **8:**452–457.
73. **Van der Ploeg, L. H. T., A. Y. C. Liu, and P. Borst.** 1984. Structure of the growing telomeres of trypanosomes. Cell **36:**459–468.
74. **Van der Ploeg, L. H. T., D. Valerio, T. de Lange, A. Bernards, P. Borst, and F. G. Grosveld.** 1982. An analysis of cosmid clones of nuclear DNA from *Trypanosoma brucei* shows that the genes for variant surface glycoproteins are clustered in the genome. Nucleic Acids Res. **10:**5905–5923.
75. **Vickerman, K.** 1985. Developmental cycles and biology of pathogenic trypanosomes. Br. Med. Bull. **41:**105–114.
76. **Wymer, B.** Unpublished observations.
77. **Zomerdijk, J. C. B. M., R. Kieft, M. Duyndam, P. G. Shiels, and P. Borst.** 1991. Antigenic variation in *Trypanosoma brucei*: a telomeric expression site for variant-specific surface glycoprotein genes with novel features. Nucleic Acids Res. **19:**1359–1368.
78. **Zomerdijk, J. C. B. M., R. Kieft, P. G. Shiels, and P. Borst.** 1991. Alpha-amanitin-resistant transcription units in trypanosomes—a comparison of promoter sequences for a VSG gene-expression site and for the ribosomal RNA genes. Nucleic Acids Res. **19:**5153–5158.
79. **Zomerdijk, J. C. B. M., M. Ouellette, A. L. M. A. Ten Asbroek, R. Kieft, A. M. M. Bommer, C. Clayton, and P. Borst.** 1990. The promoter for a variant surface glycoprotein gene expression site in *Trypanosoma brucei*. EMBO J. **9:**2791–2801.