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Brief note

An UPF3-based nonsense-mediated decay in *Paramecium*

Julia Contreras, Victoria Begley, Sandra Macias, Eduardo Villalobo*

Departamento de Microbiología, Universidad de Sevilla, Av Reina Mercedes 6, 41012 Seville, Spain

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Abstract

Nonsense-mediated decay recognises mRNAs containing premature termination codons. One of its components, UPF3, is a molecular link bridging through its binding to the exon junction complex nonsense-mediated decay and splicing. In protists UPF3 has not been identified yet. We report that *Paramecium tetraurelia* bears an *UPF3* gene and that it has a role in nonsense-mediated decay. Interestingly, the identified UPF3 has not conserved the essential amino acids required to bind the exon junction complex. Though, our data indicates that this ciliate bears genes coding for core proteins of the exon junction complex.

Keywords: Exon junction complex; Nonsense-mediated decay; *Paramecium tetraurelia*; Premature termination codon; Splicing; *UPF3*

1. Introduction

Messenger RNA is under the surveillance of diverse quality-control pathways [1], one of them being Nonsense-Mediated Decay (NMD). NMD recognises and rapidly degrades mRNAs containing a Premature Termination Codon (PTC). NMD core involves in most eukaryotes a set of three proteins: UPF1, UPF2, and UPF3 [2,3]. UPF1 is an RNA helicase; UPF2 binds both UPF1 and UPF3; UPF3 binds UPF2 and the Exon Junction Complex (EJC), which deposits on the mRNA during and after splicing [4]. PTC recognition by NMD can be independent or dependent on the EJC [2,3]. In the latter case, UPF3/EJC binding constitutes a molecular nexus between NMD and splicing [5], since miss-splicing is a potential source of PTC-containing mRNA (PTC-mRNA). PTC-mRNA is degraded via the exonuclease XRN1, and the exosome [2,3].

Despite the importance of NMD in eukaryotes, little is known in protists. *UPF1* has been identified and characterised in *Trypanosoma brucei* [6], *Giardia lamblia* [7], and *Paramecium tetraurelia* [8]. *UPF2* has been identified and characterised in *T. brucei* [6] and *P. tetraurelia* [8]. The search for *UPF3* homologues in *T. brucei* and *G. lamblia* failed [6,7]. Maybe, these latter protists do not need a nexus between NMD and splicing because they are intron-less organisms. We undertook this study due to the lack of information on *UPF3* in intron-bearing protists. We chose *P. tetraurelia* since this ciliate contains roughly 90,000 introns and more importantly, some degree of miss-splicing: intron retention [8].

We report here that *P. tetraurelia* bears an *UPF3* gene, as expected. Our results indicate UPF3 is involved in NMD in this ciliate. We provide data indicating that the identified UPF3 has not evolutionary conserved the essential amino acids implicated in EJC binding. However, we show this ciliate possesses the genetic information needed to assemble an EJC.

2. Materials and methods

2.1. Cultures

P. tetraurelia strain d4-2 was grown at 27 °C in buffered wheat grass medium inoculated with *Escherichia coli* HT115

* Corresponding author.
E-mail addresses: juli_bio_87@hotmail.com (J. Contreras), vsbegley@gmail.com (V. Begley), sanmacben@hotmail.es (S. Macias), evpolo@us.es (E. Villalobo).

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and supplemented with 0.4 μg/ml β-sitosterol. *E. coli* strain (HT115) was grown in LB or LB agar supplemented with 10 μg/ml tetracycline. Whenever required, ampicillin, X-Gal, and IPTG were added to LB media. For silencing by the feeding method [9], paramecia were grown for three days in silencing medium, which was inoculated with HT115 *PtUPPF3*::pL4440 and induced by IPTG. Control (unsilenced) paramecia were grown for three days in control medium, which was inoculated with HT115-*PtUPF3*::pL4440. Cease of the silencing condition, was achieved by simply feeding the silenced culture for three extra days with medium (no IPTG) inoculated with HT115-*PtUPF3*::pL4440. For paramecium number determination, nine cells were collected, transferred to depression slides (three cells each depression), and grown at 27 °C in the appropriate media. After 24 h, cells were counted under a dissecting microscope.

Actinomycin D (AMD) was dissolved in DMSO. Both unsilenced and silenced cultures were treated with 1 μg/ml AMD for 1, 2, and 3 h.

2.2. Molecular biology methods

To obtain genomic DNA, paramecia were sedimented and lysed in 10 mM Tris pH 8, 5 mM EDTA, 100 mM NaCl and 1% SDS. Proteins were eliminated by the addition of proteinase K and treatment with a phenol-chloroform-isoamyl alcohol mixture buffered at pH 8. Nucleic acids were precipitated with ethanol in the presence of salts. Contaminant RNA was removed by treating with RNase in EDTA-containing buffer. To obtain total RNA, paramecia were sedimented and lysed by adding TriReagent LS and 1-bromo-3-chloropropane. The RNA-containing aqueous phase was further purified submitting the whole phase volume to silica column, including the in-column DNase-treatment step and according to supplier's instructions (Direct-zol RNA Miniprep, ZymoResearch). To obtain cDNA, a reverse transcription reaction was carried out according to supplier's instructions (Transcriptor First Strand cDNA Synthesis Kit, Roche) using 1 μg of total RNA.

Semi-quantitative amplifications were performed in 25-μl PCR reactions, using pre-mixed master strips (DNA AmpliGel Master Mix-Strips, Biotools) in order to avoid repeated pipetting. Different amounts of template cDNA, annealing temperatures (see primers in Table 1), and PCR cycles were done depending on gene targets. For intron-containing gene targets, the forward primer was always designed within the intron sequence (see Fig. S1).

DNA insert (*PtUPF3*) was ligated to pL4440 (kindly provided by Andrew Fire, Carnegie Institution of Washington, Baltimore, Maryland, USA) using T4 Ligase (Promega), according to supplier's directions. Bacterial (HT115) DNA transformation was done by heat shock using purified *PtUPF3*::pL4440 plasmid. For DNA quantifications, DNAs were electrophoresed onto 3% low melting agarose. Equal loads were applied to each well.

2.3. Bioinformatics

Sequences were retrieved from GenBank, *Paramecium* Database, and *Saccharomyces* Genome Database. Multiple sequence alignment was done using the Muscle Tool at the EBI. Amino acid sequence was deduced using the Open Reading Frame Finder tool at NCBI. Pairwise alignment was done using the BlastP tool at NCBI. Protein domains were searched using sequence similarity by Hidden Markov Models at Pfam. Protein structure prediction was done using a template-based homology modelling using the Phyre2 server at the SBG.

For DNA quantifications, pictures were digitally taken by the Quantity One 1-D Analysis software (BioRad), avoiding pixel saturation and subtracting background. For each gene under study and sample, the trace quantity value was normalised against the trace quantity value of *PtND7* gene (Table 1) in the corresponding sample. *ND7* is transcribed as a PTC-free mRNA, therefore it can be considered as insensitive to any change in NMD. For silencing experiments ($n = 3$), quantification data (mRNA relative levels) was calculated as fold change between silenced and unsilenced samples. Values were expressed as mean ± standard deviation. For cease of silencing experiments ($n = 2$), mRNA relative levels were calculated separately, and attributing to the silenced data the arbitrary value of 1. For AMD treatment ($n = 2$), mRNA relative levels were calculated separately.

Statistical significance was evaluated with SPSS software. Data normality was evaluated by the Shapiro–Wilk's test. Equality of variances was evaluated by the Levene's test. Means were compared by the t-Student test, and differences were considered with statistical significance at P-values ≤ 0.05.

3. Results and discussion

The deduced amino acid sequence of the well-characterised *UPF3* gene of *Saccharomyces cerevisiae* was used as bait to identify a gene homologue in *P. tetraurelia* genomic database. After BlastP search, we obtained a hit (*Paramecium*DB accession number GSPATP00001393001), with an alignment score of 46 and an E-value below 10^{-5} that covered 44% of budding yeast amino acid sequence with a 41% of similarity. GSPATP00001393001, which has no ohnologues, showed 66.7% of identity with the highly conserved sequence KLVIRLLPP (yeast sequence), an RNA recognition motif that is distinctive of most UPF3s (Fig. 1). After BlastP search, GSPATP00001393001 deduced amino acid sequence hit with scores above 40 more than 65 sequences, among them that corresponding to *S. cerevisiae* UPF3 and two sequences respectively annotated as *Tetrahymena thermophila* and *Oxytricha trifallax* UPF3 (see Fig. S2 for details). Template-based homology modelling showed that 50% of GSPATP00001393001 modelled with 100% confidence to d1uw4 (Pfam entry), the template featuring the UPF3 family of proteins (not shown). UPF3 has no enzymatic activity but it is characterised by its ability to bind