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Epigenetic and classical activation of *Entamoeba histolytica* heat shock protein 100 (EHsp100) expression

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Abstract The protozoan parasite *Entamoeba histolytica* expresses a cytosine-5 DNA methyltransferase (Ehmeth) that belongs to the DNMT2 protein family. The biological function of members of this DNMT2 family is unknown. In the present study, the 5′ region of *E. histolytica* heat shock protein 100 (5′*EHsp100*) was isolated by affinity chromatography with 5-methylcytosine antibodies as ligand. The methylation status of 5′*EHsp100* was confirmed by sodium bisulfite sequencing. We showed that the expression of *EHsp100* was induced by heat shock, 5-azacytidine (5-AzaC), an inhibitor of DNA methyltransferase and Trichostatin A (TSA), an inhibitor of histone deacetylase. The effect of TSA on *EHsp100* expression was rapidly reversed by removing the drug from the culture. In contrast, EHsp100 expression was still detectable one month after removing 5-AzaC from the media. Whereas 5-AzaC and TSA caused demethylation in the promoter region of *EHsp100*, no demethylation was observed following heat shock. Remarkably, DNA that includes three putative heat shock elements identified in the promoter region of *EHsp100* bound to a protein of 37 kDa present in the nuclear fraction of heat-shocked trophozoites but absent in the nuclear fraction of 5-AzaC and TSA treated trophozoites. Our data suggest that *EHsp100* expression can be regulated by both a classical and an epigenetic mechanism.

Keywords: Heat shock protein 100; DNA methylation; Histone deacetylase; DNA methyltransferase; *Entamoeba histolytica*

1. Introduction

Entamoeba histolytica is a gastrointestinal protozoan parasite that poses a serious health problem, with 50 million annual infections throughout the world [1]. The amoebic trophozoites normally reside in the human large bowel and occasionally invade the intestinal mucosa, disseminating to other organs, mainly the liver [2,3].

In higher eukaryotes, DNA methylation regulates a number of important biological functions including chromatin structure [4], silencing of gene expression [5], parental imprinting and chromosome X inactivation in females [6], and development and protection from selfish genetic elements [7]. Methylation occurs in cytosine C5 at the CG sequences and around 60–90% of CG sequences are methylated. Methylation of CG sites in the promoter regions of genes usually leads to a reduction of gene expression. Knowledge of the role of DNA methylation in protozoa is scanty. In the ciliate *Stylonychia lemnae* de novo cytosine methylation occurs in transposon-like elements in the course of macronuclear differentiation [8]. We recently provided evidences of 5-methyl cytosine (m5C) in the protozoan parasite *E. histolytica* and of an active DNA methyltransferase (Ehmeth) that belongs to the DNA methyltransferase 2 (DNMT2) family [9]. This m5C-MTase family lacks the large N-terminal regulatory domain present in the maintenance DNA methyltransferase (DNMT1) and in 'de novo' methylases (DNMT3a, DNMT3b) [10,11]. Human DNMT2 and *Drosophila melanogaster* DNMT2 are catalytically active and cytosine present in at CpT/A dinucleotides is their primary target of methylation in contrast to CpG dinucleotide for DNMT1 and DNMT3 [12–14]. Downregulation of *D. melanogaster* DNMT2 (dDNMT2) by RNA interference (RNAi) had no effect on the fly embryonic development and resulted in a complete loss of DNA methylation [12]. Overexpression of dDNMT2 in the fruit fly leads to an extended life span [15]. DNMT2 deficient mouse embryonic stem cells are viable and do not show any obvious difference in the DNA methylation pattern [16].

The identification of genes targeted by DNA methylation catalyzed by members of the DNMT2 protein family represents the first step towards understanding their biological functions. We previously described in *E. histolytica*, the presence of methylated cytosine within the 3′-end of EhMRS2, a DNA that includes a scaffold/matrix attachment region (S/MAR) [17] and within Ribosomal DNA (rDNA) [9]. In this study, we performed affinity chromatography using m5C antibodies as ligand on *E. histolytica* genomic DNA depleted of rDNA. One of the putative methylated DNA identified by this approach encodes a homolog of heat shock protein 100 (Hsp100). Hsp100/Clp family proteins are molecular chaperones involved in the desegregation of proteins from aggregates. They employ an ATP-dependent protein unfoldase activity to solubilize protein aggregates or to target specific classes of proteins for degradation [18,19]. Hsp100/Clp proteins have been involved in virulence of *Listeria monocytogenes* [20] and *Leishmania donovani* [21]. The presence of m5C in the promoter region of *E. histolytica Hsp100* (*EHsp100*) provides an opportunity to investigate the effects that methylation and other potential regulators have on *EHsp100* expression. In this study, we showed that *Ehsp100* expression was

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induced by heat shock and by drugs that inhibit DNA methyltransferase and histone deacetylase activities.Whereas heat shock activation was associated with the binding of a 37 kDa nuclear protein to the promoter region of *EHsp100*, 5-Azacytidine and Trichostatin A activation followed a distinct mechanism.

2. Materials and methods

2.1. Parasite culture conditions

Trophozoites of the *E. histolytica* strain HM1:IMSS were grown under axenic conditions in Diamond's TYI-S- 33 medium [22] at 37 °C. Trophozoites in log phase of growth were used in all experiments.

2.2. Exposure to 5-Azacytidine (5-AzaC), Trichostatin A (TSA) and heat-shock treatment

Trophozoites were grown in presence of 5-AzaC (23 μM) for one week. This concentration of 5-AzaC does not significantly interfere with the growth of the parasite and it induces a major demethylation in the genomic DNA of trophozoites [9]. Trophozoites were grown in presence of TSA (65 nM) for 48 h. When 5-AzaC and TSA were used in combination, trophozoites were grown in presence of both drugs for 72 h. For heat-shock treatment, trophozoites in Diamond's TYI-S-33 medium were incubated at 45 °C for one hour before preparation of total RNA or protein lysate. This temperature was chosen as the optimal temperature that allows the detection of EHsp100 expression by both RT-PCR and Western blot analysis with our anti-EHsp100 antibody. Following one hour of exposure at 45 °C the viability of *E. histolytica* measured by the exclusion of trypan blue by viable cells is 65% (data not shown). Control trophozoites for the experiments with 5-AzaC or TSA are grown in absence of the drug. Control trophozoites for the experiments with heat shock are incubated at 37 °C for one hour.

2.3. Affinity chromatography using m5C antibodies as ligand

E. histolytica genomic DNA (7 μg) was cleaved with I-*Ppo* I (Promega) to linearize rDNA [23] and the rDNA was then physically separated from genomic DNA by long run electrophoresis on 0.6% agarose gel. Genomic DNA was then purified from the agarose gel using DNA isolation kit (Biological Industries, Beit Haemek). DNA Affinity chromatography using m5C antibodies as ligand was performed on this genomic DNA according to [9].

2.4. Sodium bisulfite reaction and strand specific PCR

Genomic DNA free of RNA contamination was prepared with the DNAeasy tissue kit (QIAGEN) according to the manufacturer's instructions. Sodium bisulfite treatment of *E. histolytica* genomic DNA was performed according to the method described by [24]. Primers (Table 1) used to amplify 5′EHsp100 following bisulfite treatment are EHsp1005′ and EHsp3′bis and EHsp5′bis and EHsp3′bis for EHsp100 promoter region. A synthetic DNA target encoding Gag of *Drosophila* [25] was mixed with *E. histolytica* genomic DNA and used as an internal control for the bisulfite reaction. The complete deamination of cytosines in Gag was a prerequisite for the analysis of EHsp100.

2.5. RT-PCR analysis

Total RNA was prepared from trophozoites using a TRI-reagent solution (Sigma). Reverse transcription was performed with the EZ-First Strand cDNA Synthesis Kit for RT-PCR (Biological Industries) according to the manufacturer's instructions. Primers (Table 1) used to amplify EHsp100 were HSP100s-cDNA and HSP100as-cDNA and rDNA5′ and rDNA3′ for *E. histolytica* rDNA. Direct sequencing of the PCR product was performed to confirm the specificity of the reaction.

2.6. Amplification and cloning of EHsp100

General molecular biology techniques were used according to Sambrook et al. [26]. *EHsp100* (locus 192.m00086 in *E. histolytica* TIGR Genome Database (http://www.tigr.org/tdb/e2k1/eha1/)) was amplified from genomic DNA with the primers rEHsp100s and rEHsp100as (Table 1) and cloned in the pGEM-T vector (Promega) to give the pGEM-EHsp100 vector. A recombinant EHsp100 (from amino acid 1 to 392) was prepared from the prokaryotic expression vector system PGEX-4T-1 (Pharmacia Biotech). This vector allows the expression of a protein fused to a GST tag. *EHsp100* was amplified by PCR from the plasmid pGEM-EHsp100 with the primers EHspEcoRI and SP6 (Table 1). The PCR product was digested with *Eco*RI and *Not*I and cloned into the PGEX-4T-1 plasmid previously linearized with *Eco*RI and *Not*I. Verification of the proper ligations and orientations in the resulting hybrid plasmids was performed by digestion with restriction endonucleases. One of the vectors that carry *EHsp100* fused to the GST tag in the correct orientation (PGEX-4T-1–*EHsp100*) was sequenced to confirm that no mutation have been introduced into EHsp100 during the construction.

2.7. Expression and purification of recombinant N-terminal part of EHsp100

For expression of GST-EHsp100, *E. coli* BL-21 (DE3) transfected with the PGEX-4T-1–*EHsp100* vector were grown overnight in Luria–Broth (LB) medium containing ampicillin (100 μg/ml). The preculture was used to inoculate (1:100) 2× YT medium supplemented with ampicillin (100 μg/ml) and grown for about 3 h at 30 °C until OD_{600} reached 0.8. Synthesis of the fusion protein was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM to the growing culture. After 3 h of incubation at 30 °C in presence of IPTG, the bacteria were harvested and lysed in BugBuster protein extraction reagent (Novagen). The recombinant GST-EHsp100 protein was purified under native conditions on gluthatione–agarose resin (Sigma). The protein was then eluted with glutathion elution buffer (Tris–HCl 50 mM, pH 8.0, glutathione (Sigma) 10 mM). The recombinant GST-EHsp100 protein was quantitated by Bradford's method [27].

Table 1
Primers used in this study

Primers	Location	Primer sequence 5′-3′	Direction	Underlined restriction sites
rEHsp100s	1[a]	atggatcaaaacaaatggacagatgc	Sense	
rEHsp100as	1176[a]	ctaaataattgccttgtcaggaagaa	Antisense	
EHspEcoRI	1[a]	ggggaattcatggatccaaacaaatgg	Sense	*Eco*RI
EHsp5′bis	17313[b]	atgaataagaaagtgtgaataatag	Sense	
EHsp1005′	17372[b]	tgagtatttaaaggaacttgaag	Sense	
EHsp3′bis	372[a]	aacattaattccactatttcctacta	Antisense	
HSP100s-cDNA	619	agaattgttaaaggggatgtt	Sense	
HSP100as-cDNA	846	gataacattggttttaaaata	Antisense	
rDNA5′	5925	atggtgaacaatcatacctt	Sense	
rDNA3′	5744	ttatcggatgtgtgagccc	Antisense	
SP6		gatttaggtgacactatag		

The position of primers used to amplify rDNA is defined according to their location in GenBank (Accession No. X65163).
[a]The position of the primers used to amplify EHsp100 ORF is defined according to their location in the locus 192.m00086.
[b]The position of the primers used to amplify EHsp100 promoter region is defined according to their location in the contig TIGR 317325.

2.8. Preparation of a polyclonal anti-EHsp100 antibody

GST-EHsp100 (0.5 mg) was emulsified in 1 ml of complete Freund's Adjuvant (Sigma) completed to 1.5 ml with phosphate buffer saline (PBS). The mixture was injected subcutaneously to a 2 kg rabbit. Injection of GST-EHsp100 (0.2 mg) emulsified with 1 ml of incomplete Freund's adjuvant (Sigma) and completed with PBS to 1.5 ml was repeated 3 more times at 4-week intervals. Two days prior to each injection, a blood sample was taken from the rabbit in order to check the level of antibodies produced against the GST-EHsp100 (anti-EHsp100 antibodies). The serum used for the different experiments was obtained from the third bleeding of the rabbit. EHsp100 antibody was purified by affinity chromatography on a column with immobilized protein A. The concentration of EHsp100 antibody after purification was 8.9 μg/μl. Immunoblot analysis showed that this antibody (1/2000) reacted specifically with GST-EHsp100 (10 ng) (data not shown). In addition, BlastP analysis of the EHsp100 fragment injected to the rabbits showed that it matched EHsp100 only without sharing any partial homology with others HSP present in the parasite (data not shown).

2.9. Western hybridization

E. histolytica cytoplasmic and crude nuclear extract were prepared according to [28], separated on 8% polyacrylamide SDS–PAGE gel and transferred to nitrocellulose membrane. Blots were hybridized with anti-EHsp100 antibody (1/2000). After incubation with the first antibody, the blots were subjected to interaction with an HRP-conjugated anti-rabbit antibody (1/10000) (Jackson) and developed by enhanced chemi-luminiscence (Lumilight, Roche, Germany).

2.10. Microscopic localization of EHsp100 in trophozoites

To record the distribution of EHsp100 in trophozoites, trophozoites (10^6 cells/ml) were washed in PBS buffer, resuspended in cold acetone for 10 s, and washed twice with PBS buffer. Fixed trophozoites were incubated for one hour in 1.5% normal goat serum in PBS, reacted with anti-EHsp100 antibody (1:300) for one hour, washed with PBS and reacted for one hour with anti rabbit/goat Ig fluorescein 5(6)-isothiocyanate (FITC) labeled antibody (Sigma) diluted 1:100. Trophozoites were then washed with PBS and, to observe the nuclei, they were reacted with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Sigma). A stock solution of DAPI (1 mg/ml in ethanol) was prepared at a concentration of 5 μg/ml in 50:50 EtOH/0.1 M HCl. DAPI staining was performed by pipetting 5 μl into each sample (200 μl), which was then incubated for 5 min at RT. Phase contrast and fluorescent images were taken using an Axioscop2 (Zeiss) epifluorescence microscope with a 63 × /1.25 Plan Neofluar oil immersion objective and a differential interference contrast filter. Images were captured using a CCD camera and computed with ImagePro@Plus software (Media Cyberneticx, USA).

2.11. Southwestern analysis of EHsp100 DNA binding to proteins in the nuclear fraction of heat-shocked trophozoites

E. histolytica proteins separated on SDS–PAGE were transferred to nitrocellulose membrane and analyzed by Southwestern hybridization with a radiolabeled *EHsp100* probe (0.03 μg/ml), in standard binding buffer (20 mM Tris–HCl, pH 8.0; 50 mM NaCl; 1 mM EDTA) in presence of unlabeled sheared salmon sperm DNA (labeled: unlabeled ratio 1:100) which was used as non-specific competitor DNA. This probe that includes three putative heat shock elements present in the promoter region of *EHsp100* was amplified from *E. histolytica* genomic DNA with primers EHsp5′bis and EHsp3′bis and its sequence was checked before usage. Non-labeled *EHsp100* DNA was used as specific competitor DNA (labeled:unlabeled ratio 1:50). Following overnight incubation at 4 °C, the membrane was washed 3 times in standard binding buffer and exposed to X ray film.

3. Results

3.1. Characterization of the methylation status of EHsp100

We recently developed a new technique, based on affinity chromatography of methylated DNA to 5-methylcytosine antibodies, to identify methylated DNA in *E. histolytica* [9]. We showed that rDNA which is on an episome with a copy number of ~200 per genome is an abundant sequence targeted for methylation in the parasite. To identify other putative methylated sequences in *E. histolytica*, we prepared genomic DNA of *E. histolytica* relatively free of rDNA circles by linearizing the rDNA circles with I-*Ppo*I and then physically separating the linearized rDNA from genomic DNA by long run electrophoresis on 0.6% agarose gel. A similar approach was used to decrease the rDNA background in the genome project of *E. histolytica* [23]. The genomic DNA of *E. histolytica* relatively free of rDNA circles was digested with *Dpn*II, bound to adaptors, and loaded on the anti-m5C affinity chromatography column. DNA purified by the column was amplified using the adaptors as primers, cloned in pGEM-T-easy vector, and 20 clones were sequenced. The sequences isolated by affinity chromatography were compared to sequences in the *E. histolytica* TIGR Genome Database (http://www.tigr.org/tdb/e2k1/eha1/) by Blast search. None of them matched rDNA which confirms the efficiency of the treatment to subtract rDNA. Among these sequences was a reverse transcriptase of LINE element (manuscript submitted) and a homolog of Heat Shock protein 100 that we called EHsp100. This sequence (from nucleotide 4 to 533 in locus 192.m00086) is localized in the 5′ region of *EHsp100* (5′*EHsp100*). To confirm the presence of methylated cytosine residues in 5′*EHsp100*, we run a bisulfite reaction followed by a strand specific PCR. This procedure converts all cytosine residues to uracil, giving rise to thymine after amplification by PCR. Only methylated cytosines are refractory to the deamination. 5′*EHsp100* was amplified from sodium bisulfite treated genomic DNA with primer EHsp1005′ and EHsp3′bis, cloned into pGEM-T easy vector and sequenced. The inability of the bisulfite treatment to replace cytosines with thymines demonstrates the presence of methylated cytosines in 5′*EHsp100* (Fig. 1A). As previously described for *E. histolytica* methylation was not restricted to CG sites [9,29].

3.2. EHsp100 expression is induced by heat shock, 5-azaC and TSA

We further tested whether by changing the epigenetic status of the chromatin, we could induce *EHsp100* expression. *EHsp100* expression was studied by semi quantitative RT PCR and Western blot analysis using a specific anti-EHsp100 antibody in trophozoites exposed to heat shock, 5-AzaC and TSA. 5-AzaC is an inhibitor of DNA methyltransferase that induces a major demethylation in the genomic DNA of trophozoites [9]. Histone deacetylation in transcriptionally inactive region is an epigenetic mechanism that complements cytosine methylation in regulation of gene expression [30]. TSA is a specific inhibitor of histone deacetylase that inhibits *E. histolytica* histone deacetylase activity [31]. *EHsp100* transcript was detected by RT PCR in heat-shocked, 5-AzaC and TSA treated trophozoites (Fig. 2A) but no amplification was observed in control trophozoites. A number of studies report the synergistic effect of 5-AzaC and TSA on the expression of epigenetically silenced genes [32–34]. This is apparently not the case for *EHsp100* expression as treatment of trophozoites with both drugs did not show any significant synergistic effect (data not shown). Immunoblot analysis shows that anti-EHsp100 antibody recognizes a band of 100 kDa in the nuclear fractions of trophozoites exposed to heat shock but not in their cytoplasmic fractions (Fig. 2B). No band was

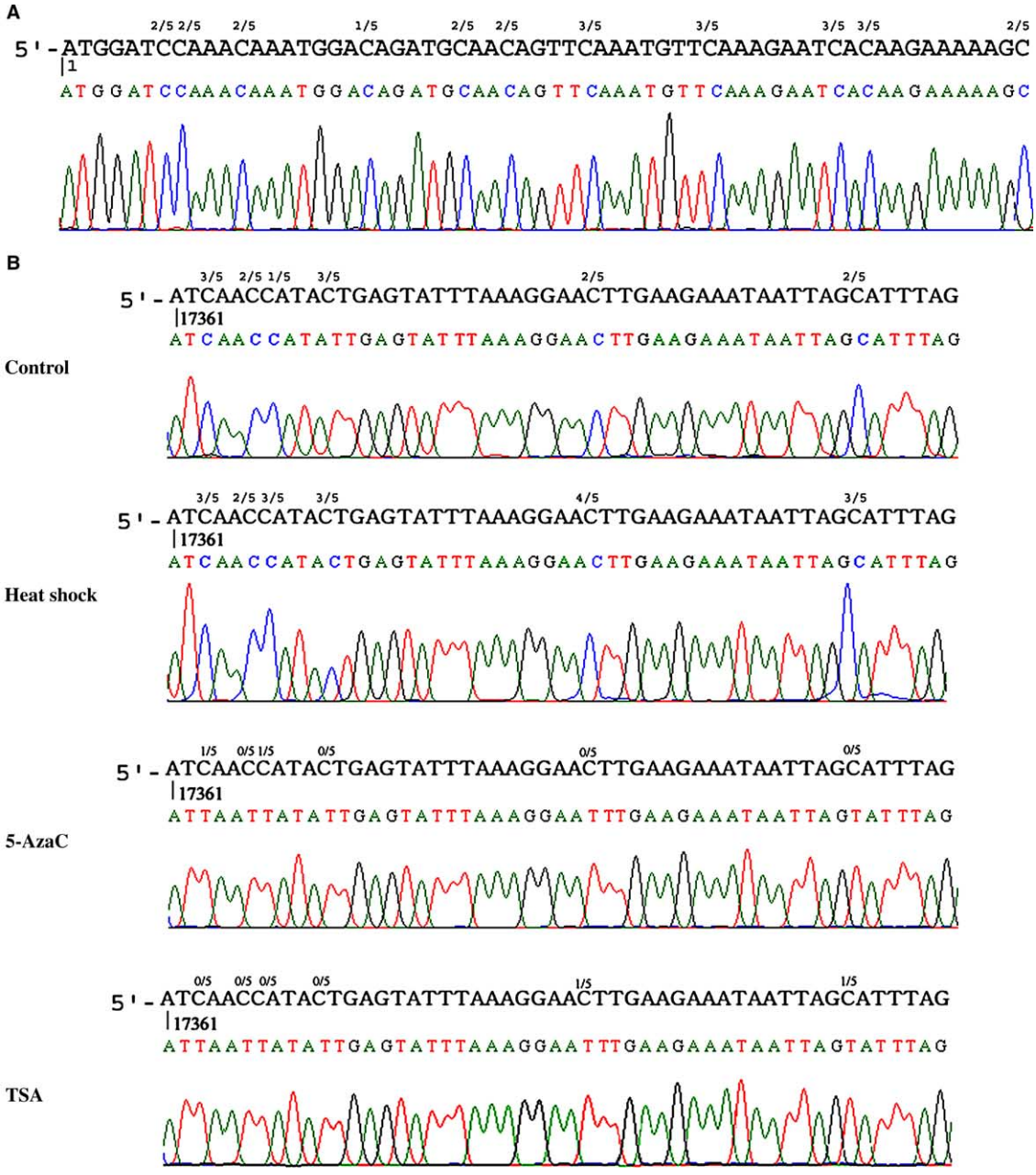

Fig. 1. Genomic sodium bisulfite analysis sequencing of 5′ *EHsp100*. (A) Nucleotide sequence of *EHsp100* (locus 192.m00086 from nucleotide 1) is shown above the chromatogram. Numbers above C residues indicate their methylation status in five independent clones. The chromatogram represent an example of nucleotide sequence of 5′EHsp100 following bisulfite treatment. (B) Nucleotide sequence of *EHsp100* putative promoter region (contig 317325 from nucleotide 17361) is shown above the chromatogram. Numbers above C residues indicate their methylation status in five independent clones. The chromatograms represent an example of nucleotide sequence of EHsp100 promoter region following bisulfite treatment in control, heat-shocked (one hour at 45 °C), 5-AzaC treated trophozoites (23 μM for one week) and trophozoites grown with TSA (65 nM) for 48 h.

detectable in the cytoplasmic and nuclear fractions of control trophozoites (Fig. 2B). These results show that EHsp100 is a nuclear protein and its expression is induced by heat shock. The nuclear localization of EHsp100 was confirmed by immunofluorescence microscopy using anti-EHsp100 antibody (Fig. 2C). Computer-assisted image overlay analysis of the signal given by EHsp100 and by DAPI, a specific nuclear stain, shows that EHsp100 is concentrated in the nuclei of heat-shocked trophozoites. No stain is observed in control trophozoites (data not shown).

In addition to the expected expression of EHsp100 under heat shock, we observed that anti-EHsp100 antibody recognizes a band of 100 kDa in the nuclear fractions of trophozoites treated with 5-AzaC and TSA (Fig. 2B).

3.3. Reversibility of TSA treatment compared to 5-AzaC treatment

The presence of methylated cytosine in the promoter region of a gene is correlated with its silent expression state (for review see [35]). The primers EHsp5′bis and EHsp3′bis were

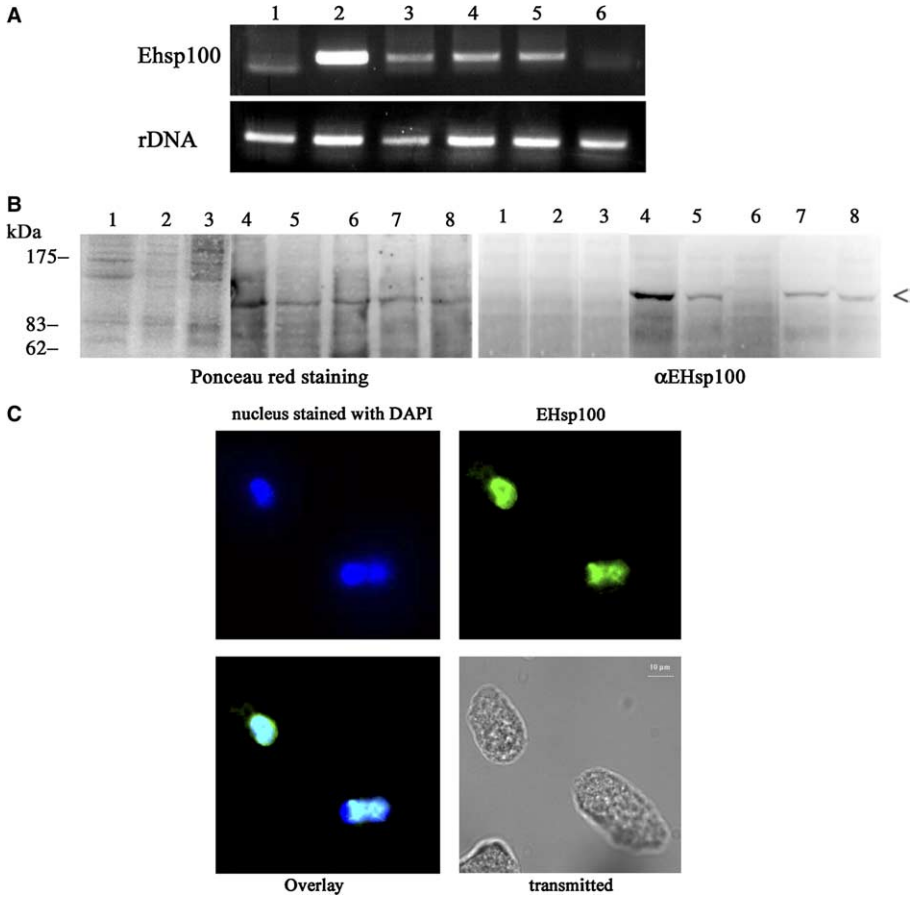

Fig. 2. Expression of EHsp100 in heat-shocked, 5-AzaC and TSA treated trophozoites. (A) RT-PCR analysis was performed on mRNA isolated from control (lane 1), heat-shocked trophozoites (lane 2), 5-AzaC treated trophozoites (lane 3), TSA treated trophozoites (lane 4), trophozoites grown with 5-AzaC for one week followed by one month of culture without 5-AzaC (lane 5), trophozoites grown with TSA for 48 h followed by 72 h of culture without TSA (lane 6). The primers used to amplify EHsp100 and rDNA are described in Table 1. (B) Western blot analysis of EHsp100 expression in trophozoites treated with 5-AZAC and TSA. Lane 1, cytoplasmic fraction of control trophozoites; lane 2, nuclear fraction of control trophozoites; lane 3, cytoplasmic fraction of heat-shocked trophozoites; lane 4, nuclear fraction of heat-shocked trophozoites; lane 5, nuclear fraction of TSA-treated trophozoites; lane 6, nuclear fraction of trophozoites grown with TSA for 48 h followed by 72 h of culture without TSA; lane 7, nuclear fraction of 5-AzaC treated trophozoites; lane 8, nuclear fraction of trophozoites grown with 5-AzaC for one week followed by one month of culture without 5-AzaC. Left panel is a Ponceau red staining of the nitrocellulose membrane. Right panel, Western blot analysis with anti-EHsp100 sera (1/2000). 40 μg of proteins was applied on each lane. (C) Cellular localization of EHsp100 in heat-shocked *E. histolytica* trophozoites by immunofluorescence microscopy. EHsp100 was detected by immunofluorescence microscopy using anti-EHsp100 antibody. EHsp100 distribution is shown in green using a primary anti-EHsp100 antibody and a secondary antibody conjugated with FITC. Nuclei (blue) were stained by DAPI. Computer-assisted image overlay analysis of the signal given by EHsp100 antibody and by DAPI shows that EHsp100 is concentrated in the nuclei of trophozoites.

used to include part of the promoter region of *EHsp100* in the bisulfite analysis. We looked at the methylation status of *EHsp100* promoter region in both heat-shocked and 5-AzaC treated trophozoites by bisulfite analysis (Fig. 1B). Whereas no significant demethylation was detected between control and heat-shocked trophozoites, a significant demethylation of the *EHsp100* promoter region was observed in 5-AzaC treated trophozoites. The demethylated state of *EHsp100* promoter region was conserved in trophozoites cultivated for one month following the removal of 5-AzaC from the culture (data not shown). In addition, no significant decrease in the expression of *EHsp100* was observed in these trophozoites compared to 5-AzaC treated trophozoites (Fig. 2A and B).

The expression of *EHsp100* is induced in trophozoites grown with TSA (65 nM) (Fig. 2A and B). Trophozoites exposed to TSA for 48 h and then grown for 72 h without the drug had the expression of *EHsp100* turned off (Fig. 2A and B). In addition to its ability to inhibit histone deacetylase, TSA causes selective loss of DNA methylation in *Neurospora* [36]. To determine if an identical phenomenon also occurs in *E. histolytica*, we looked at the methylation status of the promoter region of *EHsp100* in trophozoites grown with TSA. Bisulfite analysis showed a significant demethylation of the promoter region of *EHsp100* compared to the control trophozoites (Fig. 1B).

These results indicate that in addition to the expected mechanism of Ehsp100 activation by heat shock, there is an epigenetic mechanism based on DNA methylation and histone deacetylase activity that controls EHsp100 expression.

3.4. Identification by Southwestern blot analysis of proteins interacting with the promoter region of EHsp100

The heat shock factor (HSF) protein family is involved in heat shock protein gene regulation [37]. Six putative HSFs have been annotated in the *E. histolytica* genome project database (Accession Nos.: XP_655877, XP_655405, XP_654362,

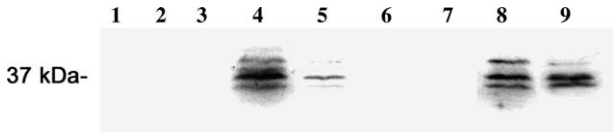

Fig. 3. Southwestern blot analysis with radiolabeled 5′EHsp100 DNA. Lane 1, cytoplasmic fraction of control trophozoites; lane 2, nuclear fraction of control trophozoites; lane 3, cytoplasmic fraction of heat-shocked trophozoites; lane 4, nuclear fraction of heat-shock trophozoites; lane 5, nuclear fraction of heat-shocked trophozoites incubated with radiolabeled 5′*EHsp100* DNA and unlabeled 5′*EHsp100* DNA (labeled:unlabeled ration 1:50); lane 6, nuclear fraction of trophozoites grown with 5-AzaC; lane 7, nuclear fraction of trophozoites grown with TSA; lane 8, nuclear fraction of trophozoites grown with 5-AzaC followed by heat shock; lane 9, nuclear fraction of trophozoites grown with TSA followed by heat shock. Sheared salmon sperm DNA (labeled:unlabeled ration 1:100) was used systematically as non-specific competitor DNA for all the assays. Identical results were obtained in three independent experiments.

XP_652580, XP_651351, XP_650767) but nothing is known about their specificity and how they regulate HSPs expression. In eukaryotes, HSF binds as homotrimer to its regulatory site, the heat-shock element (HSE), composed of inverted repeats of the consensus 5-bp sequence 5′-nGAAn-3′ [38,39]. The presence of putative HSEs in EHsp100 promoter region (contig TIGR 317325 between nucleotides 17329 and 17336; 17346 and 17353; 17385 and 17393) suggests that HSF could interact with this DNA. Southwestern hybridization showed interaction between EHsp100 promoter region with a 37 kDa protein present in the nuclear fraction of trophozoites exposed to heat shock (Fig. 3). This protein could correspond to the only HSF with a size of 37 kDa present in the genome of *E. histolytica* (Accession No. XP_652580).

The binding to the 37 kDa protein is inhibited by unlabeled *EHsp100* DNA. In contrast no protein interacts with the radiolabeled probe in nuclear and cytoplasmic fraction of control; 5-AzaC and TSA treated trophozoites (Fig. 3). Interestingly, the presence of the 37 kDa protein in the nuclear fraction of trophozoites grown with 5-AzaC or TSA and further exposed to heat shock indicates that the classical pathway is still functional in these trophozoites. Indeed, this result confirms the presence of two parallels mechanisms that control EHsp100 expression.

4. Discussion

Ehmeth is a 5-cytosine methyltransferase responsible for genome methylation in *E. histolytica* [9]. Ehmeth belongs to DNMT2, a very intriguing family of DNA methyltransferase of unknown biological function. Our recent studies showed that a putative role for Ehmeth is the control of repetitive elements including rDNA and S/MAR elements [9,17]. In an effort to characterize other genes targeted by DNA methylation, we used m5C affinity chromatography on *E. histolytica* genomic DNA depleted of rDNA. One of the putative methylated DNA identified by this approach encodes a homolog of Hsp100. Clp/Hsp100 chaperones are ATP-dependent protein unfolding machines [19,40,41]. They are important components of the protein quality control system and integral parts of the regulatory arsenal controlling the intracellular levels of global regulatory proteins [42,43]. We observed that EHsp100 has a nuclear localization by both microscopy and protein fractionation. This result was unexpected as Hsp100 proteins have been localized for other eukaryotes in the cytoplasm [21], in mitochondria [44] or in both the cytosol and the nucleus [45]. Therefore, EHsp100 could have evolved in *E. histolytica* to specifically restore and/or repair heat-induced nuclear alterations.

EHsp100 is induced by heat shock and its expression is correlated with the presence of a 37 kDa protein in the nuclear fraction that binds to EHsp100 upstream region. This result strongly suggests that under heat shock the expression of EHsp100 is regulated by a classical pathway that involves the binding of HSF to HSE. This HSF could correspond to the only HSF with a size of 37 kDa present in the genome of *E. histolytica* (Accession No. XP_652580).

EHsp100 transcript can be detected less than 10 min following exposure of the trophozoites to heat shock (data not shown). The rapidity of this response is incompatible with a mechanism of regulation that involves DNA demethylation. In agreement with this remark, we observed that EHsp100 expression under heat shock occurs without demethylation of the 5′EHsp100 region. The presence of m5c in the 5′ region of EHsp100 and its expression induced by 5-AzaC suggest that EHsp100 expression is also regulated by an epigenetic mechanism. This mechanism is distinct from the classical mechanism of regulation as no protein interacting with 5′EHsp100 has been detected in the nucleus extract of trophozoites grown with 5-AzaC and TSA. Information about epigenetic regulation of Hsp expression is scanty. DNA methylation is involved in the silencing of H11 Hsp, an eukaryotic homolog of herpes simplex virus protein [46] and Hsp47 in human neuroblastoma [47]. In both cases, the expression of these Hsps is reduced in tumor cells by DNA methylation and their reactivation by 5-AzaC lead to apoptosis [46] or inhibition of cells growth [47]. Histone acetylation is associated with transcriptional activation of hsp22 in fly [48], hsp70 in fly [49] and hsp70 in *Xenopus* [50]. DNA methylation silence genes either by directly inhibiting the interaction of transcription factors with their regulatory sequences [51,52] or by attracting methylated DNA-binding proteins, which in turn recruit histone deacetylases and histone methyltransferases, resulting in a inactive chromatin structure [53,54]. An additional model of genes silencing based on recent evidence from *Neurospora crassa* and *Arabidopsis thaliana* has recently emerged. In this model deacetylation of histones followed by methylation of the lysine residue K9 in histone protein H3 can direct DNA methylation [36,55,56]. The lack of classical methylated DNA-binding proteins in *E. histolytica* (according to our bioinformatics analysis; data not shown), the reversibility of TSA treatment compared to 5-AzaC treatment and above all the demethylation of EHsp100 promoter region induced by TSA treatment strongly suggest that histones modifications occur upstream to DNA methylation in the EHsp100 promoter region.

The dual regulation of gene expression by both heat shock and DNA demethylation is not unique to our study. Alu element, a short interspersed repeated sequence that belongs to transposable element, is strongly methylated and its expression is also induced by both demethylating agents like 5-AzaC [57] and heat shock [58]. Although the increased accessibility of chromatin following induction is involved in the expression of Alu [58], the precise mechanism that leads to Alu expression under various conditions is far to be understood. Heat shock

proteins are expressed during the differentiation of a number of protozoan parasites including *E. histolytica* [59], *Giardia lamblia* [60] and *Toxoplasma gondii* [61]. The epigenetic regulation of EHsp100 expression may serve during the differentiation of the parasite whereas an unexpected and stringent stress requires the quick expression of EHsp100 that allows the classical pathway.

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