EhPgp5 mRNA Stability Is a Regulatory Event in the *Entamoeba histolytica* Multidrug Resistance Phenotype*

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The multidrug resistance (MDR) phenotype in *Entamoeba histolytica* is characterized by the overexpression of the EhPgp5 gene in trophozoites grown in high drug concentrations. Here we evaluated the role of EhPgp5 mRNA stability on MDR using actinomycin D. EhPgp5 mRNA from trophozoites growing without emetine had a half-life of 2.1 h, which augmented to 3.1 h in cells cultured with 90 μM and to 7.8 h with 225 μM emetine. Polyadenylation sites were detected at 118-, 156-, and 189-nucleotide (nt) positions of the EhPgp5 mRNA 3′-untranslated region. Interestingly, trophozoites grown with 225 μM emetine exhibited an extra polyadenylation site at 19 nt. The 3′-untranslated region sequence is AU-rich and has putative consensus sequences for RNA-binding proteins. We detected a RNA-protein complex in a region that contains a polypyrimidine tract (142–159 nt) and a cytoplasmic polyadenylation element (146–154 nt). A longer poly(A) tail in the EhPgp5 mRNA was seen in trophozoites grown with 225 μM emetine. Emetine stress may affect factors involved in mRNA turnover, including polyadenylation/deadenylation proteins, which could induce changes in the EhPgp5 mRNA half-life and poly(A) tail length. Novel evidence on mechanisms participating in *E. histolytica* MDR phenotype is provided.

*Entamoeba histolytica*, the protozoan parasite responsible for human amoebiasis, presents the multidrug resistance (MDR) phenotype (1) described first in mammalian cells (2) and then in several protozoan parasites (3, 4). MDR is associated with the overexpression of a 170-kDa membrane molecule known as P-glycoprotein (PGP), an energy-dependent pump that extrudes drugs from the cells (5, 6). In *E. histolytica*, MDR phenotype is given mainly by overexpression of the *EhPgp1* and *EhPgp5* genes, which are finely regulated by transcriptional factors (7–9). Although *EhPgp1* is constitutively expressed in drug-resistant trophozoites of clone C2, *EhPgp5* gene is overexpressed only when C2 cells are grown in a high emetine concentration (10, 11). Both genes are also amplified in the presence of a high drug concentration (12).

Transcriptional regulation of eukaryotic mdr genes has been considered as the major control point for PGP synthesis, although gene amplification mechanisms also participate in this event (12, 13). Moreover, there is growing evidence of pivotal post-transcriptional (14–17) and post-translational (18–20) regulation of the PGP expression. On the other hand, mRNA stability has recently emerged as a critical control step in determining cellular stationary mRNA levels. The abundance of a particular mRNA can fluctuate many folds due to alterations in mRNA stability without any change in the transcription rate (21). The mRNA half-life is determined by a complex set of protein interactions at the 3′-untranslated region (3′-UTR) depending on conserved cis-element sequences and secondary structures (for review, see Ref. 22). The 3′-UTR also contains consensus sequence elements that mediate mRNA nuclear export, cytoplasmic localization, translation efficiency, and polyadenylation control (23, 24). The pre-mRNAs are polyadenylated in a reaction involving 3′ endonucleolytic cleavage followed by poly(A) tail synthesis (25). Poly(A) tail is also a modulator of mRNA stability and translation (26, 27). Strict control of poly(A) tail length is achieved by the concerted interplay of key factors, including poly(A) polymerase, deadenylases, and poly(A)-binding protein activities (25).

Several reports have addressed the importance of mRNA stability on the mdr genes expression regulation. *Pgp1* and *Pgp2*, and *Pgp3* mRNAs have a higher half-life in rat tumor cells than in normal cells (15), whereas rat MDR hepatocytes in culture present a higher amount of PGp2 protein due to a post-transcriptional mechanism controlling mRNA stability (14). Human *MDR1* mRNA has a half-life of 30 min, which is prolonged to more than 20 h upon treatment with cycloheximide, suggesting that protein synthesis inhibition may influence the stability of certain mRNAs (16, 17). However, molecular mechanisms controlling mdr mRNA stability remains to be elucidated.

In *E. histolytica*, mRNA stability mechanisms have not been studied yet. The presence of higher levels of EhPGP5 protein in the multidrug-resistant trophozoites of clone C2 could be influenced by both transcriptional activation and increased mRNA stability. In this paper, we measured the EhPgp5 mRNA half-life in trophozoites of clone C2 grown at different emetine concentrations. Our data showed that EhPgp5 mRNA stability is increased at high emetine concentrations, indicating that mRNA half-life is also regulating the MDR phenotype. In addition, here we initiated the study of the mechanisms involved in mRNA turnover in this parasite.
**E. histolytica Cultures**—Trophozoites of the clones A (drug-sensitive) and C2 (drug-resistant) (strain BH11MSS) (28) were axenically cultured in RPMI-1640 medium (29). Trophozoites of clone C2 were cultured without emetine (C2) or with 90 μM (C2(90)) and 225 μM (C2(225)) emetine. Logarithmic phase growing cultures were used in all experiments. All assays presented here were performed at least three times by duplicate.

**Transcriptional Inhibition by Actinomycin D**—Actinomycin D (Roche—Nutraceuticals) was added to the trophozoites for 2 more hours in the absence of actinomycin D. Immediately, total RNA was isolated by TRizol® (Invitrogen). Incorporation of [3H]UTP in 20 μg of total RNA was measured by liquid scintillation counting system (Beckman) in duplicate samples, and data obtained were plotted. Cytotoxicity of actinomycin D and MeSO was checked by cell viability using trypan blue and measuring the growth rate of the treated cultures.

**Reverse Transcriptase (RT)-PCR Experiments**—100 ng of total RNA from trophozoites of clones A, C2, C2(90), and C2(225) were preincubated at 37 °C for 15 min with 10 units of RNA-free dNase I (Stratagene). Single-stranded cDNAs were synthesized using 10 μM each dNTP and 100 ng of oligo(dT18) in diethyl pyrocarbonate-treated water. After preincubation, the reaction was made up to a final concentration of 10 μg/ml of medium, and cells were incubated at 37 °C for different times. Fresh medium supplemented with [3H]UTP (10 μCi/ml) was added to the actinomycin D-treated trophozoites for 2 more hours in the absence of actinomycin D. Immediately, total RNA was isolated by TRizol® (Invitrogen). Incorporation of [3H]UTP in 20 μg of total RNA was measured by liquid scintillation counting system (Beckman) in duplicate samples, and data obtained were plotted. Cytotoxicity of actinomycin D and MeSO was checked by cell viability using trypan blue and measuring the growth rate of the treated cultures.

**In Vitro Transcription—Templates for transcriptase synthesis were prepared from pBluescript II SK (+) phagemid (pBS) (Stratagene), which contains the T7 a phage promoter. The PSI-AS, PSII-AS, PIII-AS, and PSIV-AS antisense DNA fragments that contain the last 100 bp of EhPgp5 open reading frame and 19, 118, 156, and 189 bp of the 3'-UTR, respectively, were PCR-amplified using 1 unit of Deep Vent DNA polymerase (New England Biolabs) and the P4 plasmid (7) as template. The primers used were EhPgp5-3'-UTR-S sense and the PSI-AS (5'-TCTATATTTATGTGACTAT-3'), PSII-AS (5'-TTATATTTATGAAATA-3'), PIII-AS (5'-AAAAGATATAAAACAAAACT-3'), and PSIV-AS (5'-TTCTATATAAACATTTAAA-3') antisense primers. Amplified products were directionally cloned into the BamHI and XhoI sites of pBS plasmid and sequenced in a ABI PRISM automated sequence.

**Cytosplasmic Extracts**—Cytosplasmic extracts (CE) were obtained as described (33) with some modifications for E. histolytica. Briefly, 1 × 10^6 trophozoites of clones C2, C2(90), and C2(225) were washed with 1 mM phosphate-buffered saline, pH 6.8, supplemented with 10 mM Tris-HCl, pH 7.9, 1.5 mM MgCl₂, 0.5 mM NaCl, 0.5 m M phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 mM dithiothreitol, and 40 units of recombinant RNasin ribonuclease inhibitor (Promega) were incubated at 0°C for 20 min. The samples were centrifuged at 10,000× g for 10 min at 4°C. The supernatant was mixed with 0.11 volume of 100 mM HEPES, pH 7.9, 20 mM MgCl₂, 250 mM KCl solution and centrifuged at 14,000× g at 4°C for 1 h. Protein concentration was determined by the Bradford method (34).

**EhPgp5 mRNA Stability Assays**—Total RNA from trophozoites of clones C2, C2(90), and C2(225) was obtained at 0, 2, 4, 8, and 12 h after actinomycin D-induced transcriptional blockage. EhPgp5 and actin mRNAs were measured by multiplex RT-PCR as described above, and intensity of the bands was quantified using Scion (Scion Corporation, Frederick, MD) NIH Image software. Amplified products and RNA fragments protected of the S1 nuclease digestion were separated by 6% PAGE at room temperature, products and RNA fragments protected of the S1 nuclease digestion were separated by 6% PAGE at room temperature, vacuum-dried, and visualized in a PhosphorImager apparatus.
at 72 °C for 7 min. PCR samples were separated on a 1.5% Tris-buffered EDTA-agarose gel then transferred to nylon membranes and hybridized with specific \textit{EhPgp5} and actin 3\text/-UTR \textit{[H]dATP}-labeled probes.

RESULTS

Actinomycin D Inhibits \textit{E. histolytica} Transcription—To study the mechanisms controlling mRNA decay in \textit{E. histolytica} we first investigated the effect of the transcription inhibitor actinomycin D on viability, growth, and mRNA synthesis in trophozoites of the drug-sensitive clone A and drug-resistant clone C2. Cell viability of trophozoites of clone C2 incubated with actinomycin D was 98%. At this time cell growth was slightly delayed in the actinomycin D-treated trophozoites in comparison with untreated cells (Fig. 1A). The effect of actinomycin D on cell growth and viability was similar in trophozoites of all clones tested (data not shown). Then we performed experiments to determine whether mRNA synthesis was affected by actinomycin D and the time required to inhibit at least 90% of mRNA synthesis. Results showed that actinomycin D affects the [3H]UTP incorporation into new synthesized RNA in a time-dependent manner (Fig. 1B). Untreated trophozoites of clones A and C2 incubated for 2 h \( (t_0) \) with [3H]UTP incorporated 16,450 and 14,660 cpm, respectively. Each value was taken as 100% incorporation for the corresponding clone (Fig. 1B). Trophozoites of clones A and C2 preincubated for 30 min with the drug and then incubated with [3H]UTP for 2 h incorporated 50.4 and 59.9% of radioactivity, respectively. One hour later, both clones presented only 12% incorporation of [3H]UTP (Fig. 1B). These low levels of RNA synthesis were maintained in trophozoites of both clones incubated with actinomycin D for up to 8 h (Fig. 1B). As a control for mRNA integrity, equivalent amounts of total RNA were isolated at each time, and rRNA were visualized in ethidium bromide-stained gels showing the recombinant RNA obtained from actinomycin D-treated trophozoites of clones A (upper panel) and C2 (lower panel) at the indicated times. DMSO, dimethyl sulfoxide.

Fig. 2. Determination of the \textit{EhPgp5} mRNA half-life. A–C and G, ethidium bromide-stained gels showing the RT-PCR products obtained at different times after actinomycin D transcriptional blockage. MW, molecular weight. D–F, densitometric analysis of RT-PCR products in A–C. H, semilogarithmic plot of the \textit{EhPgp5} and actin mRNA levels quantified by densitometry in A–C. The graphics showed the results of a representative assay of three independent experiments. Actin: \( \bullet \), C2; \( \square \), C2(90); \( \bigtriangleup \), C2(225). \textit{EhPgp5}: \( \bullet \), C2; \( \square \), C2(90); \( \bigtriangleup \), C2(225).
TABLE I

|                | Theoretical mRNA half-life | Experimental mRNA half-life |
|----------------|-----------------------------|-----------------------------|
|                | EhPgp5 | Actin  | EhPgp5 | Actin |
| C2             | 1.2    | ND     | 2.1    | ND |
| C2 (90)        | 2.7    | ND     | 3.1    | ND |
| C2 (225)       | 5.6    | ND     | 7.8    | ND |
| CA             | ND     | ND     | ND     | ND |

a According to the decay equation: $t_{1/2} = \ln 2/K$.

b According to the semilogarithmic data plotted in Fig. 2H.

EhPgp5 mRNA Stability in Higher in Trophozoites of the 
Clone C2 Cultured with Emetine—To investigate the EhPgp5 mRNA stability in trophozoites growing with different emetine concentrations, we determined the EhPgp5 mRNA half-life in actinomycin d-treated trophozoites of clones C2, C2(90), and C2(225). The EhPgp5 mRNA was measured from 0 to 12 h by semiquantitative RT-PCR assays in total RNA. We included actin primers in all reactions as internal control. Results showed that EhPgp5 mRNA was present in untreated trophozoites of clone C2, and the signal diminished progressively at 2 and 4 h after the transcriptional blockage (Fig. 2A). In clone C2(90), the EhPgp5 transcript was detected up to 8 h after actinomycin D treatment (Fig. 2B). Interestingly, in clone C2(225) the EhPgp5 mRNA was detected even 12 h after the transcriptional blockage (Fig. 2C). These data indicated that EhPgp5 mRNA amounts are reduced in a time-dependent manner in actinomycin D-treated trophozoites, but they are maintained for a longer time in the emetine-cultured cells. In contrast, we did not detect the EhPgp5 mRNA in untreated trophozoites of the wild type drug-sensitive clone A (Fig. 2G), confirming that EhPgp5 gene is not transcribed in the drug-sensitive trophozoites (36).

The EhPgp5 and actin mRNAs were quantified by densitometry and arbitrary expressed in pixels (Fig. 2, D–F). Pixels given by actin at $t_0$ were taken as 100% in each clone, and the EhPgp5 percentage was expressed with respect to the actin mRNA levels. At $t_0$, the actin amount appeared almost unaltered, whereas EhPgp5 varied in the three clones. In trophozoites of clone C2, the EhPgp5 mRNA was 63% of the actin RNA (Fig. 2D), and in C2(90) it was 81% (Fig. 2E), whereas clone C2(225) exhibited similar levels of both transcripts (Fig. 2F). The other bands seen in the gels were not related to the EhPgp5 transcript, as probed by Southern blot hybridization using the EhPgp5 probe (data not shown).

To determine the experimental mRNA half-life, the results of normalized EhPgp5 mRNA levels were plotted in a semilogarithmic scale against the exposure time to actinomycin D (Fig. 2H). In these calculations, the amount of EhPgp5 and actin mRNA at $t_0$ was taken as 100% in each clone. In trophozoites of clone C2, the experimental EhPgp5 mRNA half-life was estimated to 2.1 h, whereas in C2(90) it was 3.1 h and 7.8 h in C2(225), confirming significant variations in the decay rates of the three clones. In addition, these experiments showed that actin mRNA decay remained with minimal changes during the 12-h course of the transcription inhibition in all clones. There are reports proposing that actin mRNA has a half-life between 24 and 33 h in mammalian cells (37). According to our experiments, E. histolytica actin mRNA has a half-life longer than 12 h.

Experimental values were close to the theoretical EhPgp5 mRNA half-life predicted from the decay equation described under “Experimental Procedures.” The theoretical EhPgp5 mRNA half-life was 1.2 h in the trophozoites of clone C2, 2.7 h in C2(90), and increased to 5.6 h in C2(225) (Table 1).

EhPgp5 mRNA Presents 3′-UTR Heterogeneity—To determine whether the distinct EhPgp5 mRNA half-lives observed...
in the trophozoites of clones C2, C2(90), and C2(225) could be influenced by changes in the 3'-UTR length, we performed RNA protection assays with S1 nuclease (Fig. 3A). Three RNA-DNA-protected fragments were found at nt 118, 156, and 189 downstream of the UAA stop codon of the EhPgp5 mRNA in all C2 clones (Fig. 3A, lanes 2–4). These fragments, corresponding to different EhPgp5 3'-UTR variants, were denoted as PSI118, PSIII156, and PSIV189, respectively. Interestingly, clone C2(225) showed an extra protected fragment at the nt 19 (PSI19) (Fig. 3A, arrow). The protected fragments detected were analyzed by densitometry (Fig. 3B). The total pixels obtained from the transcript species in clone C2(225) were taken as 100%, and those from C2(90) and C2 clones were 53 and 47%, respectively. In clone C2, the PSIV189, PSIII156, and PSI118 variants showed levels of 26, 23, and 31 pixels, respectively (Fig. 3C). In clone C2(90), PSIV189 and PSIII156 appeared in similar amounts (36 and 34 pixels), whereas PSI118 gave only 21 pixels (Fig. 3D). In clone C2(225), the transcript PSI19 was 40% of total variants (68 pixels), whereas PSIV189, PSIII156, and PSI118 gave 43, 39, and 23 pixels, respectively (Fig. 3E). In clone C2(225), the PSIII156 and PSIV189 species increased almost 2-fold with respect to PSI118, whereas PSI19 was 3.3-fold, suggesting that they could be more stable (Fig. 3E).

The presence of various polyadenylation sites suggested that mRNA variants could influence the EhPgp5 mRNA steady-state levels, as has been reported for other cells (38–40). Differences in sequence at the 3'-UTR could be involved in the EhPgp5 mRNA stability and polyadenylation site selection. The comparative analysis of the first 189 bases in the 3'-UTR EhPgp5 DNA showed that 3'-UTR sequences were identical in clones A, C2 (Fig. 4), and C2(225) clones (data not shown), indicating that differences in sequence do not account for the EhPgp5 mRNA half-life or for the polyadenylation site choice.

The EhPgp5 3'-UTR is 85% AU-rich, and it presents several putative consensus binding sequences for regulatory proteins. There are five putative consensus polyadenylation signals (PS) with the UA(A/U)U sequence described for E. histolytica (41) at nt 1, 17, 98, 127, and 174 (Fig. 4, open boxes). We also found several eukaryotic 3'-UTR elements including the canonical polyadenylation signal (AAUAAA) at nt 90 (Fig. 4, shadowbox) and three putative AU-rich elements (AUREs) conforming to the conserved AU(A/U)U sequence described for E. histolytica (41) at nt 118, 76, and 31 downstream from the UAA codon (Fig. 4, ellipses). In addition, two polypyrimidine tracts (Py) were detected at nt 44 and 142–159 (Fig. 4, discontinuous underlined) and a consensus cytoplasmic polyadenylation element (CPE), (UUUUUAU) at nt 146–154 (Fig. 4, continuous underlined). The CPE seems to promote cytoplasmic polyadenylation in eukaryotic cells (43).

**Fig. 5. RNA-protein complex formation in the EhPgp5 3'-UTR mRNA.** A schematic representation of the putative cis-acting elements present in the 3'-UTR of the EhPgp5 fragments used for RNA electrophoretic mobility shift assays. EPS, eukaryotic polyadenylation signal; UAA, stop codon. ORF, open reading frame. B–D, [α-32P]UTP-labeled PSI19, PSII118, PSIII156, and PSIV189 probes were incubated with CE from trophozoites of the different clones, as described under “Experimental Procedures.” Lane 1, free PSI19; lane 2, PSII118; lane 3, PSIV189; lane 4, PSI19; and lane 5, PSIII156 probes. E and F, competition experiments. The PSIII156 (E) and PSIV189 (F) riboprobes were incubated with CE from the trophozoites in the presence of free probe (lane 1), no competitor (lane 2), specific competitor (SC; lane 3) (350-fold molar excess of homologous unlabeled fragments), and unspecific competitor (UC; lane 4) (350-fold molar excess of tRNA). Arrows denote RNA-protein complex.
In contrast, we did not find any RNA-protein complex when we used PSI19 and PSI118 fragments (Fig. 5, B–D, lanes 4 and 5).

To delimitate the region in which the RNA-protein complex was formed, we carried out cross-competition experiments using the PSIV189 transcript as labeled probe and the PSI19, PSI118, and PSI1156 RNAs as competitors. In the three clones the complex formed in the PSIV189 region was specifically competed by the same probe and by PSIII156 (Fig. 6, A–C, lanes 5 and 6) but not by PSI19 and PSI118 RNA fragments, as expected (Fig. 6, A–C, lanes 3 and 4). These results suggest that RNA-protein interaction takes place in a region of 38 nt (nt 119–156), which is shared by PSIII156 and PSIV189 fragments. This region contains a PS (nt 127–131) and a 15-nt Py (nt 142–156) sequences, including a CPE (nt 146–154) motif, which could be targets for regulatory RNA-binding proteins (43–46).

The EhPgp5 mRNA Poly(A) Tail Is Longer in C2(225) Trophozoites—Poly(A) tail is an important modulator of mRNA turnover, and its length is subjected to cellular control throughout the life span of the mRNA (25). We investigated the poly(A) tail length of EhPgp5 mRNA from trophozoites of clones C2, C2(90), and C2(225) by LM-PAT (35), as described under “Experimental Procedures.” We observed three well defined bands corresponding to the PSI118, PSI1156, and PSIV189 predicted transcripts plus 100 bp of the EhPgp5 open reading frame, respectively (Fig. 7A). In these assays, we could not amplify the PSI19 variant, probably because EhPgp5 PSI19 transcript has a very short poly(A) tail. The identity of the amplified products was confirmed by Southern blot hybridization with a DNA probe containing the last 100 bp of the open reading frame and the first 19 nt of the EhPgp5 mRNA 3′-UTR (Fig. 7B). The signal appeared as a smear ranging from 218 to 300 nt in mRNA from trophozoites of clones C2 and C2(90) (Fig. 7B). Interestingly, in clone C2(225) the hybridization signal showed a longer smear spanning from 218 to 500 nt. The same membrane hybridized with the actin 3′-UTR (Fig. 7C). In contrast, in the actin control assays, we detected a defined 130-bp band corresponding to actin 3′-UTR (30 nt) plus 99 bases of the 3′-actin open reading frame and a short smear spanning 130–150 nt in all clones (Fig. 7, D and E). The actin control membrane gave no signal with the EhPgp5 3′-UTR probe (Fig. 7F).

These LM-PAT patterns represent an enlargement of the poly(A) tail length of the EhPgp5 mRNA in the trophozoites of clone C2(225) or, alternatively, a shortening in C2 and C2(90) cells, suggesting that changes in poly(A) tail length are involved in EhPgp5 mRNA half-life.

**DISCUSSION**

Previously, we demonstrated that the EhPgp5 gene is over-expressed in *E. histolytica* trophozoites grown in the presence of high drug concentration (30). Transcriptional factors partic-
ipate in the EhPgp5 gene promoter activation (11). Results presented here show novel evidence that post-transcriptional EhPgp5 gene regulation occurs in the drug-resistant trophozoites. EhPgp5 mRNA is more stable in trophozoites grown in 225 µM emetine than in those grown in 90 µM or without drug (Fig. 2). Additionally, the EhPgp5 mRNA 3′-UTR length is heterogeneous (Fig. 3A), which may influence the mRNA half-life. The PSIII156 and PSIV189 mRNA variants augmented when the emetine dose was increased (Fig. 3, A–E). Their predicted secondary structure suggests that they have exposed a Py tract and a CPE motif (data not shown). Furthermore, a RNA-binding protein complex was detected in their 30-nt shared region (Figs. 5 and 6). In other organisms, polypyrimidine tract-binding proteins have been involved in splicing and stability control of the mRNA, whereas CPE-motif interacting proteins target specific mRNAs to cytoplasmic polyadenylation, producing the translational activation of the transcripts (43–46). Interestingly, the EhPgp5 mRNA presents a longer poly(A) tail in clone C2 (225) (Fig. 7), and it is well known that large poly(A) tails give higher stability to mRNA and promote a more efficient translation (39). Emetine stress could affect the expression of many factors, including the polyadenylation/deadenylation proteins involved in the poly(A) tail length control.

mRNA half-life and translation are linked in ways that are not completely understood. In cells exposed to translation inhibitors some mRNAs are stabilized in several ways, including alterations in polyadenylation rates (22). For example, in mamalian cells, cycloheximide prolongs c-myc mRNA half-life by slowing the deadenylation process but does not promote degradation of the mRNA body once deadenylation is being completed (47). The heterogeneity of the EhPgp5 transcripts is explained by the alternative usage of several polyadenylation signals detected in the 3′-UTR (Fig. 4), as has been well documented for other systems, including other mdr genes (40, 48). Mouse mdr1a mRNA shows length variations at both 5′ and 3′ ends, and mRNA variants have very large 3′-UTRs, which are differentially overexpressed in multidrug-resistant cell lines (48). Interestingly, the EhPgp5 mRNA also presents heterogeneity in the 5′ end of trophozoites of clones C2 and C2(225) (11). All these data indicate that EhPgp5 gene is a complex transcriptional unit whose regulation produces multiple transcript sizes at the 3′ and 5′ ends.

Emetine partially inhibits protein synthesis in trophozoites of clone C2 (225) (data not shown). This could induce a stabilizing mRNA effect (22, 49) affecting certain EhPgp5 transcript variants. The PSIV189, PSIII156, and PSII118 mRNA variants were detected in all clones, whereas the PSI19 was observed only in clone C2(225). PSI19, PSIII156, and PSIV189 transcripts were more abundant in the trophozoites of clone C2(225). PSI19 transcript, which has a very short poly(A) tail length or is not polyadenylated, was almost 2-fold the amount of PSIII156 and PSIV189 in clone C2(225) (Fig. 3). It is possible that the expression of the PSI19 transcript has been independent of the poly(A) tail contribute to the PSI19 transcript stability. However, additional experiments are required to confirm this hypothesis.

AURE motifs have been involved in destabilization of mRNAs with a short half-life (42). However, Prokopczak et al. (16) find that AUREs at the 3′-UTR of human MDR1 mRNA is an inefficient promoter of mRNA decay, which suggests that AURE-dependent mRNA stability regulation may not operate in certain cases, such as the MDR1 and EhPgp5 mRNAs. This assumption is supported because under the experimental conditions reported here, we did not detect any RNA-protein complex in the AURE motifs present in the EhPgp5 3′-UTR mRNA, suggesting that they do not act as cis-regulatory elements in the EhPgp5 mRNA half-life control.

We found a RNA-protein complex in the proximity of the Py tract in the PSIII156 and PSIV189 transcripts (Figs. 5 and 6) that may contribute to their stability in C2(225) cells. However, the same complex was also detected in clones C2 and C2(90), suggesting that other factors present only in clone C2(225) are required to stabilize certain EhPgp5 mRNA variants. The identity of the 3′-UTR EhPgp5 mRNA-interacting protein(s) detected here remains to be elucidated. Interestingly, the EhPgp5 mRNAs from trophozoites of clone C2(225) present longer poly(A) tails than those from C2 and C2(90) cells, suggesting that polyadenylation and deadenylation events, occurring at different rates, could be affecting the EhPgp5 mRNA half-life.

Our working hypothesis assumes that trophozoites of clone C2 grown without emetine have some factors that maintain short poly(A) tails, which may contribute to a shorter EhPgp5 mRNA half-life (Fig. 8). Some of these factors could be affected by emetine in C2(225) cells, and emetine-responsive factors could both induce an enhanced polyadenylation of EhPgp5 mRNAs. The expression and activity of some proteins involved in 3′ to 5′ exonucleolytic mRNA degradation and polyadenylation may also be participating in the longer EhPgp5 mRNA half-life in C2(225) cells. Hence, the putative role of other 3′ end processing and polyadenylation/deadenylation factors cannot be discarded.

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