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Upstream open reading frames regulate the cell cycle-dependent expression of the RNA helicase Rok1 in *Saccharomyces cerevisiae*

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ABSTRACT

The RNA helicase Rok1 plays a role in rRNA processing and in control of cell cycle progression in *Saccharomyces cerevisiae*. We identified two upstream open reading frames (uORFs) within the *ROK1* 5′ untranslated region, which inhibited Rok1 translation. Mutating uATG to uAAG or generation of a premature stop codon in the uORFs resulted in increased Rok1p levels. Rok1 protein levels oscillated during the cell cycle, declining at G1/S and increasing at G2. The *uAAG1* mutation caused a constitutive level of Rok1 proteins throughout the cell cycle, resulting in significant delays in mitotic bud emergence and recovery from pheromone arrest. Our study reveals that the Rok1 protein level is regulated by uORFs, which is critical in cell cycle progression.

1. Introduction

RNA helicases are a large family of proteins exhibiting both ATP hydrolysis and double-strand RNA unwinding activities [1,2]. It has become apparent that RNA helicases also displace proteins from RNA molecules and thereby remodel RNA–protein complexes [3,4]. RNA helicases play essential roles in many cellular processes involving RNA metabolism, including translation, pre-mRNA splicing, ribosome biogenesis, RNA transport, and RNA degradation.

Rok1 is a member of the DEAD-box RNA helicase family in *Saccharomyces cerevisiae* [5,6]. Proteins of this family are characterized by nine conserved motifs that include the amino acid sequence Asp-Glu-Ala-Asp (DEAD) [3,7]. The human DEAD-box family, comprised of 36 members, has been given the abbreviation *DDX*. *DDX52* is a Rok1 ortholog with 44% identity [8]. A number of reports have revealed that Rok1 is involved in controlling cell cycle progression and rRNA processing [5,9,10]. Depletion or overexpression of the Rok1 protein causes cell cycle arrest at the G1/S stage. Rok1 is essential for the 18S rRNA synthesis and the protein itself appears to be associated with the 40S pre-ribosomes [11].

The translational efficiency of eukaryotic mRNAs is often influenced by structural features of the 5′ untranslated region (UTR). Structural features of the 5′ UTR that can impact translational efficiency include length, secondary structure, and the presence of upstream open reading frames (uORFs) [12–15]. The contributions of uORFs to translational regulation have been described for a number of genes including *S. cerevisiae CLN3* and *GCN4*, mouse MOR, and human *bcl-2*, *BACE1*, and p27 [16–21]. In general, eukaryotic ribosomes scan for AUG codons and initiate translation at the proximal 5′ AUG codon according to the scanning model [12,13,22]. The presence of uORFs frequently decreases the translational efficiency of a downstream main ORF because these uORFs inhibit ribosome scanning for the downstream main AUG [15,23].

In this study, we showed that two uORFs present in the *ROK1* 5′ UTR were inhibitory to Rok1 translation. Mutations in uORFs resulted in increased Rok1p levels. Our results provide evidence that translational regulation of the DEAD-box RNA helicase Rok1 is critical for its functions in regulating cell cycle progression.

2. Materials and methods

2.1. Yeast strains and plasmids

The *S. cerevisiae* strains and plasmids used in this study are listed in Table S1. Integration of *ROK1-myc* or *uAAG1-ROK1* into yeast genome was performed as previously described [24]. Strain JK406 (*ROK1-myc*) was derived from JK147 by use of a 3 kilobase

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(-kb) ClaI fragment from pJI376, which contained the *URA3* gene inserted at the *ROK1* 5′ UTR as well as the myc-tagged N-terminal region of *ROK1*. *URA3* was removed by culturing in 5-FOA media. For construction of the *uAAG1* strain (JK407), a 1.7-kb URA3-uAAG1-ROK1-myc cassette was PCR amplified from plasmid pJI376 using primers R443 and R840 (Table S2). The resulting PCR product was transformed into the JK406 strain, and *URA3* was removed by culturing in 5-FOA media. The presence of the myc epitope tag or *uAAG1* mutation was confirmed by DNA sequence analysis of PCR amplified fragments.

2.2. Site-directed mutagenesis

To generate the *uAAG* and premature stop codon mutations, a set of four primers was designed for each mutation, and PCR mutagenesis was carried out as previously described [6]. To generate the *uAAG* mutations, the following primers were used as mutagenic primers: R442 and R443 for *uAAG1*, R444 and R445 for *uAAG2*, R775 and R776 for *uAAG1,2*. The final PCR products were used to replace the EcoRI-SalI fragment of pJI244 to generate pJI248, pJI249, and pJI250, respectively. To generate the uORF premature stop codon mutation, the following primers were used as mutagenic primers: R896 and R897 for *uATG1-S*, R922 and R923 for *uATG2-S*, R926 and R927 for *uATG1,2-S*. The presence of the mutations was confirmed by DNA sequence analysis.

2.3. Cell cycle analysis

Strains were grown to early-exponential phase in SC medium and then treated with 5 μM α-factor for 2 h to allow cell synchronization in G1 phase. Cells were subsequently washed twice with pre-warmed media and then resuspended in pre-warmed media to allow cell cycle progression. Budding index (N_{budded}/N_{total}) was measured as previously described [25]. Cells with visible buds were counted; for each case, at least 200 cells were counted. For evaluating budding index during release from stationary phase, cells were grown in SC media for 2 days (OD_{600} = 5.0) and then inoculated into fresh media to allow release from G1-arrest.

2.4. Primer extension

Total RNA was isolated with the RNeasy kit (QIAGEN). A *ROK1* gene-specific primer R19 (50 pmol) was end-labeled with 50 μCi of [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs) and hybridized to 50 μg of total RNA. Primer extension reactions were performed using 50 units of AMV-reverse transcriptase (Roche). Sequencing reaction was carried out using Sequenase (version 2.0).

2.5. mRNA quantification by real-time PCR

Total RNA was prepared with TRIzol (Invitrogen) according to the manufacturer's instruction. Residual DNA was removed by DNase I (RQ1 RNase-free DNase, Promega) treatment and cDNA synthesis was performed using oligo dT and TOPscript™ cDNA synthesis kit (Enzynomics). Quantification of the *ROK1* mRNA levels was performed by real-time PCR using SYBR green dye. All values were normalized to the level of *ACT1* mRNA. *ROK1* gene-specific primers (R1052 and R1053) and *ACT1* gene-specific primers (R1054 and R1055) were used to amplify a 92-bp fragment of the *ROK1* coding sequence and 100-bp fragment of the *ACT1* coding sequence, respectively.

3. Results

3.1. ROK1 5′ UTR contains two uORFs

Previous studies have shown that *ROK1* expression is regulated at the posttranscriptional level [26]. The *KEM1/XRN1* gene which encodes a multifunctional protein with a 5′–3′ exoribonuclease and microtubule-binding activities participates in this regulation [27–29]. When the 5′ upstream sequence of the *ROK1* ORF was

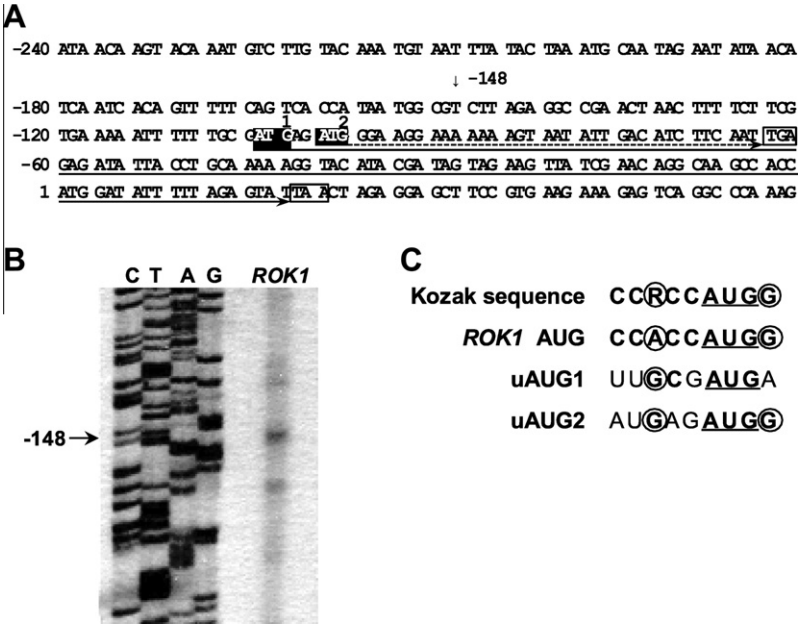

Fig. 1. Analysis of the *ROK1* 5′ UTR. (A) Structures of the *ROK1* 5′ UTR. The transcription start site is indicated by the arrow at −148. The uORF initiation and stop codons are indicated with boxes. uORF1 is underlined; uORF2 is dotted underlined. (B) Mapping the 5′ end of the *ROK1* transcript by primer extension. The arrow indicates the position of the extended product in the *ROK1* sequence. Two minor bands were non-specific because they were also detected in P_{GAL}-*ROK1* (pJI251, Fig. S1) (data not shown). (C) Sequence context of upstream and authentic *ROK1* AUGs as compared with the Kozak sequence. AUGs are underlined. Nucleotides that match the Kozak sequence are represented in bold. The most conserved nucleotides, R at −3 and G at +4, are shown in circles.