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Post-transcriptional regulation of ribosomal protein genes during serum starvation in *Entamoeba histolytica*

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ABSTRACT

Ribosome synthesis involves all three RNA polymerases which are co-ordinately regulated to produce equimolar amounts of rRNAs and ribosomal proteins (RPs). Unlike model organisms where transcription of rRNA and RP genes slows down during stress, in *E. histolytica* rDNA transcription continues but pre-rRNA processing slows down and unprocessed pre-rRNA accumulates during serum starvation. To investigate the regulation of RP genes under stress we measured transcription of six selected RP genes from the small- and large-ribosomal subunits (RPS6, RPS3, RPS19, RPL5, RPL26, RPL30) representing the early-, mid-, and late-stages of ribosomal assembly. Transcripts of these genes persisted in growth-stressed cells. Expression of luciferase reporter under the control of two RP genes (RPS19 and RPL30) was studied during serum starvation and upon serum replenishment. Although luciferase transcript levels remained unchanged during starvation, luciferase activity steadily declined to 7.8% and 15% of control cells, respectively. After serum replenishment the activity increased to normal levels, suggesting post-transcriptional regulation of these genes. Mutations in the sequence −2 to −9 upstream of AUG in the RPL30 gene resulted in the phenotype expected of post-transcriptional regulation. Transcription of luciferase reporter was unaffected in this mutant, and luciferase activity did not decline during serum starvation, showing that this sequence is required to repress translation of RPL30 mRNA, and mutations in this region relieve repression. Our data show that during serum starvation *E. histolytica* blocks ribosome biogenesis post-transcriptionally by inhibiting pre-rRNA processing on the one hand, and the translation of RP mRNAs on the other.

1. Introduction

Ribosome biogenesis in any cell type is a highly energy consuming process. It requires the coordinated regulation of all three RNA polymerases (Pol I, II, and III) to produce equimolar amounts of the four rRNAs (18S, 5.8S, 28S and 5S rRNAs), ~80 ribosomal proteins (RPs) and more than 200 additional proteins [1,2]. In most model organisms rDNA transcription ceases under conditions of growth stress, as the requirement for ribosomes goes down [3,4]. We find that this is not the case in the early branching protist *Entamoeba*. We have earlier shown that in *Entamoeba histolytica*, a human parasite, rRNA transcription continues during serum starvation but the processing of pre-rRNA slows down, and unprocessed pre-rRNA accumulates to high levels [5]. In *Entamoeba invadens*, a reptilian parasite, when trophozoites are transferred to low glucose medium to induce cyst formation, unprocessed pre-rRNAs accumulate to high levels in the cyst, along with the RP gene transcripts [6,7]. Thus *Entamoeba* has evolved somewhat different mechanisms to regulate ribosome biogenesis during cellular growth stress and differentiation.

In model organisms there is crosstalk between the transcription machinery of RP and rRNA genes so that both may be coordinately regulated during growth stress [8,9]. Studies with *Saccharomyces cervisiae* have demonstrated a number of mechanisms leading to coordinate regulation of RP and rRNA genes [10]. One of them is mediated by the transcription factors: Rap1, Fhl1, and Ifh1 needed for RP gene transcription. Ifh1 is part of a complex with CK2, Utp22 and Rrp7 (CURI). Of these Utp22 and Rrp7 are essential for pre-rRNA processing. During unfavourable growth conditions when rRNA synthesis slows down, free Utp22 and Rrp7 sequester CK2

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and Ifh1 in the CURI complex, and non-availability of Ifh1 slows down RP gene transcription. Conversely, depletion of Utp22 and Rrp7 results in increased levels of RP mRNA [11]. Another possible mechanism could be through the high mobility group (HMG) protein, Hmo1 (high mobility group protein1), which binds both to promoters of RP genes and to the rRNA gene locus [12]. The target of rapamycin (TOR) pathway which controls cell growth and proliferation in response to environmental signals could also contribute to the cross talk. In *S. cerevisiae* it has been shown that this pathway regulates H3K56 (histone H3 lysine56) acetylation which, in turn, regulates the binding of Hmo1 to rDNA [13].

Coordinate regulation of rRNA and RP gene expression has not been studied in parasites. Since unprocessed pre-rRNA accumulates in *E. histolytica* during serum starvation we were interested to know whether RP mRNAs also accumulate under the same conditions, and whether these mRNAs are translated. Here we show that RP mRNAs persist during starvation but their translation is inhibited by sequences in the 5′ untranslated region (UTR).

2. Materials and methods

2.1. Cell culture and growth conditions

Trophozoites of *E. histolytica* strain HM-1: IMSS (clone 6) were axenically maintained in TYI-S-33 medium supplemented with 15% adult bovine serum (PAA laboratories, Austria), Diamond's Vitamin Tween 80 solution (Sigma–Aldrich) and antibiotics (0.3 units/ml penicillin and 0.25 mg/ml streptomycin) at 35.5 °C. For serum starvation, media from mid log phase grown trophozoites were replaced with TYI-S-33 medium containing 0.5% adult bovine serum for indicated time period. Replenishment was achieved by decanting total media after indicated time period and filled with complete TYI-S-33 medium for indicated time periods. G-418 (Sigma) was added at 10 μg/ml for maintaining the transfected cell lines [14].

2.2. RNA isolation and northern hybridization

E. histolytica trophozoites were grown for 48 h and transferred to low-serum medium for serum starvation. Cells were removed at different time points. Total RNA from $\sim 5 \times 10^6$ cells was purified using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Poly A+RNA was isolated using poly A Tract mRNA isolation system (Promega) as per manufacturer's protocol. For northern analysis 10–15 μg of total RNA was resolved on 1.2% formaldehyde agarose gel in gel running buffer [0.1 M MOPS (pH 7.0), 40 mM sodium acetate, 5 mM EDTA (pH 8.0)] and 37% formaldehyde at 4 V/cm. The RNA was transferred on to Gene-Screen plus R membrane (PerkinElmer). α-P^{32}dATP labeled probe was prepared by random priming method using NEBlot kit (NEB). Hybridization and washing conditions for RNA blots were as per manufacturer instructions.

2.3. Luciferase reporter construct and stable transfection

Upstream sequence (900 bp) of RPS19 and RPL30 genes were amplified from genomic DNA of *E. histolytica* by PCR using primers AJF and AJR2, and ALF and ALR1 (all primers used in this study are listed in Supplementary Table 1). Primers ALF4 and ALR1 were used for amplifying the wild type (244 bp) upstream sequence of RPL30 gene for luciferase expression. To mutate the motif1 (Mut 1) an amplicon of 219 bp was obtained with primers ALF4 and ALR2 M and an amplicon of 151 bp was obtained with primers ALF5 and ALR which overlap by 27 bp. (Primers ALR2 M and ALF5 contained the mutated sequence). The two amplicons were stitched and a 344 bp fragment was obtained with ALF4 and ALR primers. Finally, using this fragment as a template, a 244 bp fragment was amplified by using primers ALF4 and ALR1. A schematic description is given in supplementary Fig. S3. To mutate the motif 3 (Mut 2) of RPL30, a 244 bp fragment was amplified by using primers ALF4 and ALR3 ACC (latter contained the mutated motif 3). To mutate the 5′ UTR motif (Mut 3), a 244 bp fragment was amplified by using primers ALF4 and ALR MUT (latter contained mutated 5′ UTR). Constructs were cloned upstream of LUC gene at *Xho*I/*Acc*651 site in pEh-NEO-LUC vector. Constructs were transfected by electroporation and maintained in presence of G418 at 10 μg/ml [14].

2.4. Luciferase assay

This was done as described previously [15]. Briefly, stably transfected trophozoites, maintained in TYI-S-33 medium supplemented with 10 μg/ml G-418, were chilled on ice, harvested and washed twice in 1XPBS (pH 7.4), and lysed in 200 μl of reporter lysis buffer (Promega) with the addition of protease inhibitors E64-C and leupeptin. Lysates were frozen overnight at −80 °C. After thawing on ice for 10 min, cellular debris was pelleted, and the samples were allowed to warm to room temperature. Luciferase activity was measured according to the manufacturer's instructions (Promega) using a Turner Luminometer (model TD-20E). Luciferase activity per microgram of protein was calculated as a measure of reporter gene expression.

2.5. Primer extension

DNase I (Roche)-treated PolyA + RNA (2 μg) was used for primer extension with end labeled oligonucleotides (listed in Supplementary Table 1). Reverse transcription was carried out using superscript III (Invitrogen) at 45 °C according to manufacturer's instructions. Sequencing reaction was run on 6% denaturing polyacrylamide gel. The gel image was produced using a Typhoon phosphor imager (GE Healthcare).

2.6. Search for the motifs in upstream region of RP genes

The online tool MEME [16] was used for prediction of the motifs present in upstream sequence. 100 bp of sequence upstream of the RP's were extracted from *E. histolytica* HM-I:IMSS genome and were analysed for the motifs. The input consisted of 188 FASTA formatted sequences of RPs with the default settings of width (minimum six and maximum 20) and the search was optimized for identifying zero or one motif for sequence and motif searched for given strand only. For control we used 150 bp upstream sequence to CDS of all *E. histolytica* HM-I:IMSS 8333 genes using Perl-coding. The motifs were searched in these sequences using CisFinder with default parameters except the search was done for forward strand only and maximum number of motif to find were set as 100 (minimum for CisFinder).

3. Results

3.1. Ribosomal protein transcripts persist during serum starvation.

Previous work had shown that unprocessed pre-rRNA accumulates in *E. histolytica* cells subjected to growth stress due to serum starvation [5]. We were interested to investigate whether transcripts of RP genes also persist during serum starvation in *E. histolytica*.

The small (40S) and large (60S) ribosomal subunits are individually made by precise assembly of RPs and rRNAs. The synthesis and turnover of RPs is highly controlled to generate equimolar amount of all RPs [17,18]. We selected three genes each from the small