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SUS1 introns are required for efficient mRNA nuclear export in yeast

Bernardo Cuenca-Bono[1], Varinia García-Molinero[1], Pau Pascual-García[1], Hernan Dopazo[2], Ana Llopis[1], Josep Vilardell[3,4,*] and Susana Rodríguez-Navarro[1,*]

[1]Centro de Investigación Príncipe Felipe (CIPF), Gene Expression coupled to RNA Transport Laboratory, Av Saler 16. E-46012, Valencia, Spain, [2]Centro de Investigación Príncipe Felipe (CIPF), Bioinformatics and Genomics Unit, Av Saler 16. E-46012, Valencia, Spain, [3]Dept. Molecular Genomics, Institut de Biologia Molecular de Barcelona (IBMB), 08028 Barcelona and [4]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

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ABSTRACT

Efficient coupling between mRNA synthesis and export is essential for gene expression. Sus1/ENY2, a component of the SAGA and TREX-2 complexes, is involved in both transcription and mRNA export. While most yeast genes lack introns, we previously reported that yeast *SUS1* bears two. Here we show that this feature is evolutionarily conserved and critical for Sus1 function. We determine that while *SUS1* splicing is inefficient, it responds to cellular conditions, and intronic mutations either promoting or blocking splicing lead to defects in mRNA export and cell growth. Consistent with this, we find that an intron-less *SUS1* only partially rescues *sus1Δ* phenotypes. Remarkably, splicing of each *SUS1* intron is also affected by the presence of the other and by *SUS1* exonic sequences. Moreover, by following *SUS1* RNA and protein levels we establish that nonsense-mediated decay (NMD) pathway and the splicing factor Mud2 both play a role in *SUS1* expression. Our data (and those of the accompanying work by Hossain *et al.*) provide evidence of the involvement of splicing, translation, and decay in the regulation of early events in mRNP biogenesis; and imply the additional requirement for a balance in splicing isoforms from a single gene.

INTRODUCTION

A significant part of mRNP biogenesis takes place co-transcriptionally, and functional links between chromatin modifications, transcriptional elongation, splicing and mRNA export have been shown (1,2). Sus1 (ENY2 in metazoans) is a small, evolutionarily conserved 11-kDa protein that is a component of two molecular assemblies with roles in both transcription and mRNA export (3,4). Sus1 can be found in the SAGA complex, involved in chromatin function, where is part of the histone H2B deubiquitinating (DUB) module together with Ubp8, Sgf11 and Sgf73 (5,6). Interactions between these four proteins are necessary to reconstitute full DUB activity *in vitro*, as revealed recently by structural studies (7,8). In addition, during transcription Sus1 can be recruited to promoters and along coding regions, where it interacts with RNA Pol II and the mRNA export factors Yra1 and Mex67 (9). At the nuclear pore, Sus1 is a component of the TREX2 complex formed by Sac3, Thp1, Sus1, Cdc31 and Sem1 (10–12). Sus1 plays a role in mRNA export from the nucleus and it is implicated in anchoring active genes at the nuclear periphery through gene gating (12,13). Moreover, in specific circumstances Sus1 can be observed at cytoplasmic structures (14). Thus, Sus1 participates in multiple stages of gene expression, with roles in chromatin function and mRNA biogenesis.

In addition, the *SUS1* gene structure is of particular interest. Unlike most yeast genes, which are typically intronless, *SUS1* bears two introns; and while most yeast introns display conserved splicing signals (15), both the 5′ splice site and branch site of the first intron of *SUS1* are non-canonical (3,16). Interestingly, *Saccharomyces cerevisiae* introns are not randomly distributed throughout the genome, but most are found in highly expressed genes (17).

*To whom correspondence should be addressed. Tel: +34963289680; Fax: +34963289701; Email: srodriguez@cipf.es
Correspondence may also be addressed to Josep Vilardell. Tel: +34934020549; Fax: +34934034979; Email: josep.vilardell@ibmb.csic.es
Present address:
Pau Pascual-García, Molecular & Cell Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA.

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

Notably, introns are found as well in a number of genes functionally relevant to RNA metabolism, such as *YRA1*, *MTR2*, *SRC1*, *NSP1* and *HRB1* (18). Interestingly, *SRC1* is a rare example of alternative splicing in yeast (19,20), and both *YRA1* and *SRC1* are functionally and genetically linked to *SUS1* (10,20). In fact, extensive work on *YRA1* points to the intricate circuitry of an mRNP life, encompassing splicing, export, and degradation (21–25).

Here we assessed a link between Sus1 function and *SUS1* unusual genomic structure. We find that *SUS1* introns are evolutionarily conserved, and their deletion leads to defects in RNA export and to slow growth. The unconventional splicing signals in *SUS1* are also important for *SUS1* function, which is affected by non-essential splicing factors such as Mud2 and modulated by the non-sense mediated decay (NMD) pathway. Thus, proper *SUS1* expression requires a delicate balance of splicing, translation and decay.

MATERIALS AND METHODS

Identification of *S. cerevisiae SUS1* orthologs and phylogenetic trees

Putative orthologs of *S. cerevisiae SUS1* gene were identified by BLASTn (26) and WU-BLAST2 (27) searches on public databases and default search parameters were used. A list of species used in our analysis can be found at Supplementary Table S1. The evolutionary transformation of three structural characters of *SUS1* (intron number, 5′ss and BS) were optimized onto the known phylogeny of 25 eukaryotes species (28,29). Ancestral and derived multistate transformations were achieved considering equal weighted unordered characters in MacClade program (30).

Yeast cultures and microbiological techniques

Growth assays to test the intron functionality were done by growing cells at 30°C on synthetic selective medium [SC: glucose 2%, ammonium sulfate 0.5%, yeast nitrogen base 0.17% and supplements (Dropout)] lacking Tryptophan (Trp) to 0.3–0.4 OD_{600}. Subsequently, 10-fold serial dilutions of an equal number of cells were made and drops spotted onto SC-trp plates. Growth was recorded after 2–3 days of incubation at 30 and 37°C. Yeast cell transformations were done by the LiAc/SS carrier DNA/PEG method (31). For wild-type (WT), *mud2Δ, upf1Δ* and *mud2Δupf1Δ* splicing pattern comparison, cells were grown at 30°C in YPD (2% glucose, 1% yeast extract and 2% peptone) and collected when 0.6 OD_{600}. To test different stress conditions influencing *SUS1* splicing pattern, cells were grown in YPD at 30°C until 0.4 OD_{600} and then divided in three flask, in order to incubate the cell cultures under different treatments: (i) cells were grown 2 h more at 30°C in YPD; (ii) cells were incubated 20 min at 42°C; or (iii) carbon source was shifted to galactose and the cells were grown 2 h at 30°C. After each treatment, cells were collected, frozen in liquid nitrogen and stored at −80°C until RNA extraction. For copper assays, cultures at 30°C were grown to 0.4 OD_{600} in SC medium lacking leucine and equal volumes were dropped onto SC-Leu plates containing $CuSO_4$ ranging from 0 to 1.0 mM (32). Plates were photographed after 3 days at 30°C.

RNA analysis

Total RNA was harvested from yeast cultures by the Hot/Acid-phenol method (33). RNA was quantified using Nanodrop and quality was checked by agarose gels with ethidium bromide (etBr). Northern blot analyses were carried out as in (34). *SUS1* and U1 snRNA detection was done using T7-transcribed riboprobes.

Reverse transcription PCRs and qRT–PCRs

RT–PCR and qRT–PCR analysis were performed using 1 μg of total RNA. After DNase-I treatment (Promega), RNAs were purified by Phenol/Chloroform extraction. Reverse transcription was performed using standard procedures, with random hexamers and M-MLV reverse transcriptase (Invitrogen). Specific pairs of primers were used to amplify *SCR1* or *SUS1* transcripts containing exons 1 and 3; using 3 μl of cDNA as template (previously diluted 1/20). Amplified products were run in a 2% agarose-EtBr gel and visualised. Specific primers for each *SUS1* transcript or *SCR1* ncRNAs were used to amplify qRT–PCR products from 3 μl of cDNA (previously diluted 1/10) and using SYBR® GreenER™ qPCR SuperMix (Invitrogen) in a 10 μl final volume. Each sample was run in duplicate with the standard curve. Real-Time PCR was performed using a LightCycler® (Roche). An activation step of 10 min at 95°C followed by 45 cycles of 10 s at 94°C, 15 s at 50°C and 20 s at 72°C were used for mRNA, intron 1 (I1) and intron 2 (I2) primer pairs. For I1 and I2 transcripts, the 72°C step was extended to 30 s. For *SCR1* RNA, 38 cycles of 10 s at 94°C, 15 s at 60°C and 20 s at 72°C were used. See the 'Materials and Methods' in Supplementary Data for a list of primers. The amount of each *SUS1* transcripts was represented in arbitrary units (AU). One arbitrary unit is equivalent to 10^{-2} fmol/mg of total RNA.

Protein purification, western blot and inmunoprecipitation analysis

For crude protein extracts, 1.5 OD_{600} from cultures on exponential growth at 30°C were collected. Cells were lysed in 150 μl of NaOH, 2 M plus 7.5% (v/v) β-mercaptoethanol and proteins were precipitated for 10 min on ice. After centrifugation, the pellet was resuspended in 50 μl of denaturing loading buffer. Samples were heated at 95°C before loading and separation in 16% SDS–PAGE gels. Western blot analysis was performed using anti-LexA and anti-GFP according to standard procedures.

For GFP-Sus1 immunoprecipitations 50 ml of WT and *mud2Δ upf1Δ* strains containing plasmids bearing GFP or GFP-SUS1g were grown on selective medium to OD_{600} 0.5. Cells were harvested, washed, and resuspended in 200 μl of ice-cold lysis buffer (50 mM HEPES-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM PMSF and protease inhibitors). The same volume of glass beads was added and cells were

broken by four pulses of vortexing during 1 min at 4°C. The supernatant were immunoprecipitated during 1 h at 4°C in a turning wheel and using anti-GFP antibody (Roche) coupled to dynabeads (Invitrogen). The immunoprecipitates were washed three times for 10 min with lysis buffer and subsequently resuspended in 50 µl of SDS–PAGE sample buffer. Western blot analysis of samples was made using anti-GFP and anti-Sus1 antibodies.

In Situ Hybridization (FISH)

Fluorescent *in situ* hybridization (FISH) against poly(A)+RNA was done growing yeast cells in 100 ml of YPD medium at 30°C to an 0.5 OD_{600}. Then, cultures were divided and a half of each culture were rapidly shifted to 37°C incubator for 3 h, and the other half was incubated at 30°C during that time. Cells were immediately fixed adding 10% of formaldehyde, 1 h at room temperature. The fixative was removed by two rounds of centrifugation and washed with 0.1 M potassium phospate (pH 6.4). Cells were resuspended in ice-cold washing buffer (1.2 M sorbitol and 0.1 M potassium phosphate, pH 6.4), and subsequently, cell wall was digested with 0.5 mg/ml of Zymolyase 100 T and samples were applied on poly-L-lysine-coated slide wells. Non-adhering cells were removed by aspiration, cells were rehydrated with 2× SSC (0.15 M NaCl and 0.015 M sodium citrate) and hybridized overnight at 37°C in 20 µl of prehybridization buffer (formamide 50%, dextran sulfate 10%, 125 µg/ml of *Escherichia coli* tRNA, 4× SSC, 1× Denhardt's solution and 500 µg/ml herring sperm DNA) with 0.8 pmol of Cy3-end-labeled oligo(dT) in a humid chamber. After hybridization, slides were washed with 1× SSC at room temperature, air-dried and mounted using VECTASHIELD® Mounting Medium with DAPI. Detection of Cy3-oligo(dT) was performed using a Leica DM600B fluorescence microscope.

RESULTS

The *SUS1* gene

Two features of *SUS1* genomic structure are of particular interest. As we previously showed, *SUS1* (ENY2 in metazoans) bears two introns, a rare feature in the *S. cerevisiae* genome (3). Only 5% of the genes in *S. cerevisiae* have introns and of those just ten carry two (18,35–37). In addition, while most yeast introns display conserved splicing signals, both the 5′ splice site and branch site of the first intron of *SUS1* are non-canonical. Interestingly, other yeast introns with regulated splicing also have non-canonical splice sites (21). Thus, we decided to explore the degree of evolutionary conservation of these features, which may indicate their biological relevance.

We identified *SUS1* homologs in different species (Supplementary Tables S1 and S2), and performed an evolutionary analysis. Our results (Figure 1) reveal the following: first, *SUS1* is likely to have evolved from an ancestral gene containing four introns (Figure 1A). While in fungi genes containing one, three or four introns are equally probable; after the branching to *C. neoformans* (lane 3 in Figure 1) a single-intron gene (*SUS1* I1) is unequivocally the most parsimonious ancestral reconstruction. Subsequent evolution leads to the addition of a second intron in most of the Saccharomycotina group of species. Second, having two introns in fungi correlates

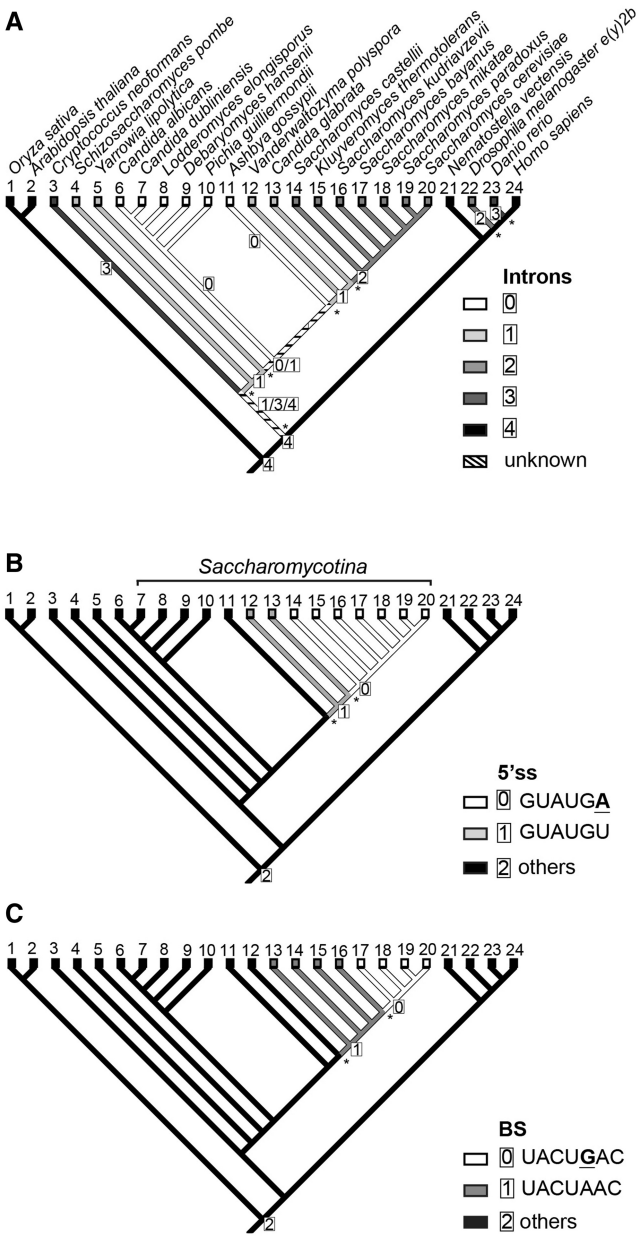

Figure 1. Evolution of three *SUS1* structural characteristics. (**A**) Evolution of the number of introns in *SUS1*. Intron number (boxes) in *SUS1* orthologs for 24 eukaryotic species is represented based on a known topology (28). According to the distribution of ancestral and derived states, seven evolutionary changes (in asterisks) are required in the tree to explain intron number diversity of *SUS1*. (**B**) The Saccharomycotina subphylum incorporates a second intron in *SUS1* orthologs when a second intron appeared. Numbers in boxes represent non-consensus sequence (0), consensus sequence (1) or other sequences for 5′ss in *S. cerevisiae* and related fungi. (**C**) A non-consensus BS appears in the I1 of *SUS1* for the most related species to *S. cerevisiase*. Numbers in boxes represent, non-consensus sequence (0), consensus sequence (1) or other sequences for BS in *S. cerevisiae* and related fungi.

Table 1. Splicing signals in *SUS1* and *SUS1* ortholog introns

Species	Introns	I1			I2		
		5′ss	BS	3′ss	5′ss	BS	3′ss
S. cerevisiae	2	GUAUG**A**	UACU**G**AC	UAG	GUAUGU	UACUAAC	UAG
S. mikatae	2	GUAUG**A**	UACU**G**AC	UAG	GUAUG**A**	UACUAAC	UAG
S. paradoxus	2	GUAUG**A**	UACU**G**AC	UAG	GUAUG**A**	UACUAAC	UAG
S. bayanus	2	GUAUG**A**	UACU**G**AC	CAG	GUAUG**C**	UACUAAC	UAG
S. castellii	2	GUAUG**A**	UACUAAC	AAG	GUAUG**A**	UACUAAC	CAG
S. kudriavzevii	2	GUAUG**A**	UACUAAC	UAG	GUAUG**A**	UACUAAC	UAG
K. thermotolerans	2	GUAUG**A**	UACUAAC	CAG	GUAUGU	UACUAAC	UAG
C. glabrata	1	GUAUGU	UACUAAC	CAG			
V. polyspora	1	GUAUGU	**C**ACUAAC	AAG			
Y. lipolytica	1	GU**GA**GU	**C**ACUAAC	CAG			
S. pombe	1	GUAU**AA**	U**UA**UAAC	UAG			

Sequence of 5′ splicing site (5′ss), Branch Site (BS) and 3′ splice site (3′ss) were described for most evolutionary related species. 5′ss, BS, and 3′ss consensus sequences in *S. cerevisiae* are GUAUGU, UACUAAC and YAG (Y:pyrimidine) respectively (45). In bold and underlined, nucleotides different from consensus splicing sequences in *S. cerevisiae*.

with carrying a non-canonical 5′ss in the first intron (Figure 1B and Table 1). Third, non-consensus BS in I1 is only present in *S. bayanus, S. mikatae, S. paradoxus* and *S. cerevisiae* (Figure 1C). Strikingly, in fungi, *SUS1* genes having I1 with the non-consensus BS UACU**G**AC correlates with the presence of a highly conserved 3′UTR of ~300 nt (Supplementary Figure S1). Altogether, these data suggest a biological relevance for *SUS1* introns in its expression and we decided to further address this question.

SUS1 splicing

We used quantitative and semi-quantitative RT–PCR to determine the relative splicing efficiencies of *SUS1* introns. Bands corresponding to the fully spliced (mRNA) and *SUS1* transcripts retaining I1, were consistently detected in WT cells (Figure 2A). This is in agreement with both data from Hossain *et al.* (16) and genome-wide studies (38,39), reporting detectable amounts of *SUS1* transcripts retaining I1. We hypothesize that splicing of *SUS1* I1 is either inefficient or subject to regulation, leading to cellular accumulation of unprocessed *SUS1* transcripts.

To test this idea, we analyzed *SUS1* splicing under different conditions of temperature and carbon source, where *SUS1* function is more determinant (10). Quantitative RT–PCRs of RNA collected from WT cells incubated 20 min at 42°C revealed that *SUS1* expression is reduced by the temperature shift, with accumulation of unspliced *SUS1* transcripts and reduction of the corresponding mRNA (Figure 2B). Notably, *SUS1* expression is induced by growth in galactose, while its splicing pattern remains unchanged (Figure 2B). Altogether, our results indicate changes in expression and splicing efficiency dependent on growth conditions and suggest that *SUS1* splicing is a regulated event.

To analyze *SUS1* splicing we generated constructs based on the *CUP1* reporter system, widely used to follow splicing efficiency *in vivo* (32). *cup1Δ* cells were transformed with one of the following reporters (Figure 3A): pACT1-CUP1, pSUS1g-CUP1 (with both *SUS1* introns), pSUS1-*I1Δ*-CUP1 (I1 removed) and pSUS1-BS-CUP1 (I1 with UACU**G**AC mutated to UACU**A**AC, rendering its BS consensus). Splicing was monitored by both assessing copper tolerance and primer extension analyses. As illustrated in Figure 3A deletion of I1 improves growth compared to the genomic construct (compare lanes 2 and 4), likely due to a better splicing efficiency of *SUS1* transcripts lacking I1, which suggests that I1 splicing is limiting for *SUS1* expression. Accordingly, the construct SUS1-BS-CUP1 displays a similar copper tolerance to the construct SUS1-*I1Δ*-CUP1 (Figure 3A, lanes 3 and 4). These results were further confirmed by primer extension (data not shown) and are consistent with those presented in the accompanying paper (Hossain *et al.* this issue). Thereby we conclude that SUS1 splicing efficiency likely is limited by the suboptimal BS in I1.

We further explored this possibility by assessing the role of Mud2 in *SUS1* expression. Mud2, the yeast homolog of U2AF, interacts with BBP, an essential factor that identifies the BS and its required for splicing of a variety of transcripts (40,41). Mud2 is not essential, so we analyzed *SUS1* splicing in *mud2Δ* cells. Northern blot analysis of RNA from *mud2Δ* cells shows accumulation of *SUS1* pre-mRNA species with a concomitant decrease of mature RNA, compared to WT (see below in Figure 4B and Hossain *et al*). Thus, we decided to further analyze *SUS1* splicing in *cup1Δ mud2Δ* cells, using our *CUP1* reporters. Consistent with the northern blot analysis, deletion of *MUD2* leads to a marked reduction in copper tolerance for cells carrying SUS1g-CUP1 (Figure 3A, compare lanes 2 and 6). Interestingly, while mutation of the I1 BS to consensus (SUS1-BS-CUP1) dramatically improves copper tolerance in *mud2Δ* cells, this tolerance is further increased by deleting I1 (Figure 3A, lanes 7 and 8). This is consistent with a requirement for Mud2 in I1 splicing in part but not exclusively due to the non-consensus BS, and the non-consensus 5′ss of I1 is likely to be involved as well (Hossain *et al.* this issue).

To further assess the dependency of *SUS1* splicing on Mud2 *in vivo*, we have quantified by qRT–PCR the different splicing forms in *mud2Δ* cells. Absence of Mud2

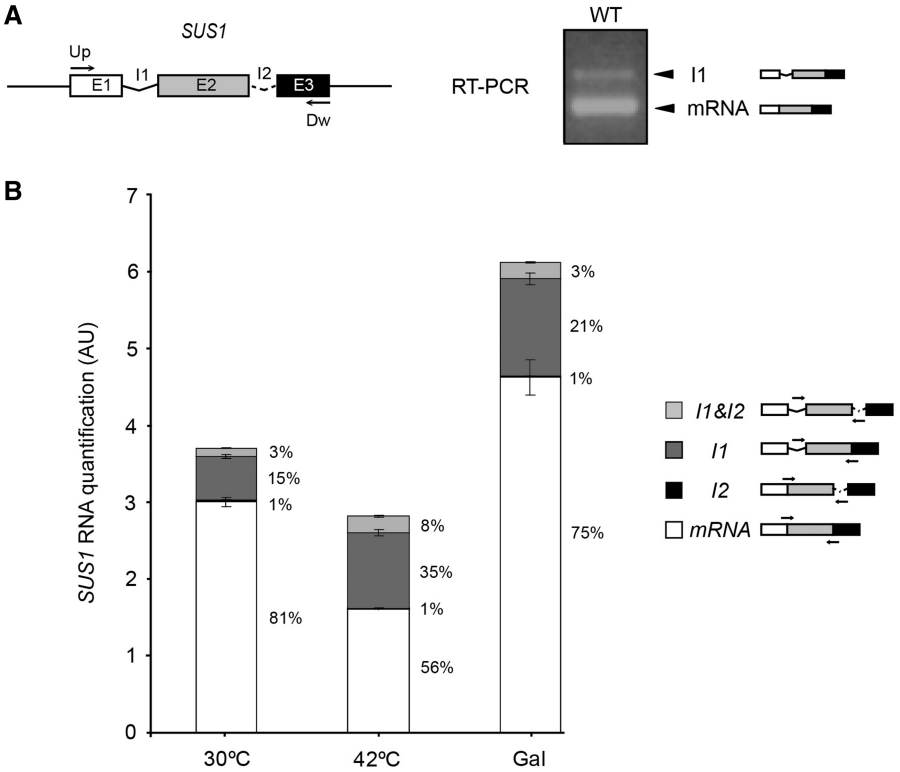

Figure 2. *SUS1* splicing is regulated. (**A**) Analysis of *SUS1* transcripts by semi-quantitative RT–PCR. Left, *SUS1* primer positions. Right, *SUS1* transcripts at 30°C. Bands corresponding to mature RNA (mRNAs) and pre-mRNA retaining I1 are indicated. (**B**) Quantitative analysis of *SUS1* splicing pattern under different growth conditions, as indicated (Gal: growth at 30°C with galactose as carbon source). The percentage of each *SUS1* transcript is indicated. Primer pairs to specifically detect each *SUS1* transcript by qRT–PCR are depicted on the right.

correlates with a substantial enrichment of pre-mRNA retaining either both introns or I1, rising from 16% of total transcripts to 68% (a 4-fold increase, Figure 3B). However, the largest change in absolute terms is the dramatic decrease in the mature mRNA species (a 10-fold change). In summary, our data support a prominent role for Mud2 in *SUS1* expression. It is likely that other factors involved in the early recognition of introns are relevant to *SUS1* expression as well (Hossain *et al.*, this issue).

SUS1 unspliced transcripts are targeted by NMD

The reduced *SUS1* expression in *mud2Δ* cells (Figure 3B) might be a consequence of the appearance of premature stop codons (PTC) in transcripts containing I1, which would trigger NMD (42). Consistent with this hypothesis, qRT–PCR and northern analyses of RNA from both WT and *upf1Δ* strains revealed a 1.5-fold increase in accumulation of intron-containing *SUS1* transcripts in *upf1Δ* cells (Figure 4A and B, lane 3).

To test whether products of *SUS1* splicing (or mis-splicing) in *mud2Δ* cells were targeted for degradation by the NMD pathway, we determined the levels of *SUS1* transcripts in *mud2Δ upf1Δ* cells (Figure 3). Northern blot and semi-quantitative RT–PCR analyses (Figure 4B) show accumulation of unspliced *SUS1* transcripts in *mud2Δ upf1Δ* cells, compared to those of WT. qPCR results (Figure 4C) indicate that this accumulation corresponds to a 6-fold increase. Thus we conclude that *SUS1* splicing depends on Mud2 and that *SUS1* unspliced transcripts are targeted by NMD.

Sus1 protein expression is regulated by its introns

Many introns regulate gene expression by a variety of mechanisms, and in yeast there are several instances of introns involved in autoregulatory loops (*YRA1* or *RPL30*) (17). Here we show that *SUS1* introns have a critical role on mRNA levels, and in order to assess whether they are necessary to maintain appropriate Sus1 amounts, we determined the quantity of Sus1 protein expressed from several constructs including those carrying modified splicing signals. Plasmids bearing SUS1cDNA or the genomic version of *SUS1* (SUS1g), N-terminally fused to LexA or GFP tags, were introduced into a mutant strain lacking endogenous *SUS1*. Levels of LexA (Figure 5A) or GFP (Supplementary Figure S2) were monitored by western blot analyses. Our results indicate that elimination of *SUS1* introns causes accumulation of Sus1 protein when compared to SUS1g (Figure 5A compare upper band in lanes 2 and 3).

To study how the presence and position of each intron contribute to Sus1 protein levels, we cloned different *SUS1* constructs in pBTM-LexA (43) under the *ADH1* promoter. Expression of the LexA-Sus1 fusion proteins was monitored by western blotting using anti-LexA antibodies (Figure 5A). Notably, the presence of I1 at

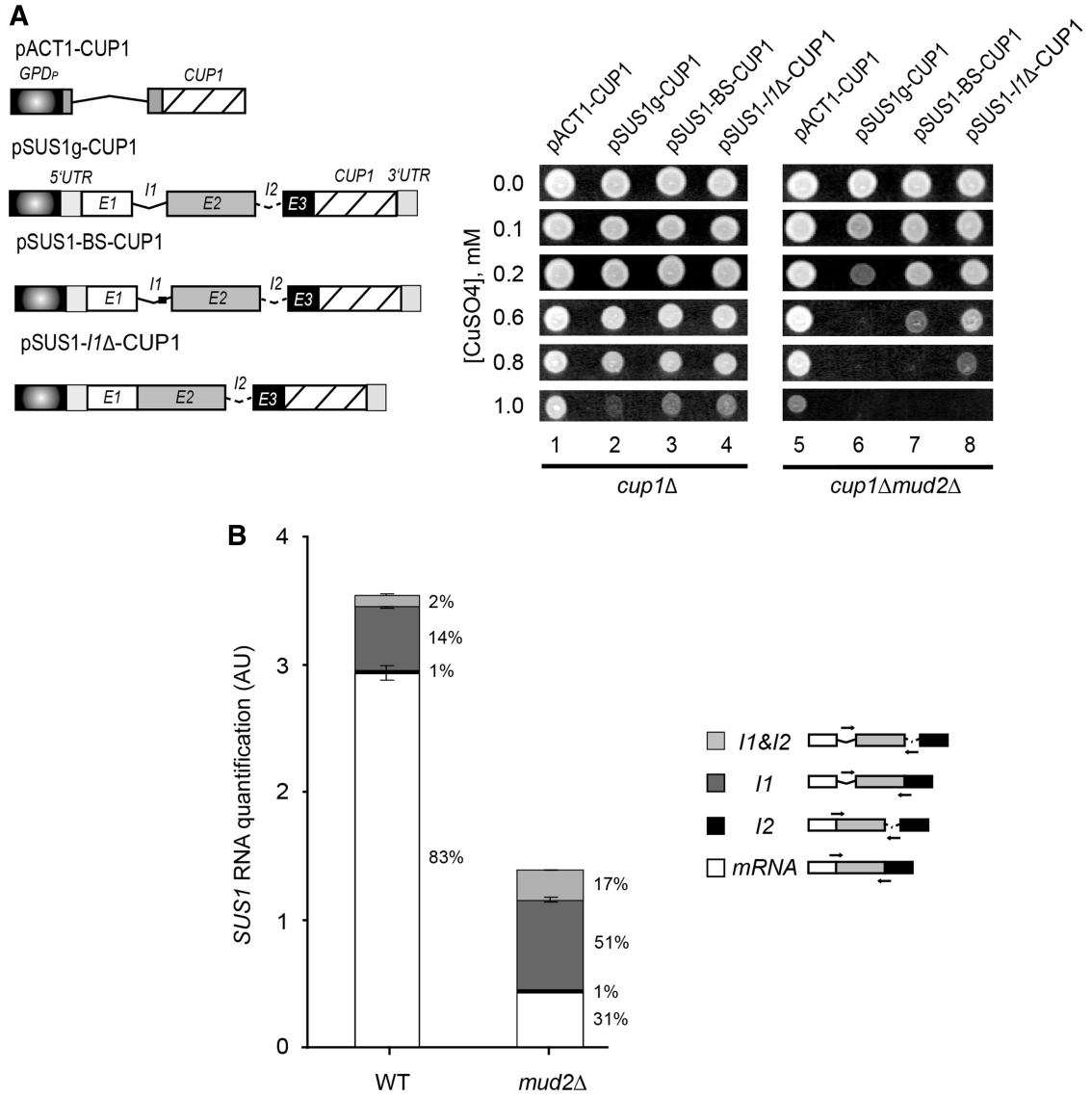

Figure 3. Mud2 is required for efficient *SUS1* splicing. (**A**) On the right, copper assays of *cup1Δ* and *cup1Δmud2Δ* cells transformed with different *SUS1-CUP1* versions (shown at the left). Comparable number of cells was spotted onto plates containing $CuSO_4$, as shown. (**B**) Quantification of *SUS1* transcripts by qRT–PCR, from RNA extracted from WT or *mud2Δ* cells. The Percentage of each *SUS1* transcript is indicated. Primers used to specifically amplify each transcript are depicted on the right.

both locations (2xI1, Figure 5A construct 4) provoked the strongest reduction in Sus1 protein levels (Figure 5A, lane 4). As expected from our splicing analysis, I1 elimination (lane 7), substitution by I2 (lane 5) or mutation of its BS signal to the consensus (lane 9), stimulated Sus1 expression to levels comparable to the SUS1cDNA version. Of note, swapping introns (lane 6) or removing I2 (lane 8) also affects Sus1 protein expression. We conclude that both the presence and the position of both introns are key determinants of Sus1 protein expression levels.

Although production of protein isoforms by alternative splicing in yeast is rare, *SUS1* potentially could be alternatively spliced to produce different proteins. N-terminal tagging of *SUS1* with both introns enables the detection of alternate forms of Sus1 protein if they exist. Strikingly, a smaller intense band similar in size to the tag alone was observed when using both antibodies (marked band with an asterisk in Figure 5A). This band is consistent with a smaller version of Sus1 protein translated from pre-mRNAs retaining either I1 or both introns. Intron 1 retention introduces a PTC, and the predicted protein translated from this sequence will include exon 1 and 6 additional residues. To assess whether this protein is not a degradation product of full-length Sus1, we hypothesized that its abundance will correlate with an increase in *SUS1* transcripts containing I1. Therefore, we determined Sus1 protein relative abundance in two different situations. First, we followed the ratio small/full-length Sus1 protein (pLexA-SUS1g) in *mud2Δ upf1Δ* cells, where unspliced versions of *SUS1* are enriched compared to WT (Figure 4B and C). Consistent with our hypothesis,

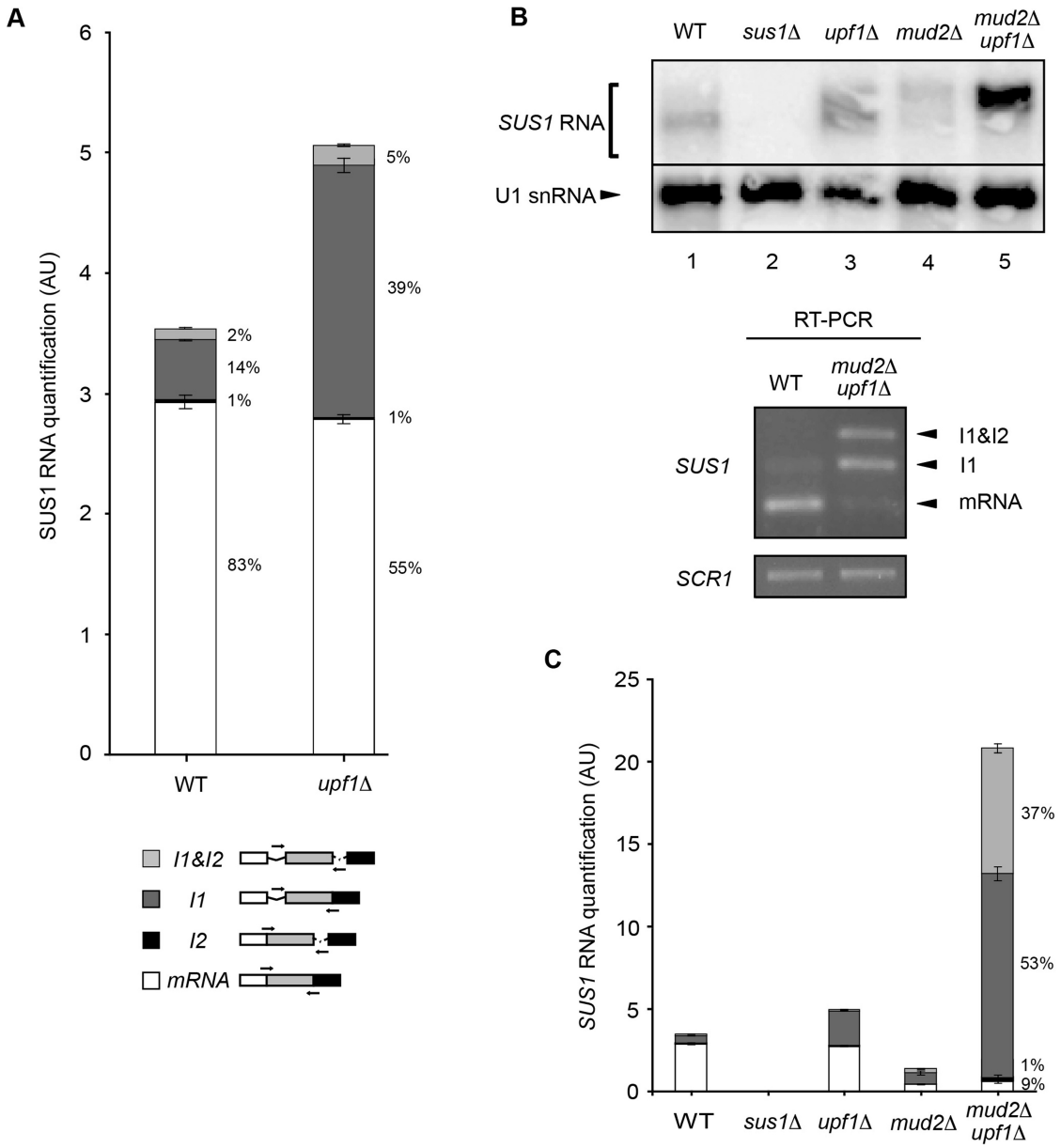

Figure 4. *SUS1* transcripts are targeted by the Non-sense Mediated Decay pathway (NMD). (**A**) qRT–PCR quantification of *SUS1* transcripts from RNA extracted from WT or *upf1Δ* cells growing at 30°C. (**B**) Northern blot (upper panel) and semi-quantitative RT–PCR (lower panel) showing the *SUS1* RNA accumulation in mutant strains affecting *SUS1* splicing. U1 snRNA and *SCR1* are used as loading controls in northern and RT–PCR analysis respectively. (**C**) qRT–PCR quantification of *SUS1* transcripts from WT, *sus1Δ upf1Δ mud2Δ* and *mud2Δupf1Δ*. The Percentage of each *SUS1* transcript is indicated for *mud2Δupf1Δ*.

we observe an accumulation of the small Sus1 in the double mutants (Figure 5B). Second, we cloned into pBTM-LexA a version of the *SUS1* gene [SL-9 mut, (10)] that has a mutated BS sequence (UACUGAC mutated to CACUGAC), which blocks splicing of I1, driving accumulation of *SUS1* transcripts retaining I1 (Figure 5C, left panel). As expected a prominent band consistent with the size of the small Sus1 protein, is recognized using anti-LexA antibodies, while no full-length Sus1 protein is observed (Figure 5C right panel). Altogether, we conclude that a small version of Sus1 is translated from *SUS1* transcripts containing I1.

To further verify that the small band detected in our western analyses contains Sus1 residues, we transfected pGFP-SUS1g in WT and *mud2Δ upf1Δ* cells and subsequently we immunoprecipitated whole cell extracts using anti-GFP antibodies. Western blotting reveals two GFP forms (Figure 5D), one (~38 kDa) consistent with full-length Sus1-GFP; and a second (~28 kDa) consistent with the translation of Sus1 exon 1 plus 6 residues, fused to GFP (GFP-Sus1p*). Consistent with our model, this form is enriched in extracts from *mud2Δupf1Δ* cells bearing pGFP-SUS1g (which leads to accumulation of *SUS1* transcripts retaining I1, lane 3). Additional confirmation that the 28 kDa band includes a truncated version of

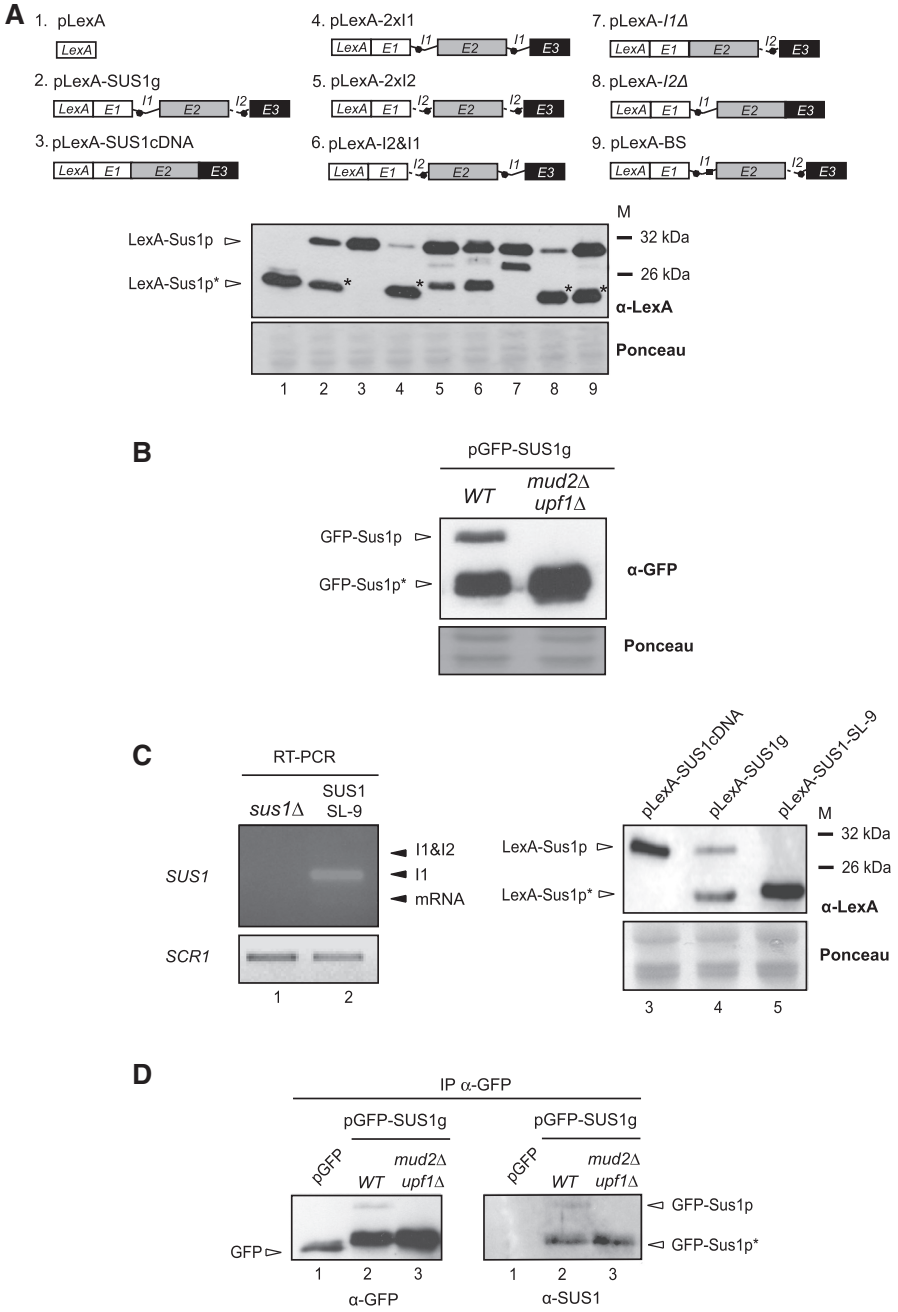

Figure 5. *Sus1* levels are regulated by the presence and position of *SUS1* introns. (**A**) Whole cell extracts from *sus1Δ* cells transformed with the indicated plasmids (top) were used to follow Sus1 protein levels by western blot using anti-LexA (bottom). Black hexagons in *SUS1* introns scheme indicate position of PTCs if the introns are not removed. The ponceau staining was used as a loading control. Asterisk indicates the putative protein translated from *SUS1* transcripts retaining I1. (**B**) Sus1 levels of whole cell extracts from WT and *mud2Δ upf1Δ* strains containing pGFP-SUS1g were analyzed by western blot using anti-GFP. The ponceau was used as loading control. (**C**) Left, *SUS1* transcripts detected by RT–PCR from and SL-9 cells. Right, Sus1 levels in whole cell extracts, from *sus1Δ* cells transformed with the indicated plasmids, measured as in (A). (**D**) Anti-GFP immunoprecipitated proteins from WT and *mud2Δ upf1Δ* strains bearing pGFP or pGFP-SUS1g plasmid were analyzed by western blot using anti-GFP (left panel) or anti-Sus1 (right panel) antibodies respectively.

Sus1 was obtained by probing the same immunoprecipitated with anti-Sus1 antibodies, producing the same result (Figure 5D, right panel).

Exonic sequences enhance *SUS1* splicing

To further decipher the molecular mechanisms controlling *SUS1* splicing, we have investigated whether exonic sequences flanking *SUS1* introns might contribute to their splicing. To do this, we have analyzed the splicing efficiency of *SUS1* introns in another exonic context, using the *TAF14* gene. *TAF14* has one canonical intron (position 10–114 nt, from the start codon). We have followed the expression of this construct by western analysis of LexA fusions (Figure 6A). As anticipated

given its reduced splicing, replacement of the *TAF14* native intron by *SUS1* I1 decreases the amount of expressed LexA-Taf14 protein (Figure 6A, lanes 1 and 2). In an attempt to recapitulate *SUS1* gene organization, we cloned I2 at the same distance from I1 that it is located in *SUS1* ORF, creating a Taf14 exon2* (see construct design in Figure 6A). Surprisingly, incorporation of I2 leads to a further splicing block, causing a strong reduction in Taf14 protein levels (Figure 6A, lanes 2 and 3). This is consistent with the observed importance of both introns in *SUS1* expression (Figure 5). Next we decided to explore the role of exon 2 sequences in *SUS1* splicing using our LexA-TAF14 construct containing *SUS1* introns (TAF14-I1&I2SUS1). Intriguingly, replacing the *TAF14* sequence between *SUS1* introns (*TAF14* exon 2*) by *SUS1* exon 2, restores splicing to almost native Taf14 levels (Figure 6A, compare lanes 1 and 4).

To better substantiate this observation, we have monitored splicing efficiency by growth in copper using a *CUP1* construct in which the original *SUS1* exon 2 was replaced by a sequence corresponding to TAF14 exon 2* (see construct design in Figure 6B). In agreement with our western results, replacement of *SUS1* exon 2 by *TAF14* sequence results in reduced copper tolerance consistent with a reduced splicing (Figure 6B, lane 3). Thus we conclude that exon 2 sequences on *SUS1* gene support proper *SUS1* splicing.

SUS1 introns are required for *Sus1* cellular function

To address the biological relevance of *SUS1* introns we have examined the phenotype of *sus1Δ* cells transformed with plasmids containing either *SUS1* with its introns (pSUS1g) or without (pSUS1cDNA). *sus1Δ* cells are sensitive to high temperature (*ts*) and their growth can be affected by the carbon source [data not shown and (10)]. Notably, while *sus1Δ* cells transformed with pSUS1g lose the *ts* phenotype, there is less suppression of the phenotype with the pSUS1cDNA (Figure 7A, see growth at 37°C). This is consistent with the differential expression profile observed for *SUS1* transcripts at high temperature (Figure 2B), and we speculate that this profile is relevant to the role of Sus1 at 37°C.

An intriguing explanation could be that *SUS1* transcripts containing I1 have functionality at higher temperatures. To assess this hypothesis, we tested the growth at 37°C of *sus1Δ* cells transformed with pSUS1-SL-9 (with an I1 containing a non-consensus BS, which renders mostly *SUS1*-I1 retained transcripts, as described in Figure 5C). As expected from its deficient splicing, cells transformed with this construct grow similar to an empty plasmid at 30°C (Figure 7A, compare growth of p-empty and pSUS-SL-9 at 30°C after 24 h). Surprisingly, this construct is able to complement the *ts* phenotype exhibited at 37°C to a larger extent (Figure 7A, compare growth of p-empty and pSUS-SL-9 at 37°C after 48 h) than the cDNA, albeit less so than the gDNA. These data

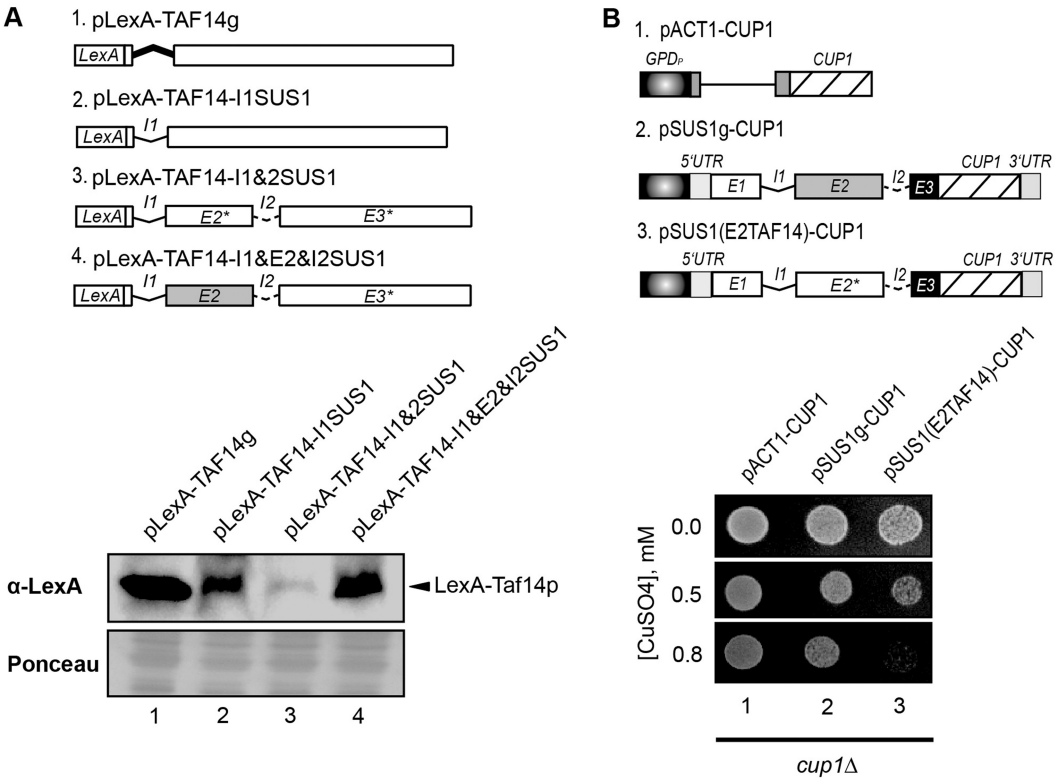

Figure 6. Exonic regions induce *SUS1* splicing. (**A**) LexA-Taf14 protein levels were monitored by western analysis of whole cell extracts, from cells transformed with the indicated plasmids and using anti LexA antibodies. The ponceau staining was used as a loading control. (**B**) Top, scheme showing the *CUP1* constructs used to monitor the role of *SUS1* exon 2 in *SUS1* splicing. In construct 3 the exon 2 of *SUS1* is replaced by *TAF14* sequences. Splicing was monitored by a copper assay, shown at the bottom.

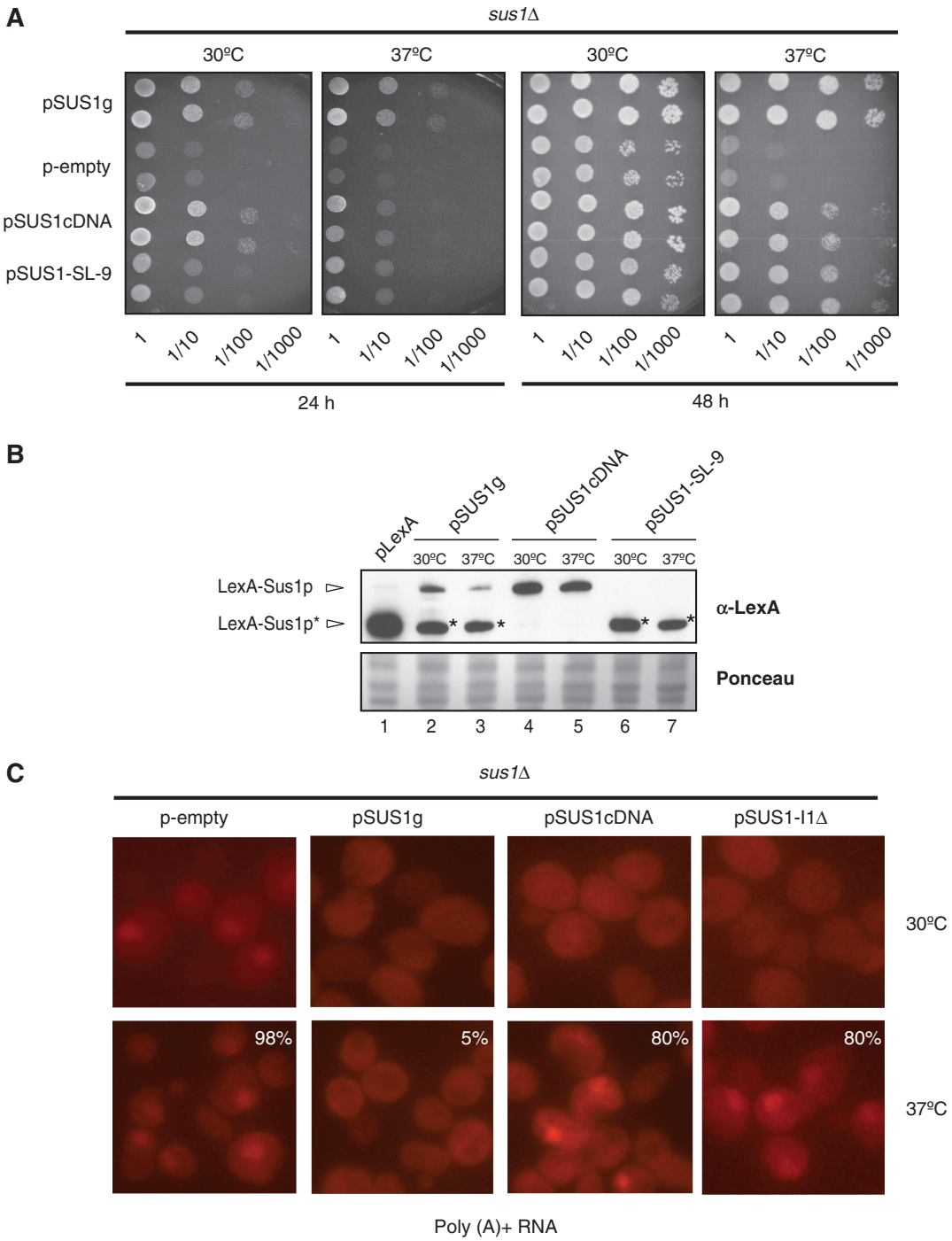

Figure 7. *SUS1* introns are required for optimal growth and mRNA export. (**A**) *sus1Δ* strains containing an empty plasmid (p-empty), the same plasmid bearing *SUS1* with its introns (pSUS1g), without introns (pSUS1cDNA) or with a suboptimal BS at I1 (pSUS1-SL-9), were spotted applying serial dilutions of an equal number of cells onto SC-trp plates and incubated at 30 or 37°C. (**B**) Whole-cell extracts from *sus1Δ* cells transformed with the indicated plasmids and grown at 30 or 37°C for 4 h, were used to follow Sus1 isoforms proteins levels by western blot using anti-LexA. The ponceau staining was used as a loading control. Asterisk indicates the putative protein translated from *SUS1* transcripts retaining I1. (**C**) FISH analysis of nuclear mRNA export in *sus1Δ* cells. Localization of poly(A)+RNA at 30°C and after shifting to 37°C for 3 h, in *sus1Δ* strains transformed with empty, SUS1g, SUS1cDNA or SUS1-I1Δ containing plasmids, was performed using Cy3-labeled oligo(dT) probe The percentage of *sus1Δ* cells with nuclear export defect is indicated for each condition.

indicate that *SUS1* introns are required for optimal growth and have a biological relevance.

To further address whether changes in Sus1 protein expression could account for the differences observed in our complementation assay, we followed the levels of Sus1 isoforms at different temperatures when expressed from the genomic version (p*SUS1*g), the cDNA (p*SUS1*cDNA), or the SL-9 BS mutant (p*SUS1*-SL-9) (Figure 7B). As expected from the results of the RNA analyses, a down-regulation of full-length Sus1p is

observed at 37°C (compare upper band in lanes 2 and 3). Although a reduction in Sus1p levels is also appreciable in cells containing the cDNA version, Sus1p levels at 37°C are still elevated when compared to those of wt both at 30 or 37°C (lanes 4 and 5 versus 2 and 3). A slight downregulation of Sus1p* also occurs at 37°C (lanes 2 and 3) and this is more apparent when *SUS1* is expressed form the SL-9 BS mutant version (lanes 6 and 7). These data support our hypothesis that the changes in the relative levels of Sus1 isoforms account for the differences observed in our complementation assays.

Sus1 is necessary for mRNA export, and in *sus1Δ* cells nuclear accumulation of poly(A)$^+$ RNA is readily detected at 30 and 37°C [Figure 7C and (10)]. To assess the effect of *SUS1* introns on this phenotype, we performed RNA *in situ* hybridization (FISH) in exponentially growing *sus1Δ* cells, transformed with p*SUS1*g, p*SUS1*cDNA, or p*SUS1-I1Δ* (Figure 7C). Consistent with the growth complementation results shown in Figure 7B, both SUS1g and SUS1cDNA were able to complement to a large extend the mRNA export defect of *sus1Δ* cells at 30°C (Figure 7C upper panel). In contrast, FISH signal from *sus1Δ* cells transformed with p*SUS1*cDNA and grown at 37°C, indicates that the *SUS1* gene lacking both introns is not able to efficiently complement the *sus1Δ* mRNA export defect. Moreover, this is replicated by a construct lacking I1. Thus we conclude that *SUS1* introns are required for the *SUS1* role in optimal mRNA export in *S. cerevisiae*.

DISCUSSION

The study presented here provides insights into the intricate mechanism that regulates *SUS1* expression, a crucial factor linking transcription and mRNA export. We found that (i) *SUS1* introns are required for its functionality, (ii) protein levels of Sus1 are controlled by *SUS1* introns, (iii) *SUS1* expression is regulated by splicing and NMD and (iv) intronic and exonic sequences participate in *SUS1* regulation. The accompanying manuscript by Hossain *et al.* in this issue exposes the relevance of *SUS1* expression as a model for understanding the role of core spliceosomal components in alternative splicing.

Discovery of the *SUS1* gene in a genetic screening revealed an intriguing genomic organization (10). *SUS1* gene (438 nt) consists of three exons (of 71, 140, and 77 nt) and two introns (80 and 70 nt). Notably, their size and position are widely conserved (Supplementary Table S2). Sus1 plays important roles during transcription elongation and mRNA biogenesis, which likely explains its high degree of evolutionary conservation, from yeast to human. Why does this small gene, with key functions at different stages in gene expression, contains two introns in yeast, where most genes contain none? A provocative hypothesis is that this allows the *SUS1* gene to be a sensor of these processes, acting in a yet unknown feedback control mechanism. We demonstrate here that splicing and decay of *SUS1* transcripts regulate expression of Sus1 protein. This strategy is reminiscent of that of *YRA1*, another factor involved in the coupling of mRNA export and transcription. Intriguingly, *YRA1* shares with *SUS1* having both an atypical intron and its expression regulated at the level of splicing, degradation and export of its transcripts, although likely by different mechanisms (22,24,25). Moreover, mutations in *SUS1* and *YRA1* are synthetic lethal, and Sus1 and Yra1 interact physically. A possible scenario is that these factors are finely tuned to work together to sense or modulate correct mRNP biogenesis, as they are sensitive to alterations in several aspects of this pathway.

Our findings reveal that I1 is the major determinant of *SUS1* splicing efficiency. Elimination of I1 or mutation of its BS leads to more efficient *SUS1* splicing. These data are consistent with the observed weak splicing efficiency of I1, largely due to its non-consensus BS and imply that I1 and its BS are crucial for *SUS1* expression. We also show that splicing of *SUS1* largely depends on the BS recognition factor Mud2. However, we see that deletion of I1 has a stronger effect than mutation of the BS on *mud2Δ* cells. These results imply that other features of I1 are dependent on Mud2, likely the non-consensus 5′ splice site of I1 (GUAUGA). In agreement with this, Hossain *et al.*, in the accompanying study have addressed the relevance of non-canonical splicing signals for I1 and I2, revealing that the 5′ splice site of I1 is in fact an important determinant of its processing.

We find an intriguing co-evolution between the presence of a non-consensus BS in I1 and the existence of a conserved long sequence at the 3′-end of the gene. In this context, transcriptome analyses showed that *SUS1* transcripts retaining I1 carry a longer 3′UTR than the fully spliced RNAs (38). We hypothesize that efficient splicing and proper 3′-end formation in *SUS1* could be linked, as it has been shown for other transcripts (44). Current work in our lab tries to address how important is this 3′-end sequence in *SUS1* regulation.

Intron 2 of *SUS1* also appears to play an important role in splicing, despite its apparent lack of unusual features. We find that removing I2 can be detrimental for protein expression for LexA-*SUS1* (Figure 5A, lane 8), and intriguingly, the sole presence of I2 provokes a splicing block when placed into the *TAF14* gene (Figure 6A), which is consistent with the data from Hossain *et al.* addressing the splicing efficiency of I2. In addition, swapping *SUS1* introns also affects Sus1 protein levels, strongly arguing for a co-dependence in splicing of both *SUS1* introns. We also have found that splicing of *SUS1* introns is also affected by exonic sequences, as evidenced by the low expression of *TAF14-SUS1* chimerical constructs (Figure 6). Remarkably this can be suppressed by replacing the *TAF14* newly-created middle-exon with that of *SUS1*. Consistent with this, substituting *SUS1* exon 2 by this *TAF14* 'exon 2' reduces Cup1 expression in *SUS1-CUP1* constructs. Thus our data suggest a positive role for exon 2 in *SUS1* splicing. Work is currently under way to address whether exonic splicing enhancers are involved.

We have verified the biological relevance of *SUS1* introns, by showing that a cDNA version of *SUS1* is not able to fully complement *sus1Δ* phenotypes in mRNA export and growth [Figure 7; we also assess that this is not due to a lack of protein production from an

intron-less construct (lanes 2 and 3 versus 4 and 5, Figure 7B)]. Similar behavior was observed for *YRA1* cDNA (21). Observations that *SUS1* splicing is inefficient under optimal growth (Figure 2A) suggest that *SUS1* splicing could be regulated. Consistently, we find that the ratios between the different species of *SUS1* RNAs are influenced by growth conditions. In fact, at higher temperatures in which *SUS1* function is more critical, pre-mRNA forms retaining the I1 or both introns (I1 and I2) are more abundant (Figure 2B). This change in the transcript ratios could account for the lack of fully complementation by SUS1-cDNA at 37°C (Figure 7B and C). In this context a striking observation is the existence of a small protein containing part of Sus1, likely translated form a transcript containing I1 (Figure 5). Western analyses are consistent with this peptide being produced, in agreement with the functional requirement of transcripts containing I1, suggesting a role of the I1 *SUS1* transcripts. Our results are consistent with the notion that the small Sus1 peptide is not a proteolytic product of the full-length protein. First, it accumulates in *mud2Δupf1Δ* cells, where full-length Sus1 production is reduced (Figure 5); and second, the small Sus1 product is absent in cells bearing an intron-less *SUS1*, where full-length Sus1 is enhanced (Figure 7). An alternate explanation, invoking a selective degradation of full-length Sus1 when encoded by an intron-containing *SUS1* gene, is difficult to conceptualize and not consistent with the abundance of the small peptide in cells bearing the *SUS1* SL-9 BS mutant, which cannot be spliced and thus cannot encode full-length Sus1. Notably, the functional requirement of transcripts containing I1 at high temperature is suggestive of a biological role for this unprocessed *SUS1* transcript. More work is needed to assign this functionality to the pre-mRNA, its product, or both.

Our results, together with those from the accompanying report of Hossain *et al*., provide compelling evidence indicating that expression of *SUS1* hinges on a balance of several factors including limited splicing and degradation pathways, modulated by intronic and exonic sequences. The question now emerges as to how this complex strategy gives *SUS1* its relevant role in multiple aspects of mRNP biogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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