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Experimental Parasitology 110 (2005) 226–232
Experimental Parasitology
www.elsevier.com/locate/yexpr

Entamoeba histolytica: Cloning and expression of the poly(A) polymerase EhPAP

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Received 14 January 2005; received in revised form 24 February 2005; accepted 24 February 2005
Available online 1 April 2005

Abstract

In eukaryotes, polyadenylation of pre-mRNA 3′ end is essential for mRNA export, stability, and translation. Here we identified and cloned a gene codifying for a putative nuclear poly(A) polymerase (EhPAP) in *Entamoeba histolytica*. Protein sequence alignments with eukaryotic PAPs showed that EhPAP has the RNA-binding region and the PAP central domain with the catalytic nucleotidyl transferase domain described for other nuclear PAPs. Recombinant EhPAP expressed in bacteria was used to generate specific antibodies, which recognized two EhPAP isoforms of 60 and 63 kDa in nuclear and cytoplasmic extracts by Western blot assays. RT-PCR assays showed that *EhPap* mRNA expression varies in multidrug-resistant trophozoites growing in different emetine concentrations. Moreover, *EhPap* mRNA expression is about 10- and 7-fold increased in G_1 and S phase, respectively, through cell cycle progression. These results suggest the existence of a link between EhPAP expression and MDR and cell cycle regulation, respectively.

Index Descriptors and Abbreviations: aa, aminoacids; CE, cytoplasmic extracts; CPSF, cleavage and polyadenylation specificity factor; mRNA, messenger ribonucleic acid; MW, molecular weigh; MDR, multidrug resistance; NE, nuclear extracts; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PAP, poly(A) polymerase; RBD, RNA-binding domain

Keywords: *Entamoeba histolytica*; Poly(A) polymerase; Pre-mRNA 3′ end processing; Polyadenylation

1. Introduction

In eukaryotes, most mature mRNAs have a poly(A) tail at their 3′ end, which participates in mRNA nuclear export, stability, and translation efficiency. Polyadenylation is a multistep nuclear reaction that involves pre-mRNA 3′ end cleavage followed by adenosine residues addition. Both processes depend on *trans*-acting factors interacting in a coordinated way with *cis*-sequence elements (Zhao et al., 1999). Poly(A) tail synthesis in the nucleus is catalyzed by the canonical poly(A) polymerase (PAP) that belongs to the DNA polymerase β-like nucleotidyl transferase superfamily (Holm and Sander, 1995). In mammals, PAP is tethered to RNA substrates by interaction with FIP1 (Kaufmann et al., 2004) and cleavage and polyadenylation specificity factor (CPSF), which is bound to the consensus polyadenylation signal (AAUAAA) (Keller et al., 1991). Moreover, PAP activity and poly(A) tail length are regulated through additional interactions with CFIm25 protein and poly(A)-binding protein II (Kerwitz et al., 2003). PAP also interacts with U1A and U2AF65 factors, establishing a functional link

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0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.02.017

between pre-mRNA splicing and polyadenylation (Gunderson et al., 1994; Vagner et al., 2000).

In cytoplasm, polyadenylated mRNAs stability depends on their degradation rate through pathways that begin with poly(A) tail elimination by specific poly(A) ribonucleases, including Ccr4, POP2, Caf1p and poly(A) nuclease (Tucker et al., 2001). However, certain mRNAs involved in early developmental processes or brain functions are readenylated in cytoplasm by a different family of PAPs referred as GLD-2, TFR4, and CID, to promote translation (Richter, 1999).

Interestingly, nuclear PAP is inactivated during mitosis through phosphorylation by p34cdc2/cyclin B, contributing to the reduction of polyadenylated mRNAs and proteins content and to an efficient cell cycle progression (Colgan et al., 1996; Zhao and Manley, 1998). Alternative RNA processing and protein phosphorylation allow the generation of multiple nuclear PAP isoforms, which are thought to be responsible for distinct functions in vivo (Thuresson et al., 1994; Zhao and Manley, 1996).

Recently, we reported the relevance of *EhPgp5* mRNA stability in the regulation of multidrug resistance (MDR) phenotype in *Entamoeba histolytica*. *EhPgp5* mRNA presents an increased half-life and a longer poly(A) tail in drug-resistant clone C2 trophozoites growing at high emetine concentrations, suggesting that emetine stress may alter polyadenylation reaction and mRNA decay rate (López-Camarillo et al., 2003). However, molecular events regulating pre-mRNA 3′ end processing are unknown in *E. histolytica*. To gain insights into mechanisms underlying mRNA 3′ end processing, stability and poly(A) tail length control in this parasite, we are currently studying the cleavage and polyadenylation machinery (López-Camarillo et al., 2005). Taking advantage of the raw genomic information obtained from the *E. histolytica* genome sequence project databases, we identified here a single gene codifying for a putative canonical nuclear poly(A) polymerase (EhPAP). Its cloning and sequence analysis show that EhPAP presents a molecular organization similar to the one reported for other nuclear PAPs and exhibits close phylogenetic relations to eukaryotic enzymes. Interestingly, *EhPap* mRNA expression varies through cell cycle progression and in drug-resistant trophozoites growing in different emetine concentrations, suggesting the existence of a link between EhPAP expression and these cellular events.

2. Materials and methods

2.1. Entamoeba histolytica cultures

Trophozoites (strain HM1-IMSS) were axenically grown in TYI-S-33 medium (Diamond et al., 1978). Multidrug-resistant clone C2 trophozoites were cultured without emetine (C2(0)) or with 90 (C2(90)) and 225 μM (C2(225)) emetine. Clone L-6 trophozoites cultures were cell cycle synchronized by incubation in 200 μg/ml colchicine for 24 h; after washing out the drug, cells were grown in fresh medium and harvested at 0, 3, 8, and 14 h corresponding to M, G_1, S, and G_2 phases, respectively (Orozco et al., 1988).

2.2. Cloning, sequence analysis, expression, and purification of rEhPAP

The complete *EhPap* ORF was PCR amplified from genomic DNA of clone A trophozoites using sense (5′-AAAGAAAATGAAAAAAGAACT-3′) and antisense (5′-TCAATTTTTTCTTGTAAA-3′) primers. Amplification was performed as follows: 94 °C for 5 min, 94 °C for 1 min, 52 °C for 90 s and 72 °C for 1 s, for 30 cycles, and a final extension step at 72 °C for 7 min, using DNA *Taq* polymerase high fidelity (2.0 U). The PCR product was cloned in frame into *Bam*HI and *Hin*dIII sites of the pRSET-A expression vector (Invitrogen) and automatically sequenced for subsequent in silico analysis. Sequence similarity searches were done using BLAST and multiple protein alignments were performed by ClustalW (http://www.expasy.org). Functional and structural domains were predicted by Prosite (http://www.expasy.org) and Pfam (http://www.sanger.ac.uk). Phylogenetic analysis was performed using the BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the Molecular Evolutionary Genetics Analysis (MEGA) (http://www.megasoftware.net) software.

The recombinant EhPAP (rEhPAP-6xHis tagged) was expressed in bacteria (*Escherichia coli* BL21-PLysS) in the presence of 1 mM IPTG for 3 h at 37 °C and purified through denaturing Ni^{2+}–NTA affinity chromatography (Qiagen) according to manufacturer recommendations. Its identity was confirmed by Western blot assays using anti-6xHis tag antibodies (Roche) and the ECL Plus Western blotting detection system (Amersham).

2.3. Inmunolocation

Purified rEhPAP (100 μg) in Freund's adjuvant was intramuscularly inoculated (four times at 14 days intervals) into pathogen free mice. The anti-rEhPAP serum was used to analyze cytoplasmic (CE) and nuclear (NE) extracts of clone A trophozoites (Schreiber et al., 1989) in Western blot assays. As a control we used Mab8 antibodies that are raised against the EhCPADH complex (Garcia-Rivera et al., 1999) in the same experiments.

2.4. RT-PCR experiments

Total RNA was obtained from multidrug-resistant clone C2 trophozoites grown in various emetine

concentrations and cell cycle synchronized clone L-6 trophozoites, using TRIzol (Gibco) reagent. Semi-quantitative RT-PCR was performed as previously described (López-Camarillo et al., 2003) using sense (5′-AGATTA AATGTATATGGA-3′) and antisense (5′-CCATAATT TAATAGTACG-3′) *EhPap* internal primers. *Actin* primers (López-Camarillo et al., 2003) were used as an internal control in the same experiments. Amplified products were separated by 6% PAGE and submitted to densitometric analysis.

3. Results and discussion

3.1. DNA and protein sequences analysis

The *EhPap* gene was identified at the locus 16.m00324 (TIGR) from nucleotide 69982 to nucleotide 71550 on assembly scaffold 00016. In agreement with previous reports about the small number of introns in *E. histolytica* (Wilihoeft et al., 2001), the 1566 pb ORF has no intron. It encodes a 522 amino-acid (aa) polypeptide with a predicted isoelectric point of 5.39 and a predicted molecular weight (MW) of 59180 Da (Fig. 1A). In silico sequence similarity searches using BLAST analysis show that the highest scoring hits (1e−35 to 2e−43) were for related eukaryotic nuclear PAPs. EhPAP presents 25–32% identity and 43–52% homology with yeast, protozoa, nematodes, plants and mammals nuclear PAPs (Table 1). Remarkably, BLAST analysis shows that EhPAP does not have high homology/identity with the cytoplasmic PAPs family (GLD-2, TFR4, and CID). Computer screening of *E. histolytica* TIGR and Sanger genome databases revealed that *EhPap* appears to be a single copy gene. Moreover, we did not find other genes codifying for canonical nuclear PAPs, suggesting that EhPAP is the unique nuclear PAP in *E. histolytica*.

Protein sequence inspection showed that EhPAP belongs to the DNA polymerase β-like nucleotidyl transferase superfamily. It has the highly conserved N-terminus that contains the characteristic PAP central catalytic domain (36–131 aa) (Pfam PF04928) with the three invariant active aspartate residues (D_{76}, D_{78}, and D_{130}) involved in nucleotide transfer and the F/YGS motif (63–65 aa) responsible for ATP binding in other PAPs (Zhao et al., 1999) (Fig. 1A). At the less conserved C-terminus, we identified a divergent RNA-binding domain (RBD) (322–444 aa) (Pfam PF04926), as well as two cAMP phosphorylation sites (425–428 aa and 516–519 aa) and other potential protein kinase C and casein kinase II phosphorylation sites, which could be important for activity regulation. Intriguingly, EhPAP has no typical nuclear localization signal (NLS), suggesting that *E. histolytica* could use other sequence signals for protein nuclear translocation. It also lacks 100 aa at the C-end corresponding to a part of the Ser/Thr-rich domain containing the NLS2 in human PAP (Zhao et al., 1999) (Fig. 1B).

Phylogenetic analysis of nuclear PAPs from protozoan parasites (*Plasmodium falciparum* and *E. histolytica*), yeast (*Saccharomyces cerevisiae*), plant (*Arabidopsis thaliana*), nematodes (*Caenorhabditis elegans*), and mammals (*Homo sapiens*) shows a progressive evolution of these proteins from lower to higher eukaryotes. EhPAP appears to be in a separated arm, suggesting that it diverged early on the evolutionary scale. However, it is closely related to protozoan parasite enzymes, such as *Plasmodium* PAP (Fig. 1C).

3.2. Expression of recombinant EhPAP

The recombinant pRSET-*EhPap* plasmid produced a rEhPAP with a 6xHis tag at the N-terminal end (Fig. 2A), which was inmunodetected by anti-6xHis tag antibodies in Western blot assays (Fig. 2B). rEhPAP was also specifically recognized by anti-rEhPAP serum generated in mice, confirming its identity (Fig. 2C). In contrast, anti-6xHis tag antibodies and anti-rEhPAP serum did not recognize any proteins in non-induced *E. coli* lysate used as negative control (Figs. 2B and C).

3.3. Subcellular location of EhPAP

To study the subcellular location of EhPAP in trophozoites, we separated CE and NE to perform Western blot analysis using anti-rEhPAP serum (Fig. 2D). In NE, specific antibodies revealed a protein of ≈60 kDa, which corresponds to the predicted MW for EhPAP. They also detected an additional 63 kDa protein, which could result from phosphorylation of EhPAP at the putative phosphorylation sites described above, as seen for other PAPs. The presence of both 60- and 63-kDa EhPAP isoforms in the nucleus, where RNA synthesis and pre-mRNA 3′ end processing take place, is in good agreement with the biological function of a factor involved in nuclear processes. Intriguingly, the same bands were immunodetected with the same intensity in CE, where proteins are synthesized. As a control of NE and CE integrity, we used Mab8 antibodies, which have been previously shown to detect the EhCPADH complex in cytoplasmic membranes of trophozoites using immunofluorescence assays (Garcia-Rivera et al., 1999). Here, Mab8 antibodies recognized a 116 kDa band corresponding to the EhCPADH complex in the cytoplasmic but not in the nuclear fraction (Fig. 2D). This demonstrates the absence of cross-contamination between both fractions and confirms that the fractionation protocol used is suitable to accurately determine the subcellular location of EhPAP.

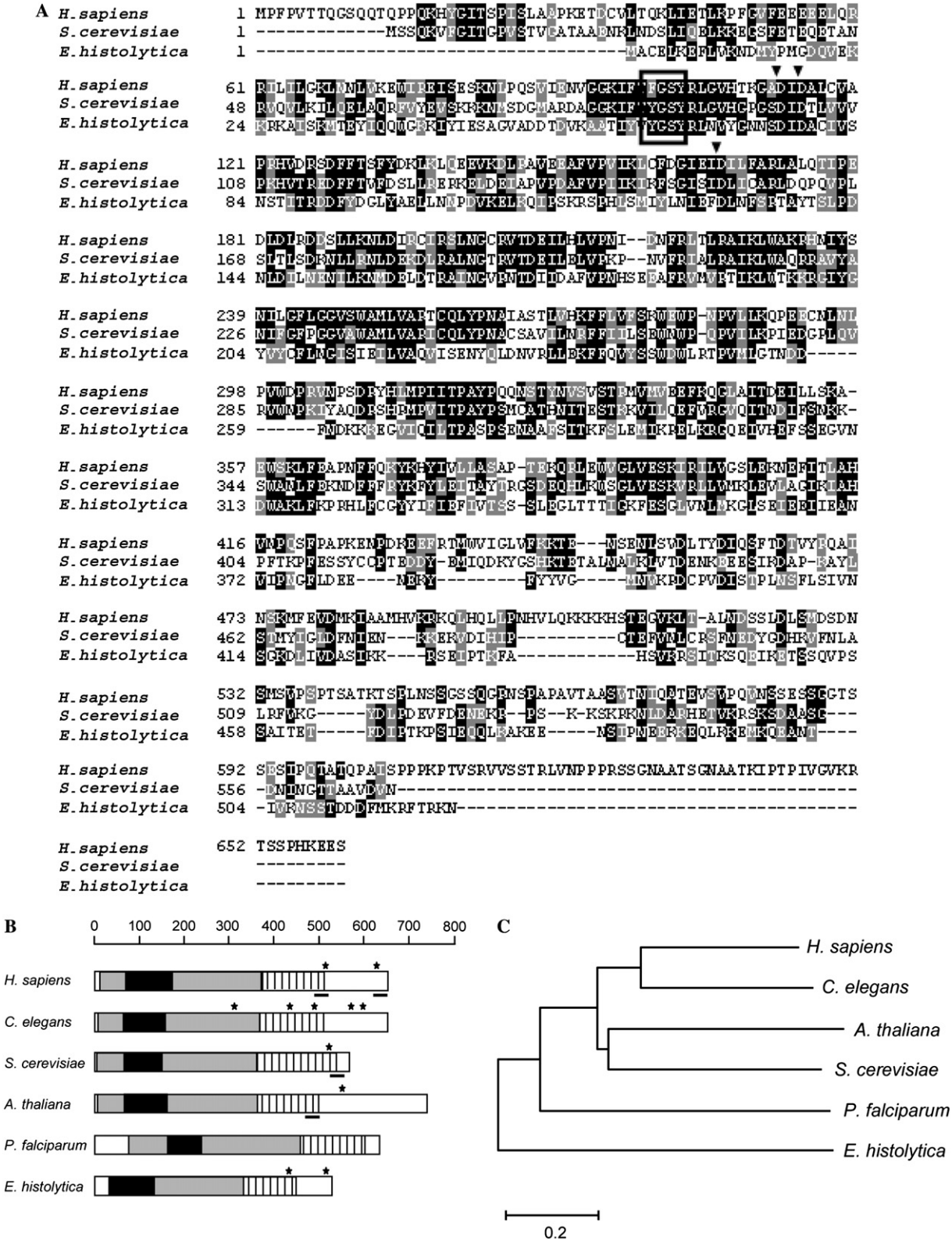

Fig. 1. Comparison of the deduced amino acids sequence of EhPAP with other eukaryotic PAPs. (A) Multiple alignments of EhPAP with *H. sapiens* PAPα and *S. cerevisiae* PAP1. Black box, identical aa; grey box, similar aa; open box, F/YGS motif; ▼, invariant aspartate residues (D_{76}, D_{78}, and D_{130}). Numbers at the left are relative to the initial methionine of each protein. (B) Molecular organization of PAPs. Grey box, PAP central domain; black box, nucleotidyl transferase domain; hatched box, RBD; underlined, NLS; asterisk, cAMP phosphorylation site. The scale at the top indicates the size in aa. (C) Phylogenetic analysis. The rooted tree of PAPs was created with MEGA program based on BioEdit alignments, using complete protein sequences. Accession numbers are indicated in Table 1.

Table 1
Comparison of EhPAP with other eukaryotic PAPs

Protein	Organism	[a]Accession number	*E*-value	Identity (%)	Homology (%)
PAP	*Caenorhabditis elegans*	Q9U2P3	2e−43	25	43
PAPα	*Homo sapiens*	P51003	6e−42	25	48
PAP	*Arabidopsis thaliana*	Q7XJ91	3e−39	26	47
PAP	*Plasmodium falciparum*	Q6LF18	1e−38	27	48
PAP1	*Saccharomyces cerevisiae*	P29468	1e−35	32	52

[a] SwissProt and TrEMBL databases.

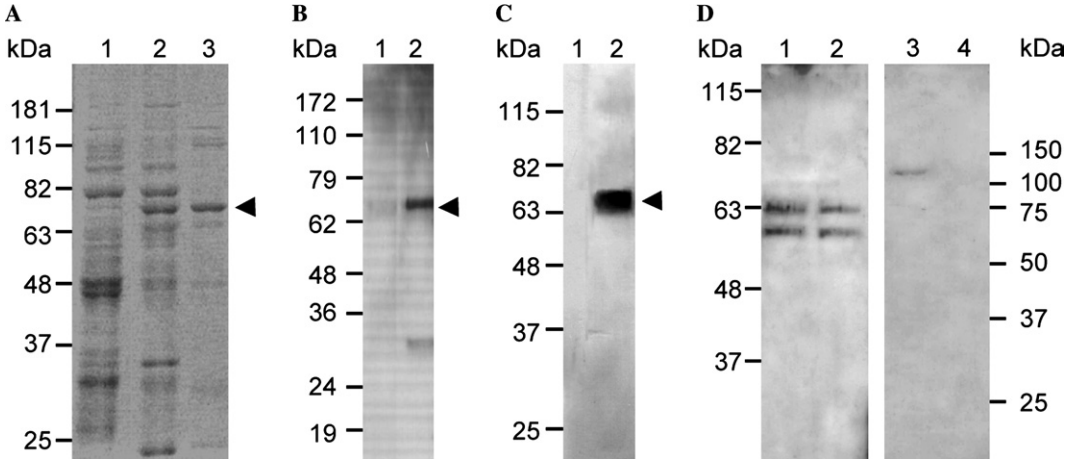

Fig. 2. SDS–PAGE and immunoblot analysis of EhPAP. (A) SDS–PAGE (10%) of rEhPAP-His 6x tag protein expressed in *E. coli.* Lane 1, non-induced *E. coli* lysate; lane 2, IPTG induced *E. coli* lysate; lane 3, purified rEhPAP. (B) Western blot of purified rEhPAP using anti-His antibodies. Lane 1, non-induced *E. coli* lysate; lane 2, rEhPAP. (C) Western blot of purified rEhPAP using a anti-rEhPAP serum. Lane 1, non-induced *E. coli* lysate; lane 2, rEhPAP. (D) Subcellular location of EhPAP using anti-rEhPAP serum (lanes 1 and 2). Lanes 1 and 3, CE; lanes 2 and 4, NE. Mab8 antibodies were used as control (lanes 3 and 4). Arrow, rEhPAP.

3.4. Expression of EhPap mRNA in drug-resistant clone C2 trophozoites

Previously, we demonstrated that *EhPgp5* mRNA half life and poly(A) tail length are progressively augmented in multidrug-resistant clone C2 trophozoites growing in the presence of increasing concentration of emetine (López-Camarillo et al., 2003). To gain insights into the possible role of EhPAP in these events, we measured *EhPap* mRNA levels by semi-quantitative RT-PCR assays using total RNA obtained from clone C2 trophozoites growing with various emetine concentrations. *Actin* mRNA was also amplified as a control. As shown in Fig. 3A, *EhPap* mRNA expression varies in a drug concentration dependent way. *EhPap* mRNA was weakly expressed in the absence of drug (C2(0)), corresponding to about 14% of *actin* mRNA expression (Fig. 3B). Interestingly, *EhPap* mRNA expression was augmented in the presence of emetine, being about 1.5- and 1.9-fold higher in C2(90) and C2(225) trophozoites, respectively (Figs. 3A and B). In contrast, *actin* gene expression remained almost constant (Fig. 3A). These results showed that emetine has some effects on the expression of *EhPap* mRNA, as well as on other factors involved in pre-mRNA 3′ end processing and polyadenylation (data not shown). We hypothesize that emetine induces a significant augmented EhPAP expression, allowing the synthesis of a longer *EhPgp5* mRNA poly(A) tail and therefore an increased *EhPgp5* mRNA stability in clone C2 trophozoites growing in increasing emetine concentrations. Therefore, the higher EhPAP expression could contribute to the MDR phenotype generation in drug-resistant trophozoites.

3.5. Expression of EhPap mRNA through cell cycle progression

It has been reported that the inactivation of nuclear PAP in mitosis is important for cell cycle progression in other organisms (Colgan et al., 1996; Zhao and Manley, 1998). To gain insights into the possible role of EhPAP in trophozoites division, we analyzed *EhPap* mRNA expression during cell cycle progression using total RNA obtained from clone L6 trophozoites arrested in M, G_1, S, and G_2 phases after colchicine treatment. Semi-quantitative RT-PCR assays showed that *EhPap* mRNA was mainly expressed in G_1 (corresponding to about 83% of *actin* mRNA expression) and S (56%) phases. Intriguingly, *EhPap* gene does not seem to be expressed in G_2 and M phases (Figs. 3C and D). However, we cannot discard that *EhPap* mRNA expression in G_2 and M phases is too low to be detected through RT-PCR assays. In contrast, *actin* mRNA expression remained almost constant through cell cycle progression (Fig. 3C). Variation

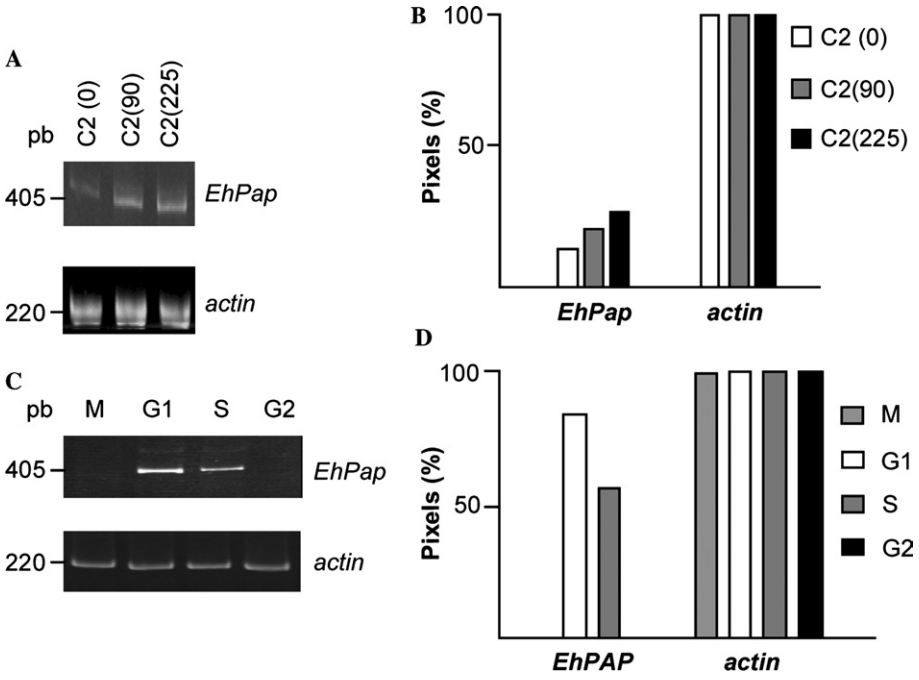

Fig. 3. EhPap mRNA expression analysis. (A and C) Semi-quantitative RT-PCR assays using *EhPap* internal primers and total RNA from clone C2 trophozoites growing without (C2 (0)), in 90 μM (C2 (90)) and 225 μM emetine (C2(225)) (A), or clone L-6 trophozoites synchronized by colchicine in M, G_1, S, and G_2 phases (C). *Actin* control is shown at the bottom. (B and D) Densitometric analysis of *EhPap* mRNA levels in clone C2 (B) and synchronized clone L-6 (D) trophozoites. Pixels corresponding to *actin* product were taken as 100% in each lane and *EhPap* product levels were expressed with respect to *actin* amount in each lane.

of *EhPap* mRNA levels suggests that EhPAP expression and functions could be regulated through cell cycle progression. It also suggests that EhPAP could play a regulatory role in this cellular process. In vertebrates, PAP is inactivated in mitosis through phosphorylation allowing an efficient cell cycle progression (Colgan et al., 1996; Zhao and Manley, 1998). Although we detected the presence of putative phosphorylation sites in EhPAP, these results suggest that the absence of EhPAP expression in M phase could be a regulatory event in *E. histolytica* cell cycle progression, probably by reducing the polyadenylated mRNA population and protein synthesis.

Based on our in silico studies, the EhPAP reported here appears to be a bona fide canonical nuclear PAP, having the well conserved aspartate residues of the PAP catalytic core region as well as the RBD. This suggest that EhPAP could have the same functions and properties as higher eukaryotes PAPs. Further studies currently in progress will help us to determine if both nuclear and cytoplasmic forms are functional and to elucidate the exact role of EhPAP in *E. histolytica* MDR phenotype and cell cycle regulation.

Acknowledgments

Preliminary *EhPap* gene sequence data were obtained from TIGR and Sanger *E. histolytica* genome sequence projects databases. The *E. histolytica* sequencing effort is part of the Genome Sequencing Project and is supported by awards from The National Institute of Allergy and Infectious Diseases, National Institute of Health, and from The Wellcome Trust. This work was supported by CGPI-IPN (20040056) and CONACyT-México (39888-M). The authors thank Mr. Alfredo Padilla-Barberi for artwork.

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