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# An exon three-way junction structure modulates splicing and degradation of the *SUS1* yeast pre-mRNA

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ABSTRACT

The *SUS1* gene of *Saccharomyces cerevisiae* is unusual as it contains two introns and undergoes alternative splicing, retaining one or both introns depending on growth conditions. The exon located between the two introns can be skipped during splicing and has been detected in circular form. This exon (E2) has also been found to influence the splicing of the flanking introns, an unusual situation in budding yeast where splicing mainly relies on intron recognition. Using SHAPE (selective 2′-hydroxyl acylation analyzed by primer extension), NMR spectroscopy, gel electrophoresis and UV thermal denaturation experiments combined with computational predictions, we show that E2 of *SUS1* comprises a conserved double-helical stem topped by a three-way junction. One of the hairpins emerging from the junction exhibited significant thermal stability and was capped by a purine-rich loop structurally related to the substrate loop of the VS ribozyme. Cellular assays revealed that three mutants containing altered E2 structures had impaired *SUS1* expression, and that a compensatory mutation restoring the conserved stem recovered expression to wild-type levels. Semi-quantitative RT-PCR measurements paralleled these results, and revealed that mutations in E2 altered splicing and transcript degradation processes. Thus, exon structure plays an important role in *SUS1* RNA metabolism.

## 1. Introduction

In eukaryotic cells, introns are eliminated from pre-mRNA molecules by the spliceosome, a dynamic multicomponent machine [1–4]. The process of alternative splicing is widespread in higher eukaryotes, where it is considered to be a main source of protein diversity [5, 6]. The pervasiveness of this mechanism has been firmly supported by transcriptome sequencing data [7, 8]. Regardless of whether it leads to functional protein isoforms, alternative splicing contributes to modulate gene expression [9, 10]. For example, regulated splicing has been shown to control the levels of key transcripts encoding proteins involved in gene expression pathways, so that the cell can respond to environmental changes *via* feed-back mechanisms [11]. In addition, the spliceosome generates circular RNA molecules (circRNA) through exon back-splicing. circRNAs are more commonly produced than initially thought, and may play important roles in gene regulation [12, 13].

Sus1 (ENY2 in mammals) is a small, evolutionary conserved 11-KDa protein involved in several processes of mRNA biogenesis [14]. In *Saccharomyces cerevisiae*, Sus1 interacts during transcription elongation with RNA polymerase II and the export factors Yra1 and Mex67 [15], and also accumulates at the nuclear pore, where it is part of the TREX-2 mRNA export complex [16, 17]. In addition, it participates in histone H2B deubiquitination as a component of the SAGA complex [16, 18].

Compared to higher eukaryotes, alternative splicing is rarer in *S. cerevisiae* cells. In this organism genes containing introns are scarce and typically contain only one intron with canonical 5′ and 3′ splice site (SS) and branch site (BS) sequences. In this context, the structure of the *SUS1* gene of *Saccharomyces cerevisiae* is remarkable: it contains two introns (Fig. 1), and the first intron (I1) exhibits non-canonical 5′SS and BS sequences [16, 19]. The possible functional role of the two introns of *SUS1* has been explored. In agreement with the presence of non-canonical 5′SS and BS, I1 is retained in > 15% of the *SUS1* transcripts, and growth conditions affect the degree of I1 retention [20, 21]. On the other hand, the second intron (I2) is efficiently spliced and forms a

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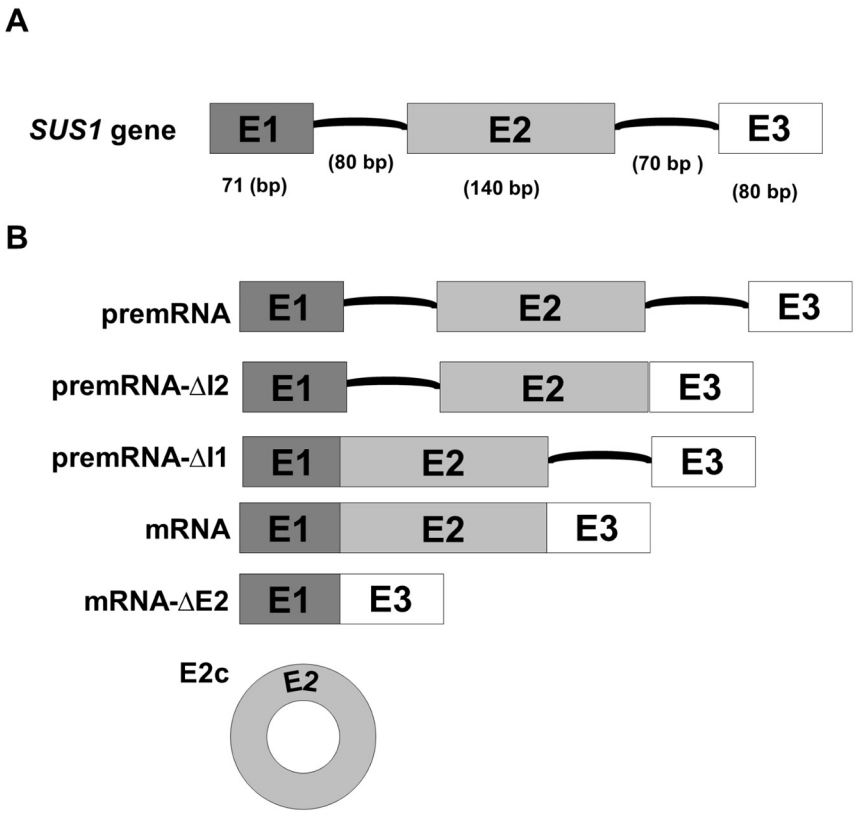


**Fig. 1.** *S. cerevisiae SUS1* gene structure and RNA transcripts. (A) Schematic representation of the gene. (B) *S. cerevisiae SUS1* RNA species detected so far: pre-mRNA, partly-spliced pre-mRNA lacking I2 or I1, fully-spliced mRNA, fully-spliced mRNA lacking E2, and circular E2 (E2c).

weakly stable stem-loop structure that increases the accessibility of the BS and 3′SS nucleotides (nt). Changes in this I2 hairpin structure were found to alter the patterns of Sus1 expression as well as *SUS1* splicing, giving rise to I1 retention and skipping of the second exon (E2) [22] (Fig. 1). Altogether, these findings suggest that the functions of Sus1 in mRNA biogenesis are modulated *via* splicing regulation.

During these studies it was detected that the sequence of *SUS1* E2 influenced the splicing of the flanking introns (I1 and I2) [20, 21]. This was also remarkable, as splicing in budding yeast relies on recognition by the spliceosome of conserved 5′ and 3′ SS and BS intronic sequences, and examples of modulation of this process by adjacent exon sequences are scarce [23]. In contrast, in higher eukaryotes the 5′ and 3′ SS are more degenerate, and splice site selection is carried out in conjunction with enhancer and silencer sequences located in introns and exons, which act as binding platforms for auxiliary proteins. The process of SS selection is also influenced by RNA structure in all eukaryotic organisms, which adds another layer of regulation. This is usually accomplished by modulation of the accessibility or spatial distribution of splicing signal sequences *via* base-pairing, but also through the presence of more complex folds like riboswitches and ribozymes [24–27]. In yeast, however, studies of the effect of RNA structure have focused on intronic sequences [28]. It was therefore relevant to evaluate whether a non-intronic sequence like *SUS1* E2 was structured and whether this structure had an impact on splicing, as this might compensate for the apparent absence of enhancer and silencer sequences in yeast pre-mRNA molecules. Our interest was also reinforced by the recent discovery that *SUS1* E2 of *Saccharomyces cerevisiae* is generated in circular form (E2c) [29] (Fig. 1). In this report we explore the role of E2 structure on *SUS1* metabolism by combining structural analyses with cellular assays evaluating the impact of mutant E2 sequences.

## 2. Materials and methods

### 2.1. Sequences

The sequences of the second exon of *SUS1* across different species of yeast were obtained from the Yeast Genome Database and NCBI (http://www.ncbi.nlm.nih.gov/). E2 RNA and protein sequence identity calculations were carried out with the SIAS web tool (http://imed.med.ucm.es/Tools/searches.html). E2 RNA and protein sequence alignments were accomplished with the T-COFFEE multiple sequence alignment web server [30]. Codon usage analyses were carried out with the CAIcal web server [31].

### 2.2. Secondary structure predictions

Secondary structure predictions were obtained with the Mfold [32], RNAfold [33], RNAStructure [34] and MC-Fold [35] web servers using default parameters. The structures were drawn using VARNA (http://varna.lri.fr/) [36]. We used the locARNA [37] web server to assess the conservation of the secondary structure formed by E2 across the seven yeast species containing two introns in the *SUS1* gene [20].

### 2.3. Preparation of RNA samples for experiments in vitro

The DNA template used for transcribing the full-length *S. cerevisiae SUS1* E2 RNA sequence used in SHAPE studies was obtained by PCR, using the primers indicated in Table S1 and a GPDp-*SUS1-CUP1* plasmid containing the wild-type *SUS1* sequence. The integrity of this DNA template was checked by sequencing. The 140-nt E2 RNA sequence was generated by T7-polymerase *in vitro* transcription and was flanked by 14- and 43-nt cassette sequences in its 5′ and 3′ sides, respectively [38]. The incorporation of these flanking sequences, designed to fold into stable hairpin structures, allowed evaluation of the