A Negative Regulatory Element Controls mRNA Abundance of the Leishmania mexicana Paraflagellar Rod Gene PFR2†

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The Leishmania mexicana PFR2 locus encodes a component of the paraflagellar rod (PFR), a flagellar structure found only in the insect stage of the life cycle. PFR2 mRNA levels are 10-fold lower in the mammalian stage than in the insect stage. Nuclear run-on experiments indicate that the change in PFR2 mRNA abundance is achieved posttranscriptionally. Deletion and block substitution analysis of the entire 1,400-nucleotide 3′ untranslated region (UTR) of PFR2 led to the identification of a regulatory element contained within 10 nucleotides of the 3′ UTR, termed the PFR regulatory element (PRE), that is necessary for the 10-fold regulation of PFR2 mRNA levels. Comparison of the half-lives of PFR2 transcripts, identical except for the presence or absence of the PRE, revealed that the PRE acts by destabilizing the PFR2 mRNA in amastigotes. The PRE was inserted into a construct which directs the constitutive expression of a chimeric PFR2 transcript. Insertion of the PRE resulted in regulated expression of this transcript, demonstrating that the regulatory element is sufficient for promastigote-specific expression. Since the PRE is present in the 3′ UTR of all L. mexicana PFR genes examined so far, we propose that it serves a means of coordinating expression of PFR genes.

In tropical areas where phlebotomine sand fly vectors are endemic, protozoan parasites of the genus Leishmania cause widespread human disease. As Leishmania parasites cycle between the insect vector and the mammalian host, they differentiate into morphologically and biochemically distinct stages that are adapted for survival in the distinct environment of each host. Insect-stage promastigotes can be readily distinguished from mammalian-stage amastigotes by the presence of a long flagellum emerging anteriorly. The flagellum is the motility organelle of promastigotes and infectious metacyclic promastigotes (1, 26). Only a rudimentary nonemergent flagellum is present in amastigotes (25). The flagellum therefore affords a unique opportunity to understand stage-specific regulatory mechanisms employed by Leishmania.

Trypanosomatid protozoans such as Leishmania have evolved cellular pathways that are fundamentally different from those of organisms that have been studied more extensively, such as bacteria, yeasts, and mammals (27). In trypanosomes, mature mRNAs are formed by processing of polycistrionic pre-mRNAs. This occurs by trans splicing of a capped 39-nucleotide (nt) miniexon near the 5′ end of the coding sequence (28). Polycistrionic transcription units often contain mRNAs whose steady-state levels are vastly different or mRNAs that accumulate at different stages of the life cycle. Coupled with a failure to identify RNA polymerase II promoters associated with Leishmania genes, this has led to a paradigm in which posttranscriptional mechanisms for regulation of gene expression predominate (29). The Leishmania mexicana genes PFR1 and PFR2, which encode the major structural components of the paraflagellar rod (PFR), conform to this posttranscriptional regulation paradigm.

The PFR, restricted to the flagella of kinetoplastids, euglenoids, and some dinoflagellates, is a massive cytoskeletal structure that runs the length of the flagellum next to the axoneme once it emerges from the flagellar pocket (32). The PFR is essential for flagellar motility in Leishmania promastigotes; however, it is absent from the attenuated flagellum of amastigotes (1, 26). Two major protein components of the PFR have been identified in many trypanosomatid species and are referred to here as PFR1 and PFR2. The genetic loci share a common organization, being composed of tandem arrays of four and three genes, respectively (23). Steady-state mRNA levels of PFR1 and PFR2 are about 10-fold greater in promastigotes, which possess a PFR, than in amastigotes, which lack a PFR. Genes flanking the PFR1 and PFR2 arrays do not display this regulation, which suggests either the presence of specialized regulated promoters for the PFR genes or a posttranscriptional means of regulation (23).

Unlike the regulation of gene expression in most prokaryotic and eukaryotic organisms, which occurs primarily at the level of transcription, gene regulation in trypanosomes is largely posttranscriptional, occurring at the level of trans splicing, polyadenylation, mRNA stability, translation, and protein stability (12, 29, 30). However, transcriptional regulation in trypanosomes has been observed in only a few cases of specialized polymerase I promoters, such as in the genes that encode variable surface glycoproteins and procyclin acidic repetitive proteins (13). In contrast to the dearth of evidence for transcriptional control in Leishmania, there are many examples of trypanosomatid genes whose mRNA levels are modulated by posttranscriptional mechanisms. Sequences within the 3′ untranslated region (UTR) (2, 4, 10, 14, 15, 33), the 5′ UTR (21),
or the coding sequence of an mRNA (33) can contribute to the regulation of a particular mRNA’s abundance.

Sequences outside the mature mRNA can also control differential expression of *Leishmania* genes. For example, Brooks et al. have shown that stage-regulated differential gene expression of the cytosine protease gene array is dependent upon the presence or absence of short sequence elements in the respective intercistronic region and that these sequence elements influence processing of precursor mRNA (3). These regulatory sequences presumably influence events in the maturation of mRNA such as trans splicing.

The expression of many proto-oncogenes, cytokines, and lymphokines in higher eukaryotes is controlled at the level of mRNA stability. AU-rich elements (AREs), present in the 3' UTRs of these mRNAs, control the decay rates of these transcripts by modulating poly(A)-deadenylation rates and subsequent decay of the mRNA (5, 22). Recently, a yeast transcript, TIEF151A, was shown to be subject to regulation by an ARE present in its 3' UTR. Both yeast and mammalian AREs promoted deadenylation-dependent mRNA decay in the yeast system, suggesting conservation of this decay process from yeasts to mammals (31). In trypanosomatids, a specific ARE has been shown to control the stage-regulated expression of the *Trypanosoma cruzi* mucin gene family (8). This suggests that AREs might be involved in regulation of other trypanosomatid gene families.

We report here that expression of the *L. mexicana* PFR2 genes is regulated posttranscriptionally by modulation of mRNA decay rates, and we have identified a 10-nucleotide (nt) AU-rich regulatory element in the 3' UTR of the *PFR2C* gene that is both necessary and sufficient for the stage-specific regulation of *PFR2* mRNA.

**MATERIALS AND METHODS**

**Parasites and cell culture.** Promastigotes of *L. mexicana* (WHO strain MNYC/BZ/62/m379) were cultured in M199 medium containing 5% (vol/vol) fetal bovine serum and 5% (vol/vol) bovine embroyonic fluid at 26°C as described previously (18). All amastigotes of *L. mexicana* used in this study were from axenic cultures and were obtained by shifting the incubation conditions of promastigotes to 33°C and pH 5.5 in a modified UM 54 medium as described previously (23). Amastigote cultures were maintained by serial dilutions (1:25) every 3 to 4 days. Axenic amastigotes were harvested at least 5 days after initial differentiation (23).

**Transfection.** Methods for electroporation of DNA into *Leishmania* and selection of transformants were described previously (18). Puromycin was the selective drug for all experiments described in this work and was maintained at a selective concentration of 10 μg/ml. Puromycin-resistant transformants were isolated by plating 106 to 107 nuclei and were assayed for regulation.

**In vitro nuclear run-on assay.** Promastigotes and amastigotes were grown to mid-log phase (5 × 107 to 1 × 108 parasites/ml) before nuclei were isolated. Intact nuclei were isolated from approximately 2 × 107 cells or about 100 to 200 ml of culture. The parasites were centrifuged at 1,000 g in a tabletop centrifuge and washed once in cold PS buffer (10 mM Na2HPO4 and 3 mM KH2PO4, at pH 8.0) (7). The pellet was resuspended in 1 ml of cold PS to a concentration of 2 × 107 parasites/ml and placed on ice. NP-40 was added to a final concentration of 0.8%. Lysis of the parasite plasma membrane but not the nuclear membrane was verified by phase-contrast microscopy.

The nuclei were pelleted and then resuspended in the nuclear membrane buffer (10 mM Hepes [pH 7.5], 50 mM NaCl, 50 mM KCl, 2 mM MgCl2, 5 μM diethylthiotreitol, 0.5 mM spermine, 1 mM spermidine, 1 mM putrescine, 20% glycerol, 1 mM ATP, 1 mM GTP, 1 mM UTP, 0.1 mM creatine phosphate, 10 μM of creatine kinase, 160 U of RNasin, 500 μCi of [α-32P]CTP at 800 Ci/mmol) and incubation at 30°C. After 10 min the reaction was stopped by the addition of 20 μl of DNease I followed by 200 μl of 20 mM Tris (pH 7.5), 20 mM EDTA, 1 mg of proteinase K per ml, and 1% SDS. The samples were incubated for 10 min at 37°C in the presence of 20 U of RNase I and DNease I. The mixture was phenolized in a 1:1 ratio and DNA was extracted with chloroform. Prehybridization of membranes and washing were as previously described (19). Membranes were hybridized to 32P-labeled DNA from plasmids pFR2C and pFR2B, which are described below.

**Plasmids.** Yeast plasmids, except for pLuc, were constructed in the pX63PAC backbone, a *Leishmania* expression vector which confers resistance to puromycin (18). Plasmid pLuc contains a segment of about 23 kb of *L. mexicana* genomic DNA that spans the PFR2 locus from a unique Dral site in the PFR2 start codon to an Asp718 site about 7 kb upstream of the PFR2A start codon (see Fig. 2), housed in pBluescript II SK+ (Stratagene), by performing the hybridization with equal molar amounts of each plasmid and subtracting the value obtained by hybridization to pBluescript II SK+ (Stratagene).

**Nuclear run-on assay.** After subtraction, the phosphorylase values were divided by the size of the insert for each clone to arrive at a value representing transcription per kilobase. Transcription across the PFR2 coding sequences was normalized to take into account the presence of three gene copies. To compare results from different experiments, all values were normalized to those for transcription of β-tubulin, whose steady-state mRNA levels are expressed constitutively (16). Plasmids p2.B1, p2.5S, p2.a2, p2.10X, and p1.9X, which contain subcloned portions of the PFR2 locus, have been described (23). Plasmid pLT-P1β, which contains β-tubulin sequences, is described below.

**pLT-P1β construction.** All the deleted plasmids, except for pLuc, were constructed in the pX63PAC backbone, a *Leishmania* expression vector which confers resistance to puromycin (18). Plasmid pLuc contains a segment of about 23 kb of *L. mexicana* genomic DNA that spans the PFR2 locus from a Clal site 7 kb downstream of the PFR2C stop codon to an Asp718 site about 7 kb upstream of the PFR2A start codon (see Fig. 2). In vitro transcription of the PFR2C stop codon to an Asp718 site about 7 kb upstream of the PFR2A start codon, housed in pBluescript II SK+. Plasmid pNC2C contains a 10.5-kb segment of the *L. mexicana* PFR2 locus, extending from a Narl site 6 bp downstream of the PFR2B stop codon to a Clal site about 7 kb downstream of the PFR2C stop codon, cloned between the HindII and Clal sites of pBluescript II SK+. The Narl 5' overhang was filled in with the Klenow fragment of DNA polymerase I to enable blunt end ligation to the HindII site in pBluescript II SK+. Plasmid pFRC2C was created by excising the 10.5-kb *Leishmania* DNA fragment of pNZC2 with *polynick* Asp718 and EcoRV sites, filling the 5' overhang of the Asp718 site with Klenow, and blunt-end ligating it to BamHI-cut and Klenow-treated pX63PAC. The PFR2C and PAC genes were in the same relative orientation in pFRC2C (see Fig. 2). Plasmid p2.CW was made by excising a 5.5-kb *AhoI* fragment from pNC2C, filling in the termini with Klenow fragment, and blunt-end ligating it to BamHI-cut and Klenow-treated pX63PAC such that the PFR2C and PAC genes were in the same relative orientation (see Fig. 2). Plasmid p2.CCH is identical to p2.CCW except that a HindIII site was inserted at the 5' end of the PFR2C stop codon by a PCR-based mutagenesis strategy analogous to that described previously (26). Plasmid p2.C3A, a derivative of p2.CCW that lacks 3' sequences downstream of the HindIII site, was made by deleting a 2-kb HindIII-BglII fragment of p2.CCW, filling in the termini with Klenow fragment, and blunt-end ligating to pX63PAC. Plasmid p2.BW was made by inserting a 1.4-kb ClaI-SmaI fragment from...
plasmid p3.8Cla that contained PFR2B 3' flanking sequence into plasmid p2BΔ3' (23).

Plasmid p2CΔ1 was derived from p2CH by deletion of a 489-bp HindIII-ClaI fragment in the 3' UTR of PFR2C. Plasmid p2CΔ2 was derived from plasmid p2CΔ1 by deletion of a 595-bp ClaI-NruI fragment in the 3' UTR of PFR2C. Plasmid p2CΔ3 was derived from p2WT by deletion of a 305-bp NruI-NotI fragment at position 1089 and an NruI site at position 1146. Plasmid p2CΔ3.2 is a derivative of p2CW with sequences deleted between NruI sites at positions 1130 and 1146. Plasmid p2CΔ3.3 is a derivative of p2CW with sequences deleted between an NruI site at position 1130 and an ApaI site at position 1245 (see Fig. 5). Plasmid p2CΔ3.4 is a derivative of p2CW with sequences deleted between an ApaI site at position 1245 and a BsmI site at position 1391 (see Fig. 5). Each of these four deletions was verified by sequencing.

To facilitate block substitution mutagenesis in the PFR2C 3' UTR, a 2.4 kb HindIII-ApaI fragment from plasmid p7NEO (26) was ligated into HindIII and Asp718-digested pUC18, creating plasmid p3UTR. The inserted fragment encompasses the PFR2C 3' UTR from a HindIII site just downstream from the PFR2 stop codon to a XhoI site further downstream. Block substitutions were generated in this plasmid by excising the 59-bp NruI-NotI fragment and replacing it with pairs of annealed oligonucleotides that spanned the region but contained the sequence AGCGGGCGCT, which contains a NotI cleavage site, in place of the corresponding fragment of p2CWT. Plasmid p1.5Δ1 was a derivative of p2CW with sequences deleted between an NruI site at position 1130 and an ApaI site at position 1245 (see Fig. 5).

Expression of L. mexicana PFR2 is posttranscriptionally regulated. PFR2 is encoded by a tandem array of three iden-

tical genes whose steady-state mRNA levels are about 10-fold higher in promastigotes than in amastigotes (23). Transcripts of two sizes are generated from these three genes, which differ in the size and sequence of their 3' UTRs. A 3.1-kb transcript arises from the first two genes of the PFR2 array, PFR2A and PFR2B, and a 3.8-kb transcript arises from the third gene in the array, PFR2C.

In order to determine whether the regulation of steady-state PFR2 mRNA levels was due to changes in the rate of mRNA synthesis, RNA polymerase density across the PFR2 locus was measured in an in vitro nuclear run-on assay (7). Plasmid clones spanning the PFR2 array extending through two adjacent upstream (U1 and U2) and downstream (D1 and D2) transcriptional elements were hybridized to 32P-labeled RNA synthesized in isolated nuclei from 107 promastigotes or amastigotes (23). Plasmid pL-β1, which contains the β-tubulin gene, was included for normalization among experiments. Table 1 summarizes the results of experiments with promastigotes and amas-
tigote nuclei. Transcription rates across the PFR2 locus were nearly constant, differing less than twofold in both promastigotes and amastigotes.

### TABLE 1. Relative rates of transcription across the PFR2 region

| DNA | Transcript | Transcription rate in: |
|-----|------------|------------------------|
|     |            | Promastigotes | Amastigotes | P/A |
| p2.5H | U2        | 6.6 ± 1.9     | 4.4 ± 1.8   | 1.5 |
| p1.5SM | U1        | 5.7 ± 0.7     | 4.8 ± 1.6   | 1.2 |
| pfla2 | PFR2      | 6.6 ± 0.7     | 4.9 ± 2.8   | 1.4 |
| p3.0X | D1        | 7.3 ± 2.0     | 6.4 ± 2.4   | 1.1 |
| p1.9X | D2        | 4.5 ± 1.6     | 4.3 ± 0.5   | 1.1 |

* In arbitrary units.

P/A, ratio of rate in promastigotes to rate in amastigotes.

### RESULTS

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This continuity of transcription is consistent with the model that the PFR2 locus is transcribed as a polycistronic precursor. The α-amanitin sensitivity profile of PFR2 transcription indicated that the PFR2 locus was transcribed by RNA polymerase II (data not shown). As there is a 10-fold difference in steady-state PFR2 mRNA levels, the differential expression of PFR2 mRNA in the two life cycle stages must be controlled by regulatory events that occur posttranscriptionally.

The half-life of PFR2 mRNA is shorter in amastigotes. The decay rates of PFR2 mRNA in promastigotes and amastigotes were determined by using the RNA polymerase inhibitor actinomycin D. Total RNA was isolated after exposure of promastigotes or amastigotes to actinomycin D for various times. The amount of PFR2 mRNA was then determined by Northern blot analysis and quantified on a phosphorimager. A plot of the quantity of promastigote PFR2 mRNA versus the time course of actinomycin D incubation revealed the expected exponential decay pattern with an observed half-life of approximately 3.9 h (Fig. 1). In contrast, the PFR2 mRNA was degraded more rapidly in amastigotes with an observed half-life of about 0.8 h. These results indicate that changes in the rate of decay of mature PFR2 mRNA can account for the large differences in steady-state PFR2 mRNA levels during the life cycle. Therefore, regulatory elements governing the observed changes in the rate of decay must reside within the mature mRNA sequence.
is 0.18 h

Promastigotes and amastigotes were treated with actinomycin D (10 μg/ml) for the time indicated. Amastigotes received additional actinomycin D at 1 h. The zero time point was taken just prior to the addition of actinomycin D. Five micrograms of total RNA from each time point was fractionated, blotted, and probed with in vitro-synthesized RNA from pPFR2 as described in the text. (A) Representative Northern blots for the promastigote and amastigote time courses. The signals for the pPFR2 and rRNA probes are displayed. (B) PFR transcript abundance was quantitated by using a phosphorimager and was normalized to the small subunit of rRNA to control for loading. For each time point, the normalized values for PFR2 mRNA ([R]P) were divided by the normalized time zero value ([R]0) to obtain a value for the fraction of PFR2 mRNA remaining ([R]t/[R]0) which was plotted against time, in hours. The plotted data were fitted to the exponential decay expression: [R]t/[R]0 = e⁻kt, where k is a rate constant, t is time in hours, and e is the inverse natural log. For the promastigote data, k is 0.18 h⁻¹; for the amastigotes, k is 0.86 h⁻¹. Values for the half-life were calculated by setting [R]t/[R]0 at 0.5 and solving for t. Each data point is the average for three independent experiments. Error bars show standard deviations.

In the course of these experiments, we found that amastigote PFR2 mRNA levels began to rise after 1 h of actinomycin D incubation. This phenomenon, which has been reported previously (4), suggests that actinomycin D might be unstable in amastigote media, since the effect could be eliminated by subsequent addition of more actinomycin D (Fig. 1).

Episomal expression of PFR2 genes recapitulates the wild-type regulation of mRNA levels. In order to map the cis elements responsible for PFR2 regulation, we took advantage of L. mexicana knockout lines in which PFR2 genes have been deleted by homologous replacement. Using these clean genetic backgrounds, we tested whether it was possible to assay regulation of transcripts arising from episomal constructs in which PFR2 genes are linked to endogenous 5’ and 3’ flanking sequences. In initial experiments, plasmids containing the entire PFR2 locus (pLocus) or the PFR2C gene with either 7 kb (pPFR2C) or 2 kb (p2CWT and p2CH) of 3’ flanking sequence were introduced into Δpfr2 promastigotes (Fig. 2). The resultant lines were converted into axenic amastigotes, and RNAs from both promastigote and amastigote forms were analyzed by Northern blotting using the PFR2 coding region as a probe. Figure 2 shows the results of Northern blots obtained with a representative clonal line harboring each plasmid; however, at least three clonal lines were assayed in each case. L. mexicana Δpfr2 promastigotes containing pLocus directed expression of the 3.8- and 3.1-kb PFR2 transcripts as expected. The steady-state level of PFR2 mRNA was about 10-fold higher in promastigotes containing pLocus than in amastigotes. The two transcripts displayed equivalent regulation. Wild-type L. mexicana displayed a 12-fold regulation of PFR2 mRNA in control experiments (Fig. 2B). Leishmania harboring plasmids pPFR2C, p2CWT, or p2CH displayed levels of PFR2C mRNA that were at least fivefold higher in promastigotes than in amastigotes. Although Δpfr2 L. mexicana harboring the described plasmids did not fully duplicate the level of regulation observed with wild-type L. mexicana, the 5- to 10-fold regulation achieved in these cell lines recapitulates the authentic regulation sufficiently to enable mapping of the regulatory elements involved in PFR2 regulation. Furthermore, the size of each mRNA was identical to that of the wild-type mRNA,
indicating that p2CWT and p2CH retained the native downstream splice site that specifies the site of 3’ polyadenylation.

3’ UTR sequences confer stage specificity to PFR2 mRNA accumulation. The 3’ UTR participates in the posttranscriptional regulation of many trypanosomatid stage-specific transcripts. In order to determine whether sequences within the PFR2 3’ UTRs were required for the stage specificity of PFR2 expression, the expression pattern of PFR2 plasmids lacking the 3’ UTR and flanking sequences was compared with that of PFR2 expression plasmids that had intact 3’ flanking sequences. Plasmid p2CΔ3’ is a derivative of p2CWT that lacks all 3’ sequence associated with PFR2C. Plasmid p2BΔ3’ is a derivative of p2BWT that lacks sequences downstream of a ClaI site 200 bp downstream of the stop codon of PFR2B. The Δpfr2 lines harboring these constructs direct the constitutive high levels of expression of a chimeric PFR2 transcript possessing a 3’ UTR derived from vector sequences due to the presence of an adventitious splice acceptor site within the vector (Fig. 3B). In contrast, plasmids p2CWT and p2BWT, which contain the endogenous 3’ flanking sequences of PFR2C and PFR2B, directed expression of appropriately regulated PFR2 transcripts whose steady-state levels were five- to eightfold higher in promastigotes than in amastigotes. These experiments indicate that 3’ sequences are necessary for the regulation of PFR2 mRNA. Since the half-life of the mature PFR2 mRNA is subject to stage-specific control, the above data strongly implicate sequences within the 3’ UTR of the mature PFR2 mRNA functioning as a regulatory element.

Identification of a cis regulatory element in the 3’ UTR. We chose to map the putative regulatory element in the PFR2C 3’ UTR. Initially, a series of three plasmids were derived from p2CWT. Plasmids p2CΔ1, p2CΔ2, and p2CΔ3 each lack a segment of the 1,400-nt PFR2C 3’ UTR but retain the intergenic region and endogenous downstream processing signal required for appropriate mRNA maturation (Fig. 4). Together, the three deletion constructs span the entire 3’ UTR. Northern analysis of L. mexicana Δpfr2 lines containing these 3’ UTR deletions revealed that lines harboring p2CΔ1 or p2CΔ2 retained the ability to produce an appropriately regulated PFR2 mRNA. In contrast, lines harboring p2CΔ3 failed to regulate PFR2 mRNA abundance (Fig. 4B). Instead, levels of PFR2 mRNA in amastigotes were elevated to the levels observed in promastigotes. This constitutive high-level expression of PFR2 mRNA indicates that the cis element required for regulation of PFR2C mRNA levels resides in region 3 of the PFR2C 3’ UTR. In each case, the size of the mRNA produced from the deletion constructs was in agreement with the predicted size, assuming that the wild-type polyadenylation site was used. Thus, the cis element does not appear to alter the site of polyadenylation.

To further map the regulatory sequences in region 3 of the 3’ UTR, four deletion constructs of p2CΔ3 were made based on unique restriction sites present in region 3. p2CΔ3.1 deletes a 60-bp NsiI-NaeI fragment, and p2CΔ3.2 deletes a 16-bp NaeI-NsiI fragment that is contained within the 60-bp deletion of p2CΔ3.1 (Fig. 5A). Plasmid p2CΔ3.3 removes a 115-bp NaeI-ApaI fragment and p2CΔ3.4 deletes a 146-bp ApaI-BsmI fragment of region 3 (Fig. 5A). Northern blot analysis of Δpfr2 lines containing these constructs (Fig. 5B) revealed that of the four constructs, only lines containing p2CΔ3.1 displayed a loss of regulation of PFR2 mRNA accumulation. This result indicates the presence of a regulatory element within the 60-nt region of the p2CΔ3.1 deletion. Furthermore, since p2CΔ3.2 overlaps 16 nt within the p2CΔ3.1 deletion but shows no loss of regulation, the regulatory element must be contained within a 44-nt segment of the p2CΔ3.1 deletion.

To further localize the regulatory sequence within the 3.1 region, a block substitution approach was used. Four con-
constructs, each containing a contiguous 10-bp replacement made sequentially across the 44-bp region defined by p2CΔ3.1, were built into the p2CWT background and designated p2BS1, p2BS2, p2BS3, and p2BS4 (Fig. 6A). These constructs were transfected into Δpfr2 promastigotes, and PFR2 mRNA abundance was quantitated as in previous experiments. Lines containing p2BS2 displayed constitutive expression of PFR2, while lines containing the three other block substitutions retained the ability to regulate expression of PFR2 mRNA (Fig. 6B). These experiments indicate that the 10-nt RNA sequence AU GUAUAGUU contains a regulatory element required for stage-specific regulation of PFR2 mRNA.

A 10-nt element is sufficient to confer promastigote-specific expression. To test whether the putative regulatory element was sufficient to generate regulation of mRNA accumulation, the 10-nt sequence defined by the BS2 block substitution was inserted into a construct which contained the PFR2 coding sequences fused to vector sequences. This construct, p2CΔ3', directs the expression of a transcript possessing a 3' UTR derived from vector sequence due to the presence of an adventitious splice acceptor site in the vector sequence. Within the vector-derived portion of the 3' UTR is a sequence containing p2BS2 displayed constitutive expression of PFR2, while lines containing the three other block substitutions retained the ability to regulate expression of PFR2 mRNA (Fig. 6B). These experiments indicate that the 10-nt RNA sequence AU GUAUAGUU contains a regulatory element required for stage-specific regulation of PFR2 mRNA.

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The 10-nt element alters the half-life of PFR2 mRNA in amastigotes. The experiments described above demonstrate that the 10-nt element is responsible for regulating PFR2 expression.
mRNA abundance. In order to demonstrate directly that the element functions by destabilizing the PFR2 mRNA in amastigotes, we measured the difference in stability of PFR2 element functions by destabilizing the mRNA abundance. In order to demonstrate directly that the regulatory element acts by destabilizing the PFR2 mRNA in amastigotes.

DISCUSSION

A negative regulatory circuit controls PFR2 expression. The PFR2 mRNA is regulated 10-fold during the Leishmania life cycle in concert with the elaboration of the PFR within the flagellum (23). Based on the data in this report, this regulation can now be explained largely by changes in the half-life of the PFR2 mRNA during the Leishmania life cycle. We have identified a single sequence element, contained within 10 nt of the 3’ UTR of the PFR2C mRNA, that is responsible for PFR2C mRNA regulation. Deletion of this element from the PFR2C transcript resulted in an increase in amastigote mRNA abundance until it coincided with levels in promastigotes, thereby eliminating regulated expression. Insertion of this element into an unregulated transcript conferred regulation of the transcript by decreasing mRNA levels in amastigotes. Thus, within the contexts examined, it appears to be both necessary and sufficient for regulation of PFR2 mRNA levels. We refer to this element defined by the 10-nt block substitution, BS2, as the PFR regulatory element (PRE). To our knowledge, the PRE represents the only cis regulatory element controlling mRNA degradation that has been mapped to this resolution in Leishmania to date.

We have demonstrated in two ways that the PRE acts by destabilizing the PFR2 mRNA in amastigotes rather than by stabilizing the mRNA in promastigotes. First, elimination of the PRE results in elevation of steady-state PFR2 mRNA levels in amastigotes but has no effect on steady-state PFR2 mRNA levels in promastigotes. Second, direct measurement of the kinetics of mRNA degradation in amastigotes indicates an alteration in the rate of degradation that depends on the presence of the PRE. Thus, the element must be part of a negative regulatory circuit that exerts its effect in amastigotes. We did not detect any function of the PRE in promastigotes.

This negative regulatory circuit could be described most simply by postulating a trans-acting factor(s), present exclusively in amastigotes, whose interaction with the PRE results in the degradation of the PFR2 mRNA. Degradation might be initiated by a direct cleavage at the site of binding or by facilitating deacylation of the poly(A) tail of the mRNA followed by the action of an exonuclease on the deacylated transcript (17). However, negative regulatory circuitry could be envisioned involving a constitutively expressed trans-acting factor that interacts with the PRE. For example, a constitutively expressed trans-acting factor could be activated either by covalent modification exclusively in amastigotes or by recruitment of additional factors exclusive to amastigotes. Alternatively, regulation could be achieved by a constitutively expressed RNA binding protein if it were selectively seques tered in promastigotes but available for binding in amastigotes. This mechanism has been postulated to account for the ability of members of the ELAV protein family to selectively act on different transcripts containing AREs (11, 24). Therefore, al-
TABLE 2. Location of the PRE in the 3′ UTRs of other PFR genes

| L. mexicana gene | GenBank accession no. | Position in GenBank sequences | Distance (nt) from stop codon | Sequence |
|------------------|-----------------------|-------------------------------|-------------------------------|----------|
| PFR2C            | U45884                | 4492                          | 1,098                         | AUGUAuAGUu |
| PFR2A            | NA                    | NA                            | 505                           | AUGUAaAGUa |
| PFR2B            | U45884                | 508                           | 505                           | AUGUAaAGUa |
| PFR1C            | AY198411              | 2809                          | 1,354                         | AUGUAaAGUg |
| PFR1D            | AY198411              | 6217                          | 778                           | AUGUAaAGUg |
| PFR4             | AY198410              | 3680                          | 117                           | AUGUAaAGAa |

Consensus: AUGUAaAGUa

*a* NA, not available.

though we believe that negative regulation must occur in amastigotes, it is not necessarily the case that the trans-acting factor that engages the element must be amastigote specific.

**Global regulation of PFR genes by a common regulatory element.** In what other contexts does the PRE regulatory sequence occur? We searched the 3′ UTR sequences of other PFR mRNAs to determine whether all or part of the 10-nt sequence defined by the BS2 block substitution is present. We have found in each of five other PFR gene 3′ UTRs (PFR2A, PFR2B, PFR1C, PFR1D, and PFR4) an exact match to an identical 8-nt subset of the 10 nt altered in BS2 (Table 2). Unlike the position- and orientation-dependent activity of the 3′ UTR regulatory element of the regulated amastin mRNA in *T. cruzi* (6), the position dependence of the PRE is not conserved in the other PFR genes. The orientation dependence of the PRE has not been tested.

A search for this 8-nt conserved element in the *L. major* Friedlin genomic sequences deposited in GenBank revealed its presence at a frequency of 1:520,296 nt, in good agreement with the expected frequency of this 8-nt sequence in a genome with a G+C content of 63% (1:296308). The occurrence of the PRE in each of the available *L. mexicana* PFR gene 3′ UTRs at a frequency that is about 370 times greater than its overall frequency in the *L. major* genome, is unlikely to be a chance occurrence. Rather, it suggests that the PRE element functions in the coordinate regulation of a group of functionally related genes. Northern analysis on *L. mexicana* total RNA from seven of these genes identified in the database search shows regulated mRNA accumulation analogous to *PFR2C* (T. R. Holzer, K. K. Mishra, and J. H. LeBowitz, unpublished data). These genes include the *PFR1* and *PFR4* genes, where the PRE is conserved between the two species. As expected, several of the other genes have been implicated in flagellar structure or biology.

A protein that specifically recognizes AU-rich instability elements involved in the stage-regulated expression of the mucin-type gene family of *T. cruzi* has been identified (9). AREs, a family of functionally and structurally distinct sequence motifs such as the AUUUA pentamer, the UUAUUUA(A/U)(U/A) nonamer, and stretches of U-rich domain that range in size from 50 to 150 bp, are destabilizing elements that are present in 3′ UTRs of higher eukaryotic early-response-gene mRNAs, including cytokines, lymphokines, and proto-oncogenes (for a review, see reference 5). In the case of the *T. cruzi* mucin SMUG gene, an RNA-binding protein, TcUBP, has been identified that is believed to be the trans-acting factor responsible for the 6- to 10-fold-lower levels of SMUG mRNA in trypomastigotes compared to epimastigotes. TcUBP-1 appears to be involved in the destabilization of SMUG mRNA. TcUBP-1 levels are 10-fold higher in amastigotes and trypomastigotes than in epimastigotes. In fact, overexpression of the protein in epimastigotes results in a lowering of SMUG mRNA in that stage (9). Thus, TcUBP appears to participate in a negative regulatory circuit similar to that observed for the *L. mexicana* *PFR2* gene.

Can the PRE be a member of the AU-rich family of regulatory elements? The *T. cruzi* AREs identified in the mucin gene family bear a striking resemblance to their mammalian counterparts. In contrast, the PRE, although it is AU rich and is imbedded in an AU-rich sequence in *PFR2C*, has not previously been reported as a member of the ARE family. However, the sequence AUGUA, which is contained in the PRE, has been shown to compete effectively with the canonical AU-rich pentamer, AUUUA, for binding to the mammalian AU-rich binding protein HuR (24). The PRE may represent a novel member of the ARE family. In this case, given that a *Leishmania* homolog of the TcUBP-1 family has been reported, it or a relative might interact with the PRE. We are currently testing this possibility.

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