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The region encompassing the procyclic acidic repetitive protein (PARP) gene promoter plays a role in plasmid DNA replication in *Trypanosoma brucei*

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ABSRACT

We have previously reported the construction and characterization of an autonomously replicating plasmid in *Trypanosoma brucei.* In this plasmid the procyclic acidic repetitive protein (PARP) gene promoter drives the transcription of a selectable marker. Deletion of this promoter incapacitates the plasmid, suggesting its utilization as a promoter-trap. Three independent libraries were created by inserting variously digested *T.brucei* genomic DNA into this promoterless construct. Transfection of these libraries into procyclic *T.brucei* and the subsequent isolation of episomes led only to the reisolation of the PARP promoter. Additionally, a ribosomal RNA promoter failed to keep the construct as an episome, although it can sustain mRNA transcription in *T.brucei* and was shown to be an efficient promoter in this construct. Finally, by using a transient replication assay involving the methylation-sensitive restriction endonuclease *Dpn*I to distinguish between input and replicated DNA, we showed that the PARP promoter-bearing construct could replicate autonomously in procyclic *T.brucei,* but the corresponding construct with the rRNA promoter could not. The close association between elements that sustain transcription and DNA replication in *T.brucei* mirrors results observed in several higher eukaryotes and their viruses and suggests an ancient origin of this feature.

INTRODUCTION

The protozoan parasite *Trypanosoma brucei* (of the order Kinetoplastida) is one of the causative agents of African trypanosomiasis. Phylogenetic analyses demonstrate that these organisms are among the most ancient eukaryotic lineages known (1–3). The parasite has a digenetic life-style, alternating between the mammalian bloodstream and the midgut and salivary glands of its insect vector (*Glossina* spp.).

Little is known about the regulation of gene expression in *T.brucei*. Transcription is apparently polycistronic and the *trans*-splicing of a 39 nucleotide spliced leader onto each mature mRNA means that the 5′ end of the primary transcript is quickly lost. Consequently, transcription initiation sites and promoters have been difficult to delineate. Only 3 promoters have been characterized: the promoters for ribosomal RNA (rRNA), the procyclic acidic repetitive protein (PARP or procyclin) and the variant surface glycoprotein (VSG) genes (4–9). PARP and VSG are the major surface proteins in the procyclic (insect midgut stage) and bloodstream forms of the parasite. All three of these promoters mediate transcription that is resistant to the drug α-amanitin, a feature characteristic of RNA polymerase I (pol I). Largely as a result of this and other evidence (for example see 10), PARP and VSG genes are thought to be transcribed by pol I. Additionally, it has been shown that the rRNA promoter can support mRNA synthesis in *T.brucei*, with the 39 nt spliced leader providing the 5′-cap that is a necessary feature of all eukaryotic mRNAs (11, 12). Although α-amanitin-sensitive transcription units are known, no RNA polymerase II (pol II) promoters have been found.

To attempt to identify additional promoters in *T.brucei*, we made use of a panel of autonomously replicating episomes that we recently constructed and characterized (13). These were made by inserting random pieces of *T.brucei* genomic DNA into a plasmid (pH51) that could not otherwise exist as a stable replicon. We call the inserted pieces of DNA the plasmid maintenance sequence or PMS. Two members of this panel, plasmids pT13-11 and pT13-41, were extensively characterized. Both exist in procyclic *T.brucei* as single-copy episomes that nonetheless demonstrate unusual stability in the absence of selection. In each of them, the PARP promoter mediates transcription of the neomycin phosphotransferase (NPT-II) gene, which constitutes the selectable marker. We sought to precisely delete this promoter from the plasmid pT13-11 and replace it with a functionally equivalent one from the *T.brucei* genome.

Our experiments were aided by a detailed knowledge of the region encompassing the PARP promoter. The promoter and signals essential for *trans*-splicing are contained within a 300 bp 5′ flanking region upstream of the PARP initiator codon (5). Linker scanning analyses indicate that the promoter consists of

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two major elements centered at positions −38 and −68 with respect to the transcription start site of PARP (7). Brown *et al.* (1992) place these elements between positions −37 to −11 and −69 to −56 respectively. Additional elements (at least one of which is further upstream of these two) play a lesser though significant role. The splice acceptor signals (SAS) map to a polypyrimidine tract, which extends between +46 and +72 with respect to the transcriptional start site and can be separated from the promoter (14).

Removal of a 108 bp region corresponding to the major elements of the PARP promoter incapacitates pT13-11, suggesting its use as a promoter-trap. However, in experiments involving three independent libraries created by inserting variously digested *T.brucei* genomic DNA into this promoterless construct, we were only able to re-isolate the PARP promoter. An investigation of this surprising outcome led us to experiments that demonstrate a role of the PARP promoter region in plasmid DNA replication in addition to its previously elucidated role in transcription.

MATERIALS AND METHODS

Culture, transfection of trypanosomes, and preparation of Hirt DNA

These were performed exactly as previously described (13).

Plasmids

The plasmid pT13-11 has been previously described (13) and is shown in Figure 1. The plasmids pJP44 and pHD30 (see below for their use) have been described earlier (7, 43) and were kindly provided to us by Christine Clayton (University of Heidelberg, Germany). The plasmid pT13-11S was constructed by exhanging the ~1.9 kb *Pfl*MI−*Eco*NI fragment of pT13-11 with the same fragment derived from a modified version of pJP44 (called pJP44-Neo). The exchanged fragments were identical except for a *Sma*I site that had been engineered into pJP44 at a position 26 bp downstream of the PARP transcriptional start site (108 bp downstream of the *Pfl*MI site). pJP44-neo is essentially similar to pJP44 except that we have replaced the coding sequence of the chloramphenicol acetyl transferase (CAT) gene with that of NPT-II. The plasmid pEV was obtained by inserting a polylinker consisting of the restriction sites *Sma*I−*Pfl*M1−*Asc*I−*Kpn*I−*Pme*I−*Sma*I (contained in the sequence CCCGGGCCATTTT-GTGGCGCGCCGGTACCGTTTAAACCCGGG) at the *Sma*I site of pT13-11S. Sequencing of this region of pEV indicated that the polylinker had gone in as an inverted dimer with the order of restriction sites in the upstream unit of the dimer being as shown above. Digestion of pEV with *Pfl*MI deletes 108 bp of the PARP promoter and the entire polylinker (due to its presence as an inverted dimer) to yield pNull-S, which bears a unique *Sma*I site. To construct pT13-11Rr and pT13-11Rw, we inserted an 519 bp *Bam*HI−*Hin*dIII fragment containing the rRNA promoter isolated from the plasmid polINeo (44) into the unique *Sma*I site of pNull-S. The protruding ends of the promoter-containing fragment were blunt ended by Klenow polymerase before ligation and the insertion was achieved in the right (i.e. with the promoter oriented towards the NPT-II gene) and wrong orientations, to give pT13-11Rr and pT13-11Rw respectively. To construct the plasmid pEV-luc, we obtained a ~2. 2 kb fragment containing the gene for firefly luciferase flanked by actin SAS and putative polyadenylation signals (PAS) from pHD30. This fragment was obtained by a polymerase chain reaction (PCR) using oligonucleotides hybridizing just upstream of the actin SAS and just downstream of the PAS (i.e. at the *Sma*I site and *Pst*I site of pHD30 respectively). The PCR primer was designed with additional *Asc*I sites at either end. The isolated fragment was digested with *Asc*I and inserted onto similarly digested pEV to yield pEV-luc. In one of these plasmids (from the several independent clones that were obtained), the expected downstream *Asc*I site (at the actin−PAS end) had been lost as a result of an aberrant ligation event. This plasmid (which behaved no differently from its siblings in every other respect tested) would prove very useful because of its unique *Asc*I site and was used in all subsequent experiments. To create pEV-Rluc, we once again obtained the *Bam*HI−*Hin*dIII fragment from pol1Neo, this time by using a PCR reaction. The PCR primers were designed such that the isolated rRNA promoter-containing fragment would bear a *Spe*I site at the upstream end and a *Kpn*I and *Asc*I site at the downstream end. Digestion of pEV-luc with *Asc*I and *Spe*I followed by ligation with a similarly digested PCR fragment containing the rRNA promoter gave pEV-Rluc. In this plasmid the entire (552 bp) PARP promoter region upstream of the position of the polylinker has been removed and replaced by the rRNA promoter-bearing fragment. In addition, because of a *Spe*I site just inside the right boundary of the PMS, 57 bp of the latter have also been removed in pEV-Rluc. We were unconcerned with the loss of this 57 bp because a larger deletion involving the entire (~1.4 kb) *Apa*I−*Apa*I fragment had been earlier shown to be able to replicate autonomously in procyclics (19). Plasmid pEV-Rluc is of approximately the same size as pEV-luc.

Preparation of a genomic library

Total genomic DNA was prepared from procyclic *T.brucei* (45). 10 μg of this DNA was digested with one of the restriction endonucleases *Rsa*I, *Hae*II or *Hpa*I, and the digested material was ligated onto *Sma*I-digested and phosphatased pNull-S. The ligated products were electroporated into the *Escherichia coli* strain DH10B and transformed bacteria were grown to saturation under ampicillin selection. Supercoiled DNA corresponding to the ensuing library of plasmid molecules (called pPL-Rsa, pPL-Hae and pPL-Hpa) was obtained by banding over a gradient of cesium chloride (46). To estimate the extent of the genome sampled by each library, we had plated a small aliquot of the electroporated samples onto selective media containing agarose. The number of colonies obtained in each case and the average size of the insert in the plasmid library indicated that each library had $\sim 1-2\times 10^7$ bp of sequence from the *T.brucei* genome. The three libraries together would cover nearly half the trypanosome genome (nuclear DNA content $\sim 7\times 10^7$ bp). Approximately 20% of the molecules in each of the libraries corresponded to re-ligated pNull-S.

Transient replication assay

Wild-type procyclics were electroporated with 5 μg of closed-circular pEV-luc or pEV-Rluc DNA obtained from *E.coli* strain DH10B (dam+). Five transfections were carried out with each of the two constructs, the electroporated cells were resuspended in media and the contents from each set were pooled. Total volume of each pooled culture was 100 ml. G418 was added to each of the cultures ~24 h following electroporation. Also, an assay for luciferase activity was performed on day 1 (i.e. ~24 h post transfection) using 1 ml of the cell suspension removed from each of the two pooled cultures. An aliquot of 20 ml was withdrawn immediately from each of these pools (the 'day 0'

sample) and on days 2, 4, 6 and 8. A small volume (10 μl) from each of these samples was used to determine the number of live cells. The remaining sample was used to obtain Hirt DNA. The procedure for obtaining Hirt DNA was similar to what has been described above, except that we used 2× volumes of each of the solutions indicated. The Hirt DNA was resuspended in 15 μl TE. 5 μl of each of the samples was then digested with *Dpn*I (two additions of this enzyme were used at intervals of 4 h) and *Bgl*II before being electrophoresed through agarose and then transferred onto a membrane of nitrocellulose. A standard amount (~10 pg) of input pEV-Rluc was also digested with *Bgl*II as a control and electrophoresed through the same agarose gel. Following transfer, the filter was cut transversely into two, and both halves were hybridized separately with the same ^{32}P-labeled probe prepared from an ~1.2 kb *Eco*RI–*Eco*RI fragment derived from the luciferase gene. We split the filter in two and hybridized the two halves separately because we were worried that the large amount of *Dpn*I-sensitive unreplicated DNA that was expected to be found at the bottom of the filter might act as a sink for the probe and reduce our chances of seeing the small signal corresponding to the replicated DNA at the top of the filter. Transfer, hybridization and washings (0.1× SSC with 0.1% SDS, 65°C, 30 min) were by standard procedures (46).

Luciferase assay

A cell pellet corresponding to ~1 ml of the transfected cell culture was washed once with phosphate-buffered saline (PBS) and then resuspended in 100 μl of lysis buffer. 5 μl of this lysate was added to 45 μl of the luciferase substrate. Activity was measured on a Turner TD-20e Luminometer. The lysis buffer and substrate were from a luciferase assay kit (Promega Corp. Madison, WI).

RESULTS

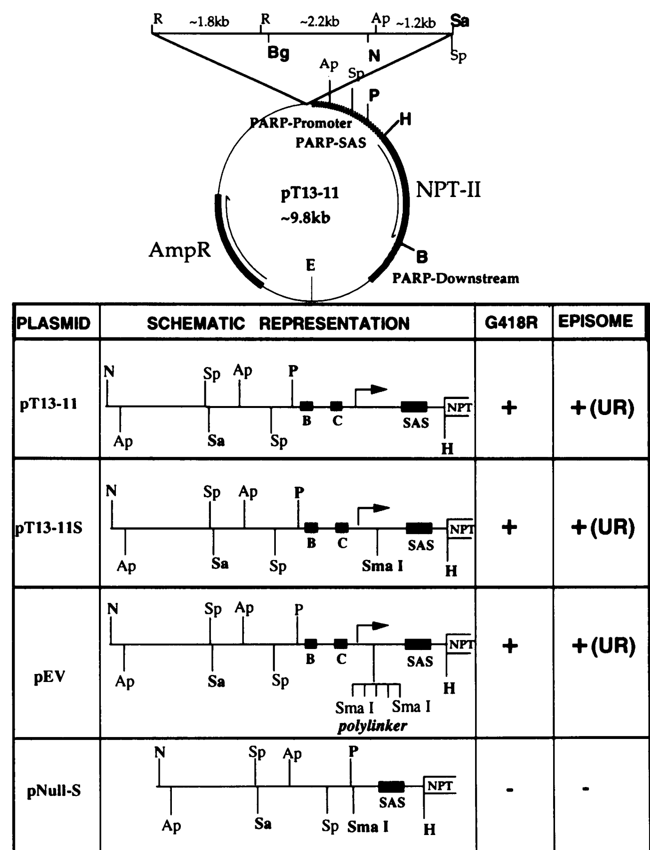
Creation of a promoter-trap

In the plasmid pT13-11 (Figure 1), as in all other members of the panel of autonomously replicating episomes that we had previously constructed, the NPT-II gene is flanked by 640 bp from the PARP 5′ upstream region and 380 bp of PARP 3′ untranslated sequence, which contains the site for polyadenylation. Our initial efforts were directed at constructing a promoter-trap by deleting the PARP promoter from pT13-11 without affecting the SAS. Towards this end, we introduced a *Sma*I site 26 bp downstream of the transcriptional start site (i.e. between the PARP promoter and SAS), to obtain the plasmid pT13-11S (Figure 1). A polylinker (containing a site for the restriction endonuclease *Pfl*MI, among others) was inserted at this site to obtain the plasmid pEV (Figure 1). Both pT13-11S and pEV replicate autonomously in procyclic *T.brucei* (Figure 1). Digestion of pEV with *Pfl*MI removed a 108 bp fragment, which includes the two major elements of the PARP promoter, creating pNull-S (Figure 1). Alteration of either of the two major elements reduces PARP promoter activity by almost 90%, as demonstrated by transient transfection assays (7). Not surprisingly therefore, procyclics transfected with closed-circular pNull-S DNA were unable to survive selection with the drug G418.

These results are consistent with the notion that the PARP promoter is essential for the transcription of the NPT-II gene on pT13-11 and suggested that fortuitous transcriptional initiation, which had been invoked for several episomes in trypanosomatids (15, 16), either does not occur on pT13-11, or occurs at a level that is insufficient to overcome G418 selection. We concluded from these experiments that pNull-S could be used as a promoter-trap where we would attempt to replace the PARP promoter with a functionally equivalent one from the *T.brucei* genome.

Use of the promoter-trap leads only to the re-isolation of the PARP promoter

Three independent genomic libraries were constructed by inserting *Hae*II-, *Hpa*I- or *Rsa*I-digested *T.brucei* DNA at the *Sma*I site of pNull-S. These enzymes were selected at random to generate a representative sampling of the *T.brucei* genome. Two of these enzymes (*Hae*II and *Rsa*I) have a 4 bp recognition sequence and were expected to generate on average smaller fragments than the other (*Hpa*I), which has a 6 bp recognition sequence. The libraries were amplified in *E.coli*. Closed-circular plasmid DNA from these libraries was transfected into procyclic *T.brucei* and G418R cells were selected. The experiments were done in duplicate and, as a control, procyclics were transfected



PLASMID	SCHEMATIC REPRESENTATION	G418R	EPISOME
pT13-11	N, Sp, Ap, P, B, C, SAS, NPT, Ap, Sa, Sp, H	+	+(UR)
pT13-11S	N, Sp, Ap, P, B, C, SAS, NPT, Ap, Sa, Sp, Sma I, H	+	+(UR)
pEV	N, Sp, Ap, P, B, C, SAS, NPT, Ap, Sa, Sp, H, Sma I, Sma I, *polylinker*	+	+(UR)
pNull-S	N, Sp, Ap, P, SAS, NPT, Ap, Sa, Sp, Sma I, H	-	-

Figure 1. The top half of the panel is a map of the plasmid pT13-11. The triangle represents the plasmid maintenance sequence (PMS). The map is not to scale. Abbreviations; Ap, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*NI; H, *Hpa*I; N, *Not*I; P, *Pfl*MI; R, *Eco*RI; Sa, *Sal*I; Sp, *Spe*I. Unique restriction sites are shown in bold type. The column labeled G418R represents selectability of transfected *T.brucei* with G418. The bottom half of the panel shows various derivatives of pT13-11 and indicates their replicative behavior in procyclic *T.brucei*. Only the region around the engineered changes are shown; the remaining sequences are identical. Boxes B and C indicate the two major elements of the PARP promoter. SAS indicates the splice acceptor signals. The arrow represents the start site of transcription. UR, indicates unrearranged episomes. Remaining abbreviations are identical to those used in the top panel.

with either pNull-S or its parent pEV. After a cumulative dilution of 10^7 to eliminate interference from surviving input DNA, low molecular weight DNA was isolated from G418R cells by the method of Hirt (17), and this DNA was used to transform bacteria. The DNA isolation protocol of Hirt is an enrichment procedure for episomal DNA molecules. As expected, transfection with pNull-S did not yield G418R trypanosomes. Hirt isolates from the positive control transfection with pEV yielded thousands

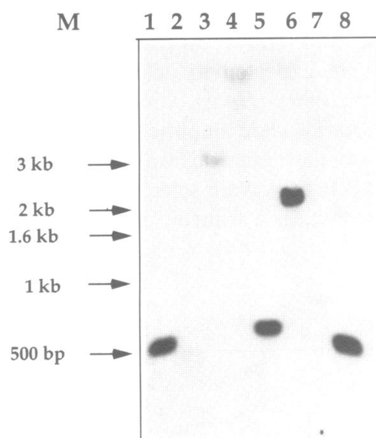


Figure 2. Southern blot analysis to detect re-isolation of the PARP promoter through the promoter-trap. Cesium-banded DNA corresponding to pEV (lane 1), pNull-S (lane 2), pPr-B (lane 3), pPr-C (lane 4), pPr-G (lane 5), pPr-H (lane 6), pPr-I (lane 7) and pPr-J (lane 8) were each digested with *Apa*I and *Hpa*I before electrophoresis and Southern transfer. The filter was probed with a 108 bp fragment corresponding to the deleted region of the PARP promoter in pNull-S. M represents a marker lane bearing a 1 kb DNA ladder (GIBCO-BRL).

of ampicillin-resistant (AmpR) bacterial colonies upon transformation of *E.coli*. Rather surprisingly, however, only 24 AmpR colonies were obtained from Hirt DNA corresponding to 5 of the 6 library transfections. All 24 colonies were grown individually to saturation density and plasmid DNA was prepared. Restriction endonuclease digestion followed by agarose gel electrophoresis indicated that the isolated plasmid DNA fell into 9 classes (as defined by the size and restriction pattern of the inserts), which we call pPr-A, pPr-B, pPr-C and so on. A majority of these seemed to bear very little resemblance to their parents and represented extensively rearranged DNA. Closed-circular DNA corresponding to each of the plasmid classes was prepared and was retested for autonomous replication in procyclics. Only two members, pPr-G and pPr-H, were able to survive as episomes and transform procyclics to G418R when assayed individually a second time.

Hirt DNA corresponding to the sixth library transfection yielded nearly 1000 AmpR colonies when used to transform bacteria. An examination of 12 independent colonies showed them to harbor identical plasmids. In addition, using a limited repertoire of restriction endonucleases, we could not distinguish this plasmid from pPr-G. Nonetheless we continued to deal with it as a member of a separate class which we called pPr-J. pPr-J was also able to survive as an episome and transform procyclics to G418R when assayed individually a second time.

To test if any of the plasmids had re-acquired the PARP promoter, we digested cesium-banded plasmid DNA with the restriction endonucleases *Apa*I and *Hpa*I. pNull-S and pEV were similarly digested as controls. The plasmids pPr-A, -D, -E and -F were not included in this experiment as they showed almost no resemblance to the input DNA, and in any case did not survive as an episome in our repeat transfection. Following Southern transfer, the filter was probed with a ^{32}P-labeled gel-isolated

TRANSFECTED DNA	SCHEMATIC REPRESENTATION OF PLASMID	Luciferase Activity	G418R Cells	EPISOME
pT13-11Rw	N Sp Ap P rRNA promot. SAS NPT Ap Sa Sp H	ND	-	-
pT13-11Rr	N Sp Ap P rRNA promot. SAS NPT Ap Sa Sp H	ND	+	8 All rearranged
pEV-luc	N Sp Ap P B C SAS NPT Ap Sa Sp A R R R H (SAS) LUCIFERASE(PAS)	9.93	+	> 300 None rearranged
pEV-Rluc	N Sp rRNA promot. SAS NPT Ap A R R K H (SAS) LUCIFERASE(PAS)	15.12	+	6 All rearranged
No DNA	NO PLASMID	0.005	-	-

Figure 3. Replicative behavior of pEV-luc and plasmids with the rRNA promoter replacing the PARP promoter in procyclic *T.brucei*. A schematic representation of these plasmids only shows the region around the engineered changes. The remaining sequences are identical to pT13-11. Maps are not to scale. SAS and PAS (polyadenylation signals) surrounding the luciferase gene are from the *T.brucei* actin locus; A, *Asc*I; K, *Kpn*I; ND, not done; Other abbreviations used are identical to those in Figure 1.

108 bp fragment corresponding to the region of the PARP promoter that had been deleted in pNull-S (Figure 2). Each of the plasmids pPr-G, -H and -J (lanes 5, 6 and 8 respectively) contained the PARP promoter, which was absent from their parent pNull-S (lane 2). In the case of pPr-H (lane 6), the PARP promoter is contained within an approximately 1.5 kb insertion at the library cloning site, giving rise to a ~2 kb *Apa*I−*Hpa*I band that hybridizes to the probe. On the other hand, in the case of pPr-G and pPr-J, the size of the hybridizing band was identical to the one obtained from the plasmid pEV (lane1), indicating that the 108 bp lesion corresponding to the PARP promoter in the parent vector pNull-S had been precisely reinstated (confirmed by sequencing). As none of the three restriction endonucleases used to create the libraries would have generated a 108 bp PARP promoter-bearing fragment, we surmise that the deletion in pNull-S has been repaired, either by an intermolecular gene conversion event between the genomic PARP locus and the PARP upstream sequences existing in pNull-S, or by intramolecular recombination between these plasmid sequences and a library insert containing the PARP promoter.

Re-isolation of the PARP promoter (especially as in pPr-H) indicated that the trap had indeed worked in the intended manner, but we failed to identify any new promoters. Each library would have screened $\sim 1\times10^7$ bp of DNA (i.e. ~1/7th of the trypanosome genome). As transcription in *T.brucei* is probably polycistronic, any restriction fragment containing a promoter would be unlikely to also bear a transcriptional terminator. Thus, a transcript originating from a correctly oriented promoter anywhere on the inserted fragment should have sufficed to generate NPT-II mRNA, G418-resistance and, indirectly, autonomous existence of the plasmid. For these reasons, our inability to rescue any other promoters from nearly half the trypanosome genome (in the 3 libraries), was deeply puzzling.

One likely explanation lay in the strength of the PARP promoter. It was possible that only a strong promoter would suffice to provide the level of transcription necessary to withstand selective pressure. Such promoters may be rare in the *T.brucei* genome and could have been missed by our screen. A second possible explanation could be that the PARP promoter element provides functions other than just transcriptional initiation and is therefore irreplaceable in the context of the plasmid. We decided to test these hypotheses by directly inserting the rRNA promoter into pNull-S. The rRNA promoter initiates transcription with an efficiency greater than the PARP promoter (8) and, as indicated before, can support mRNA transcription in *T.brucei*.

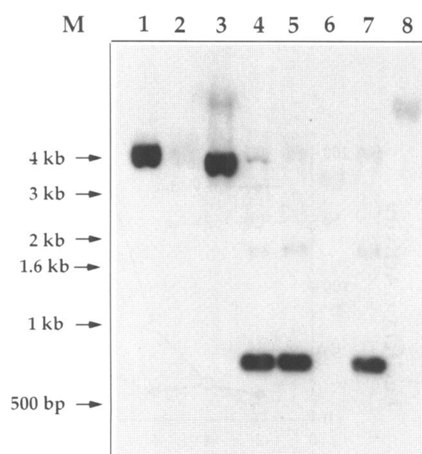


Figure 4. Southern blot analysis to detect reappearance of the PARP promoter in the rearranged episomes derived from pEV-Rluc. Plasmid DNA corresponding to pEV-luc (lane 1), pEV-Rluc (lane 2), and all six rearranged episomes isolated (lanes 3−8) were digested with *Eco*RI before electrophoresis and Southern transfer. The filter was probed with a 108 bp fragment corresponding to the deleted region of the PARP promoter in pNull-S. M represents a marker lane bearing a 1 kb DNA ladder (GIBCO-BRL).

The rRNA promoter cannot functionally replace the PARP promoter on these episomes

Three constructs were made (Figure 3). The plasmid pT13-11Rr and pT13-11Rw, for example, were constructed by inserting a 519 bp fragment containing a previously characterized rRNA promoter, in either orientation, into the unique *Sma*I site of pNull-S. For the remaining construct, we started by inserting the gene for firefly luciferase (surrounded by the SAS and putative polyadenylation signals derived from the *T.brucei* actin locus) into the polylinker of pEV, creating pEV-luc. Removal of the entire PARP promoter region upstream of the polylinker, followed by insertion of the rRNA promoter, gave us pEV-Rluc. The luciferase gene in pEV-luc and pEV-Rluc enabled us to quantitate the activities of the PARP or rRNA promoters following transfection.

All four plasmids were electroporated into procyclic *T.brucei* in duplicate. Approximately 24 h later, an aliquot corresponding to 10% of the volume of the transfected culture was removed from the cultures transfected with the plasmids pEV-luc and pEV-Rluc and assayed for luciferase activity. Results (Figure 3) indicated that both promoters were active, with the rRNA promoter being about 1.5-fold stronger than the PARP promoter, agreeing with previous work (8).

Stable transfectants were selected with G418. Following a cumulative dilution of $>10^7$, Hirt DNA was prepared from each of the G418R samples and this DNA was used to transform *E.coli*. As shown in Figure 3, Hirt DNA derived from pEV-luc-transfected procyclics gave rise to several hundred AmpR bacterial colonies. An examination of the plasmid DNA from 12 of these colonies indicated that the plasmid was identical to the input DNA. We concluded that pEV-luc, like its parent pEV, is able to exist as an autonomously replicating plasmid in procyclic *T.brucei*. The plasmid pT13-11Rw was unable to transform procyclics to G418R. As the rRNA promoter-driven transcription in this construct is directed away from the NPT-II gene, this was expected. The plasmids pT13-11Rr and pEV-Rluc produced G418R cells. However, Hirt DNA isolated from these cultures gave a total (from the duplicate experiments) of only 8 and 6 AmpR colonies respectively, when used to transform bacteria. An examination of plasmid DNA from each of these colonies showed that these did not resemble the input DNA and had undergone substantial rearrangements (data not shown). These rearrangments were fairly complex and involved the acquisition of new sequences, as indicated by the presence of new restriction sites. Similar experiments with pEV-Rluc have been repeated twice (each time in duplicate) with essentially the same results. Although a far greater number of AmpR colonies (~100) were recovered in one of these experiments, an examination of plasmid DNA from five of these colonies once again showed them to be unlike their parent. We believe the large number of colonies simply reflects the propagation of an early rearrangement event,

a particularly stable form, or both. The observation that the plasmid DNA from each of the five colonies was identical, was consistent with this notion. We concluded, from this set of experiments, that neither pT13-11Rr nor pEV-Rluc is able to exist stably as an episome, despite the proven activity of the rRNA promoter. The G418 resistance must therefore arise largely as a consequence of these plasmids integrating into the genome.

Plasmid DNAs corresponding to the group of 6 colonies derived from pEV-Rluc-transfected culture (Figure 3) were each digested with *Eco*RI, electrophoresed through agarose, blotted onto a membrane of nitrocellulose and probed with the 108 bp PARP promoter fragment. Cesium-banded pEV-luc and pEV-Rluc DNAs were included as controls. Results are shown in Figure 4. Amazingly, in 4 of the 6 colonies examined (lanes 3, 4, 5 and 7), the PARP promoter had reappeared!

The inability of any of the rRNA promoter-containing constructs to survive as an unrearranged episome suggested that the deleted PARP promoter fragment might harbor functions necessary for plasmid maintenance, in addition to its expected role in promoting transcription. As our assay for stable autonomous existence was carried out after a cumulative dilution of $>10^7$ following electroporation, a function (or functions) in plasmid replication and/or episomal stability was conceivable.

The PARP promoter plays a role in plasmid DNA replication

To determine if the PARP promoter region played a role in plasmid replication, we performed two different assays. In the first, we measured the amount of luciferase activity in cultures transfected with either pEV-luc or pEV-Rluc over an 8 day period. We reasoned that, if the plasmid replicated, the amount of luciferase activity would steadily increase during this period. On the other hand, a non-replicating episome would be indicated by either an unchanging (see below for explanation) or a steadily declining activity curve. As even a non-replicating plasmid can integrate into the genome or rearrange to a replicating form (see previous result), this type of assay would only be informative at early time points.

Transfected cells were resuspended in 20 ml of media and G418 was added ~24 h later (day 1). 1 ml of the culture suspension was withdrawn on days 2, 4, 6 and 8 and assayed for luciferase. A small aliquot from the withdrawn sample was used to determine cell density. Results are shown in Figure 5. While the pEV-luc transfected culture showed a steady increase in luciferase activity, as expected for a replicating plasmid, luciferase activity in the pEV-Rluc transfected cultures increased only marginally over the 8 day period. In contrast, the number of G418R cells was similar in both cultures throughout the course of the experiment. Note that, as G418 kills non-plasmid-bearing (sensitive) cells rather slowly (taking upto 5 days to kill wild-type procyclics, at the concentration used), the cell counts on day 4 or day 6 may reflect a mixture of sensitive and resistant organisms. Nevertheless, the cell counts clearly demonstrate what we have consistently observed. Both pEV-luc- and pEV-Rluc-transfected organisms give rise to G418R cells at an approximate equal initial frequency, such that we needed to split each of the samples around 10 days following transfection. Numbers of G418R cells in both cultures at this point of time were very nearly equal. However, after this initial split (which involved a 1:10 dilution of the original culture) we noticed that the growth rate of the pEV-Rluc-transfected cultures in media containing G418 usually declined precipitously for a while before the doubling time recovered to that of wild-type cells growing in the absence of drug (data not shown). We interpret these observations to mean that transfected cells that survive selection receive several copies of the input DNA carrying the selectable marker. Even if this DNA cannot replicate (our hypothesis for pEV-Rluc), a crisis is averted until a certain number of cell divisions has served to dilute the initial number of copies. After this, only cells where the non-replicating input DNA has integrated into the genome (usually a much smaller number of the total population) will survive. The luciferase assay supports this notion. Luciferase activity remains essentially unchanged in the initial stages while the input DNA is just being partitioned to new cells and no new pEV-Rluc DNA is being synthesized. The small increase seen could be due to the few integrated copies that are expected.

In our second experiment, we directly tested the ability of pEV-Rluc to replicate by means of a transient-replication assay. We relied on a fairly standard procedure (for example see (18), which involves the use of a methylation-sensitive restriction endonuclease to discriminate between the large amount of residual input DNA that was expected to be encountered and newly replicated plasmid molecules. *Dpn*I digests DNA at the sequence GATC only if the adenine in the sequence is methylated. Input DNA is methylated at just that sequence as a result of the action of *E.coli dam* methylase and is consequently sensitive to this

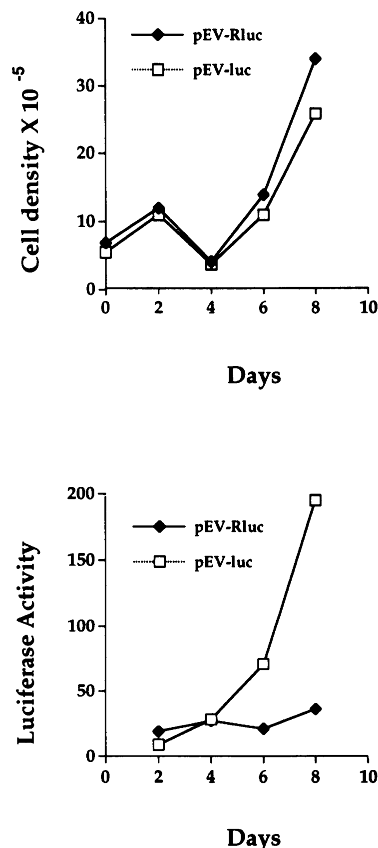


Figure 5. Live cell density and luciferase activity in cultures transfected with either pEV-luc or pEV-Rluc. G418 was added to the transfected cultures on day 1 (i.e. ~24 h after electroporation). Luciferase activity is in luminometer units and cell density is in cells/ml.

enzyme. Newly replicated DNA is resistant, as eukaryotes lack this methylase.

For the experiment, procyclics were transfected with closed-circular pEV-luc or pEV-Rluc DNA. Five transfections were done with each of the constructs and their contents were pooled. Luciferase activity was estimated for each pooled cell suspension ~20 h post transfection. As before, procyclics transfected with the plasmid pEV-Rluc showed a 1.5- to 2-fold greater amount of luciferase activity than corresponding samples from the pEV-luc transfected culture. A fifth of the volume from each of these pooled cultures was withdrawn immediately after transfection (day 0) and on days 2, 4, 6 and 8. Cell counts showed that the numbers of live cells withdrawn from either of the two flasks on any particular day were similar. Hirt DNA was prepared from each of these samples and subjected to digestion by *DpnI* and by *BglII*, which would linearize any replicated DNA that was untouched by *DpnI*. Following electrophoresis and Southern transfer, the filters were hybridized with a ^{32}P-labeled probe corresponding to the luciferase gene (Figure 6). The intensely hybridizing smear at the bottom of the filter represents unreplicated input DNA digested by *DpnI* to small fragments. Even at day 8, a substantial amount of input DNA remains associated with the transfected cells. This DNA is somehow sequestered, as it is immune to repeated washings and to added DNAse I (unpublished observations). Linearized, *DpnI*-resistant pEV-luc, but not pEV-Rluc, DNA is clearly visible on day 4 and increases thereafter, indicating that the former can replicate but the latter cannot. The absence of an observable signal before day 4 is attributed to the low transfection efficiency of these cells and the limits of sensitivity of the Southern assay. This experiment (which has been repeated two other times with identical results) confirms a role for the PARP promoter region in autonomous plasmid replication in procyclic *T.brucei*.

The PARP promoter alone is insufficient to support plasmid replication

As we have previously reported, this panel of autonomously replicating plasmids was obtained by inserting random pieces of genomic DNA into a plasmid (pH51) that had the PARP promoter but was not a stable replicon (13). Deletion analysis indicates that a large section of the PMS in pT13-11 is necessary for stable autonomous existence in procyclic *T.brucei* (19). Thus, either the PARP promoter acts in consort with an element (or elements) in the PMS to support replication, or the twin properties of autonomous replication and stability demonstrated by these episomes are separately coded by the two regions, and are both necessary for extrachromosomal existence. To test the former possibility, we carried out an experiment similar to the one described above using the plasmid pH51. Results (Fig. 7) indicate that pH51 is incapable of transient replication in procyclic *T.brucei*, demonstrating the necessity of the PMS for plasmid replication.

DISCUSSION

The experiments described in this paper indicate a role for the PARP promoter region in plasmid DNA replication in addition to its previously elucidated role in transcription. Although the sequence mediating these two processes in pT13-11 may be different, it is likely that the region supporting plasmid DNA replication overlaps with the 108 bp fragment bearing the major elements of the PARP promoter, as its removal and replacement by the rRNA promoter (in pT13-11Rr) renders the plasmid incapable of stable extrachromosomal existence. Thus, a combination of strong promoter activity and a role in plasmid replication makes the PARP promoter region difficult to substitute in the context of these episomes and a fundamental redesign of the promoter-trap will be necessary.

The ease with which a non-replicating plasmid molecule is able to rearrange to a putatively replicating form warrants comment. We had previously noted this phenomenon (13), which indicates an efficient recombination machinery in this organism. The process seems more active when the input plasmid bears a functional promoter that drives expression of the selectable marker (as in pEV-Rluc) than when there is no such promoter (as in pNull-S), perhaps because a cell bearing the former can

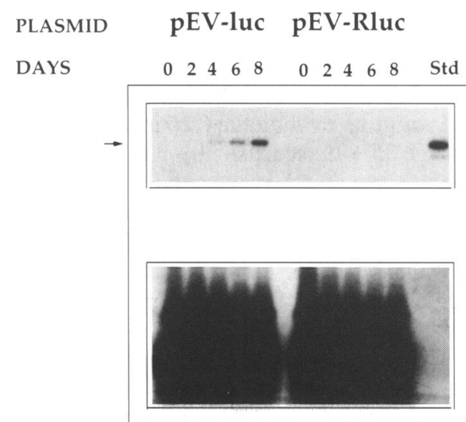


Figure 6. Southern blot analysis of Hirt DNA from pEV-luc- and pEV-Rluc-transfected procyclics to detect replicated plasmid DNA. In each case, samples were digested with *DpnI* and *BglII* before electrophoresis and Southern transfer. Std, refers to standard pEV-Rluc DNA which was digested only with *BglII*. The filter was probed with an ~1.2 kb *EcoRI*–*EcoRI* fragment from within the luciferase coding region. Exposure was for 3 days with intensifying screen. The arrow indicates the position of linearized pEV-luc or pEV-Rluc DNA. Note, pEV-luc and pEV-Rluc have similar sizes.

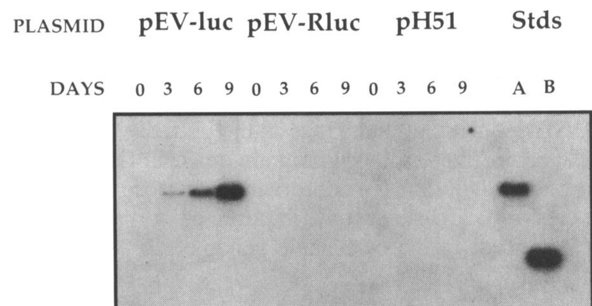


Figure 7. Southern blot analysis of Hirt DNA from pEV-luc-, pEV-Rluc- and pH51-transfected procyclics to detect replicated plasmid DNA. Hirt DNA was digested with *DpnI* and *BglII* (pEV-luc and pEV-Rluc) or *DpnI* and *BamHI* (pH51) before electrophoresis and Southern transfer. Stds, refers to standard pEV-luc (**A**) and pH51 (**B**) DNA which were digested with *BglII* or *BamHI* respectively. The filter was probed with an 810 bp *HpaI*–*BamHI* fragment corresponding to the NPT-II gene. Exposure was for 3 days with intensifying screen. Unlike in Figure 6, only the top half of the filter was probed.

survive selection for an extended period even when the plasmid cannot replicate.

We are unable to say, at the present time, whether the PARP promoter region contains an origin of DNA replication (ori) or matrix attachment sites, nuclear retention signals, etc. Several recent reports cite the latter attributes as important players in mediating DNA replication in the nucleus (20–24). Studies with the Epstein–Barr virus indicate that for stable extrachromosomal existence, this virus or plasmids derived from it require two regions, one of which is an origin of replication while the other provides 'nuclear retention function'. The modular nature of elements constituting a stable replicon (also seen in *Saccharomyces cerevisiae*) seems analogous to the situation with pT13-11, and we shall be attempting to map the ori on this episome. As DNA replication has long been considered to be regulated at initiation in both prokaryotes and eukaryotes, the definition of an ori will constitute a key step in the elucidation of the DNA replication apparatus in trypanosomatids.

The association between elements that support transcription and DNA replication in an ancient eukaryotic lineage such as *T.brucei* mirrors observations in several higher eukaryotes and their viruses, and suggests evolutionary conservation of this feature (for reviews see 25–29; see also 30–32). Transcription *per se*, either through or into an ori, may not be critical (33, 34). Rather, it is thought that proteins binding to transcriptional regulatory elements either act indirectly, by excluding core histones and thereby opening the DNA for the assembly of the replication initiation complex, or directly, by binding with or stabilizing the initiation complex at the ori.

A quite different idea has emerged from studies with the ARS1 ori in *S.cerevisiae*. Linker-scan analysis indicates that the ARS1 ori consists of multiple elements, one of which is a binding site for the transcription activating factor ABF1 (35). A protein complex (the origin recognition complex or ORC) has been isolated that binds to the ARS1 ori in *vitro* and in *vivo* (36, 37). Definitive experiments link one member of this ORC to transcriptional repression (38–40). Furthermore, the ORC has been shown to bind to each of the four silencers at the two silent mating type loci HML and HMR (38). These findings, coupled with earlier results indicating the importance of DNA replication in the establishment of silencing in *S.cerevisiae* (41, 42), anchor an intriguing proposal that origins of DNA replication and the proteins that bind them may also be involved in the formation of transcriptional domains on eukaryotic chromosomes (38). Essentially, this hypothesis is an inversion of the previously postulated relationship between elements involved in transcription and DNA replication, and suggests the primacy of the latter in modulating the transcriptional program of a cell. Our experiments allude to the evolutionary conservation of close links between these elements, and suggest the importance of maintaining such an arrangement in the eukaryotic genome.

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