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Elements in the 3′ Untranslated Region of Procyclin mRNA Regulate Expression in Insect Forms of *Trypanosoma brucei* by Modulating RNA Stability and Translation

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Procyclins are the major surface glycoproteins of insect forms of *Trypanosoma brucei*. We have previously shown that a conserved 16-mer in the 3′ untranslated region (UTR) of procyclin transcripts functions as a positive element in procyclic-form trypanosomes. A systematic analysis of the entire 297-base 3′ UTR has now revealed additional elements which are involved in posttranscriptional regulation: a positive element which requires the first 40 bases of the 3′ UTR and at least one negative element between nucleotides 101 and 173 (the LII domain). Deletion of either positive element resulted in a >8-fold reduction in the amount of protein but only an ~2-fold decrease in the steady-state level of mRNA, suggesting that regulation also occurred at the level of translation. In contrast, deletion of LII caused a threefold increase in the steady-state levels of both the mRNA and protein. LII–16-mer double deletions also gave high levels of expression, suggesting that the 16-mer functions as an antirepressor of the negative element rather than as an independent activator. All three elements have an effect on RNA turnover. When either positive element was deleted, the half-life ($t_{1/2}$) of the mRNA was reduced from ~50 min (the $t_{1/2}$ of the wild-type 3′ UTR) to <15 min, whereas removal of the LII element resulted in an increased $t_{1/2}$ of ~100 min. We present a model of posttranscriptional regulation in which the negative domain is counteracted by two positive elements which shield it from nucleases and/or translational repressors.

The differentiation of bloodstream forms of *Trypanosoma brucei* into procyclic forms which replicate in the tsetse fly midgut is marked by the synthesis of a new surface coat composed of procyclins (otherwise known as procyclic acidic repetitive proteins [PARPs]) and the shedding of the variant surface glycoprotein (VSG) coat, which covers the parasites in the mammalian host (31, 42, 54). It has been estimated that each cell is covered by approximately six million procyclin molecules (9) which are attached to the surface membrane by glycosylphosphatidylinositol (GPI) anchors (15, 16). The parasites divide by binary fission, with a population doubling time of ~9 to 10 h in culture, so there is a constant requirement for high levels of procyclin synthesis in order to maintain the density of the coat.

Like the majority of genes in trypanosomatids, the procyclin genes form part of polycistronic transcription units (reviewed in reference 50). The trypanosome strain *T. brucei* 427 contains four procyclin expression sites which are located on separate chromosomes (43). Each expression site consists of tandemly linked procyclin genes (α and β), followed by a locus-specific procyclin-associated gene (PAG) (6, 28, 51). Two expression sites also contain an additional gene, GRESAG 2 (gene related to ESAG 2), which is very similar to a gene in the VSG expression site (4).

When bloodstream form trypanosomes are triggered to differentiate into procyclic forms, there is a 5- to 10-fold increase in transcription initiation from the procyclin promoter and a concomitant stimulation of transcription elongation (36, 49). Procyclin mRNAs can be detected within 2 h after the onset of differentiation (42) and transiently exceed the levels found in established procyclic forms (37). Although procyclin and PAG mRNAs are derived from the same primary transcript, additional controls must also operate posttranscriptionally since there is more than a 100-fold difference in their steady-state levels (28, 51). This may be due, at least in part, to differences in RNA processing. The procyclin transcripts are efficiently *trans* spliced and polyadenylated at specific sites (23, 24, 46). In contrast, both splicing and polyadenylation of PAG 1 transcripts occur at several alternative positions spanning 2.5 kb (51).

Expression can also be regulated by sequences within the 3′ untranslated regions (UTRs) of trypanosome messenger RNAs (5, 20, 22, 25). It has recently been shown that an element in the last 97 bases of the VSG mRNA, extending from within the coding sequence to the polyadenylation site, stimulated expression of a reporter gene in bloodstream forms to a level about threefold higher than in a construct lacking trypanosome sequences at the 3′ end and reduced it by a similar amount in procyclic forms. In bloodstream forms, the VSG sequences increased RNA stability relative to that of the control, but in procyclic forms, there were no differences in the rates of turnover of the two transcripts, indicating that down-regulation in this life cycle stage is effected by a different mechanism (5).

The 3′ UTRs of procyclin transcripts can be classified according to whether they stem from the α gene (for examples, see references 13, 32, and 34) or β gene within a transcription unit (33, 41). Within a group, the sequences are extremely similar (~86% identity), but the longest conserved sequence that occurs in both the α and β 3′ UTRs is a 16-mer which is predicted to form part of a stem-loop structure (20). The same sequence, with the same theoretical secondary structure, has also been found in the analogous gene from another species of trypanosome, *Trypanosoma congolense*, which codes for a major surface glycoprotein known as GARP (glutamic acid- and

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alanine-rich protein) (1, 2). Although the procyclin and GARP proteins exhibit no sequence similarity, it is possible to express the latter in *T. brucei*, where it is correctly routed to the plasma membrane (21).

We have previously demonstrated by transient transfections that both the sequence and the structure of the 16-mer are required for efficient expression of a reporter gene in procyclic form trypanosomes (20). Deletions of this element resulted in a 12-fold drop in chloramphenicol acetyltransferase (CAT) activity but no detectable differences in the levels of total and cytoplasmic mRNAs, suggesting that regulation occurred at the level of translation. Elements in the 3′ UTR may also play a role in regulating stage-specific expression. In parallel with their studies of VSG regulatory sequences, Berberof et al. (5) demonstrated that a construct with a truncated form of the procyclin 3′ UTR was two- to threefold more efficiently expressed in procyclic forms than the control construct with plasmid-derived sequences at the 3′ end and that the situation was reversed in bloodstream forms. It was not established which elements were responsible, however, or at what level they were acting. To examine the different regulatory sequences more closely, we have made systematic deletions of the procyclin 3′ UTR and analyzed them in transient and stable transfections using two different reporter genes. Using this approach, we have identified elements in three separate domains which have an influence on the levels of steady-state mRNA, RNA turnover, and translation.

MATERIALS AND METHODS

Trypanosome strains. *T. brucei* 427 procyclic forms (10) were used for both transient and stable transfections. Cells were cultured in SDM-79 supplemented with 5% fetal bovine serum (8).

3′ UTR deletions. (i) Exonuclease III deletions. A plasmid containing suitable sites for exonuclease III digestion from the first base of the procyclin α 3′ UTR was generated by digestion of a CAT construct containing the entire wild-type 3′ UTR (CAT-wt [20]) with *Bam*HI and insertion of the synthetic oligonucleotide GATCCGGTACCATTCAGGATATCC and its complement GATCGGATAT CCTGAATGGTACCG. The underlined bases produce 5′ overhangs when the oligonucleotides are annealed. In the correct orientation, these oligonucleotides reconstitute the *Bam*HI site on the 5′ side of the insertion and introduce an exonuclease III-resistant restriction site (*Kpn*I) and a sensitive site (*Eco*RV). The plasmid was digested with *Kpn*I and *Eco*RV, and the linearized DNA was treated with exonuclease III, religated, and used to transform *Escherichia coli* according to the manufacturer's (Pharmacia, Lucerne, Switzerland) instructions. The deletions in individual clones were determined by nucleotide sequence analysis.

(ii) Internal deletions. A CAT construct with a specific deletion of a conserved motif in the 3′ UTR (originally known as Δ16 but now designated CAT–Δ16-mer) has been described previously (20). A plasmid with a deletion of the central region of the 3′ UTR (101 to 173, ΔLII) was constructed by amplification of the upstream portion of the 3′ UTR with the primers LII-anti (GATATCGGAAA CAAACACGAT) and #2 (20) and the downstream portion with the primers LII-sense (GATATCAATGCCTTATTAACC) and BS-forward (20). Restriction sites introduced to facilitate cloning are underlined. The products were initially cloned into the *Sma*I site of pUC18. The inserts were then excised by digestion with *Bam*HI and *Eco*RV (upstream) and *Eco*RV and *Xba*I (downstream), gel purified, and ligated simultaneously between the *Bam*HI and *Xba*I sites of pBluescript (KS). Once the correct assembly of the insert had been confirmed by sequencing, it was transferred to the CAT and pGAPRONE vectors. Double mutants were constructed as follows. A 3′ end with the double deletion Δ40Δ16-mer was amplified from the 3′ end of CAT–Δ16-mer with the oligonucleotide ATGGATCCGCTTTGAAAAGG and the BS-forward primer (20). The product was digested with *Bam*HI and *Xba*I and ligated between the corresponding sites in CAT-wt. For the Δ40ΔLII mutant, the first 101 bp of CAT-ΔLII were removed by digestion with *Bam*HI and *Eco*RV and then replaced by a fragment spanning positions 41 to 101 of the 3′ UTR. This fragment was amplified from CAT-Δ40 with a primer specific for the 3′ end of CAT (20) and LII-anti, digested with *Bam*HI to remove CAT sequences, and gel purified prior to ligation. For the ΔLIIΔ16-mer mutant, CAT-ΔLII was digested with *Eco*RV and *Xba*I to remove the 3′ UTR and intergenic sequences downstream of position 173. This section was replaced by a fragment which was amplified from the Δ16-mer construct with the primers LII-sense and BS-forward and subsequently digested with *Bam*HI. All double mutants were sequenced before the 3′ ends were transferred to pGAPRONE as *Bam*HI-*Xba*I fragments. As predicted from the cloning procedure, an additional 3 bases (ATC) were present immediately downstream of the *Eco*RV site.

Construction of pGAPRONE. (i) Construction of insert 1. To construct insert 1 (Fig. 1A), we used plasmid pKON (44), which consists of the procyclin promoter and splice acceptor site, a neomycin resistance gene, and the last 19 bp of the procyclin β gene 3′ UTR followed by the intergenic region and 426 bp of PAG 1 (28). The *Bam*HI site at the junction between the coding region and the 3′ UTR was remodelled to a *Cla*I site by digestion with *Bam*HI, fill in of the 5′ overhang with Klenow fragment, and religation. The G residue immediately preceding the cleavage site (GGATCGATCC) creates a recognition site for Dam methylase that allows Dam methylase to modify the first A residue. The *Cla*I site is therefore resistant to digestion unless the plasmid is propagated in a *dam* mutant host. After this conversion was verified, the same procedure was used to transform the unique *Hin*dIII site immediately upstream of the coding region into an *Nhe*I site. Finally, the *Xba*I site was destroyed by digestion, followed by repair with Klenow fragment and religation. The 1,377-bp *Nhe*I-*Not*I fragment containing the neomycin resistance gene and downstream sequences was termed insert 1.

(ii) Construction of insert 2. To construct insert 2 (Fig. 1B), the 5′ UTR of the procyclin β gene was amplified from plasmid pAP4 (27) with the universal −20 primer (catalog no. 1211; Biolabs) and oligonucleotide PCH (GTAAGCTTGT GAATTTTACT), which corresponds to a conserved sequence immediately upstream of the procyclin coding region. A synthetic *Hin*dIII site is underlined. The resulting 389-bp product was digested with *Bam*HI and *Hin*dIII and cloned into the corresponding sites of pBluescript KS+ to yield the plasmid p5′UTR. The *Hin*dIII site was converted to an *Nhe*I site as described above, and the *Not*I site in the multiple cloning site was eliminated by cleavage, fill in, and religation. A new *Not*I site was created by inserting the synthetic oligonucleotide GCGGC CGC into the *Hin*cII site. Insert 1 was then cloned between the *Nhe*I and *Not*I sites of p5′UTR; digestion of the resulting plasmid with *Xba*I and *Not*I yielded the 1,766-bp insert 2. This additional step was necessary because *Nhe*I and *Xba*I create compatible cohesive ends, which complicated the direct double ligation of the 5′ UTR and insert 1 into the pGAPRONE scaffold.

(iii) Construction of the scaffold and the pGAPRONE vector. To construct the scaffold and the pGAPRONE vector (Fig. 1C), the neomycin resistance gene from pKON was replaced by a 782-bp *Hin*dIII-*Bam*HI fragment containing the coding region of GARP (originating from plasmid pGARP-PCR [21]). In the final step, insert 2 was subcloned into the corresponding sites of the scaffold plasmid to give the 7-kb pGAPRONE vector. The name represents the origins of the vector modules: GA stands for the GARP reporter gene, PRO stands for the promoter and processing signals derived from a procyclin transcription unit, and NE stands for the selectable marker that confers resistance to neomycin. The entire 4-kb insert can be excised by digestion with *Kpn*I and *Not*I and is designed to integrate via homologous integration with the Pro A locus or Pro B locus (27). Derivatives of pGAPRONE containing different 3′ UTR sequences downstream of GARP were produced by replacing the *Bam*HI-*Xba*I fragment in the original construct with the *Bam*HI-*Xba*I fragment from the CAT constructs described above.

Stable and transient transfection. Transient transfections and CAT assays were performed as described previously (20, 55). Stable transformation was performed according the procedure of ten Asbroek et al. (48). Transformants were selected on the basis of resistance to G418. The inserts of all pGAPRONE derivatives used for stable transformation were excised by digestion with *Kpn*I and *Not*I. This series of transformants is designated by a G (for GARP) followed by the type of 3′ UTR, e.g., G-wt.

Southern, Northern, and Western blot analysis. DNA and RNA were isolated as previously described (42, 53). Northern and Southern blot analyses were performed by standard procedures (45), and hybridization signals were quantified with a PhosphorImager (Molecular Dynamics). RNA turnover was measured in the presence of 10 μg of actinomycin D ml^{-1} (5). A PCR product corresponding to the GARP coding region (21) and a tubulin genomic clone containing one copy each of α- and β-tubulin (47) were used as probes. Rabbit polyclonal antisera raised against a GARP–glutathione *S*-transferase fusion protein were generously provided by D. Jefferies and J. D. Barry (Wellcome Unit of Molecular Parasitology, Glasgow, Scotland). Western blot analysis was performed as described previously (21) with anti-GARP antibodies at a dilution of 1:5,000. Alternatively, quantitative analysis was performed with a Tropix Western-Star protein detection kit and light emission was measured with a Bio-Rad molecular imager.

In vitro transcription and translation. To produce templates for in vitro RNA synthesis, the promoter region and 5′ UTR were deleted from the plasmids G-wt, G–Δ16-mer, and G-Δ40. The plasmids were digested with *Kpn*I and *Hin*dIII (Fig. 1), and the 5′ overhangs were filled in by treatment with Klenow fragment. The appropriate fragments were gel purified and self-ligated. The 5′ boundaries of the resulting constructs were sequenced to confirm that the start of the GARP coding region was still intact. Templates were linearized by digestion with *Xba*I, and in vitro transcription was performed with an RNA transcription kit from Stratagene. RNA was translated in reticulocyte lysates (Promega) containing 20 μCi of $[^{35}S]$methionine (1,200 Ci $mmol^{-1}$; NEN).

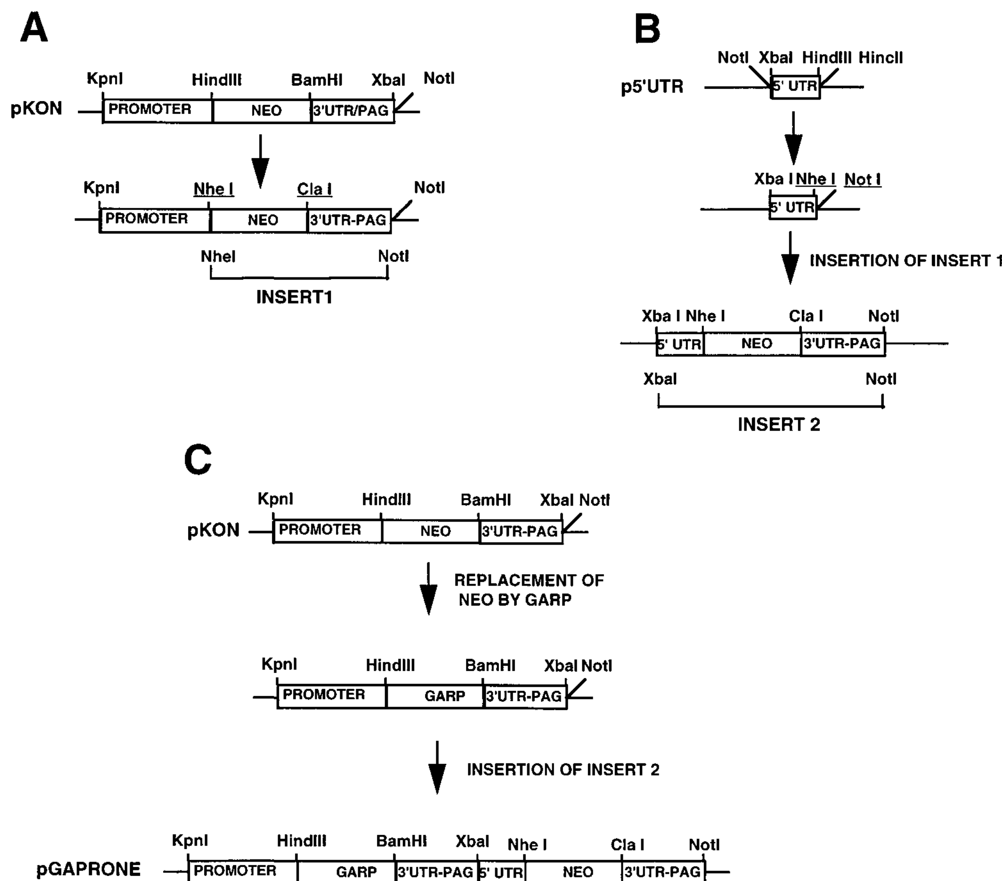

FIG. 1. Construction of the bicistronic vector pGAPRONE. The plasmid contains several unique restriction sites which allow the exchange of different modules. The individual steps are described in detail in Materials and Methods. Remodelled or new restriction sites are underlined the first time they appear. NEO, neomycin resistance gene. The promoter fragment contains the splice acceptor site and 5′ UTR of the procyclin α gene. 3′ UTR-PAG contains the last 19 bp of the procyclin β gene, the intergenic region, and the first 462 bp of PAG 1 (28, 44).

RESULTS

Positive and negative elements within the procyclin 3′ UTR. The basic wild-type construct (CAT-wt) that was used for transient transfections consists of the procyclin promoter and the α gene 5′ UTR, followed by the coding region for CAT and the full 297-bp 3′ UTR. This construct also contains sequences from the intergenic region which ensure correct polyadenylation (46). Two derivatives, a construct with a specific deletion of a conserved 16-mer from positions 204 to 219 (now designated CAT–Δ16-mer) and a construct with only the last 7 bp of the 3′ UTR, including the polyadenylation site (CAT-ΔUTR), have also been described previously (20, 46). In order to determine what additional elements might be present in the 3′ UTR, we created a nested set of deletions by starting at the first base after the stop codon and proceeding downstream (Fig. 2). Individual clones were sequenced and named according to the deletion; for example, CAT-Δ40 lacks the first 40 bp of the 3′ UTR.

The effect of longer deletions was tested by transient transfection and CAT assays (Fig. 2). Enzyme activity is expressed as a percentage of that obtained with CAT-wt. Deletion of the first 40 bp of the 3′ UTR (CAT-Δ40) and larger deletions (Δ51, Δ68, and Δ69) resulted in a decrease in CAT activity to 7 to 13%. Deletions downstream of nucleotide 69 (Δ96, Δ126, and Δ131) restored activity to ~50%, and with further deletions from position 164 onwards, the activity exceeded that of the wild type by approximately twofold. In agreement with our earlier results, the construct with an internal deletion of the conserved 16-mer produced only 8% of the level of wild-type activity. These results suggest that the 3′ UTR can be divided into three domains: (i) a positive element, which is affected by the deletions of the first 40 bp, (ii) one or more negative elements between positions 69 and 164, and (iii) a second positive element including the conserved 16-mer.

Influence of deletions on levels of steady-state mRNA and translation. To establish the level at which the different domains of the 3′ UTR influence expression, stable transfectants were produced. For these experiments we constructed a bicistronic plasmid (pGAPRONE) which contains the genes for GARP and Neo (Fig. 1 and 3A). This construct is designed to integrate by homologous recombination with either the Pro A or Pro B locus and to simultaneously replace tandemly linked procyclin genes. The rationale behind using GARP as the reporter gene was that, as the analogous surface glycoprotein from a related species of trypanosome, it might give a more accurate reflection of procyclin regulation than a soluble protein such as CAT. As will subsequently become clear, however, the two reporter genes give essentially the same results. A series of mutant 3′ UTRs were transferred to pGAPRONE from the CAT constructs described above. Transformants were analyzed for the presence of GARP (data not shown) and the deletion of a pair of procyclin genes. A set of clones that had

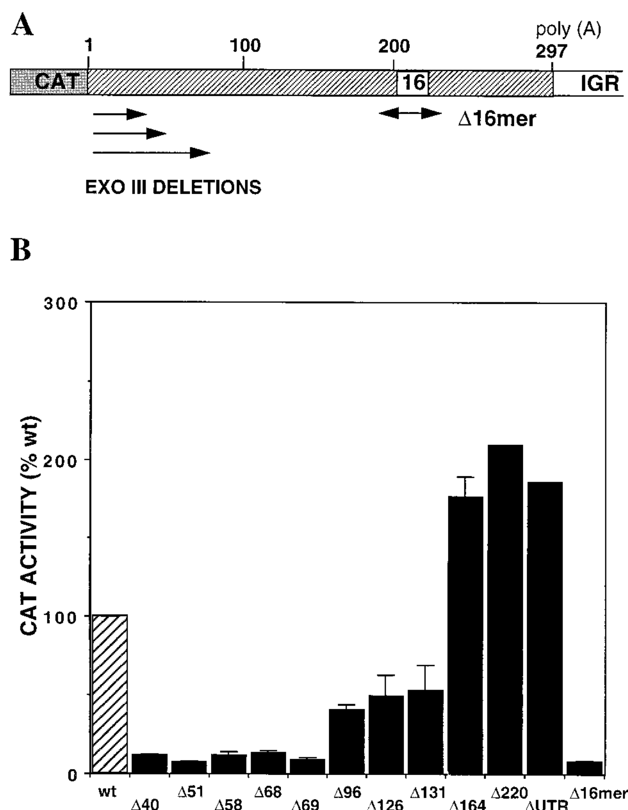

FIG. 2. (A) Schematic representation of exonuclease III deletions introduced into the CAT-wt construct. The hatched box represents the procyclin α gene 3′ UTR. CAT, CAT coding region; IGR, intergenic region between the procyclin α and β genes that is required for accurate polyadenylation of the CAT mRNA (46). A set of clones with deletions of between 40 and 164 nucleotides from the start site of the 3′ UTR was produced by digestion with exonuclease III. A construct with an internal deletion of a conserved 16-mer (CAT–Δ16-mer) has been described previously (20). (B) Effect of exonuclease III deletions on CAT activity in *T. brucei* procyclic forms. Each set of transient transfections was performed with a group of five constructs, of which the first and the last to be electroporated were identical constructs with a wild-type 3′ UTR. The difference between the two wild-type controls was always ≤10%. Filled bars represent mean values ($n = 3$) of the CAT activities of mutant constructs relative to the activity obtained with a wild-type 3′ UTR (hatched bar). wt, wild type.

all integrated into the Pro A locus (Fig. 3B) were chosen for further analysis.

A comparison of the relative steady-state levels of GARP mRNA (Fig. 4A) revealed that the transformants G-Δ40 and G–Δ16-mer had reduced amounts compared to that of G-wt (47 and 40%, respectively). This increased to 95% in G-Δ96 and exceeded wild-type levels in G-Δ164 (159%), again consistent with the removal of one or more negative elements. G-ΔUTR gave rise to two alternatively polyadenylated transcripts, presumably due to competition from the downstream polypyrimidine tract which directs 5′ processing of the Neo^r transcript. When the amount of GARP protein produced by each transformant was monitored by Western blot analysis, we found that it reflected the amount of steady-state mRNA for G-wt, G-Δ96, and G-Δ164 (Fig. 4A and B). G-ΔUTR transformants contained less mRNA than G-wt cells but produced more protein, as was previously found to be the case in transient transfections with CAT constructs (46). In contrast, G-Δ40 and G–Δ16-mer produced substantially less protein (12 and 4%, respectively) than would have been predicted from the levels of steady-state mRNA.

The results obtained with G-Δ40 or G–Δ16-mer confirm the presence of two positive elements and suggest that they might have an influence on both RNA abundance and translation. As a further control, RNA was transcribed in vitro from the corresponding constructs and translated in rabbit reticulocytes (Fig. 4C). Very similar amounts of GARP protein were obtained with RNA from both mutants and G-wt, whether 3′ UTR sequences were present or not (data not shown), indicating that the effect on translation must be specific for trypanosomes.

Dominance of an element within the central stem-loop. Computer predictions of the most stable secondary structures of the procyclin α and β 3′ UTRs give similar conformations: each folds into three main stem-loops (LI, LII, and LIII), with the 16-mer forming the top of the third stem-loop. When the positions of the exonuclease III deletions were mapped (Fig. 5A), the results obtained with both transient and stable transfections were consistent with a negative element within the central stem-loop of the procyclin 3′ UTR. To confirm this, a new plasmid was constructed with an internal deletion from nucleotides 101 to 173 (ΔLII). This slightly larger deletion was made in order to preserve the (theoretical) structures of the two outer stem-loops. When tested in transient transfections, a CAT-ΔLII construct gave rise to seven times more CAT activity than that of CAT-wt (Fig. 5B). To address the issue of whether the positive and negative elements interact with each other, three double mutants were constructed. In two of these (Δ40ΔLII and ΔLIIΔ16-mer constructs), combinations of positive and negative elements were deleted. Transient transfections with CAT-Δ40ΔLII gave results similar to those with CAT-Δ164 (180%), whereas results with CAT–ΔLIIΔ16-mer, which was nine times more active than the wild-type construct, more closely resembled those with CAT-ΔLII. Thus, although constructs with the two mutations Δ40 and Δ16-mer behave very similarly in all other respects, they are not equivalent with

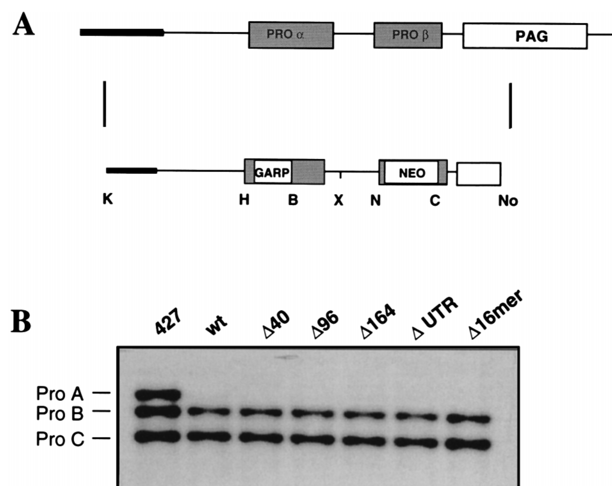

FIG. 3. Analysis of stably transfected clones of *T. brucei* procyclic forms. (A) Schematic representation of the integration of *Kpn*I-*Not*I fragments from pGAPRONE into the Pro A locus via homologous recombination. PRO α, procyclin α gene; PRO β, procyclin β gene; NEO, neomycin resistance gene. Several unique restriction sites in the plasmid allow the exchange of different modules. K, *Kpn*I; H, *Hin*dIII; B, *Bam*HI; X, *Xba*I; N, *Nhe*I; C, *Cla*I; No, *Not*I. (B) Southern blot analysis, confirming the replacement of the Pro A locus in individual clones transformed with derivatives of pGAPRONE. Genomic DNAs from *T. brucei* 427 and stably transformed deletion mutants were digested with *Pst*I. The blot was hybridized with a radiolabelled probe consisting of the procyclin α gene coding region. Lanes are labelled with the type of 3′ UTR downstream of the GARP coding region. wt, wild type.

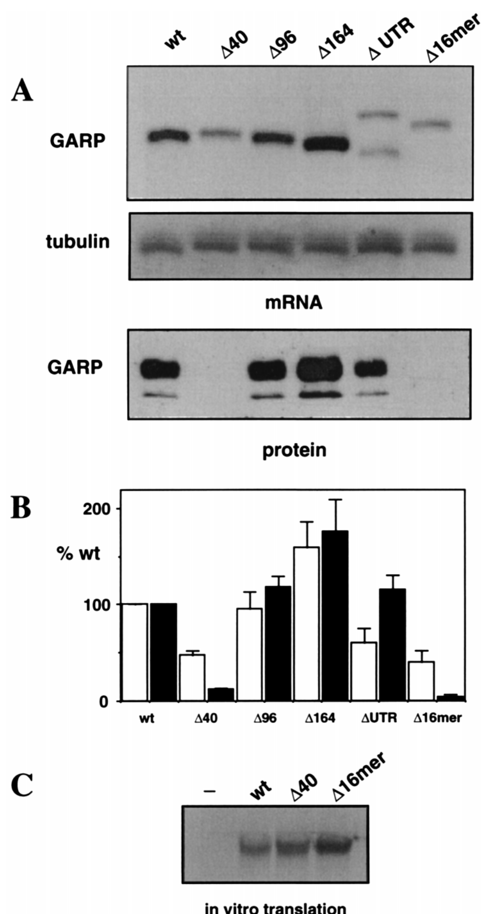

FIG. 4. Effects of deletions in the 3′ UTR on the steady-state levels of GARP mRNA and protein. (A) Northern blots in which 10 μg of total RNA was loaded per lane are shown. The blots were hybridized with a radiolabelled probe corresponding to the GARP coding region. In a Western blot, total cell lysates from 10^6 cells were loaded in each lane. GARP was detected with a polyclonal rabbit anti-GARP antiserum. The antibodies recognized the GARP precursor (29 kDa) and mature glycosylated forms ranging in size from 32 to 37 kDa (21). GARP was detected only in G-Δ40 and G–Δ16-mer when the blots were overexposed or at least 4×10^6 cells were used. (B) Comparison of the amounts of steady-state mRNA (open bars) and protein (filled bars) relative to those of G-wt transformants (100%). Results are presented as mean values ± 1 standard deviation. The mRNA data were compiled from four independent RNA isolations. The values obtained for GARP transcripts were normalized against α- and β-tubulin transcripts. Protein data were compiled from four separate Western blots. (C) Translation of in vitro-transcribed RNA in rabbit reticulocyte lysates produced a single polypeptide of 29 kDa corresponding to the GARP precursor. The first lane (−) contains no input RNA. The Δ40 and Δ16-mer deletions have no influence on translation in this system. wt, wild type.

regard to ΔLII. Finally, we examined the effect of deleting both positive elements. If the two elements interact with each other directly by base pairing or pseudoknot formation, we would not expect to see a further reduction in the double mutant. In this case, however, assays with CAT–Δ40Δ16-mer gave less enzyme activity (2 to 3%) than either mutation individually, suggesting that the two elements function independently.

To further analyze the effect of ΔLII, a new series of stable transformants was produced with derivatives of pGAPRONE (Fig. 6). In G-ΔLII trypanosomes, the deletion was sufficient to increase the levels of both steady-state mRNA and protein approximately threefold over those of the wild-type 3′ UTR. The double mutants G-Δ40ΔLII and G–ΔLIIΔ16-mer also contained more mRNA than G-wt cells (118 and 191%, respectively) and this was again mirrored by the amount of protein. In no case was the level of overexpression as pronounced as for CAT in the transient transfections, but there might be a limit as to how much GARP the stably transformed cells can express and accommodate on the surface. It is clear, however, that the ΔLII deletion is able to override the block on translation that is exerted by the other two mutations.

Effect of mutations on RNA stability. Cells transformed with constructs carrying the ΔLII mutation, whether singly or in combination with a second mutation, invariably contained more steady-state mRNA than cells with the wild-type 3′ UTR, suggesting that a destabilizing element had been removed. To test if this was indeed the case, we examined the effect of transcription inhibitors on the turnover of GARP transcripts. Cells were treated with 10 μg of actinomycin D ml^{-1}, at which concentration transcription is blocked by >99% (5). RNA was isolated at various time points, and the relative amounts of procyclin, tubulin, and GARP transcripts were quantified on Northern blots (Fig. 7). The tubulin RNAs were the most stable (half-life [$t_{1/2}$], ~120 min), followed by procyclin ($t_{1/2}$, ~60 min) and GARP-wt ($t_{1/2}$, ~50 min). In contrast, G-Δ40 and G–Δ16-mer transcripts were considerably less stable, with $t_{1/2}$s decreasing to <50% within 15 min and thereafter to levels that were too low to quantify accurately. The opposite effect was observed with G-ΔLII, however, which had an increased $t_{1/2}$ of ~100 min and clearly detectable levels of transcripts 3 h after exposure to actinomycin D.

DISCUSSION

A conserved 16-mer that is present at the same position in the 3′ UTRs of procyclin mRNAs is known to function as a positive element in procyclic forms of the parasite (20). Nested deletions of the 297-base 3′ UTR have now revealed the presence of at least two new elements which have a profound influence on expression. Removal of the first 40 bases after the stop codon destroyed a positive element, reducing CAT activity >9-fold relative to that of the wild-type 3′ UTR in transient transfections. Further deletions between nucleotides 69 and 164 caused a stepwise increase in activity to almost twice that of the wild type, consistent with the removal of one or more negative elements. An internal deletion from positions 101 to 173 (ΔLII) was even more effective, resulting in a sevenfold increase in CAT expression over the level of the wild type.

The level at which each deletion influenced expression was established with stable transformants in which GARP, the *T. congolense* analog of procyclins, was used as the reporter gene. The deletions Δ40 and Δ16-mer caused a marked reduction in the amount of protein (8-fold and 25-fold, respectively) but a relatively modest decrease in the relative amounts of steady-state mRNA (~2-fold), suggesting that translational repression is an important component of down-regulation. In contrast, removal of the LII element caused an increase in the level of steady-state mRNA that was matched by an increase in the level of protein. Deletions upstream of LII (between positions 69 and 96) resulted in a partial restoration of expression. It is possible that there are additional negative elements outside the domain defined by the ΔLII deletion; alternatively, there might be a indirect effect if the secondary structure is perturbed. It is worth noting that the results obtained with the two reporter genes are in excellent agreement: this indicates, first, that the 3′ UTR functions independently of the coding region and, second, that translations of a soluble protein (CAT) and a GPI-anchored protein (GARP) are regulated in the same way.

The effects of Δ40 and Δ16-mer on translation appear to be

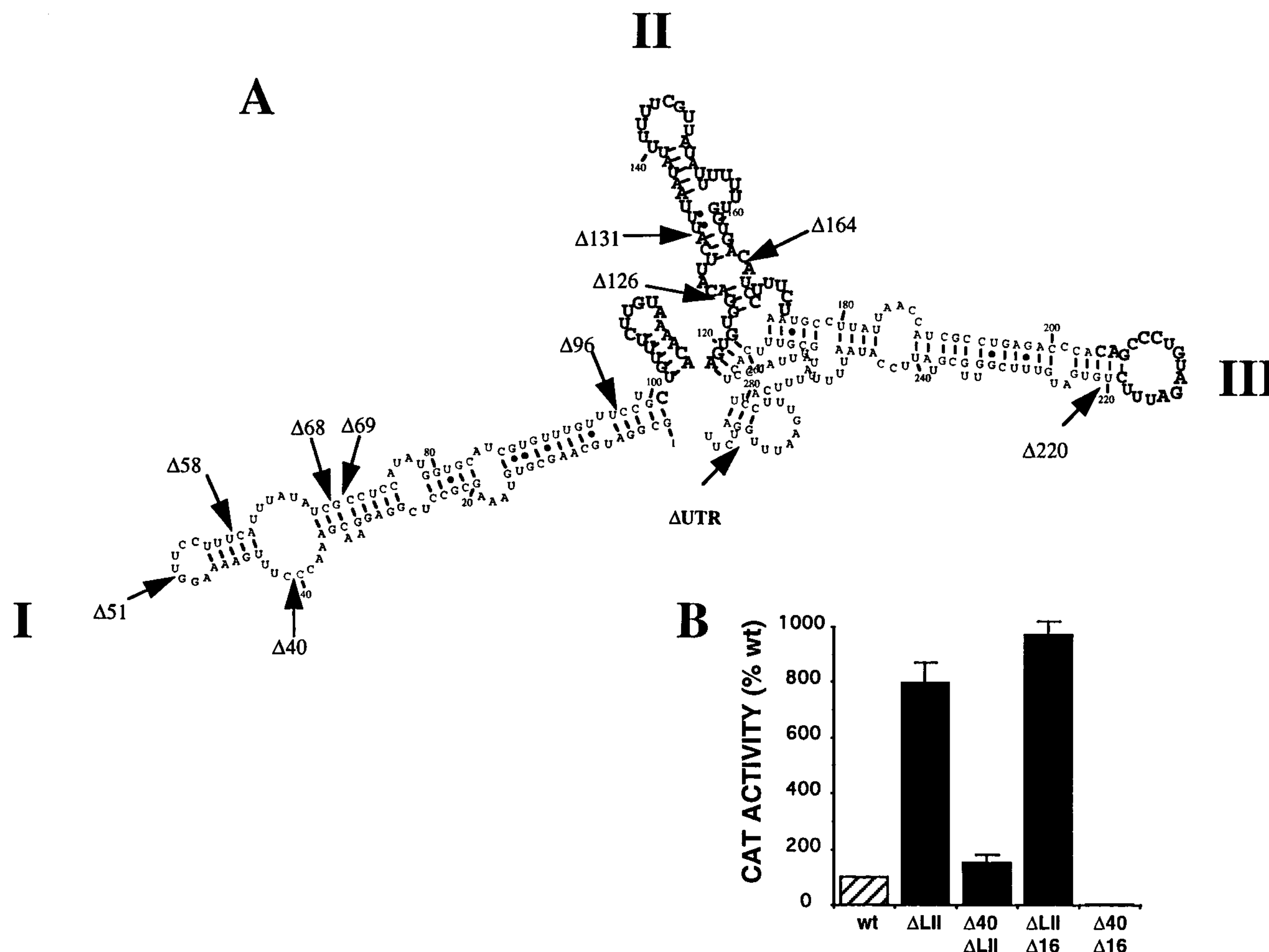

FIG. 5. (A) Predicted minimum energy secondary structure of the procyclin α gene 3′ UTR sequence according to the method of Zuker and Stiegler (56). The mRNA theoretically folds into three stem-loops (LI to LIII). Arrows indicate the positions of the exonuclease III deletions. The LII region from nucleotides 101 to 173 and the conserved 16-mer from nucleotides 204 to 219 are in boldface type. The internal deletion ΔLII was designed in such a way that the predicted structures of the two outer loops were maintained. In general, the domain structure appears to be very stable. All exonuclease III deletions up to position 164 have no effect on the structure of LIII. The majority of deletion mutants up to position 96 also have no effect on the structure of LII. In the case of Δ40, there is some alteration of the base pairing within LII, but the characteristic single-stranded "uridine bulges" also appear in this structure. (B) Effects of the LII deletion and double deletions on transient CAT expression in *T. brucei* procyclic forms. Sets of transfections with the appropriate controls were performed as described in the legend to Fig. 2. Filled bars represent mean values ($n = 3$) ± 1 standard deviation of the CAT activity of each mutant construct relative to the activity obtained with CAT-wt (hatched bar). wt, wild type.

specific for trypanosomes. When GARP RNAs were transcribed in vitro and translated in rabbit reticulocyte lysates, similar amounts of product were obtained whether the transcripts contained the wild-type, Δ16-mer, or Δ40 3′ UTR, indicating that regulation requires factors that are absent from the cell-free lysate. Factors which bind to 3′ UTRs from higher eukaryotes, such as those modulating the translation of 15-lipoxygenase (35) and protamine (30), are known to be tissue specific but are able to exert an effect when they are added to in vitro translation reaction mixtures. In preliminary experiments, however, we have found that supplementing reticulocyte lysates with cytoplasmic fractions from trypanosomes merely resulted in a general inhibition of translation (51a).

Inhibition of RNA synthesis by actinomycin D revealed that transcripts carrying the Δ16-mer and Δ40 mutations were considerably more labile than transcripts with an intact 3′ UTR ($t_{1/2}$, <15 and ~50 min, respectively). In contrast, the ΔLII mutation increased the stability of the RNA ($t_{1/2}$, ~100 min). It is not clear whether the effects on translation and RNA stability are linked or separate phenomena. Translation per se is not a prerequisite for RNA stability in trypanosomes, since RNAs without proper open reading frames, such as polyadenylated forms of the miniexon-derived RNA (38) and a subset of PAG 1 transcripts (51), can readily be detected. Furthermore, procyclin transcripts accumulate in bloodstream forms treated with protein synthesis inhibitors (13, 19) and we have recently obtained evidence that procyclin transcripts with the Δ164 deletion are expressed at high levels in bloodstream forms although the protein cannot be detected (46a).

The results we have obtained are summarized in a model which is shown in Fig. 8. Factors—which may be two domains of one protein or discrete polypeptides—bind to the two positive elements defined by Δ40 and Δ16-mer and shield the internal negative element so that the mRNA is both stable and efficiently translated. Destroying either of the positive elements exposes sequences that are targets for RNA degradation. This might entail endonucleolytic cleavage within the 3′ UTR, as is the case for the mammalian transferrin receptor

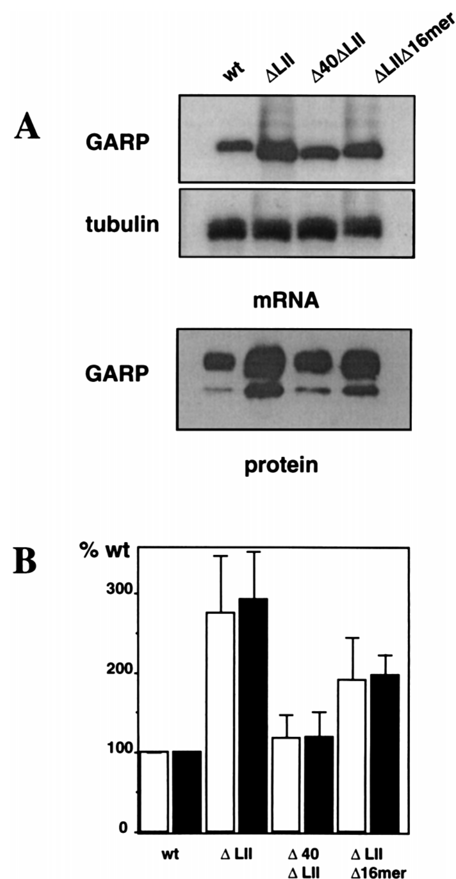

FIG. 6. Effect of the LII deletion, singly and in combination, on GARP expression in stable transformants. (A) Northern and Western blot analyses were performed as described in the legend to Fig. 4. (B) Relative amounts of steady-state mRNA (open bars) and protein (filled bars) compared to those of G-wt (set at 100%) presented as mean values ± standard deviation. The data were compiled from three independent RNA and protein isolations. wt, wild type.

mRNA (3, 7), or poly(A) tail shortening, as has been demonstrated for c-*fos* mRNA (52). The destabilization of the RNA may be sufficient to account for the reduction in the level of protein; alternatively, additional factors may mediate translational repression. These might even have the dual effect of protecting the RNA against further degradation while sequestering it in a form that is not accessible for translation. It is not unprecedented for a single element to have two functions, since UA-rich sequences in the 3′ UTR of interferon β mRNA affect both its stability and the efficiency of translation (29). Although the two positive elements in the procyclin 3′ UTR counteract the negative element, there are subtle differences. Constructs with either the ΔLII or ΔLIIΔ16-mer 3′ UTR produced similarly high levels of either CAT or GARP, indicating that the 16-mer is redundant once the LII sequence is removed. The simplest explanation is that this element functions as an antirepressor of the negative element rather than an independent activator. The situation with the other positive element may be more complex, however, since Δ40ΔLII results in a level of expression that is intermediate between those of the two single mutations.

There is a growing number of examples, most notably for *Drosophila melanogaster* (14, 26), where translational repression plays a key role in the programmed development of multicellular organisms. Translational control elements within the 3′ UTRs often function coordinately with additional elements that reinforce a specific pattern of expression. In the developing embryo, the bicoid (39) and nanos (17) mRNAs are translated at the anterior and posterior poles, respectively, and give rise to protein gradients. This is not only due to the fact that the mRNAs themselves are localized at the poles (17), since it has recently been shown for nanos that separate elements dictate translational repression and localization and can be uncoupled (11, 18). Similarly, there are discrete elements in the 3′ UTR of the cyclin B mRNA which determine its localization to the posterior pole and temporal control of translation (12).

Trypanosomes are unicellular organisms which differentiate in order to adapt to changes in environment (such as the transition from mammalian circulation to the tsetse fly midgut) rather than undergoing a predetermined set of developmental changes. In this regard, expression of the correct surface coat is of central importance (40). We have recently shown that specific forms of procyclin enhance survival in the fly (44), but these same molecules would be deleterious if they were expressed on the surfaces of bloodstream forms, as they would result in the recognition and subsequent destruction of the parasites by the host immune system. Since there is, at most, a 20-fold difference in the levels of activity of the procyclin promoter between the procyclic and the bloodstream forms of the life cycle, there is a further requirement for posttranscriptional control to achieve optimal expression by insect forms while ensuring that no protein is produced by bloodstream forms.

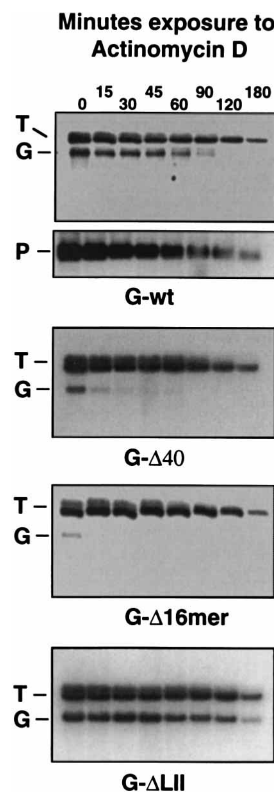

FIG. 7. Kinetics of GARP, tubulin, and procyclin mRNA decay after exposing stable transformants to 10 μg of actinomycin D ml^{-1}. Cells were harvested at the indicated time points, and total RNA was isolated. The blots were simultaneously hybridized with radiolabelled probes for α- and β-tubulin (T) and GARP (G). Total RNA from G-wt cells was also hybridized with a procyclin probe (P).

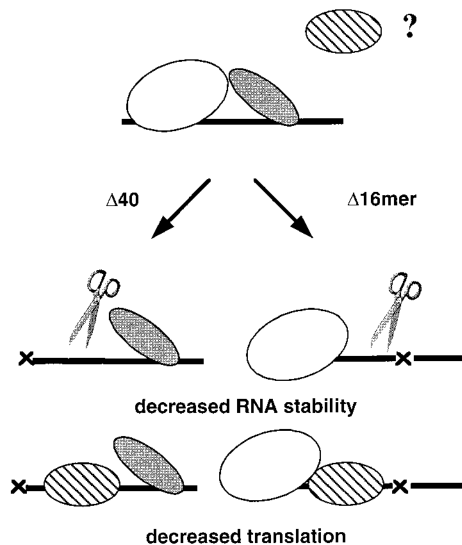

FIG. 8. Model summarizing the results obtained with transiently and stably transfected trypanosomes. Two factors (open and shaded ovals), which might be separate proteins or two domains of a single polypeptide, shield the negative LII element by interacting with positive elements defined by the Δ40 and Δ16-mer deletions. Deletion of either region prevents binding and leads to a destabilization of the RNA. Additional factors (hatched oval) may also repress translation.

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