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A conserved stem–loop structure in the 3′ untranslated region of procyclin mRNAs regulates expression in *Trypanosoma brucei*

(surface glycoprotein/RNA secondary structure/translational control/stage specific)

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ABSTRACT **African trypanosomes that cycle between mammalian hosts and the tsetse fly vector must be poised to survive in different environments. The control of stage-specific gene expression is undoubtedly one of the keys to successful adaptation, but no regulatory elements have been defined to date. Procyclins (also known as procyclic acidic repetitive proteins) are specifically expressed on the surface of procyclic and epimastigote forms in the fly. Procyclin genes are already transcribed in bloodstream forms, but stable mRNA, and later the protein, are first detected when the parasites begin to differentiate into procyclic forms. We have now identified a region of 16 bases that forms part of a predicted stem–loop structure in the 3′ untranslated regions of different procyclin mRNAs; both the sequence and the secondary structure of this 16-mer appear to be required for efficient translation of a reporter gene in procyclic forms. The level of steady-state mRNA, its polyadenylylation, and its distribution in the cell are all unaffected by the presence or absence of this element. Deletion of the 16-mer alone reduces expression more than removal or reversal of the entire 3′ untranslated region and flanking region, suggesting that there are additional negative regulatory elements in the same 3′ untranslated region.**

The protozoan parasite *Trypanosoma brucei*, which is transmitted between mammals by the tsetse fly, alternately expresses two major types of surface glycoprotein. Procyclic forms in the tsetse fly midgut are covered by an invariant glycoprotein coat composed of procyclins (1–4) whereas the bloodstream forms, which multiply in the mammalian host, are shielded by a dense surface coat of variant surface glycoproteins (VSG; for reviews, see refs. 5 and 6).

Bloodstream-form trypanosomes ingested by the tsetse fly differentiate into procyclic forms within a few days (7). Trypanosomes cultured in the appropriate medium can also be induced to differentiate *in vitro* (8). Procyclin genes are already transcribed in bloodstream forms (9), but virtually no steady-state mRNA is detectable before differentiation begins (10). Depending on the strain of trypanosomes, an increase in procyclin mRNA can be observed as soon as 15 min after triggering differentiation (11) and the proteins can be detected within 4 h (12). In a set of experiments where both mRNA and protein were monitored, there was a lag of several hours between the appearance of the mRNA and its translation (10). In parallel with the induction of procyclin expression, VSG-specific mRNA and protein synthesis are repressed (13, 14) and VSG is shed from the parasite surface (15).

Procyclin genes are located on four large chromosomes (16) in tandem arrays of two or three copies (3, 17). The 5′ untranslated regions (UTRs) and the coding regions of the various copies are highly conserved (73–93% identity) and differ principally in the length and composition of an internal repeat. The 3′ UTRs are more heterogeneous, however, and the longest region of identity is a stretch of 16 nt that is present in all copies (17), beginning 92–94 bases upstream of the polyadenylylation site (2, 3, 11, 18, 19).

Little is known about the regulation of procyclin expression. While there is clearly some control at the level of transcription initiation (9), this is not sufficient to explain the absence of procyclin mRNA in bloodstream-form trypanosomes. There is also evidence that a procyclin 3′ UTR can abolish expression of a reporter gene in bloodstream forms (20), although it is not known at what level this occurs. More recently, work from two groups (11, 21) has indicated that specific protein factors might regulate the levels of procyclin mRNA.

The occurrence of a conserved motif of 16 bases at the same position in the 3′ UTRs of different procyclin mRNAs suggested to us that it might be important in regulating expression. We have started by examining whether this sequence has an influence on expression in procyclic form trypanosomes. Here we show that this 16-mer is required for efficient expression of a reporter gene and that both the sequence and the correct secondary structure are necessary for its function. The region that we have identified provides an example of a defined regulatory element in the RNA of a protozoan parasite.

MATERIALS AND METHODS

Trypanosome Strains, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. Procyclic forms of *T. brucei* (strain 427; ref. 22) were cultivated in SDM-79 as described (23). Transfections were performed as described by Zomerdijk *et al.* (24) using 5×10^7 trypanosomes and 5 μg of CsCl-purified plasmid DNA unless otherwise stated. CAT assays were performed essentially as described (24), except that the assay buffer was supplemented with bovine serum albumin (50 μg/ml). Each transfection was performed in duplicate. Mean values (percent of wild type, ±1 SD) are derived from at least three transfection experiments.

Quantitative determination of the amount of CAT protein was performed using a CAT-ELISA kit (Boehringer Mannheim) by following the manufacturer's instructions.

Construction of Recombinant Plasmids. A CAT construct with the procyclin promoter and wild-type 3′ end was assembled from the *Pvu* II fragment of pAP2 (9, 17) that had been subcloned into Bluescript+ (Stratagene). A 3.5-kb fragment containing the procyclin promoter (25) was amplified using a primer specific for Bluescript (BS-back, see below) and primer 1 in which the initiation codon of the procyclin gene was mutated to create a *Hin*dIII site. The product was digested with *Hin*dIII and cloned upstream of the coding sequence of the CAT gene (26). A 562-bp fragment containing the procyclin 3′ UTR and 265 bases of the intergenic region was amplified from pAP2 using a primer specific

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Abbreviations: UTR, untranslated region; VSG, variant surface glycoprotein; CAT, chloramphenicol acetyltransferase.

for Bluescript (BS-forward) and primer 2, which starts 1 base downstream of the procyclin stop codon, with a point mutation to create a *Bam*HI site. The fragment was digested with *Bam*HI and cloned downstream of the CAT gene. This fragment was also cloned in the opposite orientation to generate the reverse construct.

Construction of the Deletion Mutant Δ16. The two halves of the 3′ end of Δ16 were generated from the wild type as follows: the *Bam*HI–*Eco*RV fragment upstream of the 16-mer was amplified with primers 2 and 3; the downstream *Eco*RV–*Not* I fragment was generated using the BS-forward primer and primer 4. The two fragments were ligated simultaneously into the wild-type plasmid that had been digested with *Bam*HI and *Not* I and gel-purified to remove the wild-type 3′ end. The construct 16+ was produced by insertion of the 16-mer 5′-CAGCCCTGTAGATTTC-3′ (and its complement), in the correct orientation, into the *Eco*RV site of Δ16. Additional constructs were derived by insertion of the 18-mer 5′-CAGCCCTGTAGATTTCTG-3′ into the *Eco*RV site of Δ16 in the correct orientation (18+) or the opposite orientation (18−).

Constructs with the VSG expression site promoter were produced by ligating a *Hin*dIII–*Eco*RI fragment from the CAT construct pD5 (20), which contained the promoter region and ESAG7 splice acceptor site and extended to an internal *Eco*RI site in the CAT gene, to either the wild-type vector or Δ16, which had been digested with *Hin*dIII and *Eco*RI to remove the procyclin promoter and the 5′ portion of the CAT gene.

Primers (with the relevant restriction sites underlined) are as follows: 1, 5′-CAGCGCCGGCAAGCTTGTGAATTTTACTTTTTGG-3′; 2, 5′-AATAGATATCGGATCCGGATGCAAGCGTGTAAAGCG-3′; 3, 5′-GGTAGTGATATCTGGGTCTCAGGCGATGG; 4, 5′-GGACAGCGATATCTGTGATGTTTCGGTTGC; BS-back, 5′-ACCATGATTACGCCAAGCTCG-3′; BS-forward, 5′-GTTTTCCCAGTCACGACGTTG-3′. All constructs were checked by a combination of restriction enzyme and sequence analyses.

Northern Blot Analysis. Procyclic-form trypanosomes (2.5 × 10^8 cells) were transfected with 50 μg of plasmid DNA. After a 7-h incubation, RNA was isolated for Northern blot analysis as described (27) and, subsequently, treated with RNase-free DNase. Total RNA (8 μg) was fractionated on 1% agarose gels and transferred to nitrocellulose (2). Radioactive antisense probes were generated by *in vitro* transcription of 250 bases of the coding region of the CAT gene (26) or 200 bases from the procyclin 3′ UTR. Hybridization was performed in 50% (vol/vol) formamide/0.5× standard saline citrate (SSC)/4× Denhardt's solution/0.08% SDS/80 mM sodium phosphate, pH 6.5/herring sperm DNA (80 μg/ml)/tRNA (80 μg/ml) at 50°C. Washes after hybridization were in 0.1× SSC at 65°C. No signal was obtained with a Bluescript-specific probe, confirming that hybridization was not due to contamination with plasmid (data not shown).

Quantitative PCR and Analysis of 3′ Ends. These procedures were performed by combining the methods of Frohman *et al.* (28) and Becker-André *et al.* (29). In each experiment, 2 × 10^8 cells were transfected with a mixture of the wild-type construct and Δ16 (40 μg of DNA in total) in the ratios indicated in the figure and RNA was isolated as described (10). cDNA was generated by reverse transcription using a hybrid T_{17}-adaptor primer (GACTCGAGTCGACATCGAT$_{17}$; ref. 28). CAT-specific cDNA was hybrid-selected prior to amplification to exclude recombination with cDNA derived from endogenous procyclin transcripts (see below). The PCR (35 cycles) was performed with Supertaq (Stehelin AG, Basel) using the following parameters: denaturation, 1 min, 94°C; annealing, 1 min, 55°C; extension, 3 min, 74°C. The primers used for amplification were the adaptor primer (GACTCGAGTCGACATCGA; ref. 28) and an oligonucleotide from the 3′ end of the CAT coding sequence (5′-GCGAATTCGGATGAATGGCA-3′). Restriction sites that could be used in cloning are underlined. The amplified DNA was diluted in the range 1:100 to 1:400 to obtain the same amount per reaction mixture and subjected to a further two cycles of amplification using 10 pmol of ^{32}P-labeled CAT primer and 25 pmol of unlabeled adaptor primer. The DNA was digested with *Eco*RV and separated on a 1.5% agarose gel. For cell fractionation experiments (30), 1.2 × 10^8 trypanosomes were cotransfected with the wild-type and Δ16 plasmids, each at 20 μg.

Analysis of Polyadenylylation Sites. CAT-specific cDNA was amplified as described above, digested with *Eco*RI and *Sal* I, and cloned into Bluescript+. Sequence analysis was performed by the dideoxynucleotide chain-termination method (31).

Miscellaneous Techniques. Predictions of RNA structure were made with the FOLD program from the GCG Sequence Analysis Software Package, version 7.2 (32). For hybrid selection of cDNA prior to PCR, a plasmid containing the CAT coding sequence (26) was bound to filters as described (33). RNA was removed from cDNA·RNA hybrids by hydrolysis in 0.1 M NaOH at 65°C for 30 min. The cDNA was neutralized to pH 7.5 with Tris·HCl and then hybridized with filters overnight at 42°C in 50% formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA/0.2% SDS/tRNA (70 μg/ml). Washes were performed at 55°C in 10 mM Tris·HCl/150 mM NaCl/1 mM EDTA/0.5% SDS. CAT-specific cDNA was eluted by boiling in 0.1% SDS.

RESULTS

A Conserved 16-mer in the Procyclin 3′ UTR Enhances Expression. To investigate the role of the conserved 16-mer motif in expression in procyclic-form trypanosomes, transient transfection assays were carried out using constructs in which the coding sequence of the procyclin gene was precisely removed and replaced by a *Hin*dIII–*Bam*HI fragment encoding the CAT gene (Fig. 1). These constructs contained the same procyclin promoter but different 3′ sequences (Fig. 2). A construct containing the wild-type 3′ UTR and a portion of the intergenic region showed high levels of activity (100%; Fig. 2); reversal or removal of the entire 3′ flanking region resulted in only a 2- to 4-fold drop in activity compared to the wild type. In contrast, a targeted deletion construct in which the 16-bp element was replaced by a unique *Eco*RV site (Δ16) gave only 7.7% (±0.7%) of the CAT activity measured with

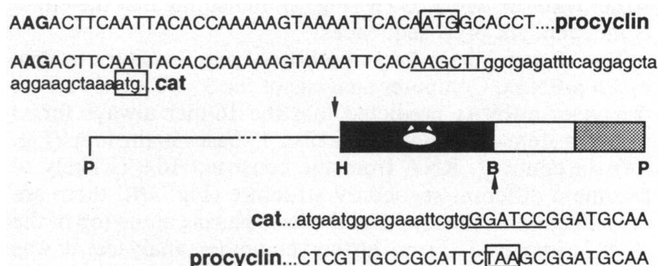

FIG. 1. Schematic representation of the wild-type CAT construct (not to scale) used in transient transfection experiments, showing the precise substitution of the procyclin coding region by CAT sequences (26). This plasmid encompasses the procyclin promoter (25) and the first of three tandemly repeated procyclin genes (17) with a 297-bp 3′ UTR and 265 bp of the intergenic region. Bases from the procyclin gene are in uppercase type, and bases from the CAT gene are in lowercase type. Initiation and termination codons are boxed. The splice acceptor site of the mature RNA is shown in boldface type; artificially introduced restriction sites are underlined. P, *Pvu* II; H, *Hin*dIII; B, *Bam*HI. The symbols used for the CAT gene, 3′ UTR, and intergenic region are as in Fig. 2.

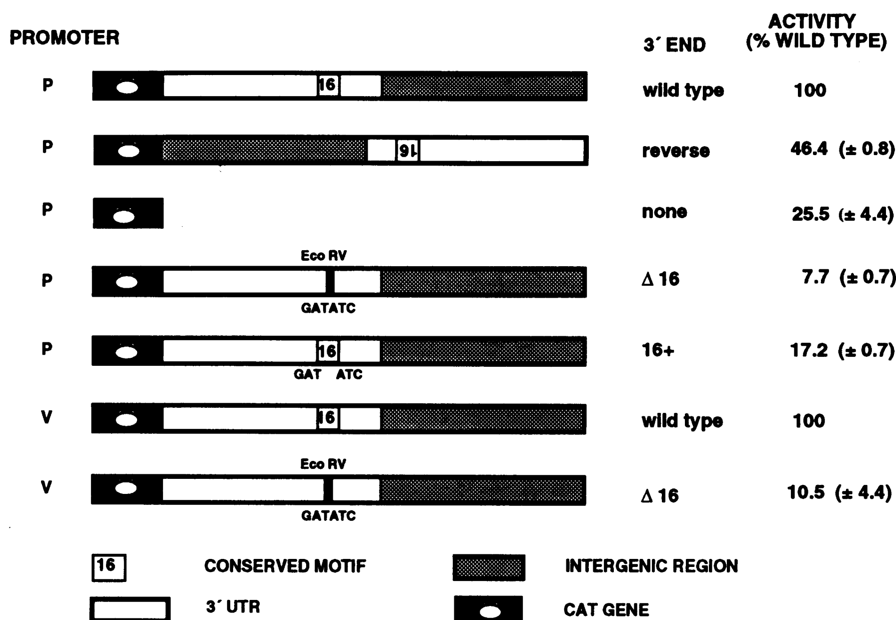

FIG. 2. Effect of 3′ flanking sequences on CAT expression. Schematic representation of recombinant plasmids used in transfection and of CAT activities relative to the wild type (100%) is shown. P, procyclin promoter; V, VSG expression site promoter.

the wild-type 3′ UTR. In a parallel experiment, the amount of CAT protein was determined by ELISA. Approximately 10 times more protein could be detected in cells transfected with the wild-type construct than with Δ16, which confirmed that the difference in enzyme activity was an accurate reflection of the amount of CAT synthesized in these cells.

In an attempt to restore the activity of Δ16 to that of the wild type, the same 16 bp that had been deleted were reintroduced in the correct orientation into the newly created *Eco*RV site. The CAT activity obtained with this construct (16+) was only 17.2% (±0.7%) of the wild type, suggesting that the secondary structure or the precise position of the 16-mer might be crucial to its function.

To ascertain whether the effect of this 3′ sequence was due to an interaction with elements upstream of the coding region, plasmids were constructed in which the procyclin promoter, splice acceptor site, and 5′ UTR were replaced by the VSG promoter, the splice acceptor site, and the 5′ UTR of the first gene in this transcription unit (ESAG7; ref. 20). Once again, a similar reduction was observed with a construct with the Δ16 3′ UTR (Fig. 2), indicating that the effect is independent of 5′ sequences.

The 16-mer Forms Part of a Stem–Loop Structure in Procyclin mRNAs. Computer analysis of the 3′ UTRs of various procyclin mRNAs predicted that the 16-mer always forms part of a stem–loop motif with 12 or 13 bases in the loop (Fig. 3*A*). In contrast, RNA from the construct 16+ is likely to assume a different secondary structure (Fig. 3*B*); there are only 10 bases in the loop and the base pairing at the top of the stem is impaired. From further computer analyses, it was predicted that the introduction of 18 bp in either orientation would restore the loop structure and increase the length of the stem by 3 bp (Fig. 3*B*). In the correct orientation (18+), both the structure and the sequence in the loop and the top of the stem correspond to the wild type (Fig. 3*B*). In the opposite orientation (18−), the structure of the stem is maintained, but the loop contains the complementary sequence and only 4 bases are in the same position as in the wild-type motif. With the 18-mer in the correct orientation, the CAT activity slightly, but consistently, exceeded that of the wild type (110 ± 1.6%). When the orientation was reversed, however, activity did not increase above that of Δ16 (6.7 ± 0.8%). These results confirm the importance of both the sequence and the secondary structure of the conserved element.

The 16-mer Does Not Affect mRNA Levels or Distribution Within the Cell. Preliminary nuclear run-on experiments indicated that the CAT gene was transcribed at similar rates in trypanosomes transiently transfected with either Δ16 or the wild-type construct (data not shown). To determine whether the differences in CAT activity reflected the levels of steady-state RNA, Northern blot analysis was performed using total RNA extracted from these cells. Hybridization with a CAT-specific probe gave signals of equal intensity (Fig. 4*A*), indicating that deleting the 16-mer does not affect the amount of RNA. In a different approach, cells were cotransfected with different ratios of the two constructs and the poly(A) RNA levels were determined by quantitative PCR (Fig. 4*B*). *Eco*RV digestion of the amplified products allowed Δ16 to be distinguished from the wild type. The results of these experiments were consistent with the Northern blot analysis, with the relative amounts of transcripts reflecting the ratio of the two plasmids. Finally, to study the distribution of the two mRNAs within the cell, trypanosomes were cotransfected with the wild-type and Δ16 constructs and separated into nuclear and cytoplasmic fractions. Since there is significantly less mRNA in the nuclear fraction, there is less of the primary PCR amplification product (data not shown), but the amounts of input cDNA from the two fractions were equalized for the second round of PCR to give comparable signals. These experiments show that the relative amounts of the two transcripts in the cytoplasm are very similar (Fig. 4*C*), and that the block in expression is not due to the selective retention of Δ16 transcripts in the nucleus.

Polyadenylylation of Transcripts. mRNAs from trypanosomes do not contain conventional polyadenylylation signals (34). To examine the effect of the 16-mer on the choice of polyadenylylation site, PCR products generated from the 3′ ends of wild-type and Δ16 CAT mRNAs (see above) were cloned and partially sequenced. Of the 10 clones analyzed (4 from the wild type and 6 from Δ16), all had exactly the same polyadenylylation site as the endogenous procyclin gene (data not shown). No significant differences were observed in the lengths of the poly(A) tails in clones derived from the two transcripts nor were there differences in the overall lengths of

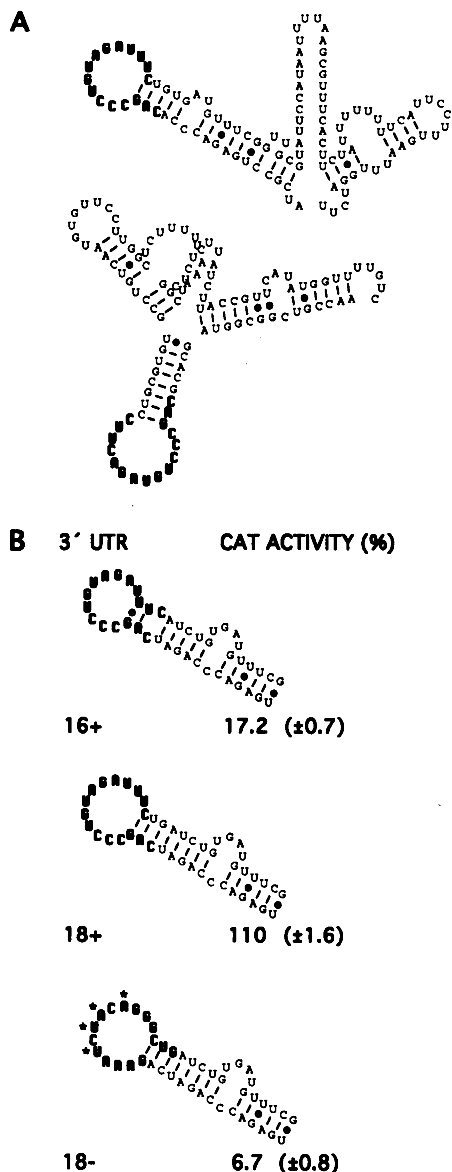

FIG. 3. (A) Conserved 16-mer CAGCCCUGUAGAYUUC (where Y is a pyrimidine) forms part of a stem–loop structure in various procyclin mRNAs. Sequences are from the cDNA clones pPS-1 (upper sequence, ref. 11) and pPRO2001 (lower sequence, ref. 2). The 3′ UTRs are 50% identical over a length of 297 bases. The position of the 16-mer is shown in boldface type. For clarity, only the last 110 bases of each 3′ UTR are shown. (B) Correlation between CAT activity and correct folding. Predicted folding of the 16-mer (boldface type) in RNAs derived from various constructs and the percentage CAT activity compared to the wild type are shown.

the transcripts (Fig. 4A). Thus these observations also rule out substantial changes in the extent of polyadenylylation.

Transcripts derived from the construct in which the intergenic region and 3′ UTR had been reversed (rev; see Fig. 2) were also analyzed in the same way. In contrast to mRNAs with the authentic 3′ end, these transcripts were polyadenylylated at different positions. Seven sites were identified from sequence analysis of 12 PCR clones (Fig. 5). No obvious sequence motifs could be discerned, however, apart from a preference for two or more adenine residues at the polyadenylylation site itself.

DISCUSSION

A conserved 16-mer in the 3′ UTR of procyclin mRNAs is required for efficient expression of a reporter gene in procyclic-form trypanosomes. Precise deletion of this motif from the wild-type construct (to yield Δ16) leads to a 10- to 12-fold decrease in CAT, whether measured as enzyme activity or as the amount of protein detectable by ELISA. Since the amount of CAT-specific mRNA, its polyadenylylation, and distribution in the cell are all unaffected, these data indicate that positive regulation must occur at the level of translation.

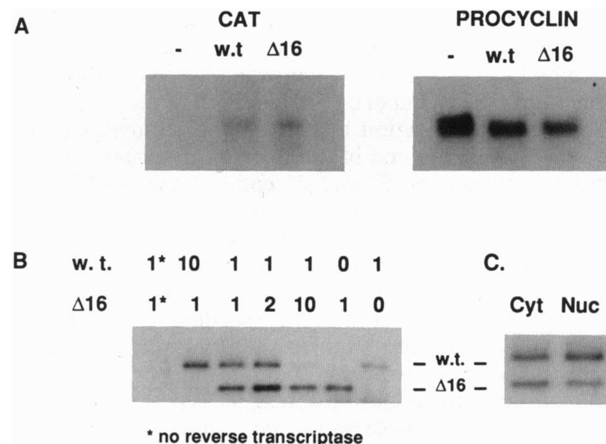

FIG. 4. (A) Equal amounts of steady-state CAT mRNA are found after transfection with equal amounts of the wild-type or Δ16 constructs. Northern blot analysis of RNA after mock transfection (−) or transfection with constructs with the procyclin promoter and wild type (w.t.) or Δ16 3′ flanking sequences. Filters were hybridized with antisense RNA corresponding to the CAT coding region or the procyclin 3′ UTR, as indicated. (B) Quantification by PCR of the relative levels of CAT transcripts after cotransfection with different ratios of the wild-type and Δ16 plasmids. CAT-specific DNA was amplified using the adaptor primer and a primer corresponding to the 3′ end of the CAT coding sequence. ^{32}P-labeled CAT primer was included in the last two cycles of amplification. The DNA was digested with *Eco*RV, which selectively cuts Δ16 PCR products, and fractionated by agarose gel electrophoresis. (C) Quantification by PCR of the relative amounts of the wild-type and Δ16 transcripts in nuclear (Nuc) and cytoplasmic (Cyt) fractions after cotransfection with equal amounts of the two plasmids.

To date, there is only one example of an element in a 3′ UTR that enhances translation. It has recently been shown (35) that two alternatively polyadenylylated forms of mRNA encoding the amyloid protein precursor are translated *in vivo* with different efficiencies and that sequences enhancing translation reside in a 258-base extension of the 3′ UTR. In contrast, elements within the 3′ UTRs of interferon β (36) and tumor necrosis factor mRNAs (37) have an inhibitory effect

FIG. 5. Transcripts with different polyadenylylation sites are generated from the rev construct, in which the intergenic region and 3′ UTR are reversed. Polyadenylylation sites are marked with arrows. The sequence shown extends 450 bases from the *Pvu* II site in the intergenic region into the procyclin 3′ UTR (reverse complement of nt 818–1267; ref. 17).

on translation. For tumor necrosis factor, the 3′ UTR also contains endotoxin-responsive elements that can override this inhibition (37). It is not immediately obvious how these elements function, but one possibility is that they might affect the rate of translocation by influencing the dissociation of ribosomes from the end of the mRNA. Alternatively, interactions between the 5′ and 3′ ends of the mRNA might regulate the initiation of translation.

The 16-mer we have identified is predicted to form part of a stem–loop structure in procyclin mRNAs with different 3′ UTRs. Our results with different constructs indicate that not only the sequence but also the secondary structure are important for function. Interestingly, a gene encoding the major surface glycoprotein of *Trypanosoma congolense* also contains this motif in the 3′ UTR, again as part of a stem–loop structure, but otherwise shows no homology to procyclins (38). This suggests that the control mechanism predates parasite speciation.

Deletion of the 16-mer alone reduces expression 3- to 6-fold more than removal or reversal of the entire 3′ UTR and flanking region. This implies that the 3′ UTR contains additional inhibitory elements. It is not yet known how these sequences affect expression, but there is a growing list of functions that can be attributed to specific elements in 3′ UTRs. In addition to regulating translation, mRNA stability (39), and polyadenylylation (34, 40, 41), 3′ elements can also influence the subcellular localization of mRNAs (42, 43).

It is clear that the 3′ UTR of procyclin plays a pivotal role in stage-specific gene expression, by down-regulating expression in bloodstream forms and by enhancing expression in procyclic-form trypanosomes. Although procyclic forms have never been observed in mammals, antigen and anti-procyclin antibodies can be detected after experimental infections (44). This would imply that some trypanosomes already differentiate in the bloodstream. It has also been shown that procyclic forms cannot survive in serum since they activate complement by the alternative pathway and are lysed (45). Defining this regulatory element in a trypanosome mRNA is just the beginning of unraveling the complex process of stage-specific gene expression. In the long term, however, this may allow the development of strategies for the control of infection by inducing premature differentiation and the subsequent destruction of parasites in the bloodstream.

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