Mechanisms of developmental regulation in *Trypanosoma brucei*: a polypyrimidine tract in the 3′-untranslated region of a surface protein mRNA affects RNA abundance and translation

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ABSTRACT

Salivarian trypanosomes are extracellular parasites of mammals that are transmitted by tsetse flies. The procyclic acidic repetitive proteins (PARPs) are the major surface glycoproteins of the form of *Trypanosoma brucei* that replicates in the fly. The abundance of PARP mRNA and protein is very strongly regulated, mostly at the post-transcriptional level. The 3′-untranslated regions of two PARP genes are of similar lengths, but are dissimilar in sequence apart from a 16mer stem–loop that stimulates translation and a 26mer polypyrimidine tract. Addition of either of these PARP 3′-untranslated regions immediately downstream of a reporter gene resulted in developmental regulation mimicking that of PARP. We show that the PARP 3′-UTR reduces RNA stability and translation in bloodstream forms and that the 26mer polypyrimidine tract is necessary for both effects.

INTRODUCTION

The salivarian trypanosomes are unicellular parasites that live in the extracellular fluids of mammals and are transmitted by tsetse flies. The bloodstream forms evade the immune response by means of a thick coat of variant surface glycoprotein (VSG) (1,2). When they enter the tsetse fly midgut, many morphological and biochemical changes ensue as they transform into procyclic forms. One change is the replacement of VSG by the procyclic acidic repetitive protein (PARP or procyclin) (3,4).

Trypanosome gene expression is unusual in that nearly all protein coding genes so far investigated are transcribed in a polycistronic fashion (5,6). The precursor transcripts are processed into monocistronic mRNAs by 3′-polycadenylation and by 5′ addition of a capped 39 nt spliced leader. Polyadenylation is both spatially and temporally coupled to the trans splicing reaction. When trans splicing is inhibited, polyadenylation ceases (7). Usually several polyadenylation sites are used, scattered over a short region ∼100 bases upstream of a trans splicing signal (8–11). Such signals commonly include a polypyrimidine tract with one or more acceptor AG dinucleotides up to ∼80 bases downstream (12,13).

There is no evidence for any regulation of RNA polymerase II-catalysed transcription in trypanosomes. Developmental regulation of mRNA levels is determined post-transcriptionally; the sequences responsible are usually located in the 3′-untranslated region (3′-UTR) of the transcripts concerned (14–19). Transcription of the VSG and PARP genes is, in contrast, subject to some regulation (5,6,20–22), but the RNA polymerase responsible is probably RNA polymerase 1 (23–25). Expression of the PARP genes is regulated at several levels. Transcription is ∼10-fold less active in bloodstream form trypanosomes than in procyclic forms (21,22,26), whereas PARP is undetectable in bloodstream forms. Indeed, expression of the antigenically invariant PARP at this stage would probably be lethal.

We have previously used a bicistronic vector bearing a CAT gene and hygromycin resistance (Hyg) cassette to study developmental regulation mediated by 3′-UTRs placed downstream of the CAT gene. 3′-UTRs from genes expressed in bloodstream forms caused bloodstream form-specific CAT expression; 3′-UTRs from genes expressed in procyclic forms, such as PARP genes, caused procyclic form-specific CAT expression (17). Actin (ACT) mRNA levels are at least 30-fold lower in bloodstream forms than in procyclic forms (18), luciferase (14) and phleomycin resistance genes (27,28).

Here we demonstrate that the sequence primarily responsible for PARP post-transcriptional regulation is a polypyrimidine tract that is conserved in all PARP gene 3′-UTRs. It acts by accelerating RNA turnover and inhibiting translation in bloodstream forms.

MATERIALS AND METHODS

Trypanosomes

Bloodstream trypanosomes (MiTat 1.2) and procyclic trypanosomes (derived from bloodstream Antat 1.1) were cultured and transfected and CAT assays performed as described (17,29). Hygromycin-resistant bloodstream trypanosome populations were generated by transfecting 10⁷ trypanosomes with 10 µg

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plasmid DNA linearized within a tubulin targeting sequence and selected with 12.5 μg/ml hygromycin and the resulting populations were analysed as soon as sufficient parasites were available (22). Alternatively, plasmids were linearized within an rRNA targeting sequence and cloned by limiting dilution. CAT assays were performed at least three times on each population. Results were reproducible when independent populations were generated with the same plasmid.

**Plasmid constructs**

Plasmids for stable transfection were based on pHD 324 (17), a bicistronic construct containing CAT (with an ACT 3′-UTR) and hygromycin resistance (HYG) genes. Different 3′-UTRs or mutated forms of them were cloned downstream of CAT; pHD 390 (Fig. 1; 17) contains the PARPα 3′-UTR. The PARPβ 3′-UTR (Fig. 2; pHD 440) was obtained by PCR with genomic DNA from procyclic Antat 1 cells as template.

Mutations in the PARP 3′-UTRs were made either by exonuclease III deletion or PCR mutagenesis, followed by cutting and pasting of the resulting mutant fragments. Complete details, together with reconstructed sequences, are available from C. Clayton on request. Trans splicing activity was tested using plasmids derived from pHD 260 (9) with various PARP 3′-UTR fragments replacing the ACT 5′-UTR upstream of CAT.

**RNA analysis**

Total RNA was isolated using TRIzol reagent (Gibco Life Technologies Inc., Eggenstein). RNA from 3.3 × 10^6 (procyclic forms) or 1–5 × 10^7 (bloodstream form) cells was separated on formaldehyde gels and blotted onto Hybond N+ membrane (Amersham, Braunschweig). In a few experiments mRNA was detected using enhanced chemiluminescence (ECL, Amersham). The exposed autoradiograms (30 s–5 h) were scanned (Agfa ARCUS II) and quantitated using NIH Image 1.52. In the other studies, including all turnover experiments, 32P-labelled probes were used and bands quantitated using a phosphorimager (Molecular Dynamics). When compared, chemiluminescence and radioactive probing yielded quantitatively similar results.

For RNase protection analysis (30), a CAT cassette cloned into pBluscript II SK+ (Stratagene) was cleaved with NcoI and

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Figure 1. Map of a PARP B locus (A), pHD 390 (B), pHD 614 (C) and the corresponding mature mRNA products. Symbols are shown in (D). (A) The PARP promoter is shown at the extreme left and successive PARP open reading frames by dark stippled boxes. Polypyrimidine tracts that can, when placed upstream of an open reading frame, act as trans splicing signals, are shown as filled black blocks. Positions of trans splicing and the corresponding polyadenylation sites (AA) are indicated by the curved arrows. A hypothetical polyadenylation site is marked as A?. The polypyrimidine tract 26 is involved in developmental regulation and tract 5 specifies polyadenylation of the PARPα mRNA. The lollipop represents the translation-enhancing stem–loop (16mer) (34). (B) pHD 390 is shown linearized and integrated into the tubulin locus. The plasmid sequence (thick line) is not to scale. The locations of 3′- and 5′-UTRs and RNA processing signals are indicated. (C) pHD 614 and its transcripts.
transcribed in the presence of [32P]UTP to yield a 290 base product, which contained 56 bases that were not present in the mature CAT mRNAs. After RNase digestion the 234 base protected product was detected on sequencing gels, with appropriate controls.

The relationship between RNA half-lives and abundance were calculated using the formula:

\[ v = \log \left( \frac{2(1/t_G + 1/t_D)}{\text{[RNA]}} \right) \]

where \( t_G \) is the measured half-life in non-growing cells and \( t_D \) is the mean division time of a growing population (8 h in bloodstream forms, 12 h in procyclics) (31). We assumed that the rate of degradation (v) of an mRNA equals its rate of synthesis.

Sequence alignments were done using align/align (32) and RNA folding assessed by MFOLD (33). Polyadenylation and splicing sites were determined by RT–PCR (9).

RESULTS

Sequence comparison of PARP 3'-UTRs

Trypanosoma brucei contains 8–12 PARP genes, arranged in direct tandem repeats containing two to three genes each (5). Figure 1A shows a simplified map of a PARP B locus with the corresponding mRNAs immediately below (PARP A loci are similar). Each locus bears an upstream (a) and a downstream (b) gene. The genes differ somewhat in the coding region but diverge in the 3'-UTRs. The untranslated regions, including both the intergenic regions and the 3'-UTRs of the mature mRNAs, contain several polypyrimidine tracts. Some of these direct splicing of mature PARP mRNAs or of unstable, non-coding RNA intermediates. Others can also be shown to direct splicing if they are placed 5' of a CAT open reading frame (9).

Poly(A) splicing tracts with known trans splicing activity are shown as filled boxes in Figure 1. Known linked upstream polyadenylation sites are also indicated.

An alignment of all PARP 3'-UTRs sequences (e.g. PARP Bα and PARP Bβ, Fig. 2) reveals two conserved regions: a 16mer that is predicted to form a stem–loop and stimulates translation in procycic forms (34) and a 26mer polypyrimidine tract ("26" in Figs 1–5). Secondary structure predictions for all available PARP 3'-UTR sequences (not shown) revealed no common features apart from the 16mer stem–loop; the 26mer poly(U) were not predicted to be involved in base pairing.

The PARP 3'-UTR mediates developmental regulation of mRNA levels and translation

To investigate the regulatory function of PARP 3'-UTRs, we used a vector designed to integrate into the tubulin locus (17; Fig. 1B). Downstream of a fragment from the tubulin locus (to enable targeted integration) is an rRNA promoter, which is not developmentally regulated (22). Next is an ACT 5'-UTR and splicing signal, followed by a CAT gene. The 3'-UTR downstream of CAT can be exchanged using unique restriction sites; it is from the PARP Bα locus in pHD 390 (Fig. 1B). Beyond this 3'-UTR is a second 5' trans splicing signal and 5'-UTR from the ACT locus (17), then a hygromycin resistance (HYG) cassette. Unless otherwise noted, the plasmid was linearized, transfected into trypanosomes and hygromycin-resistant populations studied. Such populations are derived from at least 20 individual transformants containing one to three copies of the construct (17,22); their use obviates the need to perform independent copy number determinations for each plasmid tested. Transcription of CAT was probably mediated by RNA polymerase II reading through from upstream (17).

CAT mRNA with an ACT 3'-UTR (CAT–ACT 3', pHD 324) shows no developmental regulation (17). Results for the CAT–ACT 3' plasmid were therefore used as a control (100% value) throughout this study. Using the PARPα 3'-UTR, CAT mRNA and CAT protein levels were similar in procyclic forms to those seen with the ACT 3'-UTR; with the PARPβ 3'-UTR CAT activity in procyclic forms was 2-fold less (not shown). In bloodstream forms (Fig. 3) CAT activity was suppressed \( \sim 100\)-fold and CAT mRNA levels \( \sim 10\)-fold by the PARP 3'-UTRs. The discrepancy between the levels of CAT–PARP 3' mRNA and the corresponding proteins indicates that the PARP 3'-UTRs not only reduce mRNA levels, but also adversely affect translation in bloodstream forms. Differentiation of bloodstream trypanosomes containing the CAT–PARP 3' transgene into procyclic forms resulted in \( \sim 100\)-fold up-regulation of CAT activity (to about half the final, procyclic level) within 48 h (35).

To check polyadenylation, CAT–PARP 3' transcripts (from pHD 390) from procyclic forms or CAT–PARPΔ26 3' (see below) transcripts from bloodstream forms were analysed by RT–PCR (9). In procyclics polyadenylation occurred either at the wild-type site or 12 bases upstream (two clones each). In bloodstream forms polyadenylation was at the wild-type site, except that one out of eight clones had poly(A) at position 173 (position 'g' in ref. 9).

Delineation of the sequences required for regulation

Deletion mutagenesis was used to localize sequences in the PARP 3'-UTRs that reduce bloodstream-form expression. Progressive 5' deletions of the PARPα 3'-UTR up to position 139 (Fig. 3A) had marginal effects on CAT activity, but somewhat elevated CAT
Figure 3. Deletion analysis of PARP 3'UTRs: deletion from the 5' side. Maps are of the PARPα (A) and β (B) 3'UTRs. Arrow, wild-type polyadenylation site. Black lines indicate the sequence retained in each construct and the extent of deletions is indicated on the left. CAT activities for stably transformed bloodstream forms (mean ± SD of at least three independent transfections) are indicated as a percentage of the values obtained with pHD324 (CAT–ACT 3'UTR). CAT assays done with cell extracts from hygR cell populations were corrected according to the protein concentration. Quantitation of RNA, expressed as a percentage of the CAT–ACT 3' control, from the blot (C) or from several experiments (for PARPα) are listed similarly. (C) Total RNA from 10^7 recombinant bloodstream cells, containing the deletion constructs indicated, was analysed by blot hybridization using a CAT probe. Hybridization with an ACT probe was done to standardize the amount of RNA. The extra, faster-migrating band in the ACT 3'UTR lane, hybridized with the ACT probe, was due to re-detection of the CAT–ACT 3' mRNA.

mRNA levels. Deletion 1–158 was the first to include the entire 26mer; suddenly CAT activity rose >100-fold to 42% of the ACT 3'UTR control. The level of CAT mRNA from this mutant was about half that seen from the control. Further deletion (Δ1–197) yielded an additional 1.5–2-fold increase in CAT activity. Results for the PARPβ 3'UTR were quite similar (Fig. 3B); 5' deletions
that did not include the 26mer had little effect; as soon as the 26mer had gone (Δ1–159) CAT rose to 85%. Further deletions of the PARPα 3′-UTR yielded only marginal increases in RNA or protein (Fig. 3B).

These results suggested that the 26mer (134–159) was very important for regulation, although, for the PARPB 3′-UTR at least, the region immediately downstream (159–197) also played a role. Further deletions confirmed this interpretation. First we deleted 36 bases, including the 26mer, from the PARPα 3′-UTR (Fig. 4, Δ123–158). This restored 45% CAT activity, with a corresponding increase in CAT mRNA. Internal deletion of the downstream sequence (Δ159–197) independently increased CAT activity (to 7%) and CAT RNA (~2-fold) (Fig. 4). When both deletions were combined (Δ123–197) CAT was 69%. A mutant (Δ26/16) which has both the 26mer and the stem–loop deleted (Fig. 4, Δ134–159, Δ203–218) but retains bases 160–203 produced less CAT mRNA and CAT protein than the Δ123–235 mutant (Fig. 4), confirming that bases 160–203 reduce expression. None of the other sequences examined appeared important for down-regulation in bloodstream forms (Figs 3 and 4). The function of the 26mer was not dependent on its distance from the coding region (Fig. 3, Δ1–122 and data not shown) nor on the distance from the polyadenylation site (Fig. 4, Δ159–197 and Δ198–235).

The plasmids bearing deletions Δ134–159 and Δ123–158 (Δ26) were compared with the parent PARPα 3′-UTR plasmid by transient transfection assays in procyclic forms. No significant differences in CAT expression were found (not shown).

Precise deletion of the 26mer from the PARPB 3′-UTR resulted in 10% CAT expression and 50% RNA in bloodstream forms, relative to the ACT 3′-UTR control (not shown). Thus, here too, the 26mer was important for down-regulation, but other sequences must contribute.

The context of the 26mer is important

To examine the behaviour of the 26mer in more detail, we first replaced the 26mer of the PARPα 3′-UTR with a BgII site. Expression of CAT in bloodstream trypanosomes with this plasmid, yielding CAT–PARPΔ26 3′ RNA, was 34% of the ACT control (Δ26, Fig. 4). Returning the 26mer element to its original location (in the artificial BgII site) restored down-regulation if the 26mer was in the correct orientation (Δ26+26, Fig. 4). When the 26mer was placed immediately downstream of the CAT cassette, either upstream of PARPΔ26 3′ (Δ26+p26, Fig. 4) or upstream of ACT 3′, it did not suppress expression in bloodstream forms (ACT +p26, Fig. 4). When a larger segment of the PARPα 3′-UTR (117–203) was inserted between CAT and the ACT 3′-UTR, CAT activity was reduced 5-fold (ACT +p87, Fig. 4). Insertion of the inverted (antisense) 26mer had only minor effects (Δ26 +r26, Fig. 4 and data not shown).

In all constructs tested so far, polyadenylation of CAT mRNAs was directed by an ACT 5′ splicing sequence downstream (Fig. 1). To restore the PARPα 3′-UTR to its ‘natural’ context, we placed the complete PARP intergenic region (including the 3′-UTR) between the CAT and HYG cassettes. An equivalent construct with the Δ26 deletion was also tested. This change in the region downstream of the 3′-UTRs had no influence on the level of CAT expressed (not shown).

The conserved 16mer stem–loop has been shown to enhance translation in procyclic forms without influencing mRNA levels (34). Similar results were obtained for bloodstream forms: deletion of the 16mer from the Δ26 construct (Δ26/16) reduced CAT 3-fold, but the RNA level was unaffected (Fig. 4).

The 26mer can function as a trans splicing signal

To see if the PARPα and PARPB 26mers can act as trans splicing signals, we transferred appropriate segments of the 3′-UTRs (125–203 and 127–206 respectively, Fig. 2) to a 5′-UTR position, upstream of a CAT gene, and measured CAT activity after transient transfection. In the absence of a functional trans splicing signal, no CAT is produced (9,12). Both fragments were capable of directing CAT production in bloodstream and procyclic trypanosomes as efficiently as the ACT splice signal.

We next deleted from pHDI 390 the segment from position 197, downstream of the 26mer in the PARPα 3′-UTR (Fig. 2) to the
beginning of the HYG gene (Fig. 1C, pH 614). This forced use of the 26mer as a trans splicing signal for the HYG mRNA (Fig. 1C). Drug-resistant bloodstream trypanosomes were easily obtained. The HYG mRNA was spliced at the AG at position 197. Polyadenylation was 74 or 57 bases upstream of the 26mer and yielded CAT RNA and CAT activities similar to those from the ACT 3′-UTR control (not shown). Thus the 26mer must be within the mature 3′-UTR to down-regulate expression in bloodstream forms.

Replacement mutagenesis

We now mutated individual bases within the 26mer. The resulting CAT and CAT mRNA levels are shown in Figure 5. In this series of experiments PARPΔ26 yielded CAT activity (black bars) and CAT mRNA (grey bars) of ~25% of the ACT 3′ control. All mutations of the 26mer, even those with only three transitions, affected regulation. CAT activity increased to at least 8% and, with one exception, RNA levels were doubled, attaining values similar to those from the Δ26 construct (Fig. 5). Mutant I and III 3′-UTRs yielded similar CAT activities to, but more RNA than, the mutant VI 3′-UTR. Mutant V has the sequence of the polypyrimidine tract that lies downstream of the PARPα polyadenylation site (9; see Fig. 1). This sequence is an active splice signal and mediates accurate PARPα polyadenylation (9), but is not as effective as the 26mer in reducing CAT expression and mRNA. The most interesting result was for mutant VI. This 3′-UTR appeared to have specifically lost the ability to down-regulate translation; the RNA level was reproducibly the same as for the wild-type PARPα 3′-UTR, but CAT expression was at least 10-fold higher.

mRNA turnover

Post-transcriptional regulation often operates at the level of RNA stability. We therefore compared turnover of selected mRNAs in cloned transgenic bloodstream and procyclic trypanosomes (see legends to Figs 6 and 7). RNA synthesis was inhibited using chloroquine (36) or actinomycin D (18) and the amounts of RNA assessed by blot hybridization or RNase protection. In all plots the negative time points show cells that were not drug treated and the zero time point represents cells that received drug 0–5 min prior to centrifugation (total time in the presence of drug 10–15 min). In procyclic forms the half-lives of CAT–ACT 3′, CAT–PARPΔ26 3′ or CAT–PARP 3′ mRNAs were similar (not shown). RNAs with PARP 3′-UTRs were consistently slightly more stable than those with ACT 3′-UTRs, but the difference was not statistically significant. There was no difference between (−) and t = 0 samples.

All mRNAs tested were much less stable in bloodstream forms, so that the time taken to process the cells after drug treatment was significant relative to the half-life. A Northern blot demonstrating
Northern blots and quantitations are shown in Figure 7. In all of the spliced leader RNA (38). (We are grateful to Dr E. Ull, RNA maturation using Sinefungin, which prevents methylation in this and every other experiment the control degradation after actinomycin D treatment is shown in Figure 6B. In this and every other experiment the control ACT mRNA and the CAT–ACT 3′ and CAT–PARPΔ26 3′ mRNAs increased immediately after drug addition, while PARP and CAT–PARP 3′ mRNA levels fell ~2-fold. Results for several independent experiments were combined for quantitation (Fig. 6C–E). ACT mRNA (Fig. 6C) had a half-life of 21 ± 12 min (mean ± SD, measured starting from the t = 0 time point) and the behaviour of the CAT–ACT 3′ and CAT–PARPΔ26 3′ transcripts was similar (Fig. 7D). In contrast, samples without drug contained twice as much PARP mRNA as samples in which drug was added just before centrifugation (Fig. 7B). From time point 0 onwards a low amount of PARP mRNA persisted; no accurate measurements of the turnover of this residual PARP RNA were possible because of its low abundance. 

To facilitate detection of the CAT–PARP 3′ mRNA we used RNase protection, with a probe from the 3′–end of the CAT gene. Results for the CAT–ACT 3′ mRNA were similar to those obtained by Northern blot (compare Fig. 6D and E). The CAT–PARP 3′ mRNA, like the PARP mRNA, declined precipitously immediately after drug addition (compare Fig. 7C and E). The results for CAT–ACT 3′ mRNA, CAT–PARP 3′ mRNA and CAT–PARPΔ26 3′ mRNA were confirmed using a probe from the 5′–end of the CAT gene (35).

It was possible that the biphasic kinetics of CAT–PARP mRNA decay was an artifact of the use of actinomycin D or chloroquine to inhibit transcription. Pulse-chase analysis with [3H]adenine cannot be used because the added nucleotide takes 1 h to equilibrate with internal pools (37). As an alternative, we inhibited RNA maturation using Sinefungin, which prevents methylation of the spliced leader RNA (38). (We are grateful to Dr E. Ull, Yale University, New Haven, CT, for suggesting this approach.) Northern blots and quantitations are shown in Figure 7.

In summary, in bloodstream forms most PARP and CAT–PARP 3′ mRNA is destroyed immediately after it is formed, in a process dependent on the 26mer.

**DISCUSSION**

Sequence requirements for regulation

We have shown that two PARP 3′-UTRs mediate >10-fold down-regulation of CAT mRNA levels and >100-fold down-regulation of CAT activity in bloodstream trypanosomes. The 10-fold post-transcriptional regulation of mRNA levels is consistent with the 100-fold PARP mRNA regulation observed in these trypanosomes (22), as PARP transcription is 5–10-fold less active in bloodstream
forms than in procyclics (22). A conserved polypyrimidine tract (26mer) is important in this regulation. The function of the 26mer did not depend on its distance from the coding region or from the polyadenylation site, but it had to be present in the mature mRNA, as transcripts polyadenylated 5′ of the 26mer were abundant and well-translated in bloodstream forms. Interactions with other sequences in the 3′-UTR were not essential, but in the PARPα 3′-UTR additional regulatory elements were present immediately downstream. The ability of the 26mer to suppress expression in bloodstream forms was dependent on context. It could not repress expression when placed at the 5′-ends of the ACT 3′ or PARPα26 3′-UTRs. Perhaps, when the 26mer is moved from its normal location its function is overridden by other sequences that either exert a positive influence on the RNA level or form secondary structures that preclude 26mer function.

Although the 26mer is U-rich, it is poorly homologous to the UA-rich nonamer that is implicated in RNA decay in mammalian cells (39,40). Trypanosomes diverged so early in eukaryotic evolution that the vague resemblance between the two sequences is most likely fortuitous. Also, the first six bases (UAUAUA) of the 26mer were not required for repression (Δ1–139, Fig. 3). In contrast, all mutations of the two pyrimidine tracts within the 26mer affected activity. As few as three base changes in these portions were sufficient to increase CAT expression and CAT mRNA to levels approaching, or equivalent to, those seen when the entire 26mer was deleted (Fig. 5). Polypyrimidine tracts are common in trypanosome 3′-UTRs, whether or not the mRNAs are abundant in bloodstream forms, so recognition of this particular one by regulatory mechanisms must be very sequence specific.

mRNA is less stable in bloodstream forms than in procyclic forms

Ehlers et al. (41) measured RNA turnover in trypanosomes by pulse-chase labelling with [3H]adenine. They found that total poly(A)+ RNA turned over faster in bloodstream forms than in procyclics. The estimated half-life of tubulin mRNA, which shows little developmental regulation, was 1.4 h in bloodstream forms and 7 h in procyclic forms (41). These values were probably artifactually long due to the large ATP pool (36,37). Using RNA synthesis inhibitors, we found that the half-life of ACT mRNA was 20 min in bloodstream forms, but 90 min in procyclics.

The levels of ACT mRNA, as a proportion of total mRNA, are approximately the same in bloodstream forms and procyclic forms (42). How can this be when the degradation rates differ nearly 5-fold? Firstly, we routinely obtain two to three times less RNA from bloodstream trypanosomes than from procyclic forms. Secondly, bloodstream forms grow at 37°C and procyclics at 27°C; perhaps everything: transcription, processing and degradation, is faster at the higher temperature.

The 26mer influences mRNA turnover

Previous reports have indicated regulation of trypanosomes mRNAs through accelerated or delayed turnover. Experiments in which V3G 5′- and 3′-UTRs were linked to a CAT gene yielded results suggesting that these sequences are capable of reducing mRNA levels in procyclic forms (18,43), with some effects on RNA stability (18). Regulation of gene A2 mRNA in *Leishmania donovani* was also shown to be mediated by elements in the 3′-UTR that affected RNA stability (44), the data being consistent with biphasic mRNA decay kinetics.

Our results indicate that accelerated turnover contributes to the low abundance of PARP or CAT–PARP 3′ mRNAs in bloodstream forms. After inhibition of mRNA synthesis, most CAT–PARP 3′ mRNA was degraded within 10 min. The CAT–PARP26 3′ mRNA, in contrast, behaved like CAT–ACT 3′ mRNA, indicating that the 26mer was required for rapid degradation. After 10–15 min the rates of degradation of all these RNAs were indistinguishable. The biphasic kinetics of PARP and CAT–PARP 3′ mRNA degradation suggest that there are two RNA subpopulations. Perhaps most of the newly synthesized RNA is degraded in the nucleus, while the portion that escapes into the cytosol is more stable? So far our attempts to test this hypothesis have been frustrated by an inability to detect CAT–PARP 3′ mRNAs after cell fractionation. In one experiment (Fig. 7) a possible degradation intermediate of CAT–PARP 3′ mRNA was seen. We do not know the structure of this band. All mRNAs investigated showed a gradual decrease in size after inhibition of RNA synthesis. This would be consistent with shortening of the poly(A) tail prior to degradation, as is seen in other eukaryotes (45,46).

**Regulation of mRNA processing or export?**

In mammalian cells and yeast binding of splicing factors to unprocessed RNAs can inhibit their export from the nucleus, leading to rapid degradation (47,48). It therefore seemed possible that the U-rich 26mer was affecting RNA abundance by this mechanism. When a segment including both the 26mer and the downstream sequence up to the next AG dinucleotide was placed upstream of an open reading frame (CAT or HYG), *trans* splicing activity was indeed detected. However, the *trans* splicing ability was not developmentally regulated and preliminary mutation results (49) have yielded no evidence so far for any correlation between *trans* splicing potentiality and the ability to reduce mRNA levels.

**Translation**

Bloodstream forms contain some mature PARP mRNA, but PARP is undetectable because the 26mer represses translation. Surveying the results from all mutants, an increase in CAT activity to a level significantly above background (2% and more) usually correlated with a rise in the amount of RNA to >20%; RNA levels of >35% yielded CAT activities >25%. One mutation of the 26mer abolished translational repression without affecting the level of mRNA (Fig. 5), suggesting involvement of separate factors (or separate specificities within one factor) that interact with the 26mer. To further investigate post-transcriptional regulation of PARP expression it will be necessary to identify and characterize these factors.

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