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Application of SHAPE reveals *in vivo* RNA folding under normal and growth-stressed conditions in the human parasite *Entamoeba histolytica*

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ABSTRACT

Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) is a versatile sequence independent method to probe RNA structure *in vivo* and *in vitro*. It has so far been tried mainly with model organisms. We show that cells of *Entamoeba histolytica*, a protozoan parasite of humans are hyper-sensitive to the *in vivo* SHAPE reagent, NAI, and show rapid loss of viability and RNA integrity. We optimized treatment conditions with 5.8S rRNA and Eh_U3 snoRNA to obtain NAI-modification while retaining RNA integrity. The modification patterns were highly reproducible. The *in vivo* folding was different from *in vitro* and correlated well with known interactions of 5.8S rRNA with proteins *in vivo*. The Eh_U3 snoRNA also showed many differences in its *in vivo* versus *in vitro* folding, which correlated with conserved interactions of this RNA with 18S rRNA and 5′-ETS. Further, Eh_U3 snoRNA obtained from serum-starved cells showed an open 3′-hinge structure, indicating disruption of 5′-ETS interaction. This could contribute to the observed slow processing of pre-rRNA in starved cells. Our work shows the applicability of SHAPE to study *in vivo* RNA folding in a parasite and will encourage the use of this reagent for RNA structure analysis in other such organisms.

1. Introduction

RNA is capable of folding into discrete structures which are critical for its biological functions. In addition to protein-coding RNAs, a large plethora of noncoding RNAs exist whose structure-function relationships are yet to be explored [1]. Knowledge of *in vivo* RNA structure is extremely useful to understand its possible cellular functions and regulatory roles. Various chemical and enzymatic probing methods have been used to obtain structural information of RNA [2–4] .The most commonly used method involves treatment with dimethyl sulphate (DMS) which methylates the base-pairing faces of A and C nucleotides of RNA, preferentially in non hydrogen-bonded regions like loops and bulges [5]. Enzymatic probing uses enzymes with varied specificity for single- or double-stranded RNA, and structure is inferred from the cleavage pattern generated by these enzymes [6]. These methods do not give structural information for each nucleotide. Moreover, although DMS can be used for probing RNA structure in living cells [5] the enzymatic methods can only be used for *in vitro* probing. More recently, sequence-independent reagents that have high reactivity with 2′-hydroxyl on the ribose sugar, (with no bias of purine or pyrimidine), in single-stranded or flexible RNA regions have been developed for chemical probing. The reagent-accessible site is detected by reverse transcription (RT) stops at the modified nucleotide. The method is called selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE). It commonly uses a variety of reagents some of which can penetrate nuclei of living cells, and are used *in vivo* [2,7–9]. Recently many new reagents are being developed with different reaction kinetics to study SHAPE reactivity, and the method is being coupled with high-throughput sequencing to obtain transcriptome-level information of RNA structure *in vivo* [10–12]. The method has been shown to work in model organisms, including *Escherichia coli, Saccharomyces cerevisiae, Drosophila melanogaster,* mammalian cells, and *Arabidopsis thaliana* [7,13]. SHAPE technology has great potential to explore the dynamic changes in RNA structure under different conditions of growth, differentiation, or host challenge. We wished to see if it could be applied to a parasitic protist *Entamoeba histolytica* which is an early branching eukaryote and a human pathogen [14]. Our lab has been working on ribosome biogenesis and pre-rRNA processing in *E. histolytica*. Pre-rRNA is co-transcriptionally processed and chemically modified into the mature rRNAs through precise endonucleolytic cleavages in the

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external- and internal- transcribed spacers (ETS and ITS respectively) [15]. These cleavages require the U3 snoRNA, which along with a large number of protein components forms a ribonucleoprotein complex called small subunit (SSU) processosome [16]. U3 is a non-canonical C/D box-containing snoRNA which performs chemical modifications of rRNAs, and has the specialized function of assisting in pre-rRNA processing and maturation [17]. It interacts with pre-rRNA by base pairing at specific sites within the 5′-ETS and the pre-18S rRNA to mediate the processing reactions at sites A0, A1 and A2, and release the mature 18S rRNA [18]. Previously we identified the snoRNAs of *E. histolytica*, including Eh_U3 snoRNA from genome sequence analysis [19], and showed that the sequence features typical of U3 snoRNAs were well conserved in *E. histolytica* when compared with yeast and human. We also studied the folding pattern of Eh_U3 snoRNA by *in vitro* SHAPE [20]. It showed the predicted two domains connected by a hinge sequence. The conserved boxes (GAC, boxes A and A') were located in Domain I, while boxes C', B, C and D were conserved motifs of Domain II. The structure is in close agreement with accepted solution structure of yeast U3 snoRNA [21].

We have earlier reported that, unlike model organisms, transcription of pre-rRNA and ribosomal protein genes continued in growth-stressed *E. histolytica* cells. The control of ribosomal biogenesis appeared to be post-transcriptional, as unprocessed pre-rRNA accumulated in these cells [22], and translation of r-protein mRNAs was down regulated [23]. The translational down regulation was controlled by the 5′-UTR of r-protein gene which is typically very short in *E. histolytica*. Such regulatory transitions may be accompanied by changes in RNA structure which could be determined by *in vivo* chemical probing. We applied *in vivo* SHAPE to study the folding of Eh_U3 snoRNA and 5.8S rRNAs during normal and serum-starved conditions. Here we show that the *in vivo* folding of Eh_U3 snoRNA and 5.8S rRNA is significantly different from that *in vitro*, and correlates with the known interactions of these RNAs with other molecules *in vivo*. Eh_U3 snoRNA also displayed a more accessible 3′-hinge structure in serum-starved cells which could contribute to the pre-rRNA processing defect. Our data establish that *in vivo* SHAPE is applicable to study RNA folding in *E. histolytica*.

2. Materials and methods

2.1. Cell culture and growth conditions

Trophozoites of *E. histolytica* strain HM-1:IMSS were axenically maintained in TYI-S-33 medium supplemented with 15% adult bovine serum (Biological Industries Israel), at 35.5 °C [24]. For serum starvation, mid log phase trophozoites were collected by centrifugation and resuspended in medium containing 0.5% serum [23].

2.2. Measurement of cell viability

Cell viability was determined by trypan blue assay. 0.1 ml of 0.4% trypan blue was added to 0.1 ml cells at room temperature for 2 min and cells were observed under the microscope at 20X magnification. Live and dead cells were counted (by trypan blue uptake) in triplicates using a hemocytometer (Neubauer, Marienfeld, Germany).

2.3. In vitro transcription

Linear DNA templates containing the sequences of U3 snoRNA and 5.8S rRNA were PCR amplified from genomic DNA of *E. histolytica* (Primers for U3 snoRNA: F-5′-TAGACCGTACTCTTAGGATC-3′ and R-5′-ATAGTCAGACACCCTAACATCAC-3′ and for 5.8SrRNA: F-5′-GAATA TTACTTTGGATAGTTTAG-3′ and R-5′-TGGATATTGAATATCAT ATAT ACCTTGAAG-3′). T7 promoter sequence was included in the forward primer and amplicons were cloned in PGEMT-easy vector. 500 ng of purified template was *in vitro* transcribed using RiboMAX™ (Promega) according to manufacturer's instructions.

2.4. Primer extension

The oligo nts were end labeled using γ-P^{32})-ATP (30 µci). DNase I (Roche)-treated total RNA (8 µg–12 µg) was used for primer extension with end-labeled oligo nts (supplementary Table 1). Reverse transcription was carried out using superscript III (Invitrogen) at 52 °C according to manufacturer's instructions.

2.5. In vitro SHAPE assay [25,26]

In vitro transcribed RNA (16 µg) in 16 µl was denatured at 95 °C for 3mins and chilled in ice-water. 8 µl of folding buffer (333 mM HEPES at pH 8.0, 33.3 mM MgCl2, 333 mM NaCl) was added to it and incubated at 37 °C for 20 mins. For SHAPE modification RNA was incubated with 50 mM NAI, or 130 mM NMIA (Sigma-Aldrich) in DMSO for 45 min at 37 °C, ethanol precipitated and resuspended in 8 µl nuclease free water. It was denatured at 95 °C for 3 mins, and chilled in ice for 3mins before primer extension.

2.6. In vivo SHAPE [7]

2.6.1. Synthesis of NAI

137 mg of (1 mmol) 2-methylnicotinic acid (Sigma) was dissolved in 0.5 ml anhydrous DMSO. 162 mg of (1 mmol) 1,1′-carbonyldiimidazole (Sigma) in 0.5 ml anhydrous DMSO was added dropwise in 2-methylnicotinic acid. The resulting solution was stirred at room temperature for 1hr. The final solution was used as a 1.0 M stock solution containing a 1:1 mixture of the desired compound and imidazole. The solution was frozen at −80 °C.

2.6.2. Acylation of RNA in E. histolytica cells

E. histolytica cells were centrifuged at 500*g* for 5 min. Cells ($\sim 2.5 \times 10^7$) were washed with PBS and resuspended in PBS, DMSO, or NAI in DMSO. After the desired time, cells were immediately centrifuged and supernatant decanted. To the pellet, 1 ml of TRIzol LS (Ambion, Inc.) was added. RNA was purified following TRIzol LS manufacturer's instructions.

2.7. Data processing and analysis

The SHAPE data were processed as described [13]. The gel images were produced using a Typhoon phosphorimager 5000 (GE Healthcare). The intensity of bands in the gel for (+) and (−) lanes were quantified by two-dimensional densitometry using Image Quant TL plus 7.0 image analysis software (GE healthcare). The nucleotide identity of each band was identified from dideoxy sequencing lanes. Normalized SHAPE reactivity was generated based on the 2/8% rule [13]. The top 2% of the most reactive nucleotide intensities were designated as outliers and removed from the pool for averaging. The next 8% of most reactive nucleotide intensities were averaged, and all nucleotide intensities, including the outliers, were divided by this average value to obtain normalized SHAPE reactivity. The normalized SHAPE reactivity was then plotted for further analysis

3. Results

3.1. Optimization of conditions for applying SHAPE to study in vivo RNA folding in E. histolytica

The SHAPE technique to study *in vivo* RNA folding has so far been used mainly in model systems like human and mouse cell lines, *Drosophila*, yeast, *E. coli*, and *Arabidopsis* [7,13]. Its use in less studied organisms is not extensively reported. The technique has not yet been applied to any parasitic protist, and the susceptibility of these