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The Promoter for the Procyclic Acidic Repetitive Protein (PARP) Genes of *Trypanosoma brucei* Shares Features with RNA Polymerase I Promoters

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All eukaryotic protein-coding genes are believed to be transcribed by RNA polymerase (Pol) II. An exception may exist in the protozoan parasite *Trypanosoma brucei*, in which the genes encoding the variant surface glycoprotein (VSG) and procyclic acidic repetitive protein (PARP) are transcribed by an RNA polymerase that is resistant to the Pol II inhibitor α-amanitin. The PARP and VSG genes were proposed to be transcribed by Pol I (C. Shea, M. G.-S. Lee, and L. H. T. Van der Ploeg, Cell 50:603–612, 1987; G. Rudenko, M. G.-S. Lee, and L. H. T. Van der Ploeg, Nucleic Acids Res. 20:303–306, 1992), a suggestion that has been substantiated by the finding that trypanosomes can transcribe protein-coding genes by Pol I (G. Rudenko, H.-M. Chung, V. P. Pham, and L. H. T. Van der Ploeg, EMBO J. 10:3387–3397, 1991). We analyzed the sequence elements of the PARP promoter by linker scanning mutagenesis and compared the PARP promoter with Pol I, Pol II, and Pol III promoters. The PARP promoter appeared to be of limited complexity and contained at least two critical regions. The first was located adjacent to the transcription initiation site (nucleotides [nt] −69 to +12) and contained three discrete domains in which linker scanning mutants affected the transcriptional efficiency: at nt −69 to −56, −37 to −11, and −11 to +12. The second region was located between nt −140 and −131, and a third region may be located between nt −228 and −205. The nucleotide sequences of these elements and their relative positioning with respect to the transcription initiation site did not resemble those of either Pol II or Pol III promoter elements, but rather reflected the organization of Pol I promoters in (i) similarity in the positioning of essential domains in the PARP promoter and Pol I promoters, (ii) strong sequence homology between the PARP core promoter element (nt −37 to −11) and identically positioned nucleotide sequences in the trypanosome rRNA and VSG gene promoters, and (iii) moderate effects on promoter activity of mutations around the transcription initiation site.

Three different types of RNA polymerases are committed to the transcription of particular classes of genes in eukaryotes: RNA polymerase (Pol) I transcribes rRNA genes; Pol II transcribes protein-coding genes; and Pol III transcribes the genes for certain small, stable RNAs, such as tRNA and 5S rRNA genes. The three RNA polymerase activities can be distinguished with the toxin α-amanitin, which can differentially inhibit transcription elongation by Pol II and Pol III (24). The protozoan *Trypanosoma brucei* also contains these three RNA polymerases (11), and most trypanosome protein-coding genes studied to date are transcribed by Pol II. However, a small subset of trypanosome protein-coding genes, the variant surface glycoprotein (VSG) genes (18) and the procyclic acidic repetitive protein (PARP) genes (6, 37), are transcribed by an RNA polymerase that is highly resistant to the drug α-amanitin at concentrations indicative of Pol I transcription. To explain this observation, it has been proposed that VSG and PARP genes are transcribed by Pol I (45, 52). Alternatively, the VSG and PARP genes may be transcribed by an α-amanitin-resistant isoform of Pol II (8, 11).

Transcription of protein-coding genes by Pol I in trypanosomes is supported by the finding that the rRNA promoter could mediate expression of chloramphenicol acetyltransferase (CAT) and *neo* genes in transfected cell lines (38). This transcription occurred in the nucleolus (a separate domain in the nucleus uniquely used for Pol I transcription) and produced stable transcripts that were capped by *trans* splicing (38). As predicted for PARP expression mediated by Pol I, our unpublished data showed that PARP promoter-mediated expression of *neo* genes in transfected cell lines also occurred in the nucleolus (5a). Finally, transcription of VSG and PARP genes, like that of rRNA genes, was shown to be highly resistant to the addition of the ionic detergent Sarkosyl, while transcription of seven other protein-coding genes, believed to be transcribed by Pol II, was completely inhibited by Sarkosyl (40). We proposed that maturation of pre-mRNA in trypanosomes by *trans* splicing, which provides a 5′ cap with the miniexon, may uncouple the requirement for Pol II-mediated transcription of protein-coding genes (38). Support for the second hypothesis, that this transcription is mediated by an α-amanitin-resistant Pol II, was derived from the observations that transcription of VSG genes, like that of Pol II-transcribed genes, is similarly affected by the addition of Mn^{2+} or 1,10-phenanthroline in nuclear run-on reactions (11). Moreover, the discovery of two different genes encoding the largest subunit of Pol II in trypanosomes (8, 47) suggested that one of these Pol II genes could encode an α-amanitin-resistant form of the enzyme (8).

The PARP genes, which are transcribed in an α-amanitin-resistant manner, encode the major surface protein of the

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procyclic (insect) stage of the trypanosome life cycle (25, 34–36). They are organized in tandem arrays of α and β genes at two distinct loci (A and B; identical A alleles and polymorphic B1 and B2 alleles [26, 27, 39]). Each polycistronically transcribed PARP gene array is controlled by a single promoter, which is located less than 1 kb upstream of the 3′ splice acceptor site of the α PARP gene (6, 15, 39). A linker scanning (LS) analysis of the region between the 3′ splice acceptor site (position +86) and the transcription initiation site (position +1) revealed nucleotide sequences of significance for *trans* splicing and demonstrated the absence of gene-internal promoter elements (15).

Pol I, Pol II, and Pol III promoters in all eukaryotes analyzed to date can be distinguished on the basis of several unique features (9, 43, 49). To identify the essential control elements of the PARP promoter and to classify the promoter, we performed a 5′ deletion analysis and LS mutagenesis on the B1-locus PARP promoter. Our results indicate that the PARP promoter has two major regions, a core promoter and an upstream control element. The relative positioning of these elements, their lack of homology to known Pol II promoter elements, and the homology of the core promoter elements to the trypanosome rRNA gene promoter lend support to a model in which PARP transcription is mediated by Pol I.

MATERIALS AND METHODS

Trypanosomes. Procyclic *T. brucei* (stock 427-60) was grown in continuous culture at 27°C in SDM-79 medium (5). The cells were passaged at late log phase every 2 to 3 days. Primer extension experiments were performed on RNA from a 427-60-derived cell line in which the *neo* gene was driven by the wild-type PARP promoter, integrated at the tubulin locus (as described in reference 15). S1 experiments were performed on RNA from a 427-60-derived cell line in which the PARP/*neo* construct was amplified episomally to about 100 copies per cell (19a).

RNA mapping experiments. S1 protection analysis was performed by a modification of the method of Berk and Sharp (2). Two restriction fragments containing the 3′ end of the PARP promoter and the 5′ end of the *neo* gene were 5′ end labeled by T4 polynucleotide kinase. Each labeled fragment (100 ng) was coprecipitated with 20 μg of total trypanosome RNA isolated from a cell line in which the PARP/*neo* construct was amplified to roughly 100 copies per cell (19a); as a negative control, 20 μg of tRNA was used in place of trypanosome RNA. The pellet was resuspended in 20 μl of S1 hybridization buffer (with 80% formamide), heated to 85°C for 10 min, and placed at 42°C for 12 h. The samples were then treated with 1,000 Units of S1 (Bethesda Research Laboratories) per ml for 30 min at 30°C, ethanol precipitated, and resuspended in Tris-EDTA-formamide loading buffer. The samples were run on a denaturing 5% acrylamide gel alongside M13 sequencing ladders (Sequenase).

***T. brucei* transfection.** All transfection experiments were carried out on wild-type procyclic cells as described by Rudenko et al. (38, 39), with modifications. All transfections were performed with limiting amounts of DNA, eliminating any concern about competition for essential factors, and CAT assays were always performed in the linear range of the assay. The cells were grown to mid-log phase (0.8×10^7 to 1×10^7 cells per ml), spun down at 4°C, washed with phosphate-buffered saline containing 0.1% glucose, and resuspended at a cell density of 10^8/ml in Zimmerman postfusion medium. All of the following steps were performed at 4°C. Five hundred microliters of cells (5×10^7 cells) was transferred to a 0.4-cm Biorad Gene Pulsar cuvette. A PARP promoter plasmid and a LacZ control plasmid (20 μg of each) were added sterilely to the cells. The cells were electroporated with a single pulse of a Biorad Gene Pulsar. The electroporation conditions were 1,600 V (deletion analysis) or 1,400 V (LS analysis) and 25 μF. Following electroporation, the cells were placed on ice for 10 min and then transferred to 25-cm^2 flasks containing 10 ml of SDM-79 medium (room temperature). The cells were incubated at 27°C for 20 to 22 h before preparation of whole-cell extracts for assay of CAT and β-galactosidase activities.

CAT and β-galactosidase assays. CAT and β-galactosidase assays were performed as described by Rudenko et al. (38), with one half of the whole-cell extract being used for each of the enzyme assays.

5′ deletion analysis. 5′ deletion analysis was performed on plasmid Bαsp, which has a 830-bp insert containing the B1-locus PARP promoter and the 3′ splice acceptor site of the α PARP gene. Plasmid Bαsp was digested with *Bam*HI and *Bst*XI and subjected to treatment with 8 U of exonuclease III (New England Biolabs) for 5 min at 37°C. Aliquots were collected at 30-s intervals, treated with 4 U of S1 nuclease (New England Biolabs) for 20 min at room temperature, and then treated with 1 U of Klenow enzyme (Boehringer Mannheim) to generate blunt ends. A 10-bp phosphorylated linker containing a recognition sequence for *Xba*I (New England Biolabs) was ligated onto the ends of all deletion plasmids, which were then digested with *Xba*I. The plasmids were separated from free linkers by electrophoresis on a 0.7% low-melting-point agarose gel, gel purified, and recircularized at the *Xba*I restriction enzyme site. The deletion mutants were cloned into plasmid BCi, which contains the CAT gene flanked at its 3′ end by the PARP α/β intergenic region, which provides signals and sites for polyadenylation.

LS mutagenesis. Ten-base-pair LS mutants were generated starting with a minimal PARP promoter deletion plasmid (extending up to nucleotide [nt] −312) giving full promoter activity. The −312 PARP promoter fragment was cloned in both orientations into pBluescript (Stratagene) to permit generation of 5′ and 3′ deletion series, using exonuclease III. A 10-bp *Xba*I linker was ligated onto the deleted end of each fragment. 5′ and 3′ deletion mutants whose deletion endpoints were separated by 11 bp were ligated together at the *Xba*I site; for each LS mutant, the 5′ deletion contributed the vector, and the 3′ deletion contributed an inserted fragment. All LS mutants were sequenced completely on one strand (Sequenase; U.S. Biochemical) to confirm the position of the linker and to rule out the possible introduction of secondary mutations during the cloning steps. Finally, the LS mutants were subcloned into plasmid BCi to generate the functional CAT constructs.

Fourteen-base-pair LS mutants were generated by digesting a 10-bp LS mutant, whose deletion endpoints were separated by 15 bp, with *Xba*I, filling in the ends with Klenow, and recircularizing. Twenty-two-base-pair LS mutants were generated by digesting 10-bp LS mutants, whose deletion endpoints were separated by 23 bp, with *Xba*I and inserting an 8-bp double-stranded oligonucleotide with *Xba*I-compatible ends. The top-strand oligonucleotide had the sequence 5′-CTAGACTTCCTT. The bottom-strand oligonucleotide had the sequence 5′-CTAGAAGGAAGT.

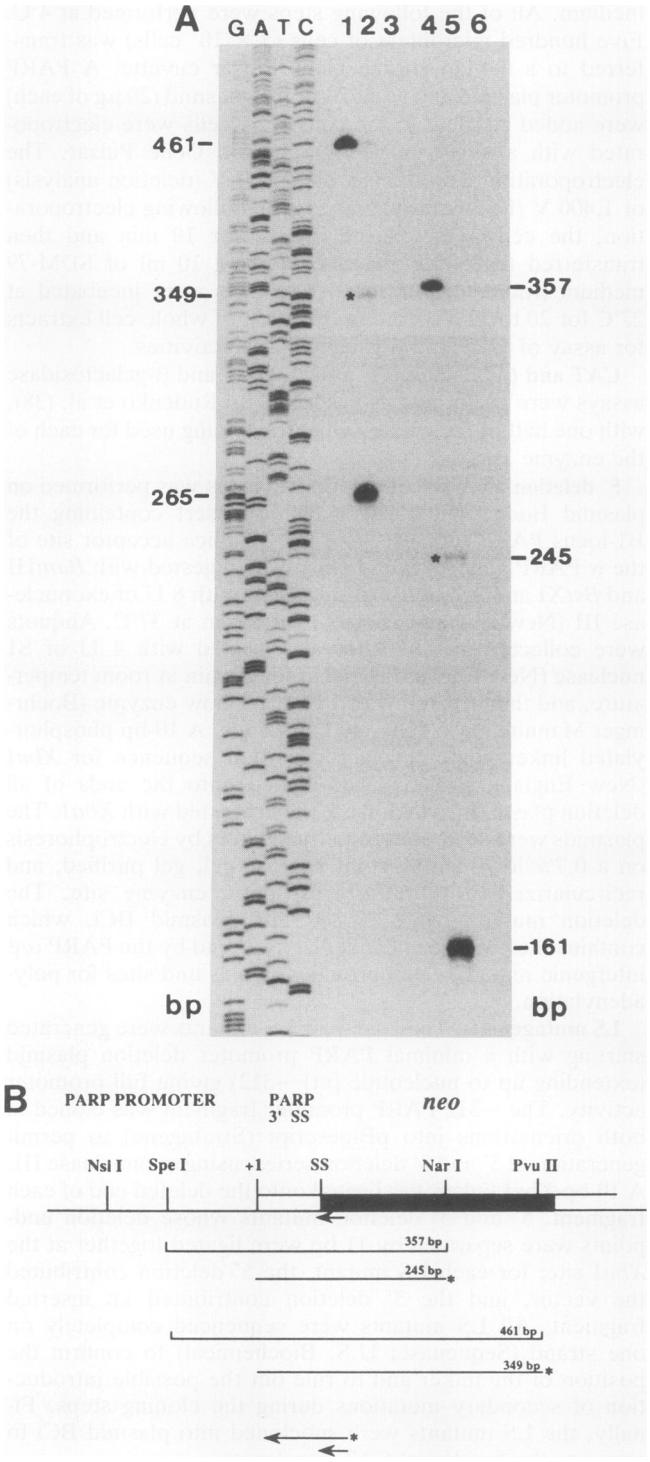

FIG. 1. Mapping of the initiation site of the B1-locus PARP promoter. (A) S1 analysis of total RNA from a cell line with an amplified PARP/*neo* construct. Two restriction fragments, *Spe*I-*Pvu*II (lanes 1 to 3) and *Spe*I-*Nar*I (lanes 4 to 6), were 5′ end labeled at the *Pvu*II and *Nar*I sites, respectively. The 5′-end-labeled probes (input in lanes 1 and 4) were hybridized for 12 h with either 20 μg of total trypanosome RNA (lanes 2 and 5) or 20 μg of tRNA (lanes 3 and 6). The samples were treated with S1, precipitated, resuspended in formamide loading buffer, and loaded onto a denaturing 5% acrylamide gel. The sizes of the fragments of the *Spe*I-*Pvu*II reaction are indicated at the left; those of the *Spe*I-*Nar*I reaction are indicated at the right. Non-*trans*-spliced, precursor RNA is labeled with asterisks. (B) Physical map of the PARP/*neo* construct, including the PARP 3′ splice acceptor site (SS). The two S1 probes, as well as two of the S1-protected fragments (marked with asterisks), are shown with their respective sizes. At the bottom, the locations of the *neo* primer (short arrow) and previously mapped extension product (long arrow with asterisk) are shown.

RESULTS

The PARP promoter transcription initiation site. All trypanosome pre-mRNAs are matured by *trans* splicing, leading to the addition of a capped 39-nt spliced leader or miniexon onto the 5′ end of every mRNA (3, 50, 53; reviewed in references 1, 4, and 19). Miniexon addition onto the 5′ end of the PARP pre-mRNA is a rapid process, making the identification of precursor RNA molecules and the mapping of the transcription initiation sites difficult. Pays et al. (32) determined the location of a potential PARP promoter transcription initiation site by primer extension of PARP pre-mRNA. They mapped a strong stop to nt −86 upstream of the 3′ splice acceptor site. Huang and Van der Ploeg (15) performed primer extension analysis on total RNA from stably transfected trypanosome cell lines, in which a CAT gene and a *neo* gene were each transcribed from a B1-locus PARP promoter. Using oligonucleotides complementary to either CAT mRNA or *neo* mRNA, they identified a strong stop corresponding to an RNA species whose PARP promoter initiation site was located 86 nt upstream of the 3′ splice acceptor site.

By S1 protection analysis of the pre-mRNA, we further confirmed that the strong stop at −86 nt represents the 5′ end of a *neo* pre-mRNA. We used RNA from a trypanosome cell line in which the PARP/*neo* construct was amplified to about 100 copies per cell (19a). Using two 5′-end-labeled restriction fragments as probes (*Spe*I-*Nar*I [357 bp] and *Spe*I-*Pvu*II [461 bp]; Fig. 1B), we observed S1 protected fragments of 245 and 349 bp, respectively, that map the location of the 5′ end of the pre-mRNA to a position at 84 nt upstream of the 3′ splice acceptor site (Fig. 1A, lanes 2 and 5; see Fig. 1B for locations of the fragments). The mature mRNA is identified by the 161- and 265-bp fragments. The 2-nt discrepancy between the location of the transcription initiation site by primer extension mapping (86 nt upstream of the 3′ splice acceptor site; see Fig. 1B for locations of primer and extended product) and the protected fragments in the S1 protection analysis (84 nt upstream of the 3′ splice acceptor site) is most likely due to S1 nibbling of the ends of the RNA-DNA hybrids or due to structure at the 5′ end of the pre-mRNA which affected the S1 protection and primer extension differently. Additional experiments using primer extension on total RNA and RNA that was pretreated with a HeLa cell S100 debranching extract (41, 42) showed that the strong stop at −86 nt was resistant to debranching activity, while control ^{32}P-labeled lariat β-globin RNA (30) was efficiently debranched (data not shown). We conclude that the transcription initiation site of the PARP promoter is located at about 86 nt upstream of the 3′ splice acceptor site.

Our estimation of the location of the PARP B-locus transcription initiation site is similar but not identical to the location of the PARP A-locus initiation site, estimated to be located 82 nt upstream of its 3′ splice acceptor site (46). It is unclear whether the 4-nt difference in the location of the transcription initiation site reflects an experimental artifact or a true difference between PARP A and B promoters.

5′ deletion analysis of the PARP promoter. We were interested in analyzing the sequences required for proper functioning of the PARP promoter. We had determined previously that the region extending from the 3′ splice acceptor site up to 830 bp upstream of the 3′ splice acceptor site conferred α-amanitin-resistant transcription onto the *neo* gene (20), showing that these sequences contained a functional PARP promoter. By contrast, sequences of significance for *trans* splicing were located between 80 nt upstream of the 3′ splice acceptor site and the splice acceptor site, and mutations in these sequences affected only *trans* splicing, having no obvious effect on promoter functioning (15). Nuclear run-on analysis had also revealed that the sequences immediately upstream of the 3′ splice acceptor site were not detectably transcribed, indicating that the transcription initiation site must be located within ~200 nt upstream of the 3′ splice acceptor site (39). These data are thus consistent with the PARP promoter extending 5′ from nt 80 upstream of the 3′ splice acceptor site. To narrow down the most 5′ border of this 830-bp fragment, we therefore first performed a 5′ deletion analysis of the putative PARP promoter region.

To narrow down the location of the PARP promoter, we subjected the 830-bp promoter and 3′ splice acceptor site containing fragment (extending 737 bp upstream of the proposed transcription initiation site) to 5′ deletion analysis using exonuclease III. A series of 16 deletion mutants was generated and cloned upstream of the *Escherichia coli* CAT reporter gene. An analysis of the CAT activity of these 5′ deletion mutants (Fig. 2A) showed that the first 580 bp of this clone (nt −737 to −250 relative to the transcription initiation site) were not essential for promoter activity, thereby localizing the 5′ end of the PARP promoter to a position approximately 250 bp upstream of the transcription initiation site. Between nt −228 and −207, CAT activity was reduced from 93% to 64%. A plateau, with a CAT activity of around 60%, was observed in the region extending between nt −207 and −154. Thereafter, a gradual reduction in CAT activity was observed in the subsequent 5′ deletion mutants, highlighting the presence of several essential PARP promoter elements.

As an internal control for the transfection efficiency, a second reporter plasmid, containing the *E. coli lacZ* gene under the control of a wild-type PARP promoter, was cotransfected with the PARP promoter deletion constructs. β-Galactosidase assays were performed on the same cell extracts as those used for the CAT assays (Fig. 2B) to ensure that the changes in the CAT activity observed in the deletion series were not the result of variations in the transfection efficiency. As expected, the β-galactosidase activities of extracts from these transfections showed only minor experimental variation, thus confirming that the 5′ deletions at the PARP promoter were responsible for the progressive reduction in CAT activity.

LS mutagenesis. The deletion analysis localized the 5′ end of the PARP promoter to a position approximately 250 bp upstream of the transcription initiation site. To determine the location of critical control elements of the PARP promoter, we performed LS mutagenesis (23) on a minimal, but fully active, PARP promoter fragment (extending up to nt −312). The LS mutants replaced 10-bp sequences in a series of 31 clones, extending from positions −272 to +12 (Fig. 3). The LS mutants were cloned upstream of the CAT gene, and CAT assays were performed as before (Fig. 4) with LacZ cotransfections as internal controls. Again, all β-galactosidase controls showed that the transfection efficiencies were

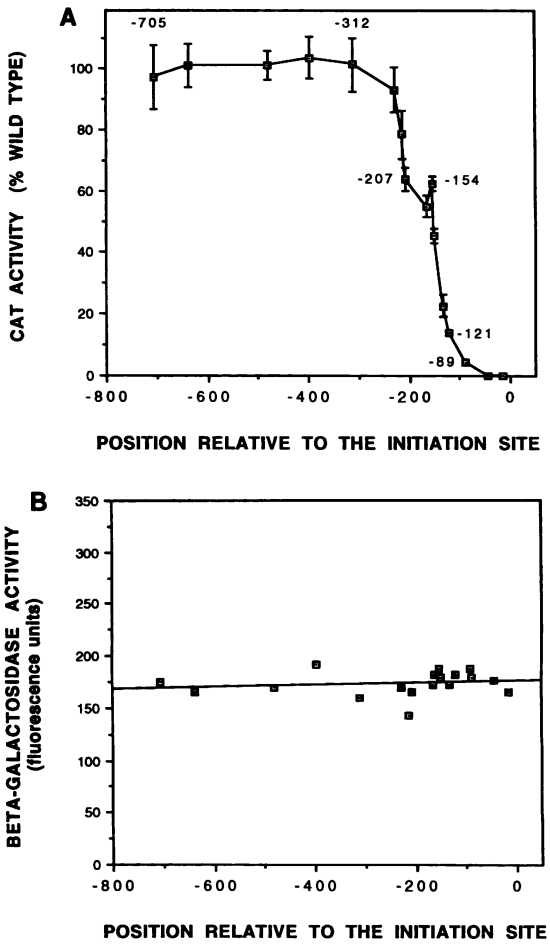

FIG. 2. 5′ deletion analysis of the PARP promoter. (A) CAT activities of 16 5′ deletion mutants of the PARP promoter. Trypanosomes (5×10^7) were transfected with 20 μg of promoter plasmid. Twenty hours after transfection, whole-cell extracts were prepared; half was used for the CAT assays, and half was used for the β-galactosidase assays (see below). CAT activities were measured after incubation of cell extracts for 2 h at 37°C, using 50 μM chloramphenicol, 250 μM butyryl coenzyme A, and 0.5 μCi [^{3}H]chloramphenicol, followed by xylene extraction and scintillation counting, as described by Seed and Sheen (44). The squares and bars represent the means and standard deviations, respectively, of three independent experiments performed in duplicate. The negative control is plasmid BCi containing the CAT gene and intergenic region alone. In a typical transfection, 20 μg of full-promoter plasmid generates a CAT activity of 250,000 cpm, compared with a background of 1,000 cpm for the CAT gene alone. To ensure that all CAT data were in the linear range of the enzyme assay, various dilutions of purified *E. coli* CAT enzyme (1 U/μl; Pharmacia) were assayed in parallel with the trypanosome extracts. The deletion endpoints are as follows: −705, −636, −479, −398, −312, −228, −215, −207, −167, −154, −150, −135, −121, −89, −45, and −15. Six deletions that are discussed extensively in the text are labeled on the graph. (B) β-Galactosidase activities of the same extracts after cotransfection with a plasmid containing the *E. coli lacZ* gene under the control of the wild-type PARP promoter. Twenty micrograms of plasmid was cotransfected with 20 μg of PARP promoter deletion plasmid. β-Galactosidase assays were performed as 30-min incubations at 37°C as described by Rudenko et al. (38). Fluorometric detection was with a Titertek Fluoroskan II. The points represent means of two independent experiments performed in duplicate.

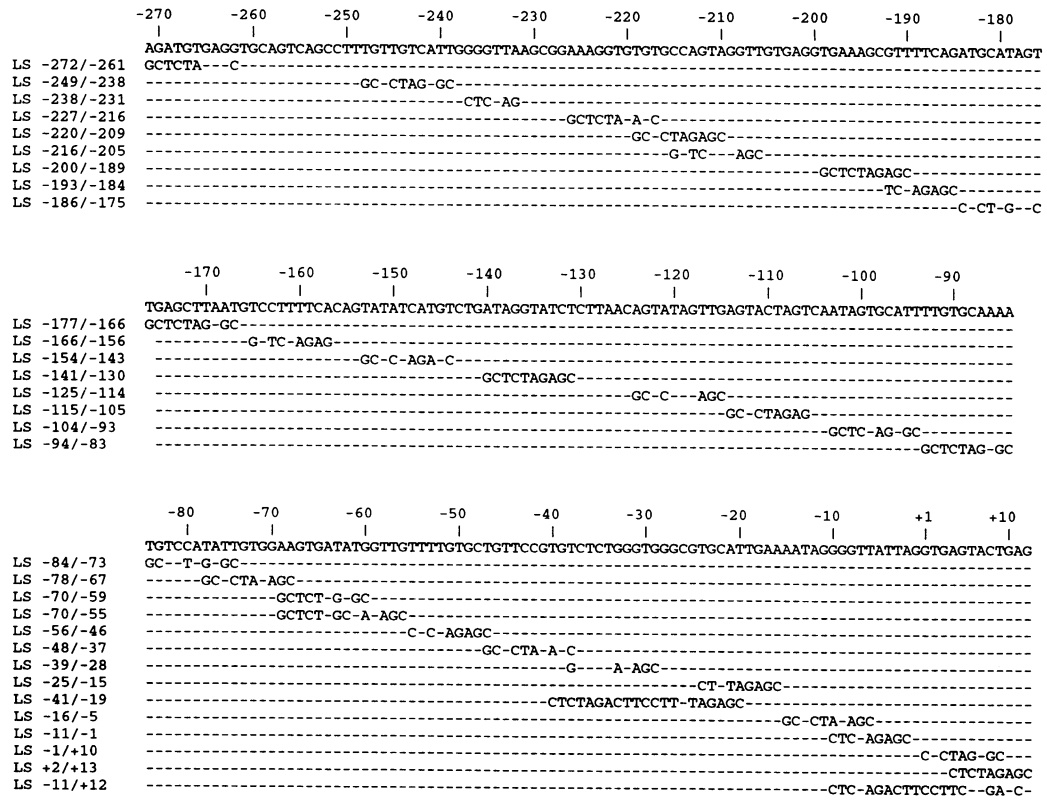

FIG. 3. Scheme for LS mutagenesis of the PARP promoter: 10-, 14-, and 22-bp replacements. LS mutants were generated as described in Materials and Methods, using a 10-bp *Xba*I linker containing the sequence 5′-GCTCTAGAGC. Two mutants, LS −41/−19 and −11/+12, were generated by using a series of synthetic oligonucleotides as described in the Materials and Methods. Dashes indicate positions of wild-type sequence, and letters show the positions of mismatch between the *Xba*I linker and the wild-type promoter sequence. The nucleotide positions in the name of each LS mutant indicate the flanking nucleotides.

comparable in all samples (data not shown), allowing a straightforward interpretation of the CAT assay results.

The majority of the LS mutants exhibited little change in CAT activity compared with the wild-type PARP promoter, and levels ranged from 83 to 123% of the wild-type CAT levels. However, LS mutations in four regions resulted in significant reductions of CAT activity. These LS mutants identified critical nucleotide sequences between nt −140 and −131, −69 and −56, −37 and −11, and −11 and +12 (Fig. 4, sequences highlighted with black boxes). From 5′ to 3′, the first potentially interesting LS mutant (−216/−205) gave a reduction in CAT activity to only 68%. The significance of this LS mutant is unclear because of its minimal reduction in CAT activity; however, this element falls in a region in which there is a 30% reduction in CAT activity, as shown by deletion analysis. LS mutant −141/−130 gave a reduction to 30% of wild-type CAT activity, and LS mutant −70/−55 gave a reduction in CAT activity to 35%. The strongest effects were seen in the region between nt −48 and −5. The approximate 5′ and 3′ borders of this region were determined by comparing a series of overlapping 10-bp LS mutants extending from nt −48 to −5, as well as a 22-bp LS mutant extending from nt −41 to −19, which gave background CAT activity (Fig. 4). The 3′ border was determined by comparing LS mutant −11/−1, which gave wild-type CAT activity, with LS mutant −16/−5, which showed only 7% of wild-type activity. We thus assume that the important nucleotides are located upstream of nt −11. Similar reasoning deduced the 5′ border located to between nt −37 and −28. Finally, three LS mutants contained nucleotide substitutions around the transcription initiation site: LS mutants −11/−1, +2/+13, and −11/+12 (Fig. 4). Only LS mutant −11/+12 showed a significant reduction in CAT activity to 44% of wild-type CAT activity. Linker scanning analysis of the PARP promoter thus identified at least two discrete regions (−140/−131 and −69/+11) whose sequences were essential for PARP promoter functioning.

DISCUSSION

Description of the PARP promoter. We have identified several critical elements of the B1-locus PARP promoter of *T. brucei*, located in the region between nt −250 and its single transcription initiation site at nt +1. Currently, this region appears to be the only area of significance for PARP promoter function. Huang and Van der Ploeg (15) had previously shown that gene-internal control elements were absent from the region between the transcription initiation site and the 3′ splice acceptor site, thus ruling out the possibility that the PARP promoter has the structure of a classical Pol III promoter with A- and B-box internal promoter elements. We therefore compared the sequences and locations of PARP promoter controlling elements with those of Pol I (rRNA) and Pol II promoters. For comparison, we also used the trypanosome Pol I promoter (38, 54, 56). Since a detailed analysis of a trypanosome Pol II promoter has not yet been performed, we will rely on known Pol II promoter elements of other eukaryotes. Given the evolutionary con-

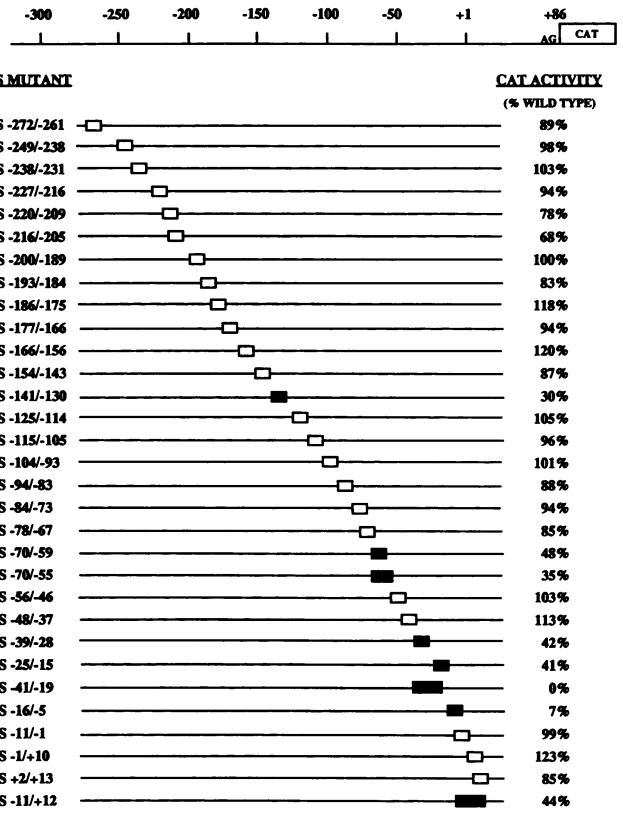

FIG. 4. CAT activities of LS mutants. CAT activities were determined exactly as described for the 5′ deletions (Fig. 2). Twenty micrograms of LS mutant and 20 μg of LacZ plasmid were cotransfected, and half of the extract was used for each enzyme assay (β-galactosidase data not shown). The CAT values represent the means of two independent experiments performed in duplicate. The variability between the two experiments was less than 10% for each point. The horizontal black lines represent the minimal PARP promoter, and the boxes represent the position and size of the linker for each mutant, as indicated by the physical map at the top. Filled boxes represent LS mutants that show significant reductions in CAT activity relative to the wild-type promoter. All linkers are 10 bp except for LS −70/−55, which is 14 bp, and LS −41/−19 and LS −11/+12, which are both 22 bp.

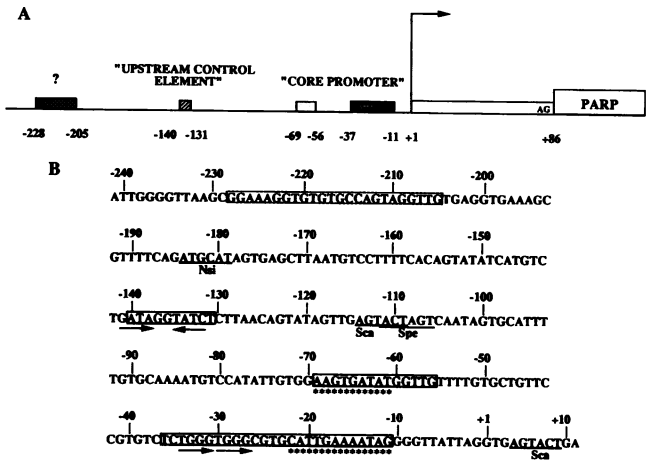

FIG. 5. Structure of the PARP promoter. (A) Schematic of the PARP promoter control elements. Boxes correspond to promoter elements described in the text. We propose that the −69/−56 element and the −37/−11 element function together to form a core promoter. The core element is stimulated by a strong upstream control element at −140/−131. The region between −228 and −205 appears critical in deletion experiments, although no discrete element is identified by LS analysis. (B) Sequence of the B1-locus PARP promoter, highlighting the control elements. Critical elements, as shown by LS analysis, are boxed. Arrows indicate repeats. The −140/−131 element has dyad symmetry. The −37/−11 element has two direct repeats in tandem. The regions underlined by asterisks show homology to the *T. brucei* rRNA gene promoter, as elaborated in Fig. 6. The *Nsi*I, *Spe*I, and *Sca*I restriction sites are underlined.

servation of the large subunit of RNA Pol II in trypanosomes and other eukaryotes (8, 47), we assume that trypanosome Pol II promoters have features in common with other eukaryotic Pol II promoters. This assumption is strengthened by the observations of Thompson et al. (51) showing that mammalian Pol II proteins lacking the C-terminal tail on the largest subunit (as is the case for the trypanosome Pol II) respond to the normal Pol II promoter elements.

The 5′ deletion analysis revealed that the 5′ border of the promoter could be mapped to nt −250. The deletion clones indicated critical control elements located upstream of nt −207 and downstream of nt −154. LS mutagenesis accurately defined two (and possibly three) essential promoter regions: one between nt −69 and +12, a second between nt −140 and −131, and a third that may be located further upstream, between nt −228 and −205. Deletion of the region between −228 and −205 generated a 30% reduction in CAT activity. However, no LS mutant in the vicinity showed very strong effects. Three other LS mutants in the region between nt −228 and −205 were tested, and none showed a reduction in CAT activity below 68% of the wild-type level (data not shown). Thus, while it would appear that the region between −228 and −205 is important for promoter activity, no strong, discrete elements are observable by LS analysis.

Three discrete elements are present in the region between nt −69 and +12: −69/−56, −37/−11, and −11/+12. A comparison of the results of the deletion series and the LS analysis revealed that while the −37/−11 element was an essential and strong promoter element, it could not function in the absence of the −69/−56 element. We therefore propose that the −37/−11 element and the −69/−56 element together function as a core promoter (Fig. 5A), generating basal promoter activity at about 5% of the wild-type promoter level (as demonstrated by a 5′ −89 deletion mutant; Fig. 2A). This conclusion is substantiated by the observation that the 5′ deletion containing only the most proximal (−37/−11) promoter element (−45 deletion clone) lacked promoter activity. To determine whether the failure of the proximal promoter element to drive CAT expression was due to inefficient transfection, we also transfected cells with higher concentrations of this deletion mutant DNA (data not shown). These experiments demonstrated that even at concentrations of DNA 10-fold higher than those used normally, the −37/−11 element was still unable to generate CAT activity. We conclude that while the −37/−11 element is an essential promoter element, it requires the −69/−56 promoter element for promoter functioning. The element at nt −140/−131, which contains an inverted repeat structure (Fig. 5B), stimulates the activity of this core promoter by about four- to fivefold (as shown by a comparison of a −154 deletion and a −121 deletion: 64%/13.7% = 4.7).

Comparison of the PARP promoter with Pol I and Pol II

promoters. We have suggested that α-amanitin-resistant transcription of trypanosome protein-coding genes may be mediated by Pol I (38, 40, 45). To investigate whether the PARP genes could be transcribed by Pol I, we compared the B1-locus PARP promoter with Pol I and Pol II promoters. Pol I promoters of diverse eukaryotes share characteristic features which differentiate them from Pol II promoters (13, 14, 16, 22, 28, 55; reviewed in references 33, 48, and 49). (i) Pol I promoters have a unique, highly conserved domain structure which differs from that of Pol II promoters. Pol I promoters consist of a large core promoter element residing between nt −70 (*Saccharomyces cerevisiae*) or nt −40 (*Xenopus*, mouse, and human) and nt +10, an upstream control element between nt −140 and −130, and a transcription terminator (allowing efficient promoter functioning) located at nt −170. These control elements are found at virtually identical positions in different Pol I promoters (29, 55), and the spacing between controlling elements in the core promoter is conserved (16, 17). (ii) In contrast to most Pol II promoters, the nucleotide sequence context near the transcription initiation site is important for Pol I promoter functioning (14, 28, 55), and the Pol I promoter elements do not resemble known Pol II and Pol III control elements (21). (iii) The various Pol I promoters lack absolute nucleotide sequence conservation across different species (12).

An overview of the analysis of the PARP promoter domains, as revealed by LS mutagenesis, is shown in Fig. 5A. This summary reveals that the PARP promoter is structurally similar to a Pol I promoter in that its core domain is a broad element located just upstream of the initiation site and that its upstream stimulatory domain occurs exactly at nt −140 to −131. The PARP promoter also contains an additional domain, at nt −69 to −56, which is similar to elements found in the *Xenopus* and *S. cerevisiae* Pol I promoters (28, 55). The −228/−205 element, while weak, may function as either a weak upstream control element or a terminator.

The nucleotide sequences of these control elements are shown in Fig. 5B. None of the sequence elements reveals similarities with common eukaryotic Pol II promoter elements, such as the TATA box, CCAAT box, Sp1 GC box, or other well-characterized Pol II enhancer or silencer elements. Both of the elements of the PARP promoter do, however, show homology with the *T. brucei* rRNA gene promoter (Fig. 6). These regions of homology are located at nearly identical positions in both the rRNA and PARP promoters. Finally, Rudenko et al. (38) showed that the nucleotide sequence context at the transcription initiation site of the trypanosome rRNA promoter was critical for promoter functioning. An analysis of LS mutants in the vicinity of the PARP promoter transcription initiation site showed that a 22-bp LS mutant between nt −11 and +12 reduced CAT activity to 44% of wild-type activity. A more detailed analysis involving point mutations is needed to understand the significance of the individual nucleotide sequence changes and their sequence context. We conclude that the PARP promoter resembles a Pol I promoter in overall domain structure, in nucleotide sequence homology with the *T. brucei* rRNA promoter, and in dependence for specific nucleotide sequences in the region surrounding the transcription initiation site.

Other notable PARP promoter sequence elements, such as the large inverted repeat at −100 to −85 and the region homologous to the human c-*fos* serum response element at −80 to −70 (7), have not been shown to be important for promoter activity. This, however, does not preclude the possibility that such sequence elements may be important for differential regulation of the PARP promoter.

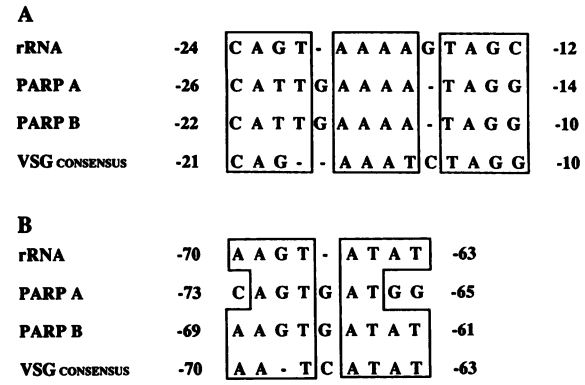

FIG. 6. Sequence homology between two PARP promoter control elements and the rRNA gene and VSG gene promoters. (A) Homology between the rRNA, PARP, and VSG gene promoters at positions −26 to −10. Conserved regions are boxed. Spaces are introduced to maximize homology. $VSG_{CONSENSUS}$ refers to the consensus sequence of the VSG 118 clone 1, VSG 221, and AnTat 1.3 promoters. For the VSG 221 promoter, the sequence positions are considered relative to the principal, downstream initiation site (called position +39 in reference 56). For the VSG 118 clone 1 promoter, the initiation site has been mapped to within 6 nt (10). Sequence data were obtained from White et al. (54) (rRNA gene promoter), Rudenko et al. (39) (PARP A and PARP B promoters), Zomerdijk et al. (57) and Zomerdijk et al. (56) (VSG 221 promoter), Gottesdiener et al. (10) and Gottesdiener (9a) (VSG 118 clone 1 promoter), and Pays et al. (31, 32) (VSG AnTat 1.3 promoter). (B) Homology between the rRNA, PARP, and VSG gene promoters at positions −73 to −61. $VSG_{CONSENSUS}$ has the same signification as in panel A.

Recently, a similar LS analysis, but of the PARP A locus, has been performed by Sherman et al. (46). Their analysis of the PARP promoter is essentially in agreement with ours except for our assignment of critical importance to the −140/−131 region, which we believe to be a major stimulatory element of the promoter. The importance of the −140/−131 element is demonstrated by (i) the discrete drop in CAT activity for the −140/−131 LS mutant (Fig. 4), (ii) the appearance of a plateau of CAT values for 5′ deletion clones upstream of this region (clones from −154 to −207 show a CAT activity of about 60% of wild-type activity [Fig. 2A]), and (iii) the finding that a 5′ deletion that removes one half of the inverted repeat (−135 deletion) reduces the CAT activity to 22%, while a 5′ deletion that removes the whole element (−121 deletion) shows a further reduction to 14% of wild-type activity. Sherman et al. also demonstrate the effects of internal insertions and deletions on promoter activity, showing that half- and full-helical-turn changes in spacing have strong effects of the PARP promoter activity. This dependence on spacing further supports the notion that the PARP promoter shares features with Pol I promoters.

Many attempts have been made to find common sequence elements between the various α-amanitin-resistant promoters of trypanosomes. One interesting feature of the homology between the core domain of the PARP promoter and the *T. brucei* rRNA promoter is the presence of similar domains in three cloned VSG promoters (Fig. 6). Strikingly, this homology occurs at nearly identical positions in all three types of the α-amanitin-resistant trypanosome promoters. A previously identified subsidiary VSG promoter (45) also exhibits extensive homology in this region. This is important

in light of the failure of previous investigators to observe any homology between these promoters (56). Thus, it is possible that these two domains identify the elusive Pol I boxes of the VSG and PARP promoters. Zomerdijk et al. (56) have recently performed a deletion analysis of the VSG 221 promoter and the trypanosome rRNA promoter. They map the 5′ border of the VSG promoter to nt −105 relative to the principal, downstream initiation site and that of the rRNA promoter to −258 relative to its initiation site. Thus, both of the homology domains outlined in Fig. 6 are contained within regions critical for promoter activity for the VSG and rRNA promoters. Since more detailed mutagenesis has not been performed on either promoter, it is impossible to deduce the domain structure of either of these promoters. However, from the deletion data, it seems reasonable to conclude that the VSG promoter is more compact than either the PARP or rRNA promoter. Furthermore, if the VSG promoter, like the PARP promoter, contains an upstream stimulatory domain, it is most likely that this domain is located closer to its core promoter than is that of the PARP promoter. Deletion analysis of the rRNA promoter demonstrates a further similarity between the PARP promoter and the rRNA promoter, with both promoters having 5′ borders in the vicinity of nt −250.

The mutagenesis data presented here support the contention that Pol I transcribes the PARP genes. This notion is further supported by the ability of trypanosome Pol I to transcribe protein-coding genes (38) and by our unpublished observations which show that the PARP promoter, driving a *neo* gene integrated at the Pol II-transcribed tubulin locus, is transcribed at the nucleolus (5a). It is therefore possible that the ability of trypanosomes to transcribe protein-coding genes by Pol I has its in vivo corollary in the transcription of the PARP and VSG genes.

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