

The odyssey of a regulated transcript

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ABSTRACT

The transcript of the *Saccharomyces cerevisiae* gene, *RPL30*, is subject to regulated splicing and regulated translation, due to a structure that interacts with its own product, ribosomal protein L30. We have followed the fate of the regulated *RPL30* transcripts in vivo. Initially, these transcripts abortively enter the splicing pathway, forming an unusually stable association with U1 snRNP. A large proportion of the unspliced molecules, however, are found in the cytoplasm. Most of these are still bound by L30, as only a small fraction are engaged in translation. Eventually, the unspliced *RPL30* transcripts escape the grasp of L30, associate with ribosomes, and fall prey to nonsense mediated decay.

Keywords: L30; mRNA, nonsense mediated decay; nuclear-cytoplasmic transport; polyribosome; regulated splicing; regulated translation; ribosomal protein

INTRODUCTION

In contrast to most bacterial mRNAs, which are transcribed and translated concurrently, the mRNA of a eukaryotic cell undergoes numerous biochemical alterations and geographic translocations during its lifetime. Splicing factors associated with the C-terminal domain of RNA polymerase II initiate spliceosome formation (McCracken et al., 1997), retaining the transcript within in the nucleus. Upon completion of splicing, or if the spliceosome has failed to form (Legrain & Rosbash, 1989; Long et al., 1995), the transcript is transported to the cytoplasm in ways that are only partly understood (reviewed in Nakiely & Dreyfuss, 1999). Once in the cytoplasm, the spliced mRNA is efficiently translated and eventually degraded, generally through shortening of its poly(A) tail, subsequent decapping, and eventual digestion by nucleases (reviewed by Caponigro & Parker, 1996). Transcripts with premature termination codons, due either to mutation or to failure of the splicing process, are subject to accelerated decay by the nonsense-mediated decay (NMD) pathway, (reviewed in Jacobson & Peltz, 1996). Several, perhaps all, of the steps in an mRNA's life can be subject to regulation.

Much of the information regarding the fate of an mRNA has been obtained using test genes containing artificial introns and/or prokaryotic coding domains. We now

exploit the opportunity offered by the transcript of the *RPL30* gene (formerly *RPL32*; Mager et al., 1997) to follow the fate of a specific, natural transcript of *Saccharomyces cerevisiae* that is subject to regulation both of splicing and of translation.

RPL30 encodes the essential ribosomal protein L30 (Dabeva & Warner, 1987). Its transcript contains a single intron that under normal conditions is effectively spliced. Its mRNA is abundant (~37 molecules per cell; Holstege et al., 1998) and relatively short-lived, with an estimated $T_{1/2}$ of 5–7 min (Li et al., 1999). The *RPL30* transcript contains a potential structure (Eng & Warner, 1991; Vilardell & Warner, 1994; Mao et al., 1999) that appears to mimic the binding site of L30 in the 60S ribosomal subunit (Fig. 1A; Vilardell et al., 2000). L30 can bind to and stabilize this structure. In doing so it inhibits splicing of the transcript at an early stage in spliceosome formation (Vilardell & Warner, 1994). Because the structure is composed largely of nucleotides in the first exon, L30 also binds the spliced *RPL30* mRNA, with somewhat lower affinity, and inhibits translation (Dabeva & Warner, 1993). Thus, molecules of L30 that are not assembled directly into ribosomes, due to some imbalance among the 80 gene products needed to construct a ribosome (Warner, 1999), employ these interactions as an effective and biologically important feedback regulation of L30 synthesis (Li et al., 1996).

We describe below the odyssey of the transcripts of *RPL30* subject to this regulation, the inhibition of splicing that leads to extended association with U1 snRNP,

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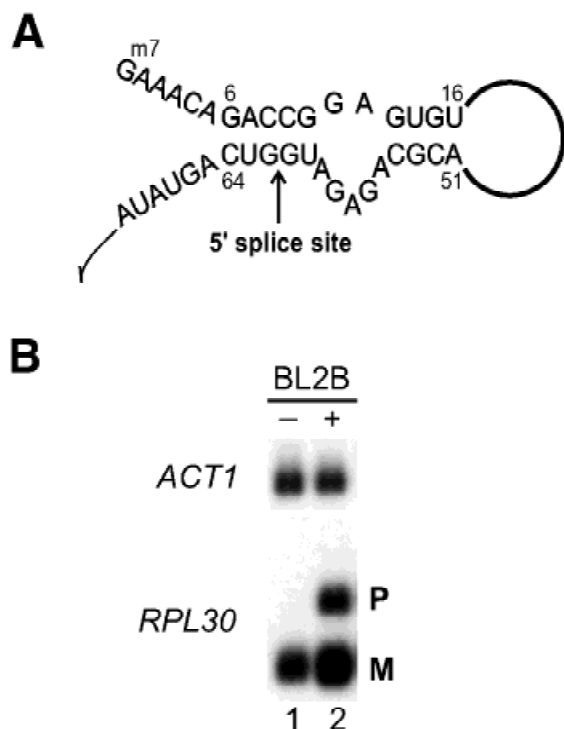


FIGURE 1. A: Structure of the 5' region of the *RPL30* transcript that binds L30 (Vilardell & Warner, 1994). The 5' splice site is indicated. The initiator AUG is just upstream of the 5' splice site. **B:** Accumulation of *RPL30* transcripts in a merodiploid. RNA was prepared from strains BL2B+ and BL2B-, fractionated on an agarose gel, and probed with riboprobes complementary to exon 2 of *RPL30* transcripts (P: unspliced precursor; M: spliced mature mRNA) and to *ACT1* mRNA. PhosphorImager quantitation, normalized to the level of *ACT1* mRNA and to U3 RNA, indicated that the ratio of mRNA in the two strains is about 2, and the ratio of pre-mRNA to mRNA in strain BL2B+ is about 0.5.

the distribution between nucleus and cytoplasm, the inhibition of translation of both spliced and unspliced forms, and the eventual degradation of the unspliced form by the NMD pathway.

RESULTS

The aim of this work was to follow the fate of a transcript that is subject to regulation of both its splicing and its translation. We constructed two otherwise isogenic strains, attempting both to perturb the cell as little as possible and to generate a true negative for in situ hybridization. In strain BL2B, the genomic *RPL30* had been replaced by a cDNA version, with the intron precisely removed (Li et al., 1996). Strain BL2B was transformed with a vector that integrated at the *URA3* locus to yield strain BL2B-, or with the vector carrying an intact *RPL30*, to yield strain BL2B+ (see Materials and Methods). To optimize the signal-to-noise ratio, we selected a transformant with two copies of the plasmid inserted in tandem. The insertions were verified by genomic PCR and Southern analysis. Both strains grow well.

The *RPL30* transcripts from strains BL2B- and BL2B+ are shown in Figure 1B. It is apparent that strain BL2B+ has accumulated unspliced transcript, as expected in a cell overproducing L30. Based on the measurement of 37 *RPL30* mRNAs per cell (Holstege et al., 1998), quantitative analysis of Figure 1B suggests that a cell of strain BL2B+ has about 35 unspliced *RPL30* transcripts.

Location of the unspliced transcript of *RPL30*

The location of the unspliced transcript of *RPL30* was determined using fluorescent in situ hybridization (FISH) with oligonucleotides complementary to intron sequences (see Materials and Methods). The intron of *RPL30* contains no snoRNAs; extensive hybridization failed to reveal any free intron, in either lariat or linear form (data not shown), due to the rapid degradation of the spliced introns (Vijayraghavan et al., 1989). Thus, the only molecules detected by FISH should be the intact, unspliced transcripts. Because there are no intron sequences in strain BL2B-, the signal in Figure 2A is a true measure of nonspecific background.

The bright foci in the nuclei of strain BL2B+ (Fig. 2B) are presumably sites of transcription (Long et al., 1995). It is evident that the tiny spots representing the unspliced mRNA are scattered throughout the cell, with the major part in the cytoplasm (Fig. 2B). This result is somewhat surprising given that in vitro the unspliced transcripts are associated in a relatively tight complex with U1 snRNP particles (Vilardell & Warner, 1994), and thus were expected to be in the nucleus. On the other hand, these results are consistent with previous findings that an artificial construct with a defective 5' splice site could be found in both cytoplasm and nucleus (Long et al., 1995).

Association of the unspliced transcript with U1 snRNP in vivo

If the unspliced transcripts are associated with U1 snRNP in vivo, as they are in vitro, the results of Figure 2B suggest either that U1 is carried to the cytoplasm by the unspliced *RPL30* transcript, or that only a fraction of the pre-mRNA is associated with U1, probably remaining in the nucleus. We attempted to detect increased cytoplasmic localization of U1 snRNA by FISH. However, only a faint cytoplasmic signal was visible in either strain, BL2B+ or BL2B- (not shown); most U1 snRNA is nuclear.

An alternative way to assess the degree of association of U1 snRNP with the unspliced *RPL30* transcript in vivo is by coimmune precipitation (co-IP) with an antibody directed against one of the proteins specific to the U1 snRNP, in this case Snu71p (Gottschalk et al., 1998). As shown in Figure 3, a fraction of the unspliced transcript is associated with U1 snRNP (lane 3), an in vivo

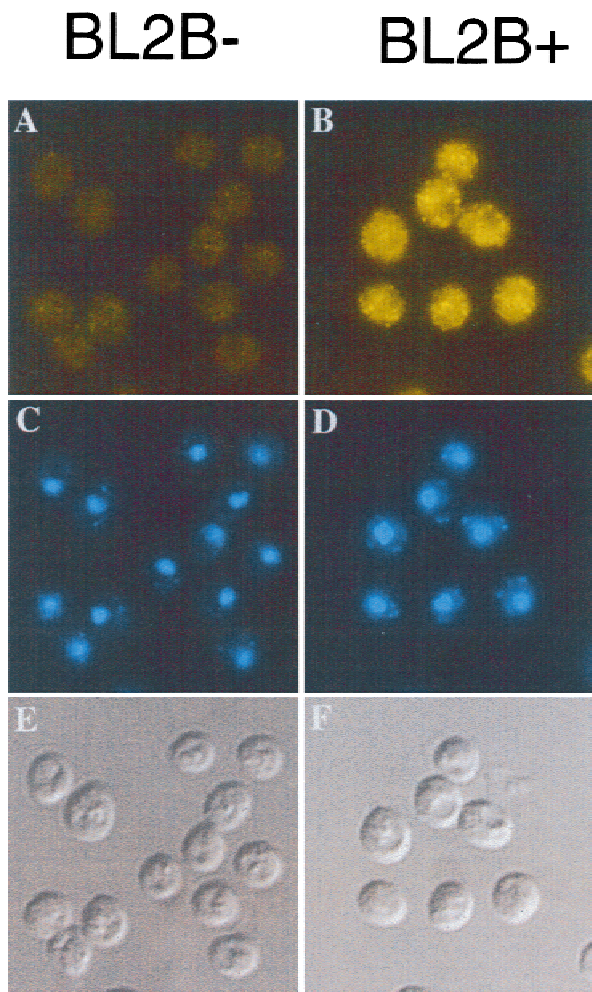


FIGURE 2. Fluorescence in situ hybridization analysis of the cellular distribution of the *RPL30* pre-mRNA intron in the yeast strains BL2B⁻ (*RPL30*Δintron) and BL2B⁺ (*RPL30*Δintron/*RPL30*). **A,B:** The fixed cells were hybridized with fluorescently labeled oligonucleotides complementary to the *RPL30* intron (see Materials and Methods); **C,D:** DAPI (diaminophenylindole) staining; **E,F:** Nomarski optical image.

confirmation of the *in vitro* observation (Vilardell & Warner, 1994). Based on the recovery of ~20% of the U1 snRNA in the immune complex, we estimate that approximately 10% of the unspliced transcript is associated with U1 snRNP. This is consistent with the proportion of unspliced transcript visualized in the nucleus (Fig. 2B), and explains the lack of an effect on the localization of U1 snRNA. Probing the blot for the constitutively spliced *RPS6B* transcript revealed vanishingly small amounts of unspliced transcript in association with U1 snRNP (Fig. 3). These data suggest that the co-IP of the *RPL30* transcript and U1 snRNP (Fig. 3) reflects not constitutive splicing but a regulatory interaction.

The unspliced transcript in the cytoplasm is not readily translated

To examine the activity of all the *RPL30* transcripts, we subjected cell extracts to sucrose gradient analysis

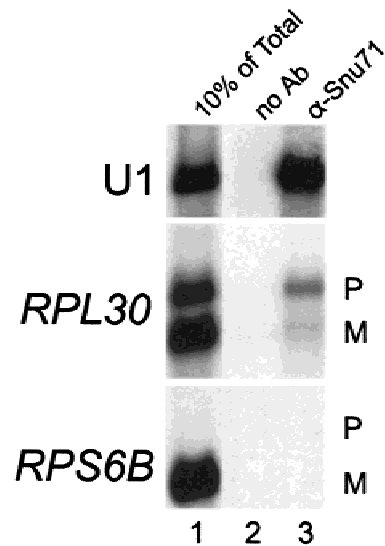


FIGURE 3. Coimmune precipitation of *RPL30* pre-mRNA with U1 snRNP. Whole-cell extracts of strain BL2B⁺ were reacted with anti-serum directed against Snu71p, a component of the U1 snRNP (see Materials and Methods). RNA was prepared from bound fractions, subjected to Northern analysis, and probed with riboprobes directed against U1 snRNA, exon 2 of *RPL30* RNA, and exon 2 of *RPS6B* RNA (P: unspliced precursor; M: spliced mature mRNA). Lane 1: 10% of total input RNA; lane 2: RNA from extract bound to protein A beads only; lane 3: RNA from extract bound to protein A beads containing anti-Snu71p.

(Fig. 4). L30 can bind to its spliced transcript (most of the binding site being within exon 1, as seen in Fig. 1; Vilardell & Warner, 1994) and inhibit its translation (Dabeva & Warner, 1993). This is evident even in strain BL2B⁻: although most of its *RPL30* mRNA is found in small polyribosomes, consistent with its 105 codon ORF, a fraction sediments at the top of the gradient, not associated with ribosomes (Fig. 4A). This probably is due to a modest overexpression of L30 that occurs because the transcript from the cDNA gene is not subject to regulated splicing. By contrast, essentially all of the mRNA encoding another ribosomal protein, S23, is engaged with ribosomes in both strains. As expected, the *RPS23* mRNA, with 145 codons, is associated with larger polyribosomes. A probe for *ACT1* yielded the same result (not shown).

In strain BL2B⁺, the pattern is strikingly different (Fig. 4B). Not only are substantial amounts of the spliced transcript of *RPL30* found at the top of the gradient, but nearly all of the unspliced *RPL30* transcript is found there as well, suggesting that it is relatively inaccessible to ribosomes. Although some of these unspliced transcripts are nuclear molecules in this whole-cell extract, the major portion must be those molecules seen in the cytoplasm in Figure 2B. Presumably L30 remains associated with these molecules during their transport from the nucleus to the cytoplasm. The high proportion of the unspliced molecules not associated with ribosomes is likely due both to the tighter binding

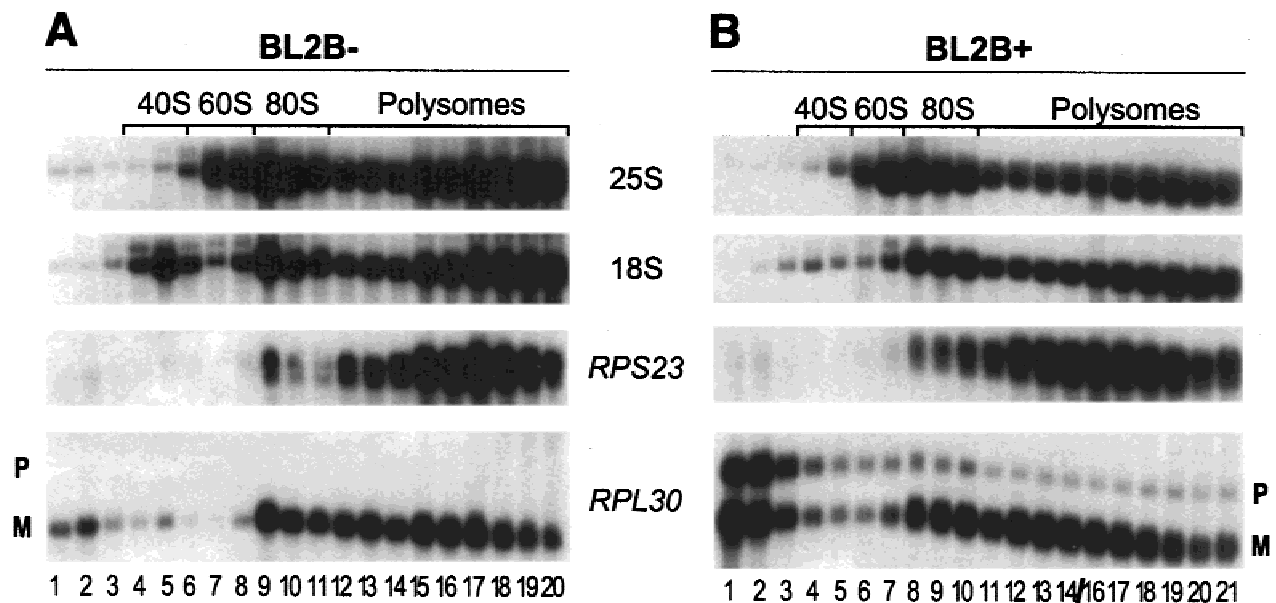


FIGURE 4. Control of translational initiation of *RPL30* transcripts. Whole-cell extracts, using a bead-beating method, were prepared from log phase cells of strains BL2B⁻ and BL2B⁺ and analyzed on sucrose gradients (see Materials and Methods). RNA was prepared from each fraction, separated on an agarose gel, and probed with oligonucleotides to detect 18S and 25S rRNAs, and with riboprobes directed against exon 2 of *RPL30* (P: unspliced precursor; M: spliced mature mRNA) and against *RPS23*. The two genes encoding S23 yield mRNAs of slightly different size, accounting for the breadth of the band (Alksne & Warner, 1993). Note that fraction #15 from the BL2B⁺ gradient was lost. The location of 40S, 60S, 80S, and polyribosomes are indicated. Centrifugation conditions were such that polyribosomes with more than four to five ribosomes ran to the bottom.

of L30 to the unspliced transcript (Vilardell & Warner, 1994) and to the loss of translated molecules to NMD (see below).

There is no sign of either spliced or unspliced *RPL30* transcripts accumulating in the ~40S region of the sucrose gradient (Fig. 4B), suggesting that bound L30 inhibits initiation at a step prior to the association of the RNA with 43S initiation complexes. This is to be expected, because the structure stabilized by the binding of L30 extends to within a few nucleotides of the CAP site (see Fig. 1). However, comparison of the spectrum of L30 mRNA from BL2B⁻ and BL2B⁺ shows essentially no difference in polysome size. This result suggests that once translation initiates, upon dissociation of L30, it proceeds with no further interference from L30. The binding of ribosomes and/or initiation factors must occlude the L30 binding site.

A surprising observation is that in strain BL2B⁺, there is a clear deficiency of free 40S subunits (Fig. 4B, lanes 3–6). Analysis of the total RNA, however, revealed no significant difference in the ratio of 40S to 60S subunits overall. The free subunits are a tiny fraction, usually <10%, of the total complement of ribosomes.

The ultimate fate of the unspliced transcript

Although some of the unspliced molecules are associated with ribosomes, most are not (Fig. 4B). We asked

whether the major part of the molecules were simply degraded, or were ultimately translated and subjected to NMD, which should occur when the ribosome encounters a stop codon at the 15th position of the intron. Strain BL2B⁺ was crossed with a strain carrying the *upf1::LEU2* knockout, which inactivates the NMD pathway. Spores were dissected and genotyped. Three spores carrying the two *RPL30* genes were selected. 1a is *UPF1*; 4a and 7a are *upf1::LEU2*. RNA was prepared from each, and the level of unspliced *RPL30* transcript was compared (Fig. 5). As a control, the transcripts of the ribosomal protein gene *CYH2* were analyzed. This transcript is constitutively poorly spliced due to interfering sequences within the intron (Swida et al., 1988), but there is no evidence of regulated translation. The $T_{1/2}$ of the unspliced *CYH2* transcript was reported as fourfold greater in a strain in which the NMD pathway is not functional (He et al., 1993). In our case, we observe a 5- to 10-fold accumulation of unspliced *CYH2* transcripts in the strains lacking *UPF1* (4a and 7a, Fig. 5). Similarly, the deletion of *UPF1* from the strain carrying two *RPL30* genes leads to about a twofold increase in the amount of unspliced transcript, showing that these transcripts are subject to NMD when they eventually do enter translation. The effect on *RPL30* transcripts appears to be less than on those of *CYH2* in a $\Delta upf1$ strain. However, this appearance is misleading because translational control leads to the accumulation, in *UPF1* cells, of a substantial amount of unspliced

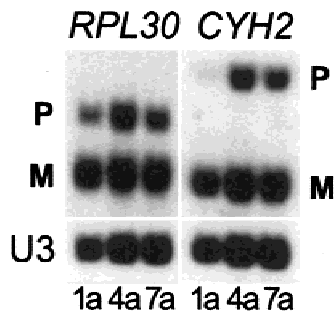


FIGURE 5. Strain BL2B+ was crossed with strain YAH01 (Hennigan & Jacobson, 1996) carrying the *upf1::LEU2* gene disruption. Spores were dissected and three spores carrying the two *RPL30* genes from BL2B+ were selected, one with the *UPF1* gene (1a) and two with the *upf1::LEU2* allele (4a and 7a). RNA was prepared, fractionated on an agarose gel, and probed with oligonucleotide JW61L to detect *RPL30* transcripts, with oligonucleotide JW425 to detect *RPL28 (CYH2)* transcripts (P: unspliced precursor; M: spliced mature mRNA), and with oligonucleotide JW449 to detect U3 snoRNA transcripts.

RPL30 transcripts that would otherwise be rapidly degraded as they entered the translational apparatus. We conclude that the unspliced transcript can be engaged by ribosomes, a necessary step for it to be degraded by the NMD pathway (Jacobson & Peltz, 1996).

DISCUSSION

It is instructive to consider the quantitative physiology of the process in which L30 binds to its transcript to autoregulate its own synthesis. Approximately 200,000 molecules of L30 are synthesized during each 100-min generation (Warner, 1999). To encode this prodigious output, the cell produces about 200 transcripts from *RPL30* per generation. [This number is based on *RPL30* mRNA at a steady state of 37 molecules per cell (Holstege et al., 1998), with a $T_{1/2}$ of 7.5 min (Li et al., 1999).] Ribosomal proteins are imported through nuclear pores, using conventional nuclear localization signals (Underwood & Fried, 1990; Schaap et al., 1991), perhaps with a specific karyopherin β (Rout et al., 1997), and are rapidly concentrated in the nucleolus (Warner, 1979). Yet little is known about the dynamics of this flow. In particular, is there a "direct route" to the nucleolus? Do the ribosomal proteins have an opportunity to interact with nuclear components on their way? In spite of 1,000 molecules of L30 flowing through the nucleus for every *RPL30* transcript produced, there is little opportunity for their interaction, because in a normally growing cell, the ratio of spliced to unspliced *RPL30* transcripts is greater than 50:1 (Dabeva et al., 1986). We suggest that there is rapid passage of newly formed ribosomal proteins to the nucleolus, where they are firmly bound. As has been found on overproduction of snoRNA molecules (Samarsky et al., 1998), only when L30 is in excess over the ribosomal precursor RNA on

which it assembles does it escape to the nucleus. Then L30 can play its regulatory role.

The number of L30 molecules involved in regulation can be estimated from the number of *RPL30* transcripts at the top of the gradient in Figure 4B, that is, about 100. This is far too small a number to be distinguished from the 200,000 copies of L30 that are present in ribosomes and thus precludes a direct demonstration of the escape of excess L30 molecules from the nucleolus.

The travels of an *RPL30* transcript in a cell overproducing L30 are summarized in Figure 6. The level of spliced mRNA (Fig. 1B) suggests that many if not most of the transcripts progress through the splicing process, either because they do not encounter an L30 molecule, or because it dissociates. However, a substantial fraction of the transcripts accumulate in an unspliced form (Fig. 1B). Some 10% of these are associated with U1 snRNP (Fig. 3), and we deduce that these are present as abortive splicing complexes bound to L30, as we have observed in vitro (Vilardell & Warner, 1994). Surprisingly, however, most of the unspliced molecules are in the cytoplasm (Fig. 2). It seems likely that

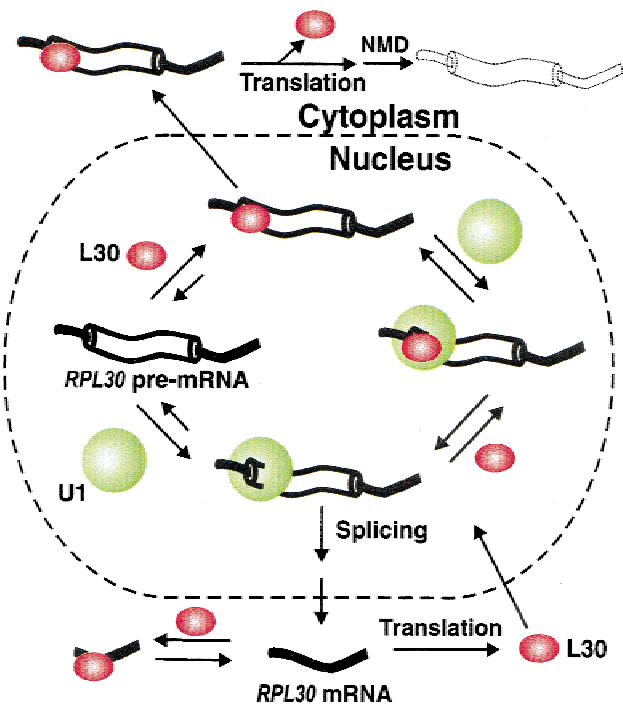


FIGURE 6. Schematic representation of the odyssey of the regulated *RPL30* transcript. Under normal conditions the production of ribosomal proteins is balanced: there is no L30 excess and the *RPL30* transcripts are efficiently spliced, exported, and translated. If an imbalance in ribosomal biosynthesis leads to an excess of L30, the autoregulatory circuitry is invoked. L30 binds to its transcript, leading to an inhibition of splicing. Unspliced transcripts can pass to the cytoplasm, and remain there largely untranscribed. Dissociation of L30 allows initiation of translation, leading to NMD. Furthermore, L30 also binds to its mature mRNA, inhibiting its translation. Cells incapable of this regulation are easily outgrown by the wild type (Li et al., 1996).

these cytoplasmic molecules migrated from the nucleus only after release from the abortive splicing complex, upon dissociation of U1 snRNP. However, some of the cytoplasmic molecules may have associated with L30 and bypassed the splicing apparatus altogether. If so, then only a small fraction of the *RPL30* transcripts associated with L30 bind to U1 snRNP, and these are retained in the nucleus until degraded by an as yet unknown mechanism. Currently we cannot distinguish between these alternatives.

The unspliced transcripts in the cytoplasm are largely untranslated (Fig. 4), suggesting continued association with L30. No accumulation of an initiation complex is seen, showing that the inhibition of translation does not permit association of the RNA with 40S subunits. Similarly, translation of the spliced transcript is also inhibited. Again no accumulation of initiation complexes is seen, and the size of the polyribosomes is the same as the control. These results suggest that once translation has started on an mRNA, the presence of translation initiation factors prevents formation of the specific structure to which L30 binds. Thus, the inhibition of translation by L30 is an all-or-none process.

A minor portion of the unspliced transcript is associated with small polyribosomes. Because of a nonsense codon only five codons into the intron, this RNA cannot be fully translated and is subject to NMD (Fig. 5).

The presence of excess L30 leads to a substantial decrease in the amount of free 40S subunits (Fig. 4B), although there is a negligible alteration in the overall ratio of 40S to 60S subunits. A possible explanation for this surprising observation derives from our recent suggestion that L30 may be responsible for stabilizing a stem-loop in the 25S rRNA that interacts with the 40S subunit (Vilardell et al., 2000). The *RPL30* transcript complexed with L30 mimics this feature of rRNA. Perhaps this complex occasionally interacts either with a pre-40S subunit to inhibit its maturation, or with an initiating 40S subunit as it awaits the 60S subunit on the mRNA. This would deplete the pool of free 40S subunits without necessarily having a major effect on overall translation.

MATERIALS AND METHODS

Strains

Strain BL2B is a derivative of W303: *MATa leu2-3, 112 his3-11 trp1-1 ura3-1 ade2-1 can1-100 ssd1-1* (Thomas & Rothstein, 1989), in which the intron of the *RPL30* gene has been precisely deleted (Li et al., 1996). Strain BL2B⁻ was generated by integrative transformation of strain BL2B using the vector pUC-*URA3*, cut within the *URA3* gene by *StuI*. pUC-*URA3* consists of pUC18 into which had been ligated the 1.1-kB *HindIII* fragment containing the *URA3* gene. Strain BL2B⁺ was generated in the same way, using pUC-*URA3* into which

had been inserted a 2.2-kB *EcoRI* fragment containing the *RPL30* gene (Dabeva & Warner, 1987). In both cases, integration at the *URA3* locus was verified by PCR. Strain YAH01, *MATa ura3 rpb1-1(ts) upf1::LEU2*, was a gift from A. Jacobson. It was crossed with strain BL2B⁺, and non-*ts* spores that contained the two *RPL30* genes, with or without the *upf1* deletion, were chosen for use.

General methods

Cultures were chilled on crushed ice and RNA was prepared and subjected to Northern analysis as previously described (Li et al., 1999), using either riboprobes or oligonucleotides: for *RPL30*, JW61L: CATCTCTGCGTATATTGATTAA; for *CYH2 (RPL28)*, JW425: GTCCAAGTTCAAGACTGGCTTCC; for U3 (*SNR17*), JW449: GGATTGCGGACCAAGCTAA; for 18S rRNA JW149: CAAGAAAGAGCTCTCAATCTGT; and for 25S rRNA: JW1120 GCGAGATTCCTACCCAC.

Extracts were prepared for sucrose gradient analysis as described previously (Dabeva & Warner, 1993). Fractions were dripped into 1% SDS, RNA prepared by extraction with phenol, precipitated, and an equal aliquot of each fraction was subjected to Northern analysis.

In situ hybridization

Cultures were grown in 50 mL of YPD medium to mid-log phase at 30 °C and the cells fixed with 1/10 volume of fresh 40% formaldehyde (Electron Microscopy Sciences, Fort Washington, Pennsylvania) for 45 min at room temperature. The cells were collected by centrifugation and washed three times with 1.2 M sorbitol and 100 mM potassium phosphate, pH 7.5. Spheroplasts were generated by incubating the cells in 1 mL of 0.1 mg/mL oxalyticase (Enzogenetics, Corvallis, Oregon), 1.2 M sorbitol, 100 mM potassium phosphate, pH 7.5, 28.6 mM β -mercaptoethanol, 60 μ g/mL phenylmethylsulfonyl fluoride, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin, 5 μ g/mL pepstatin, 20 mM vanadyl ribonucleoside complex (VRC) (Gibco BRL), 120 U/mL RNase inhibitor (Boehringer Mannheim) for 8 min at 30 °C. Spheroplasts were washed, adhered to coverslips pre-coated with 0.01% poly-L-lysine, and stored in 70% ethanol at -20 °C.

Each coverslip for in situ hybridization was removed from 70% ethanol and rehydrated twice in 8 mL of 2 \times SSC for 5 min and once in 2 \times SSC/40% formamide for 5 min. Coverslips were inverted onto 24 μ L of a solution containing 40% formamide, 2 \times SSC, 2.5 mg/mL BSA, 10 mM VRC, 60 U of RNase inhibitor, 10 ng of each Cy3-labeled probe, 10 μ g of sonicated salmon sperm DNA, and 10 μ g of yeast tRNA. Hybridizations were performed overnight at 37 °C. Following hybridizations, each coverslip was washed twice at 37 °C for 15 min in 40% formamide/ 2 \times SSC, once in 2 \times SSC/0.1% Triton X-100 for 15 min, twice in 1 \times SSC for 15 min, and once in 1 \times PBS for 15 min. Coverslips were mounted in phenyl-enediamine containing glycerol and DAPI.

Two fluorescently labeled oligonucleotides complementary to sequences in the *RPL30* intron were used for in situ hybridization:

L30I-1: 5'-GCC TTC TXG CTAATC CCA XGAAAG AAX AAA GCG AAA XAG TTA TAA AAX CA-3'

L301-2: 5'-TTX TTG CAT ATC XCA CTT TTA TXT CAC AGT CAX GGA GAA GCA XCCTTT GA-3', where X = Cy3-labeled 6-amino dTTP.

Images were captured using CellScan software (Scanalytics, Fairfax, Virginia) on an Optiplex GXpro computer (Dell, Austin, Texas) with a CH-250 16-bit, cooled CCD camera (Photometrics, Tucson, Arizona) mounted on a Provis AX70 fluorescence microscope (Olympus, Melville, New York) with a PlanApo60x, 1.4 NA objective (Olympus) and HiQ band-pass filters (Chroma Technology, Brattleboro, Vermont). Single-plane images were captured at 3 s exposure time for Cy3 and 0.5 s for DAPI and Nomarski, then analyzed and processed using Adobe Photoshop.

Immunoprecipitation

A 500-mL culture of strain BL2B+ was grown in YPD until OD₆₀₀ reached 0.5, immediately chilled on ice, the cells recovered by centrifugation, washed, and resuspended in 1 mL of 10 mM HEPES-KOH, pH 7, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, and 1 mM PMSF. A 150- μ L aliquot was transferred to an Eppendorf tube and the cells broken by vortexing with glass beads at 4°C for four 1-min pulses keeping them on ice in between. The tube was spun again for 5 min and the supernatant was subdivided into three aliquots. To each aliquot, 85 μ L of IP buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 0.5 mM DTT, 0.05% Nonidet NP40) and 20 μ L of protein A-Trisacryl beads, with or without anti-Snu71p antibodies (see below), were added. After 1 h incubation at 4°C the beads were recovered from the extracts and washed three times with IP buffer, each for 15 min at 4°C. After the last wash, the beads were resuspended in 100 μ L of 50 mM EDTA, pH 8, 1% SDS, 100 ng/ μ L *Escherichia coli* tRNA, 100 ng/ μ L Proteinase K (Boehringer), transferred to a new tube, and digested 20 min at 37°C. Bound RNA was purified by phenol/chloroform extraction and subjected to Northern analysis, using riboprobes against U1 and *RPL30* transcripts. The protein A beads were prepared as follows. Protein A beads (Pierce) were washed in IP buffer containing 100 ng/ μ L of BSA and *E. coli* tRNA, incubated with or without anti-Snu71p antiserum (10 μ L serum: 20 μ L beads) (Gottschalk et al., 1998) for 1 h at 4°C, and finally washed with IP buffer containing 100 ng/ μ L of *E. coli* tRNA.

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