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Characterization of a Transcription Terminator of the Procyclin PARP A Unit of *Trypanosoma brucei*

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The polycistronic procyclin PARP (for procyclic acidic repetitive protein) A transcription unit of *Trypanosoma brucei* was completely characterized by the mapping of the termination region. In addition to the tandem of procyclin genes and GRESAG 2.1, this 7.5- to 9.5-kb unit contained another gene for a putative surface protein, termed PAG (for procyclin-associated gene) 3. The terminal 3-kb sequence did not contain significant open reading frames and cross-hybridized with the beginning of one or several transcription units specific to the bloodstream form. At least three separate fragments from the terminal region were able to inhibit chloramphenicol acetyltransferase expression when inserted between either the PARP, the ribosomal, or the variable surface glycoprotein promoter and a chloramphenicol acetyltransferase reporter gene. This inhibition was due to an orientation-dependent transcription termination caused by the combination of several attenuator elements with no obvious sequence conservation. The procyclin transcription terminator appeared unable to inhibit transcription by polymerase II.

In *Trypanosoma brucei*, the genes seem to be generally organized in polycistronic transcription units (8, 21). Because of this particular organization, only two trypanosomal promoters for protein-encoding genes are known, namely, those for the major stage-specific surface antigens, the variant surface glycoprotein (VSG) and procyclin (9, 12, 23, 29, 32). These promoters may recruit an RNA polymerase of the ribosomal type, although this remains equivocal (7, 25). To date, no DNA sequence able to terminate transcription has been found in these organisms.

Procyclin is the major surface glycoprotein of the procyclic form of *T. brucei* (27). The procyclin genes are organized in tandem pairs present in two diploid loci termed PARP (for procyclic acidic repetitive protein), A and PARP B, with the possible presence of an additional gene copy in some trypanosome strains (9, 14, 29). The transcription promoter is present immediately upstream from the first gene copy of each locus, and it seems that the different loci are transcribed simultaneously (9, 14, 29). In each locus, transcription is polycistronic, with at least one gene associated with the tandem of procyclin genes, GRESAG 2.1 in PARP A and PAG (for procyclin-associated gene) 1 in PARP B (2, 15). The approximative extent of each transcription unit was estimated to be less than 10 kb, contrasting with the larger size of units for other genes (2, 9, 15, 25, 29).

In this paper, we report the complete characterization, from the promoter to the termination region, of the PARP A unit. There appear to be no more than four genes in this unit. Interestingly, the 3′-end region of the PARP A unit was found to cross-hybridize with the 5′-end region of at least one bloodstream-specific transcription unit. The termination region appeared to extend over 2 kb. Different fragments from this region efficiently inhibited chloramphenicol acetyltransferase (CAT) expression driven by either the PARP, the ribosomal, or the VSG promoter. We provide evidence that this effect is due to an orientation-dependent transcription termination which appears to be restricted to polymerase I (pol I)-like polymerase, since it was not observed with pol II.

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MATERIALS AND METHODS

Trypanosomes. Procyclic forms were obtained by in vitro cultivation of isolates from the midgut of flies infected with the EATRO 1125 stock of *T. brucei*. They were grown in SDM-79 medium (6) supplemented with 15% heat-inactivated fetal calf serum. Bloodstream forms (AnTat 1.1 variant) were passaged in mice.

Plasmids. The plasmid clones containing the PARP A locus were obtained as follows. A 400-bp *Dde*I-*Eco*RI fragment specific to GRESAG 2.1 (2) was used as a probe to clone a 13.4-kb *Bam*HI genomic fragment containing the PARP A locus in an EMBL 3 phage vector. This fragment was cleaved in a unique *Hpa*I site, generating a 7.2-kb *Bam*HI-*Hpa*I and a 6.2-kb *Hpa*I-*Bam*HI fragment, which were subcloned in pUC18. The resulting plasmids were termed pPROC 7 and pPROC 6, respectively. For transient CAT activity assays, plasmids derived from either pCAT-tub (procyclin promoter) (4) or pD5 (VSG promoter) (12) were used. For stable CAT expression, pCAT-tub-derived plasmids were constructed. pCAT-tub was obtained by insertion of the 1.5-kb *Ecl*136II-*Hpa*I CAT gene-containing fragment from pD5 in the *Xho*I site of plasmid pTSA-HYG2 (5). A variant of pCAT-tub was generated by insertion of the 1.9-kb *Stu*I-*Hpa*I CAT gene-containing fragment from pD5 in the *Eco*RV site of pTSA-HYG2. The resulting construct, termed pCAT-tub2, contained the ESAG 7 splice acceptor site instead of the procyclin splice site upstream from the CAT gene. A *Bgl*II site was inserted upstream from the procyclin promoter in pCAT-tub, enabling the replacement of the 1.7-kb *Bam*HI-*Mlu*I fragment of pCAT-tub2 with the 2.4-kb *Bgl*II-*Mlu*I fragment of pCAT-tub. The resulting construct, termed pCAT2, contained a second procyclin promoter between the CAT and hygromycin resistance (Hyg^r) genes. The 3.3-kb *Xho*I fragment containing the putative termination region from the PARP A locus was isolated from pPROC 6 and inserted into the *Xho*I site of pCAT-tub2 in both orientations, generating the pCAT2-term (sense) and pCAT2-mret (antisense) plasmids. The 1.1-kb *Ecl*136II-*Sna*I fragment was removed from pCAT2-term in order to delete its first procyclin promoter, resulting in a plasmid termed pCAT2-delta-term. To obtain the constructs shown in lines 1 and 2 of Fig. 5, the 2.5-kb *Sna*I-*Ssp*I fragment of pPROC 6 was inserted in the unique *Ecl*136II site of pCAT-tub in the sense and antisense orientations, respectively. To generate the constructs shown in lines 8 to 12 of Fig. 6, annealed synthetic oligonucleotides, flanked 5′ and 3′, respectively, by *Xho*I and *Hin*dIII protruding ends, were inserted in the *Xho*I and *Hin*dIII sites of pCAT-tub2. These oligonucleotides correspond to the regions extending from nucleotides (nt) 4285 to 4400 (construct 8), 4401 to 4500 (construct 9), 4501 to 4600 (construct 10), 4601 to 4715 (construct 11), and 4567 to 4690 (construct 12) in Fig. 1. The sequence of the double-stranded oligonucleotide contained in construct 13 of Fig. 6 is 5′-TCGAGTATACGTATTTTTTCTTTTTTTTCTTTTTTCCTCTC CTATCGCGAATTCTTTTCAAACAGATATCA-3′ (the CAAAC box is underlined; see Results). To obtain the construct shown in line 2 of Fig. 7, the 0.9-kb *Sal*I-*Stu*I fragment of pD5 (12) was substituted by the 2.9-kb *Xho*I-*Ssp*I fragment of pCAT2-term. To generate plasmid pD5Rib, the *Eco*RV-*Stu*I fragment of pD5A, a mutant version of pD5 missing the VSG promoter, was replaced by the 2-kb *Hin*dIII fragment of plasmid pEH5.8, which contains the

ribosomal promoter (10). The 1.4-kb *Bgl*II-*Nco*I fragment of pD5Rib was then replaced by the 3.4-kb *Bgl*II-*Nco*I fragment of pCAT2-term, generating the construct shown in line 3 of Fig. 7 (the *Nco*I site is present within the CAT gene in both plasmids). This construct contains the 2.5-kb *Bgl*II-*Xho*I fragment from the PARP A locus, located downstream from the ribosomal promoter and upstream from the splice acceptor site of ESAG 7 and the CAT gene.

Stable transformation. The procyclic forms were harvested at mid-log phase. Cells (10^7) were resuspended in 500 μl of Zimmerman postfusion medium (12) and mixed with 100 to 500 ng of plasmid linearized by *Bss*HII cleavage. Electroporation was performed by two pulses of 1.5 kV at 25 μF, using a Bio-Rad gene pulser. Electroporated trypanosomes were inoculated into 4 ml of culture medium. At 48 h after electroporation, hygromycin (25 μg/ml) was added. The drug concentration was raised to 100 μg/ml after 5 days. Transgenic trypanosomes could be detected 2 to 3 weeks after electroporation.

CAT assays. The transient CAT activity assays were performed as described previously (12).

DNA and RNA analysis. The procedures for DNA and RNA isolation, Southern and Northern (RNA) blot hybridization, and DNA cloning were as described elsewhere (24).

Run-on transcription. Run-on transcription assays were conducted as described by Murphy et al. (20). The standard assay (1 ml) contained 500 μg of DNA in nuclei, 12.5% (vol/vol) glycerol, 0.8 mg of heparin, 5 mM spermidine, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM Tris-HCl (pH 8), 0.5 mM ATP, UTP, and CTP, and 1 mCi of [α-^{32}P]GTP (2,000 Ci/mmol). The nuclei were usually incubated for 30 min at 30°C. Titration experiments indicated that under the conditions used for hybridization of the run-on transcripts, the DNA blotted on the filters was in large excess over the probe.

UV irradiation. Bloodstream forms isolated by DEAE chromatography were incubated for 30 min in Baltz medium at 37°C at a concentration of 1×10^7 to 4×10^7 cells per ml. Procyclic forms were used when a similar concentration was reached in SDM medium. Samples (125 ml) were irradiated at 254 nm (1 J/s/m^2) in sterile square dishes (22 by 22 cm; Bio-Assay; Nunc, Roskilde, Denmark) for 1 min with agitation. The irradiated cells were transferred to culture flasks and kept for 30 min at either 37°C (bloodstream forms) or 27°C (procyclic forms) in the dark until centrifugation at 4°C.

Nucleotide sequences. The nucleotide sequences presented in this report have been submitted to the EMBL database with accession numbers Z36931 and Z36932.

RESULTS

The 3′ environment of the PARP A locus. In the PARP A locus, the procyclin genes are associated with a gene termed GRESAG 2.1, which encodes a minor surface protein (2, 3). Previous evidence indicated that this unit is transcribed to a point at least 2 kb downstream from GRESAG 2.1 (2). The nucleotide sequence of the 5-kb region downstream from GRESAG 2.1 is shown in Fig. 1A. The largest open reading frame was 447 bp (nt 2737 to 3184; Fig. 1B). This 5-kb region cross-hybridized with a few other sequences in the genome. As shown in Fig. 2A, B, and D, different probes from this region (1.64-kb *Sca*I-*Bgl*II, 2.44-kb *Bgl*II-*Eco*RI, and 3.29-kb *Xho*I-*Xho*I) hybridized not only with their cognate genomic fragments (arrowheads) but also with two to five others. This observation cannot result from incomplete cleavage of DNA, since in some digests such as with *Hpa*I or *Bgl*II, fragments smaller than those expected from the map of the cloned sequence were observed. The sequences related to the 3′ region of the PARP A unit were not present in the PARP B unit or in minichromosomes, as determined by hybridization of 3′ probes from the PARP A unit with a plasmid containing the 3′-end region of the PARP B unit or with chromosomes separated by pulsed-field electrophoresis (data not shown).

Run-on mapping of the end of the transcribed region in procyclic forms. Run-on transcription assays were performed to delineate the end of the PARP A unit. As shown in Fig. 3A, in procyclic forms, the region containing the procyclin genes and GRESAG 2.1 was efficiently transcribed by an RNA polymerase resistant to α-amanitin (compare lanes ct and am). Moderate UV irradiation did not inhibit transcription in this region (lane uv), presumably because of the small distance to the promoter, hence the small size of the DNA targeted by UV. Compared with these data, the hybridization of the same run-on transcripts to restriction digests of the region downstream from GRESAG 2.1 (Fig. 3B and C, PF) appeared to be quite different. The majority of the transcripts hybridizing to this region were synthesized by an α-amanitin-sensitive RNA polymerase (compare lanes ct and am) and were thus presumably not from the procyclin locus but from related sequences (results presented below support this conclusion). The weak α-amanitin-resistant hybridization (lanes am) probably represented transcription at the end of the procyclin locus. As expected, it was higher in the 5′-terminal fragments B1, B3, and C1 than in B2 or C2 (hybridization to the small fragment C5 was detectable after long exposure). The lack of hybridization of α-amanitin-resistant transcripts to fragments C3 and C4, even after longer exposure (not shown), suggested that transcription terminates in the region of the *Eco*RV-*Eco*RI fragment C2. UV irradiation blocked transcription in this region, in accordance with the relatively long distance from the promoter. In conclusion, these results mapped the termination region in an *Eco*RV-*Eco*RI fragment located between 7.5 and 9.5 kb downstream from the promoter (B2 and C2 in Fig. 3).

The 3′ end of the PARP A unit contains sequences related to the beginning of a bloodstream-specific unit. As previously described (2, 23), the promoter-proximal region of the PARP A unit was still transcribed in the bloodstream form (Fig. 3A, BF, fragment 1 in lanes ct and am). The hybridization to fragment A3 was partially due to a cross-hybridization with the transcripts of the ESAG 2 gene present in the VSG unit active in the bloodstream form (2). UV irradiation appeared to stimulate the transcription of fragment A1 (Fig. 3A, BF, lane uv) as a result of a transient inhibition of RNA degradation (10). In sharp contrast with the situation in procyclic forms, the run-on transcripts of bloodstream forms hybridized strongly with the 3′-end region of the PARP A unit (Fig. 3B and C, BF). These transcripts were exclusively synthesized by an α-amanitin-sensitive RNA polymerase (lanes am), suggesting that they were not from the procyclin locus (confirmed by cDNA analyses; see below). Moreover, UV irradiation did not inhibit this transcription and even induced an apparent stimulation of transcription in some cases (fragments B3 and C5, covering a 900-bp region between *Xho*I and *Sal*I sites). Such a stimulation is the hallmark of the proximity of a promoter, although so far this has been observed only for units transcribed by pol I (10, 11, 22, 23). Taken together, these results indicated that the 3′-end region of the PARP A unit (between 5.5 and 8.5 kb downstream from the promoter) was not transcribed in bloodstream forms but cross-hybridized with the beginning of one or several transcription units specifically active in the bloodstream form.

Northern blotting confirms the run-on data. To verify the conclusions drawn from the analysis of the run-on data, probes from successive fragments of the PARP A locus were hybridized with Northern blots of poly(A)$^+$ RNA from procyclic and bloodstream forms (Fig. 4). While the 5′-terminal 5-kb region of the procyclin unit contains closely packed genes transcribed into procyclic-specific RNAs (procyclin, PAG 3, and GRESAG 2.1 genes; references 2 and 14 and Fig. 4, probes A and B), a probe for the region located between 6 and 6.7 kb from the promoter (*Hpa*I-*Xho*I fragment) did not reveal steady-state transcripts in the procyclic form (Fig. 4, probe C). This probe hybridized weakly to heterogeneous transcripts specific to the bloodstream form. Similar observations were made with a probe from the contiguous *Xho*I-*Eco*RV fragment (between 6.7 and 7.6 kb from the promoter), except that the detection of the heterogeneous bloodstream-specific transcripts was much stronger (Fig. 4, probe D). Other probes for the region further downstream from the *Eco*RV site confirmed these results (2.3-kb *Eco*RV-*Xho*I fragment [probe E in Fig. 4]; 1.3-kb

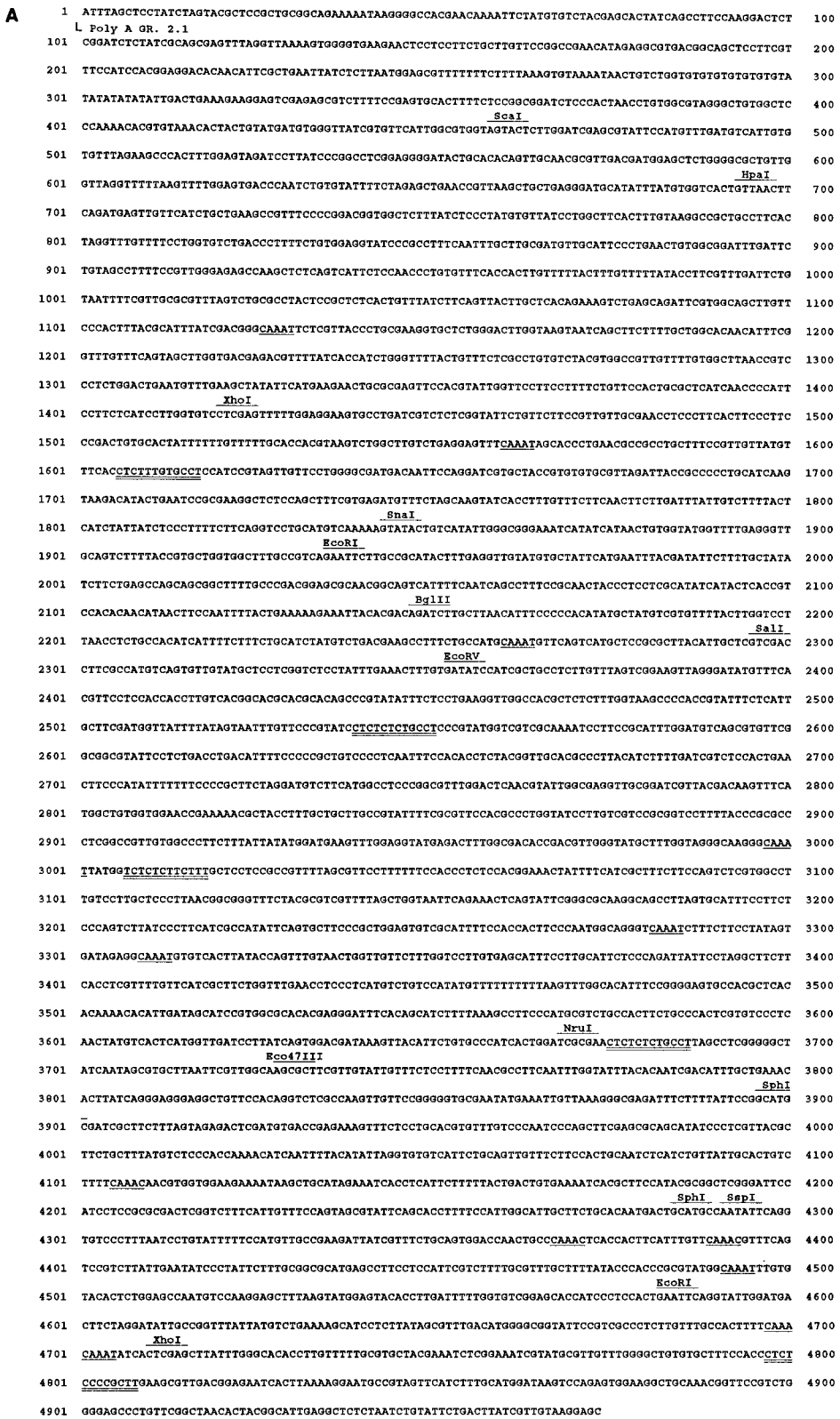

FIG. 1. (A) Nucleotide sequence of the 5-kb region downstream from the GRESAG 2.1 gene in the PARP A locus of *T. brucei*. The first nucleotide is the polyadenylation site of the GRESAG 2.1 mRNA (2). Relevant restriction sites, as well as 5-mer and 12-mer oligonucleotides (single and double underlined, respectively) discussed in the text, are indicated. The open reading frames of the coding strand are shown below the sequence. (B) Diagram of the PARP A transcription unit, with an arrow for the start site and boxes for the genes (Pα and Pβ, α- and β-procyclin genes).

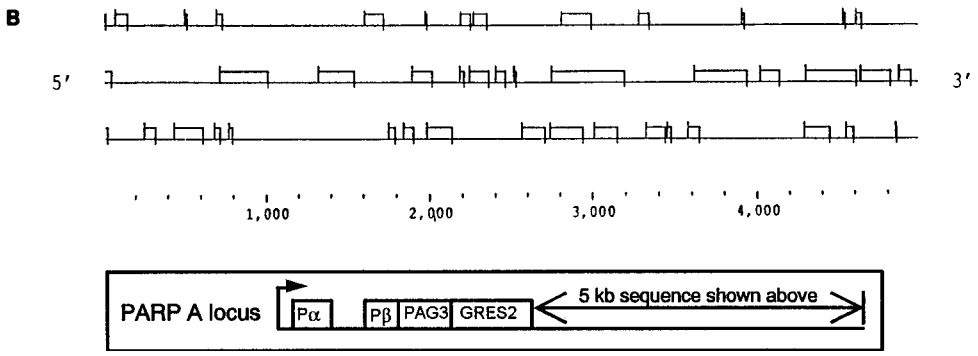

FIG. 1—*Continued.*

*Eco*RV-*Nru*I and 1-kb *Nru*I-*Xho*I fragments [not shown]). These observations were in agreement with the results from run-on assays, which suggested the following: (i) a reduced rate of primary transcription in the 5.5- to 8.5-kb region downstream from the promoter and (ii) a cross-hybridization of transcripts from the beginning of a bloodstream-specific transcription unit with the region between the two *Xho*I sites of the procyclin locus.

cDNA analyses confirm the run-on data. A probe specific to the 3′-end region of the PARP A locus (1.7 kb *Sca*I-*Bgl*II fragment; probe A in Fig. 2) was used to clone cDNAs from bloodstream and procyclic libraries. Only a few cDNAs were obtained in the latter case. The largest cDNAs, one from procyclic forms and three from bloodstream forms, were analyzed by restriction endonuclease digestion. None of these cDNAs showed the restriction map characteristic of the PARP A locus (data not shown). These results prove that sequences related to, but different from, the 3′-end region of the procyclin unit are transcribed in both forms of the parasite, although the level of transcription is clearly higher in the bloodstream form. This finding probably accounts for the α-amanitin-sensitive transcription observed in the run-on assays.

Effect of the termination region in transient activity assays. Different fragments from the region presumed to contain the

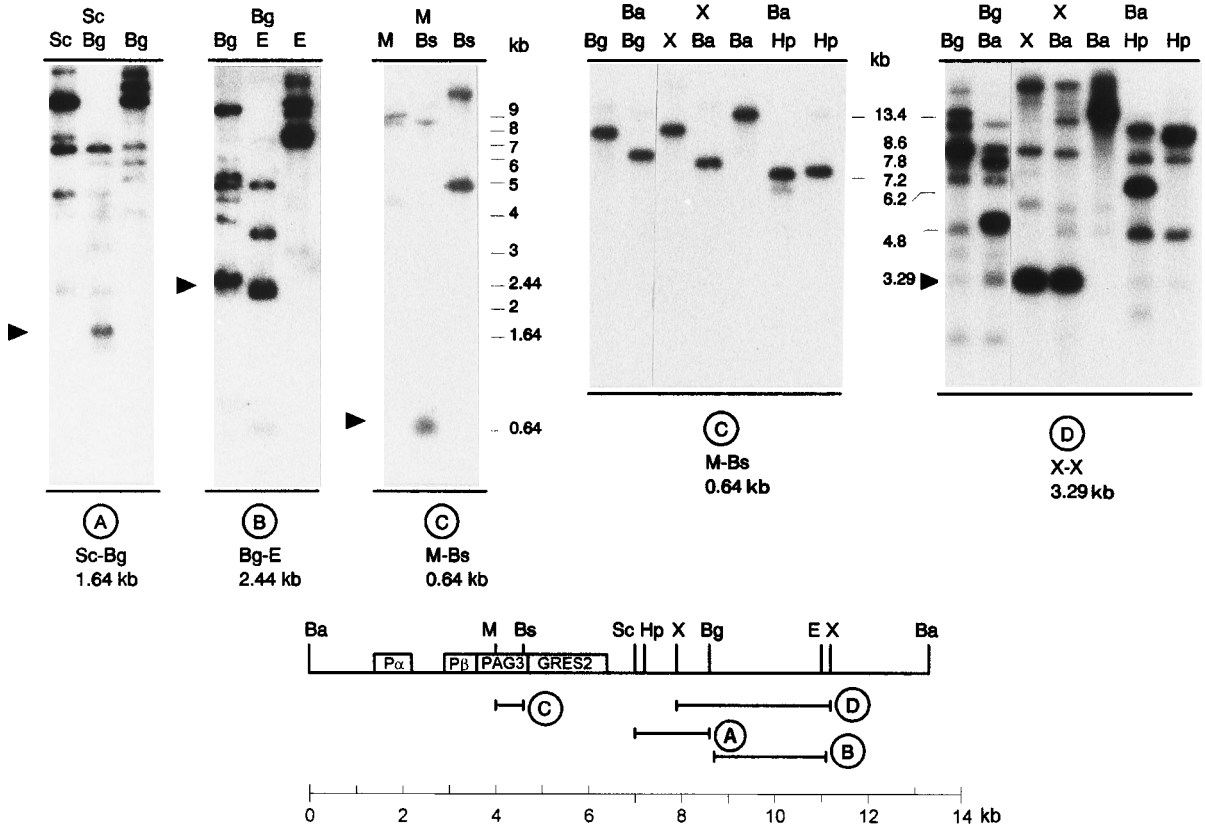

FIG. 2. Southern blot analysis of the 3′ end of the PARP A locus. Restriction digests of *T. brucei* AnTat 1.3A DNA were hybridized under stringent conditions (washings in 0.015 M NaCl at 65°C) with different probes from the PARP A unit, as indicated below the map. The bands marked by arrowheads contain the genomic fragments corresponding to the probes. Abbreviations for restriction endonucleases in all figures are as follows: B, *Bsm*I; Ba, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*BI; Bss, *Bss*HII; E, *Eco*RI; Ec, *Ecl*136II; E4, *Eco*47III; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; N, *Nru*I; S, *Sal*I; Sc, *Sca*I; Sn, *Sna*I; Ss, *Sst*I; V, *Eco*RV; X, *Xho*I; Z, *Ssp*I.

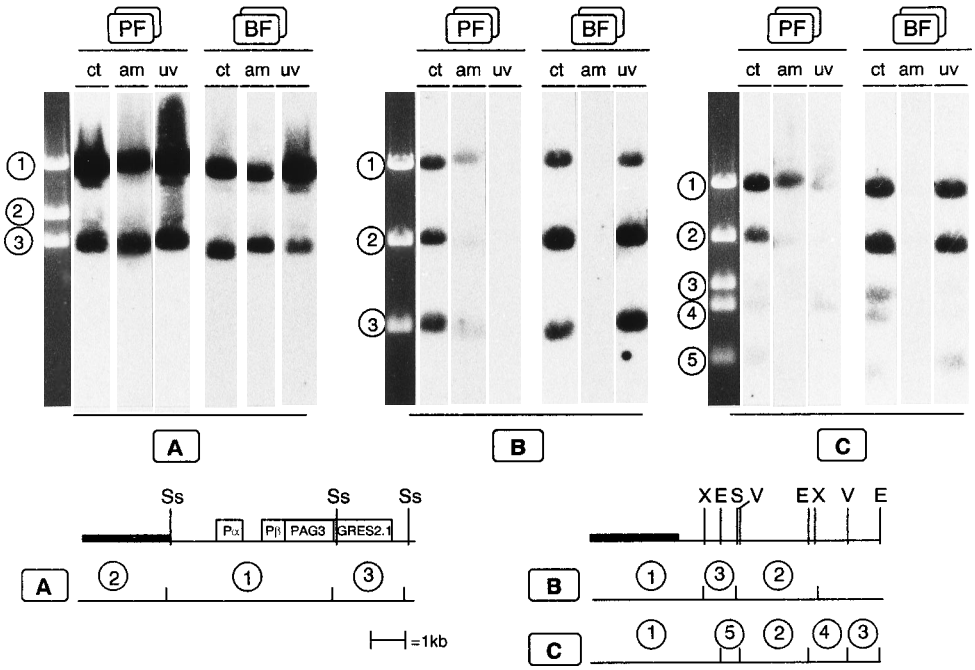

FIG. 3. Run-on transcription assays of the PARP A locus. Run-on transcription was conducted in nuclei from procyclic and bloodstream forms (PF and BF, respectively) in the absence (ct) or presence (am) of 1 mg of α-amanitin per ml. A portion of the trypanosomes were subjected to UV irradiation (uv). The labeled transcripts were hybridized to Southern blots of the restriction digests of plasmids pPROC 7 (A) and pPROC 6 (B and C), which bear two contiguous fragments of the PARP A locus. The first lane of each panel shows the ethidium bromide staining of the digests. Numbers refer to positions of the fragments in the respective plasmid maps. The black boxes in these maps represent plasmid DNA.

terminator of the PARP A unit were inserted between the PARP A promoter and a CAT reporter gene in a plasmid termed pCAT-tub2. These constructs were transfected into procyclic forms, and the transient CAT activity was measured. As shown in Fig. 5 (lines 3 and 4), the 3.3-kb *Xho*I fragment led to a strong reduction of CAT activity when inserted in the same orientation as that in the PARP A unit, whereas a full CAT activity was conserved when this fragment was inserted in the reverse orientation. This fragment did not affect CAT activity when the fragment was present in either orientation immediately upstream from the promoter (lines 1 and 2). In addition, an irrelevant sequence such as the 1.5-kb *Sal*I fragment encompassing the actin gene was not inhibitory when inserted between the promoter and the CAT gene, even if present in the same orientation as that in the original transcription unit (data not shown). The 3.3-kb *Xho*I fragment was divided into a 3′-terminal 2.41-kb and a 5′-terminal 0.88-kb fragment by *Sal*I digestion. Both fragments were still strongly inhibitory (lines 5 and 6). Therefore, the *Xho*I fragment contained at least two separate elements that repressed CAT activity independently. If shortened to 0.73 kb by *Bgl*II cleavage, the 5′ element lost some of its inhibitory effect (line 7). The inhibition was further reduced by a shortening to 0.42 kb by *Sna*I cleavage (line 8), suggesting that several weakly inhibitory elements are contained within the 0.88-kb *Xho*I-*Sal*I fragment. In the inverted 3.3-kb *Xho*I sequence, the reversal of the 0.88-kb *Xho*I-*Sal*I fragment into its original orientation restored some inhibition (line 9), confirming the inhibition capacity of this fragment (line 6). A strong inhibition was conserved when the 0.51-kb *Sna*I-*Eco*RV fragment was removed from the *Xho*I sequence (line 10), confirming that an inhibitory element is present downstream from the *Sal*I-*Eco*RV region (line 5). Deletion of either the 1.8-kb *Sna*I-*Nru*I or the 2.2-kb *Xho*I-*Nru*I fragment did not suppress the inhibition (lines 11 and 12), indicating that efficient inhibitory sequences are present downstream from the *Nru*I site. However, the 1.8-kb *Sna*I-*Nru*I fragment was also inhibitory since, when restored in its correct orientation within the inverted 3.3-kb *Xho*I fragment, it abolished CAT activity (line 13). When the 0.51-kb *Sna*I-*Eco*RV fragment was restored in its correct orientation in the inverted 3.3-kb *Xho*I fragment, it was ineffective (line 14).

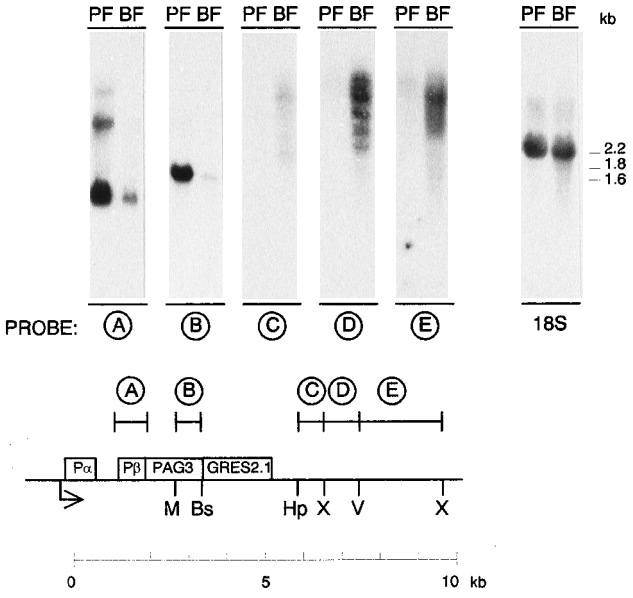

FIG. 4. Northern blot analysis of the PARP A locus. For details, see the legend to Fig. 2. The probes were all from double-stranded fragments. PF, procyclic forms; BF, bloodstream forms; 18S, 18S ribosomal gene.

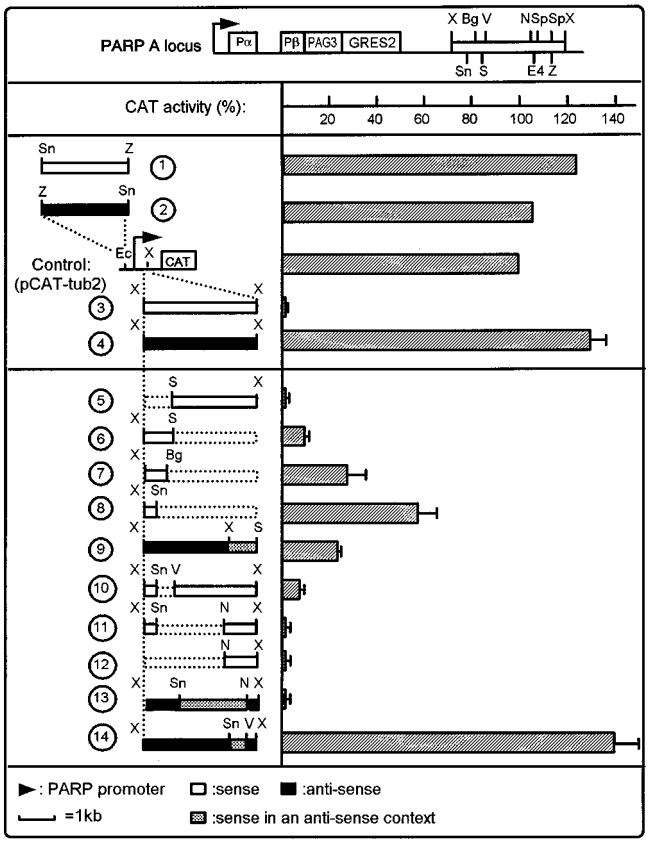

FIG. 5. Effects of fragments from the 3′-end region of the PARP A locus on transient CAT activity assays in procyclic forms. Activity is expressed as a percentage of that measured in the absence of the fragment. The 3.3-kb *Xho*I fragment is analyzed.

Therefore, the activity present in the *Sna*I-*Nru*I fragment must be located between the *Eco*RV and *Nru*I sites.

Taken together, these results suggested that in the termination region, located between 7.5 and 9.5 kb downstream from the promoter, at least three elements are able to suppress CAT activity. The first one is present in the 0.88-kb *Xho*I-*Sal*I fragment and seems to contain several weakly inhibitory elements. The second is contained in the 1.3-kb *Eco*RV-*Nru*I fragment, and the third is in the 1.05-kb *Nru*I-*Xho*I fragment (map at the top of Fig. 5). These results confirmed the run-on data which suggested that the termination region is essentially downstream from *Eco*RV (Fig. 3).

The largest sequence element conserved in the different fragments with inhibitory activity was the 12-mer CTCTCTCT GCCT (double underlining in Fig. 1). This element is present in two identical copies and three slightly divergent versions. The possible involvement of this sequence in termination was evaluated in two ways. First, the 60-bp *Nru*I-*Eco*47III fragment encompassing a 12-mer copy was deleted from the inhibitory 1.05-kb *Nru*I-*Xho*I fragment (Fig. 6, line 1). The resulting 1-kb *Eco*47III-*Xho*I fragment conserved a strong inhibition capacity (line 2). Second, synthetic 12-mer oligonucleotides were inserted in a single or in two direct copies between the PARP promoter and the CAT gene. As shown in lines 3 and 4, these sequences did not abolish CAT activity. Taken together, these results ruled out the possibility that the 12-mer was responsible for the inhibition. To define more precisely the DNA sequences able to inhibit CAT expression, the 1.05-kb *Nru*I-*Xho*I fragment was analyzed into more detail. As shown in line 5, the deletion of the internal 0.38-kb *Sph*I-*Sph*I sequence only mildly affected the inhibitory capacity of this fragment. In contrast, inhibition was largely lost when the last 0.43-kb *Sph*I-*Xho*I fragment was removed (line 6). Finally, the 0.43-kb *Sph*I-*Xho*I fragment in combination with the 0.17-kb *Eco*47III-*Sph*I fragment was strongly inhibitory (line 7). Since the *Eco*47III-*Sph*I sequence is not from an inhibitory region (line 6), it is concluded that most of the inhibition activity of the 1.05-kb *Nru*I-*Xho*I fragment is contained in the terminal 0.43-kb *Sph*I-*Xho*I sequence. This activity was dissected by assaying different synthetic oligonucleotides covering the entire 0.43-kb region (nt 4285 to 4400, 4401 to 4500, 4501 to 4600, 4601 to 4715 and 4567 to 4690; lines 8 to 12, respectively). Four of these oligonucleotides showed a weak inhibition capacity, suggesting that, as was the case for the 0.88-kb *Xho*I-*Sal*I fragment (Fig. 5, lines 6 to 8), the inhibitory effect contained in the 0.43-kb *Sph*I-*Xho*I fragment is due to the combination of several weak elements. The only conserved sequence shared by these oligonucleotides, as well as by the other inhibitory regions, was CAAA(T/C) (underlined in Fig. 1). This sequence is generally preceded by a pyrimidine-rich region. On the basis of the characteristics of terminator sequences from other eukaryotes, which consist of a protein-binding stretch preceded by a pyrimidine-rich region (13, 18), we assayed the inhibition capacity of a synthetic oligonucleotide containing a polypyrimidine stretch followed by a CAAAC box (see Materials and Methods). As shown in line 13, this sequence conferred a very poor inhibition. Therefore, it would appear that the PARP A termination region contains several elements able to reduce CAT activity when inserted between the promoter and the CAT gene. Individually, each of these elements exhibits only weak activity, but their combina-

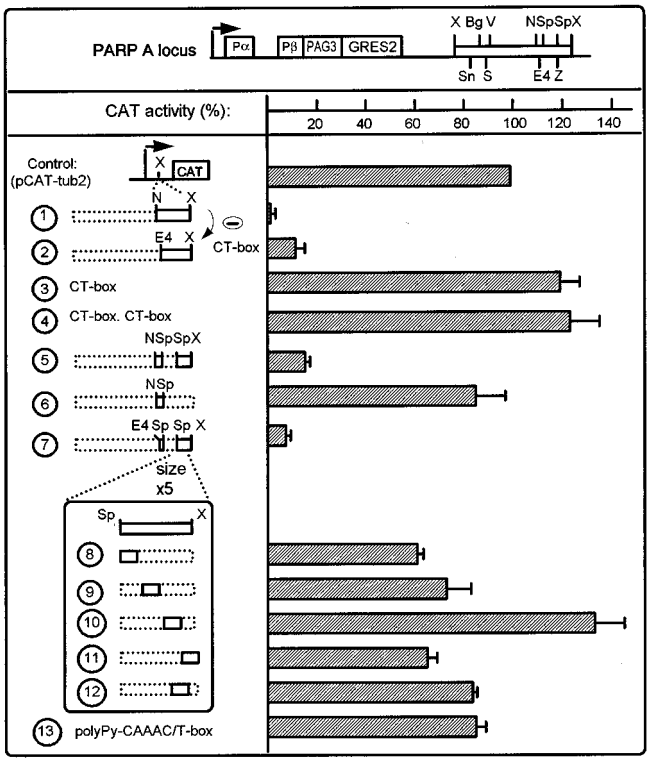

FIG. 6. Effects of fragments from the 3′-end region of the PARP A locus on transient CAT activity assays in procyclic forms. Activity is expressed as a percentage of that measured in the absence of the fragment. The 3′-terminal third of the 3.3-kb *Xho*I fragment (1.05 kb, *Nru*I-*Xho*I) is analyzed in detail.

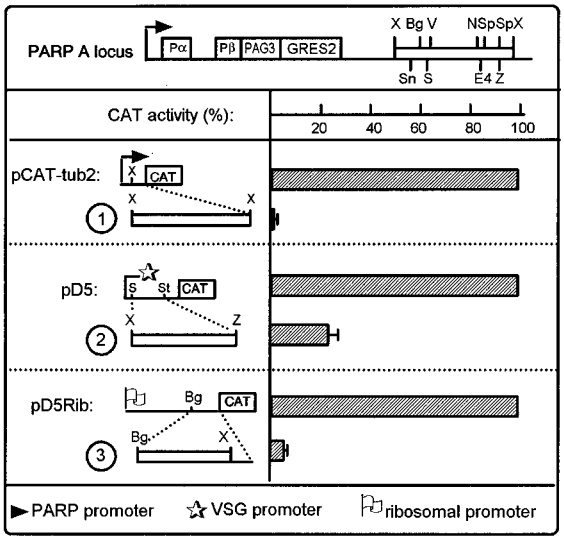

FIG. 7. Effects of fragments from the 3′-end region of the PARP A locus on transient CAT activity assays in procyclic forms. Activity is expressed as a percentage of that measured in the absence of fragment. The effect on transcription driven by different promoters is analyzed.

tion is strongly inhibitory. No characteristic or conserved sequence element seems to be associated with these effects.

Since the procyclin and VSG transcription units may share the same RNA polymerase (7), we investigated the effect of the putative PARP A termination region on transcription driven by the VSG promoter. A 2.9-kb *Xho*I-*Ssp*I fragment encompassing the procyclin termination region was found to inhibit CAT activity driven by the VSG promoter, although this effect was not complete (Fig. 7, line 2).

The same analysis was performed with the ribosomal promoter. As shown in Fig. 7, line 3, the 2.56-kb *Bgl*II-*Xho*I fragment strongly inhibited this activity.

In conclusion, when inserted between the promoter and the CAT gene, the region presumed to terminate transcription of the PARP A locus was able to inhibit CAT activity driven by either the procyclin, the VSG, or the ribosomal promoter. This effect was obtained only with DNA fragments inserted in the original orientation with respect to the promoter, strongly suggesting that CAT inhibition was due to transcription termination.

The terminal sequences of the PARP A locus inhibit CAT activity by termination of transcription. To determine the nature of the CAT inhibition by the putative PARP A termination region, different CAT constructs were targeted into the trypanosome genome by homologous recombination. These constructs contained the CAT gene and a Hyg^r gene, each placed individually under the control of a procyclin promoter. The pCAT2-term and pCAT2-mret constructs contained the putative terminator (3.3-kb *Xho*I fragment) inserted between the CAT gene and its promoter, in the sense and antisense orientations, respectively, and the pCAT2-delta-term construct was obtained by deletion of the first promoter from pCAT2-term (Fig. 8B). The plasmids were linearized by *Bss*HII cleavage in a fragment from the intergenic region between the β- and α-tubulin genes, present downstream from the Hyg^r gene. Procyclic transformants were generated by electroporation of different amounts of the plasmid DNAs. Transformants containing a single integration in the tubulin locus were selected. As shown in the Southern blots of Fig. 8, each of the three selected trypanosome transformants showed the specific fragments predicted for a single integration event. No evidence for a tandem integration, such as 11.3- and 10.4-kb *Bam*HI fragments (pCAT2-term and pCAT2-delta-term, respectively) was observed in addition to the 5′-flanking *Bam*HI fragment (9.8 and 8.8 kb, respectively).

Run-on transcription assays were performed with the nuclei of these transformants. Labeled transcripts synthesized in the presence or absence of 1 mg of α-amanitin per ml were hybridized with different restriction digests of the pCAT2-term construct in order to assess the level of primary transcription of the integrated construct in the transformed trypanosomes. As expected, in the three transformants the Hyg^r gene appeared to be transcribed under the dependence of the procyclin promoter (Fig. 9). The hybridization to fragments A3, B2, and C1 revealed similar high levels of α-amanitin-resistant transcription in the three cases, which is characteristic of the RNA polymerase recruited by this promoter, contrasting with the low level and α-amanitin sensitivity of transcription by RNA pol II (control digests for pol II in Fig. 9D). In contrast, the transcription characteristics of the CAT gene markedly differed in the three transformants. The transcription level of this gene was much lower in pCAT2-term than in pCAT2-mret (compare fragment A4 in lanes 1 and 2 and lanes 3 and 4). Liquid scintillation counting of the relevant nitrocellulose areas revealed that the difference was approximately sevenfold. This transcription was totally resistant to α-amanitin in pCAT2-mret, while it was partially sensitive in pCAT2-term (compare fragment A4 in lanes 3 and 4 and in the overexposed lanes 1 and 2). The residual transcription of the CAT gene in pCAT2-term was similar to that measured if the procyclin promoter was deleted (pCAT2-delta-term), although in the latter case the transcription was fully α-amanitin sensitive (compare fragment A4 in the overexposed lanes 1 and 2 and in lanes 5 and 6). These results indicated that when inserted in the correct orientation, the 3.3-kb *Xho*I fragment was able to

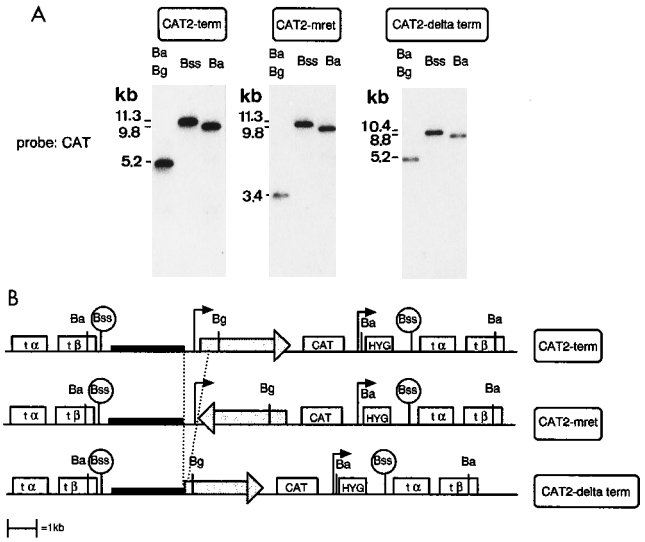

FIG. 8. (A) Targeting of CAT reporter constructs in the tubulin locus of *T. brucei* procyclic forms. Southern blots of the transformed trypanosomes were hybridized with a probe of the CAT gene. (B) Maps representing the insertion of plasmids pCAT2-term, pCAT2-mret, and pCAT2-delta-term in the *Bss*HII site of the tubulin genes. The encircled *Bss*HII sites are those used for the linearization of the plasmids. Thick arrowed boxes represent the putative terminator (3.3-kb *Xho*I fragment) and its orientation in the construct; thin arrowed bars represent the procyclin promoters; black boxes represent plasmid vector DNA. tα and tβ, α- and β-tubulin genes.

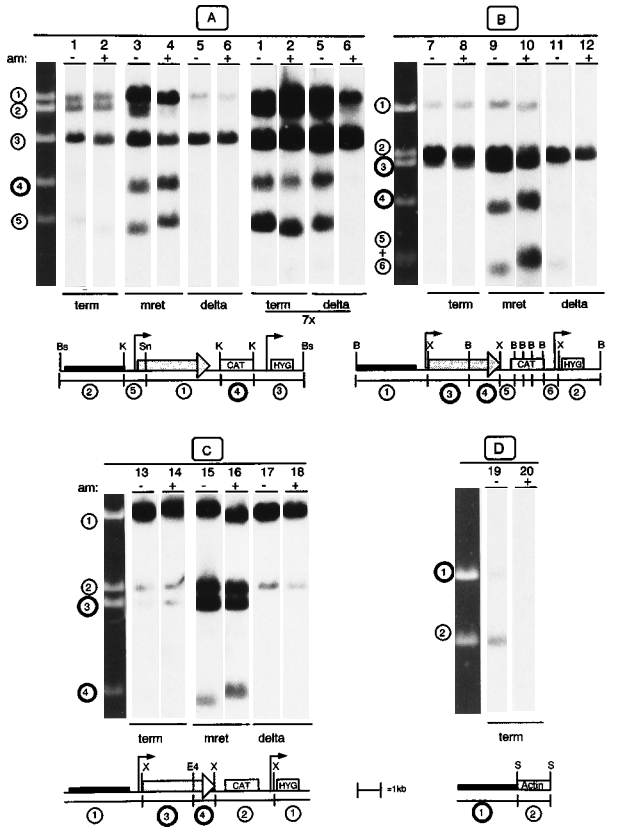

FIG. 9. Transcription termination by the 3′ end of the PARP A locus, as revealed by run-on transcription. Run-on transcription was conducted in nuclei from procyclic forms of different trypanosome transformants (pCAT2-term [term], pCAT2-mret [mret], and pCAT2-delta-term [delta]), in the absence (−) or presence (+) of 1 mg of α-amanitin (am) per ml. The labeled transcripts were hybridized to restriction digests of plasmid pCAT2-term (A to C) and a plasmid containing the actin gene (1) (D). The first lane in each panel shows the ethidium bromide staining of the digests. Numbers refer to positions of the fragments in the respective plasmid maps. The bands whose numbers are encircled with a thick line are particularly significant and are discussed in the text. The same exposure time was applied to all panels except for the last four lanes of panel A (sevenfold-longer exposure). The symbols in the maps are the same as for Fig. 8.

reduce at least sevenfold the transcription driven by the procyclin promoter. The residual α-amanitin-sensitive transcription is discussed below. The transcription pattern of the 3.3-kb *Xho*I fragment confirmed the conclusions drawn about the CAT gene. When present in the correct orientation downstream from the promoter (pCAT2-term), this sequence was only weakly transcribed, and transcription was detected only in its 5′ portion (fragments B3 and B4 in lanes 7 and 8; fragments C3 and C4 in lanes 13 and 14). The resulting pattern was similar to that observed in the absence of procyclin promoter (pCAT2-delta-term) (lanes 7 and 8 compared with lanes 11 and 12; lanes 13 and 14 compared with lanes 17 and 18). In sharp contrast, when inserted in a reverse orientation (pCAT2-mret) the same region was clearly transcribed by the RNA polymerase from the procyclin promoter, as judged by both the high level and complete α-amanitin resistance of transcription (fragments B3 and B4 in lanes 9 and 10; fragments C3 and C4 in lanes 15 and 16). These results confirmed that the *Xho*I fragment possesses an orientation-dependent termination activity. Similar results were obtained in two independent pCAT2-term transformant cell lines.

In Fig. 9, the interpretation of the hybridization to the fragments not discussed above is less straightforward because of their composite nature, but it is entirely consistent with the conclusions drawn. The relatively low α-amanitin-resistant hybridization to fragments A5, B1, B6, and C2 is due to the presence in these fragments of a short stretch of sequence downstream from the transcription start site of the procyclin promoter (the absence of α-amanitin-resistant hybridization to A5 and B1 in pCAT2-delta-term is probably related to the absence of the first procyclin promoter in the plasmid construct). Similarly, the α-amanitin-resistant hybridization to fragments A1, B5, and C2 is at least partially due to the presence in these fragments, upstream from the CAT gene, of the ESAG 7 splice acceptor region which is transcribed by a pol I-like polymerase driven by the VSG promoter still active in procyclic forms (23). Finally, the α-amanitin-resistant transcription of fragment A2 in pCAT2-term is due to the presence in this fragment of a sequence from the tubulin locus, whose transcription in this transformant was rendered largely α-amanitin resistant presumably because of readthrough transcription from the construct inserted at the beginning of the locus (data not shown).

The PARP A terminator does not inhibit transcription by pol II. In the pCAT2-delta-term transformant, the CAT gene was still transcribed (Fig. 9, fragment A4 in overexposed lane 5). As this transcription was fully sensitive to α-amanitin (compare fragment A4 in overexposed lanes 5 and 6), it appears that the PARP A terminator does not block the RNA polymerase active in the tubulin locus. The transcription level by pol II in the tubulin unit was assessed in the run-on experiments described above. As the plasmid vector region of the targeted construct is located immediately downstream from the tubulin genes (black boxes in Fig. 8B), the measurement of the hybridization level of transcripts from this region should reflect the transcription rate in the tubulin locus. The quantitative measurement of the data in Fig. 9 (fragments A2 in lane 5 and D1 in lane 19) indicated that the transcription rate on the plasmid DNA was only slightly higher than that on the CAT gene (fragment A4 in lane 1) (about 0.25 and 0.19 cpm/bp, respectively), a difference which may be due to the high instability of CAT transcripts in trypanosomes (5, 30). It was concluded that the PARP A terminator does not significantly affect transcription by pol II.

The PARP A unit contains four genes. In addition to the tandem of procyclin genes, the PARP A unit was shown to contain another gene termed GRESAG 2.1 (2). This gene seemed to encode a minor surface glycoprotein, presumably anchored by a glycosylphosphatidylinositol (GPI) (3). Since the 3′ end of the PARP A unit was now identified and did not appear to contain additional genes, we undertook the determination of the total number of genes of this unit by sequencing the remaining uncharacterized 1,480-bp region between the β-procyclin gene and GRESAG 2.1. As shown in Fig. 10, this region was found to contain a 0.6-kb open reading frame, termed PAG 3. The sequence of PAG 3 did not reveal significant homology with known genes. Southern blot analysis indicated that this gene was not unique, since hybridization of *Mlu*I-*Bst*BI digests of genomic DNA with the 0.64-kb *Mlu*I-*Bst*BI fragment which contains the PAG 3 open reading frame revealed an additional sequence (Fig. 2C). This was confirmed by hybridization of the same probe with different digests of the PARP A unit. As shown in Fig. 2, weakly hybridizing fragments were always detected besides those expected from the restriction map of this region. The PAG 3 mRNA was found to be about 1.5 kb long, 0.9 kb longer than the open reading frame (Fig. 4, probe B). Cloning of the PAG 3 cDNA indicated that this difference is mainly due to the presence of an unusually

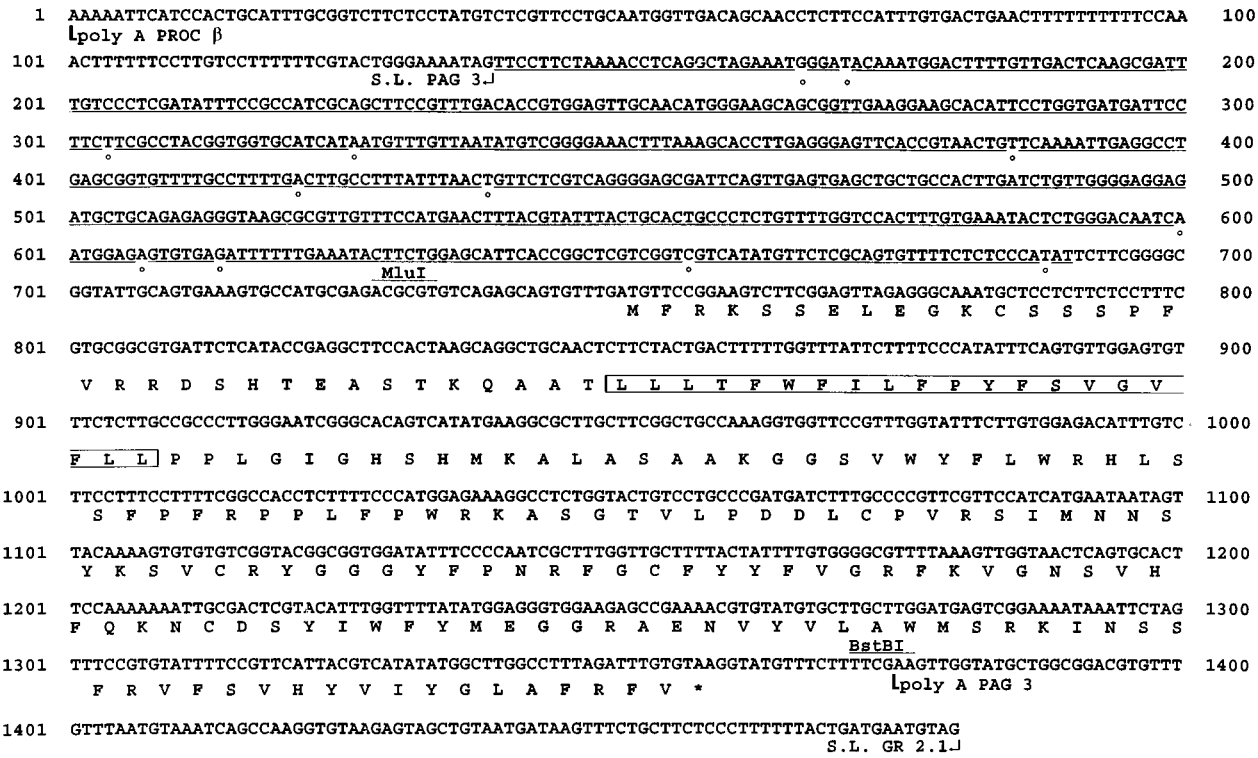

FIG. 10. Nucleotide sequence of the region between the β-procyclin gene and GRESAG 2.1 in the PARP A locus. The first nucleotide is the polyadenylation site of the β-procyclin gene (pRP2001 in reference 27). The underlined sequence is identical to that of the 5′ UTR of the PAG 1 mRNA (15); the dots indicate the differences. S.L., splice leader acceptor site. The boxed sequence is predicted to span a membrane (16).

long 5′ untranslated region (UTR) (Fig. 10). The PAG 3 splice leader addition site was found to be 128 nt downstream from the polyadenylation site of the β-procyclin mRNA (pRP2001 in reference 27), and its polyadenylation site was 103 nt upstream from the splice site of GRESAG 2.1 (Fig. 10). Interestingly, the splice site and the first 650 nt of the PAG 3 mRNA were virtually identical to those of the longest transcripts of gene PAG 1 from the PARP B transcription unit (identical sequences are underlined in Fig. 10). The amino acid sequence of the protein encoded by PAG 3 predicted a hydrophilic protein with an isoelectric point of 10.7. This protein contains a hydrophobic stretch long enough to span a membrane (boxed in Fig. 10).

DISCUSSION

As the trypanosome genome appears to be generally organized in long polycistronic transcription units, the full characterization of such an unit, from the promoter to the terminator, was not achieved. The 45-kb AnTat 1.3A VSG unit was largely sequenced (19), but its termination region may prove difficult to characterize since it lies in telomeric DNA. The procyclin units seem to be exceptionally short and are not telomeric, which facilitates their complete description. The delimitation of their termination region is interesting from two points of view: (i) it allows an additional comparison between the transcriptional characteristics of the α-amanitin-insensitive RNA polymerase active on the procyclin and VSG units, and (ii) it may allow the construction of plasmids with separate trypanosome transcription units. We report the delimitation of the procyclin PARP A transcription unit, including the characterization of the termination region.

The PARP A termination region was able to inhibit CAT activity when inserted between the PARP A promoter and the CAT gene. This effect was likely to be due to transcription termination, as it was observed only with DNA fragments from the region presumed to terminate in vivo, and it was absolutely dependent on the correct orientation with respect to the promoter. An orientation-dependent termination activity was actually demonstrated for the 3.3-kb *Xho*I fragment, using run-on transcription assays in stably transformed trypanosomes. In these assays, transcription termination was found to occur in the beginning of the *Xho*I fragment, as transcription was detectable but strongly reduced in the first half of this fragment. The exact percentage of transcription inhibition was difficult to measure, as a residual α-amanitin-sensitive transcription obscured the data. However, this inhibition was at least sevenfold. This estimate is lower than that determined by transient activity assays (98% inhibition), which may be related to the chromosomal location of the reporter construct.

In the transient activity assays, the inhibitory effect was not limited to a single discrete element, since three distinct fragments from the 3.3-kb *Xho*I region were equally inhibitory. The largest sequence shared by these fragments was the 12-mer CTCTCTCTGCCT, but the involvement of this element in transcription termination was ruled out. A detailed analysis of one of the inhibitory fragments led to the conclusion that several elements, each individually weakly effective, contribute to achieve a full inhibition. No obvious sequence characteristic could be linked to the termination activity. The termination may depend on the secondary structure of the transcripts, which is difficult to evaluate in the absence of a precise mapping of the termination sequence. The characterization of the termination region of the PARP B locus, as well as that of the

ribosomal units, may help to pinpoint the important elements required for termination. It is worth mentioning that the PARP A termination region did not show homology to any known termination site for eukaryotic RNA pol I (17, 26). This observation is in keeping with the absence of homology between these terminators (26).

As expected from the similarities of transcription between the procyclin, VSG, and ribosomal units (7), we found that the PARP A termination region was able to inhibit CAT activity driven by the VSG and ribosomal promoters, although this effect was reproducibly weaker in case of the VSG promoter. This last observation may suggest differences between the procyclin and VSG transcriptional machineries, which indeed show differential elongation activities at each developmental stage (30). Since no promoter for pol II has been characterized with certainty as yet, the sensitivity of pol II transcription to the PARP A terminator was determined by run-on analysis of stable transformants where the reporter construct was inserted in the tubulin unit. Under these conditions, the procyclin terminator was unable to block transcription by RNA pol II. This differential effect of the terminator may be taken as an additional argument that the procyclin unit is not transcribed by pol II. Together with data suggesting that the procyclin promoter can be bypassed by pol II (9, 33), the observation that the procyclin terminator also does not stop this polymerase opens the interesting possibility that the procyclin units are simultaneously transcribed by two RNA polymerases, namely, that recruited by the procyclin promoter together with that from the upstream pol II units. In such a situation, there would be no need for pol II promoters downstream from the pol I units, and the role of the pol I terminator would be limited to prevent a high level of stage-specific transcription of genes located downstream.

The PARP A termination region showed sequence homology with at least one pol II transcription unit specific to the bloodstream form. This is worth noting, since so far only a single transcription unit in *T. brucei*, that of the VSG gene, has exhibited this characteristic. The steady-state transcripts from this bloodstream-specific unit appeared to be very heterogeneous (between 2 and 10 kb), which is reminiscent of the transcription pattern of the trypanosome repeated sequence (TRS) transposable elements (20). However, no significant sequence homology was found between the PARP A termination region and those elements. Further studies are required to clarify this question.

The new procyclin-associated gene described in this work (PAG 3) seems to represent the last one to be discovered in the PARP A unit. Thus, this unit contains four closely packed genes in the first 5 kb, with intergenic regions of only 100 to 150 bp, while the last 3 to 4 kb do not appear to synthesize mRNAs and are devoid of significant open reading frames. The function of PAG 3 is unclear, as its nucleotide sequence does not show homology with known genes. Interestingly, the PAG 3 mRNA contains a long 5′ UTR which is virtually identical to the first half of the 5′ UTR of the longest PAG 1 transcript from the PARP B unit (15). The open reading frame of PAG 3 begins approximately where the nucleotide sequence of the PARP A and B units start to diverge. As the UTRs are likely to be involved in posttranscriptional controls of gene expression (5, 25), and since the presence of long 5′ UTRs is unusual in trypanosomes, it is possible that the PAG 1 and PAG 3 mRNAs are subject to similar controls. In particular, PAG 1 transcripts are processed by alternative splicing (31). It remains to be seen if the same holds true for PAG 3 transcripts.

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