Ribosomal Protein L32 of Saccharomyces cerevisiae Regulates Both Splicing and Translation of Its Own Transcript*

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Ribosomal protein L32 of Saccharomyces cerevisiae regulates the splicing of its own transcript (1, 2) apparently by interacting with a structure composed largely of the 5′ exon. However, even in strains overproducing L32 mRNA, e.g. from a cDNA copy of the gene, little accumulation of L32 is observed after a brief pulse label. When the 5′ leader of the RPL32 mRNA is replaced by an exogenous leader, the amount of pulse-labeled L32 increases severalfold, suggesting that L32 regulates the translation of its own mRNA, acting through sequences in the 5′ region. This conclusion was confirmed by the observation that in cells carrying a chimeric gene in which the L32 leader is fused to LacZ coding sequences, the presence of a second gene that overexpresses L32 itself reduces the level of β-galactosidase by 50%, in spite of a doubling of L32-lacZ fusion mRNA, presumably due to stabilization of the message. Mutations within the 5′ leader that abolish the regulation of splicing also abolish the regulation of translation, suggesting that the regulation of translation by L32 involves a structure similar to that proposed for the regulation of splicing. In cells overproducing L32-mRNA about half the excess mRNA was found in ribonucleoproteins of <25 S, unassociated with ribosomal particles. Much of the rest was found in ribonucleoproteins of 80-120 S.

The regulation of the synthesis of ribosomal proteins at the level of translation is a widespread phenomenon. It appears to be the fundamental level of control of ribosomal protein synthesis in Escherichia coli (reviewed in Refs. 3 and 4). The ribosomal protein operons are transcribed constitutively. For each operon there is one ribosomal protein that, if it does not bind to a molecule of newly formed ribosomal RNA, can bind to its mRNA and prevent translation. In some but not all cases, it is possible to show that the binding site on the mRNA mimics the binding site for that protein on the rRNA.

In the cells of higher eukaryotes, including mammals, Xenopus, Drosophila, and Dictyostelium, regulation of translation plays a key role in the control of ribosomal protein synthesis (reviewed in Refs. 5 and 6). In vertebrates the 5′ leader of the mRNA contains a polypurine tract that regulates the mobilization of this mRNA onto polyribosomes (6–8). A potential regulatory protein has recently been identified (9). On stimulation of growth by addition of growth factors (10, 11) or by partial hepatectomy (12), these mRNAs are effectively recruited into polyribosomes, perhaps by activation of elf 3 or 4 (7, 12). In Dictyostelium the onset of differentiation causes the mRNA for ribosomal proteins to leave the polyribosomes, probably due to selective loss of poly(A) tails (13).

By contrast, in Saccharomyces cerevisiae, the general control of ribosomal protein synthesis (reviewed in Ref. 14) appears to occur through coordinate transcription of the ribosomal protein genes (15–19) whose promoter strengths are such to yield roughly equimolar amounts of mRNA (20). While the coordinate transcription is presumably due in part to the Rap1p and Abf1p binding sites found in the upstream activating sequences of all ribosomal protein genes (21–23), the details are far from clear.

In addition to this coordinate regulation of ribosomal proteins, there are mechanisms to modulate the accumulation of individual proteins. For the most part, this is due to very rapid degradation of any ribosomal protein synthesized in excess (24–27), a situation that also occurs in mammalian cells (20). However, we have identified one ribosomal protein, L32, that controls its own synthesis by regulating the splicing of the transcript of its own gene (1). This appears to occur through interaction with a specific structure found in the 5′ exon and the first few nucleotides of the intron (2). We now show that L32 can also regulate the translation of its own mRNA, and that the sequences involved in the regulation of splicing are also responsible for the regulation of translation. L32 stabilizes the mRNA, and prevents at least some of it from interacting with ribosomal particles.

**MATERIALS AND METHODS**

YEAST STRAINS—S. cerevisiae strains S150–2B (MATα, trpl–389, his3–1, ural–3–2, leu2–3, 11) and W303 (Matα, ade–2–1, his3–11, 15, leu2–3, 112, trpl–1, ural–3–1, can1–100) were used. Plasmids—Five plasmids overexpressing L32 were constructed (Table I): A, a subclone of pYERPL32 (29) of about 1.8 kilobase pairs, containing the complete RPL32 gene; B, a cDNA clone isolated from a cDNA library (30), modified by fusing the authentic L32 terminator to the coding sequences; C, a chimeric RPS10-RPL32 gene (described in Ref. 2) in which the fusion point is in the middle of S10-L32 intron (this gene encodes the complete L32 protein except for an additional lysine residue at the N terminus); D, a chimeric plasmid in which the expression of L32 was under GAL10 promoter and the RPL32 leader was substituted by the CYC1 leader. For construction of these plasmids, the multicyclop vectors Yep351 andYep352 (31) were used. Plasmids overexpressing β-galactosidase from LacZ of E. coli were based on the Yep354, Yep357, and Yep367 vectors (32). The expression of LacZ was driven by the RPL32 or ADH1 promoter. The L32 leader was fused to the LacZ gene either 5 nucleotides (plasmid E) or 120 nucleotides (plasmid F) after the beginning of exon 2. The β-galactosidase activity of the transformants was determined as described (33).

An integrating version of plasmid E was mutagenized by replacing RPL32 sequences with sequences derived from previously constructed mutants (2) by polymerase chain reaction. One mutant (E2C+T; see Table III) was newly made. Mutant regions were sequenced to verify the mutation.

**Northern and Southern Blot Analysis**—Total genomic yeast DNA was isolated and digested with restriction enzymes and blotted to a nitro-
cellulose membrane as described before (34). Northern blot analysis was carried out as previously described (1, 2). The blots were probed with RNA or oligonucleotide probes as appropriate.

Polyribosome Analysis—To 100 ml of culture at A_{600} = 1.0, 150 µg/ml cycloheximide was added. The cells were chilled, poured over ice-cold water, and collected in 20 ml portions with 20 ml of LHB buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 30 mM MgCl₂), containing 50 µg of cycloheximide, 200 µg of heparin, and 0.2 µl of DEPC/ml.

The final pellet was suspended in 0.5 ml of LHB as above, and 1 ml of glass beads was added. The cells were broken by discontinuous vortexing for 210 s, interrupted by cooling in ice. Then 1.5 ml of LHB buffer containing 0.2% Nonident-P-40 was added and the suspension vortexed for another 30 s and centrifuged for 10 min at 10,000 rpm. The supernatant was recentrifuged for 10 min at 12,000 rpm.

The supernatant after the second centrifugation (about 1.2–1.3 ml) was overlaid on top of 11.5 ml of 7–47% (w/v) sucrose gradient made in 50 mM Tris-acetate, pH 7.0, 100 mM NaCl, 12 mM MgCl₂, 1 mM dithiothreitol and centrifuged for 170 min at 39,000 rpm and 4 °C in an SW 41 rotor.

Thirty-three fractions of 375 µl were collected, and a 20-µl aliquot of each was loaded on a native 1.5% agarose gel in 0.5 × TBE buffer, containing 1 mM MgCl₂. The electrophoresis was run for 5 h at 100 V with circulating buffer. The gel was blotted to Nytran under denaturing conditions (6% formaldehyde, 10 × SSC) for 16–18 h. The blots were UV cross-linked and baked before hybridization.

Synthesis of Ribosomal Proteins—A culture was labeled with either [³⁵S]methionine or EXPRESS³⁵S (Du Pont-New England Nuclear) for the time indicated, and then poured over crushed ice to stop incorporation. The cells were collected by centrifugation and broken with glass beads, and the total protein was extracted in 67% glacial acetic acid.

The cells were transformed with either the vector, or plasmid C, overproducing a chimeric mRNA in which the L32 5' leader is replaced by another 5' leader (Table I, constructs C and D). Yeast cells were transformed with plasmid C, overproducing a chimeric mRNA. RNA was prepared from the cells and subjected to Northern analysis (Fig. 1D). Cells were pulse-labeled with [³⁵S]methionine for 3 min and the total protein fractionated on two-dimensional gels (Figs. 1, panels E and F).

Fig. 1F demonstrates that the presence of a foreign 5' leader leads to substantially greater production of L32. This could be due to either an intrinsically higher level of translation initiation or to a resistance to the autogenous regulation of translation. Certainly, the result in panel F suggests strongly that the lack of overexpression of L32 in panel C is not due to extremely rapid turnover of excess L32. However, the protein produced from construct C begins M-K-A instead of the M-A of authentic L32 (Table I); the former might be more stable, although proteins with lysine at their N terminus are considered to turn over more rapidly than those starting with alanine (35).

Therefore cells were transformed with plasmid D, in which L32 mRNA coding sequences were fused to the CYC1 5' leader and transcription is driven by the conditional GAL10 promoter (Table I). This construct codes for authentic L32. It is clear in Fig. 1 that massive overproduction of this CYC1-RPL32 fusion mRNA (panel G) leads to massive overproduction of L32 (panel I).

The results shown in Fig. 1 suggest that the translation of the RPL32 mRNA is under autogenous regulation through sequences within the 5' exon. While they do not rule out the possibility that the initiation of translation on the 5' leader of the L32 mRNA is inherently less efficient than that of S10, we have shown that generally the RNAs for different ribosomal proteins are roughly equimolar (20). Since they make equimolar amounts of protein, they are likely to be translated with equal efficiency.

**Translational Regulation of L32 Involves the 5' Leader of L32**

### RESULTS

**L32 Is Not Overproduced in Strains Synthesizing Its mRNA in Excess**—The synthesis of ribosomal protein L32 is regulated in part by a feedback inhibition of splicing (1) in which L32, acting through a structure composed largely of the 5' exon of the RPL32 transcript, blocks the formation of a splicing complex (2). Nevertheless, in cells containing several extra copies of RPL32, some excess spliced mRNA is present, but little detectable accumulation of excess L32 occurs. This observation could be due to the rapid degradation of excess ribosomal proteins observed by many laboratories (24–27). However, since the RNA structure postulated to be involved in the regulation of splicing (2) is largely conserved in the spliced mRNA (see below), we asked whether we could detect the regulation of translation of the mRNA for L32. Cells were transformed with a multi-copy plasmid carrying a cDNA version of RPL32 (Table I, construct B) or with the vector. Fig. 1A shows that the amount of L32 mRNA is increased substantially in the strain bearing the cDNA clone.

The proteins of the same cultures were pulse-labeled for 1 min with [³⁵S]methionine, and the total labeled proteins were analyzed on two-dimensional acrylamide gels (Fig. 1, B and C). Almost all the spots, other than those in the upper left quadrant, represent ribosomal proteins. L32 is indicated. It is one of the smallest ribosomal proteins and does not run in a compact spot. Nevertheless, it is clear that there is no detectable overexpression of L32 in strains with excess mature L32 mRNA. Similar results were obtained after a 3-min pulse (data not shown).

These results suggest that the translation of L32 is regulated, but cannot exclude the extremely rapid degradation of excess L32.

**The Leader of RPL32 mRNA Is Involved in the Translational Regulation**—To prove that the expression of L32 is translationally regulated, we constructed plasmids from which L32 is encoded by a chimeric mRNA in which the RPL32 5' leader is replaced by another 5' leader (Table I, constructs C and D).

**Table I**

| Plasmid | Promoter | Leader | Intron | Coding Region | Terminator |
|---------|----------|--------|--------|---------------|------------|
| A       | RPL32 (500 nt) | RPL32 (61 nt) | RPL32 (230 nt) | RPL32 (315 nt) | RPL32     |
| B       | ADH1      | RPL32  | RPL32  | RPL32         | RPL32     |
| C       | RPS10     | RPS10  | RPS10  | RPS10/RPL32   | RPL32     |
| D       | GAL10     | CYC1   | 0      | RPL32         | RPL32     |
| E       | RPL32     | RPL32  | 0      | AUGCCCA/LacZ  | RPL32     |
| F       | ADH1      | RPL32  | 0      | 120 nt from RPL32/LacZ | RPL32     |

**Plasmids overexpressing L32 or β-galactosidase**

A–D are plasmids overexpressing L32. The vectors Yep351 or Yep352 (31) were used for construction of the following plasmids (see Materials and Methods.). A is a genomic clone of RPL32; the size of each element is indicated. B is a CNA clone of RPL32, lacking the intron. Transcription is driven by the ADH1 promoter. C is a chimeric RPS10-RPL32 gene, fused at the middle of the introns of the two genes. The construction of this gene has been described in detail by Ref. 2. The fusion gene encodes a protein identical to L32 except for the initial amino acid. (The methionine is removed.) The transcript of this gene is not subject to the regulation of splicing (2). For plasmid D, the GAL10 promoter drives transcription of a CYC1 5' leader fused to the complete RPL32 coding sequence. E and F are plasmids overexpressing β-galactosidase. Two different constructs were introduced into vectors Yep354 or Yep357 (32). E is a partial cDNA clone of RPL32 fused to LacZ 5 nucleotides (GCCCGC) beyond the exon 1-exon 2 boundary. Transcription is driven by the RPL32 promoter. F is a partial cDNA clone of RPL32 fused to LacZ 120 nucleotides after the initiator to LacZ. Transcription is driven by the ADH1 promoter. nt, nucleotide(s).
mRNA—If the 5’ leader of L32 mRNA is involved in the autogenous regulation of L32 translation, then mRNA expressing a protein from the L32 leader will be subjected to negative regulation in the presence of excess L32.

Two fusion plasmids were constructed expressing β-galactosidase from chimeric genes that include the 5’ leader of L32 mRNA. One construct (E) contained about 120 nucleotides of the coding sequence fused to the ADH1 promoter. The plasmids were introduced into strains overexpressing L32 mRNA and analyzed to compare the β-galactosidase activity in control and overexpression conditions.

In strains overproducing L32, the activity of β-galactosidase is reduced in both cases by 50%. However, the amount of the L32-LacZ fusion transcript is 2-fold greater, suggesting that L32 enhances the stability of the fusion mRNA, as it did for the unspliced transcript of RPL32 (2). Thus overproduction of L32 reduced the activity of the mRNA containing the LacZ sequences fused to the 5’ sequences of RPL32 by about 75% (Table II). We conclude that the 5’ leader of RPL32 mRNA is involved in the autogenous translational regulation of L32 expression. Sequences more than a few nucleotides downstream of the initiation codon are not involved in this regulation.

Similar 5’ Structures Regulate Both Splicing and Translation—The data presented above suggest that the 5’ leader of the RPL32 mRNA plays an important role in the regulation of its translation. The 5’ leader also plays an important role in the regulation of splicing of the RPL32 transcript (2). We have proposed a structural model of the 5’ leader and shown its involvement in the regulation of splicing (Fig. 2A). The spliced mRNA can be folded into a structure that differs only slightly from that of the unspliced transcript (Fig. 2B). To ascertain if the structure as shown in Fig. 2B is realistic, we asked whether mutations that abolish regulation of splicing also abolish regulation of translation. The results are shown in Table III. It is evident that, qualitatively, mutants that reduce the regulation...
of splicing also reduce the regulation of translation. It is strik-
ing that the alteration of a single base, more than 60 nucle-
otides from the initiation codon, e.g. 9C→G or 10G→A, can
have such a profound effect on translation. Further confirma-
tion that a structure of this type is involved in the regula-
tion comes from mutating +2C→T, which would re-establish the left stem (Fig. 2B) and leads to the most extensive inhibition of transla-
tion.

Our working hypothesis, derived from Ref. 2 and from the
data reported above, is that L32 can bind to the structures shown
in Fig. 2, stabilizing them to prevent the assembly of spliceosomes or of a translation initiation complex.

L32 mRNA Produced in Excess Is Not Engaged in the Poly-

somes—To determine the distribution of mRNA for L32 in cells
overexpressing L32, we analyzed cell extracts on a 4%–15% poly-

acrylamide gradient. These molecules are not engaged with ribosomes at all. On the other hand, a substantial proportion migrates be-
tween 80 and 150 S. These could represent small polyribosomes,
aberrant initiation complexes, or some other form of RNP not associated with ribosomal particles.

As a control, we analyzed extracts of cells overproducing the
mRNA for ribosomal protein L29. In this case, nearly all the
mRNA is found in polyribosomes, which on average are slightly
larger than those translating L32, reflecting the difference in
length of the two coding regions.

These results suggest that L32 can prevent its mRNA from
interacting with ribosomal particles and may inhibit the for-
mation of active polyribosomes even if an initiation complex is
formed.

DISCUSSION

The data presented above demonstrate that ribosomal pro-
tein L32 can regulate the translation of its own mRNA. It does
so apparently by interacting with a structure largely within the
5′-untranslated region, a structure nearly identical to that in-
volved in the regulation of splicing of the transcript. The ques-
tion may arise that with the accumulation of L32 regulated at
the level of splicing, of translation, and of turnover, how does
enough excess L32 accumulate to do the regulating? The an-
swer is that under normal conditions each L32 mRNA is re-
sponsible for the synthesis of about 50 protein molecules (20).
Thus an imbalance of 2% between the molar synthesis of RNA
and L32 would provide enough protein to cause detectable reg-
ulation.

Since these experiments have been carried out in vivo, we
have no definitive proof that L32 itself interacts with the 5′
leader of the mRNA. However, two experiments suggest that it
does. (i) In attempting to epitope-tag L32, we inserted 8 amino
acids at several positions. In each case the modified L32 was
inactive, not only in ribosome assembly but also in the regula-
tion of splicing and of translation. (ii) By fusing L32 to the C
terminus of the maltose-binding protein (36), we have been able
to show that it binds with high affinity to the 5′ end of the
RPL32 transcript.3 It also binds to the spliced form of the
transcript, but with much lower affinity (data not shown). This
is just what we would predict from a comparison of panels A
and B of Fig. 2, and from our observation that L32 inhibits
splicing far more effectively than it inhibits translation.

At present, the case of L32 seems unique in S. cerevisiae. No
other ribosomal proteins have been shown to regulate the splic-
ing of the transcript of their own gene. No other ribosomal
proteins have been shown to regulate the translation of their
own mRNA. However, the measurements to establish such lev-
els of regulation are often difficult, requiring both the accurate
determination of mRNA concentration and the quantitation of
labeling of a protein with a very short lifetime. In the case of
L32, the binding of the protein appears to stabilize both the
unspliced transcript (2) and the spliced mRNA (Table II). The
RNA is therefore apparent. If unsplicable or untranslated
RNA were rapidly degraded, it would be nearly impossible to
determine whether that were due to some feedback effect. In-
deed, in experiments where the number of genes within a cell is
artificially elevated, e.g. through a multicycoplasmid, rarely
does the level of mRNA match the number of additional genes
(24–27, 37). Therefore it remains to be seen whether the pheno-
omena described for L32 are representative of the regulation of
many other ribosomal proteins.

A key point that we have been unable to address concerns the
intracellular location of the untranslated mRNA. It is exceed-
ingly difficult to prepare pure cytoplasmic or nuclear fractions

1 The abbreviation used is: RNP, ribonucleoprotein.

2 J. Vilardell and J. R. Warner, unpublished data.
Fig. 3. Analysis of mRNA distribution in polysomes. Cells were transformed with: multicopy vector (Yep24') (A), Yep24' carrying RPL32 (construct A in Table I (B), or Yep24' carrying RPL29 (CYH2) (C). See "Materials and Methods." A, fractionation on a sucrose gradient of total yeast lysate (migration from right to left); ordinate, optical density. B-D, 20 μl of each fraction was analyzed on a native RNP gel (see "Materials and Methods") and blotted to a Nytran membrane. B and C, native RNP gel from control cells (B) and cells carrying construct A (C) were blotted and analyzed by hybridization with oligonucleotide probe specific for mature L32 mRNA (complementary to 9 nucleotides of exon 1 and 9 nucleotides of exon 2). D, native RNP gel from cells overexpressing L29 mRNA blotted and analyzed by hybridization with oligonucleotide probe specific for mature L29 mRNA (complementary to 9 nucleotides of exon 1 and 9 nucleotides of exon 2).

Table IV
Distribution of mRNA in polysome profiles in strains overexpressing L32 or L29

| Strain               | Polysomes | Light particles | Free particles |
|----------------------|-----------|-----------------|----------------|
| Overproducing L32    | %         | %               | %              |
| Control              | 28        | 41              | 31             |
| Overproducing L29    | 86        | 14              | 3              |

of yeast. It is possible that L32 sequesters the mRNA in the nucleus, unavailable to the ribosomes. Indeed, if the efficiency of translation is only 25% of normal (Table II), Table IV suggests that not enough of the mRNAs is at the top of the gradient. Thus it seems likely that the RNA found in fractions 11–16 of Fig. 2B is not being translated but is in some unidentified RNP, either cytoplasmic or nuclear. The same problem pertains with the unspliced RNA (2). We cannot distinguish whether the L32 permits the transcript to avoid the splicing apparatus and proceed directly to the cytoplasm, as most yeast mRNAs do, or whether the L32 binds to the transcript, and keeping it within the nucleus but permanently sequestered from the splicing apparatus.

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L32 Regulates Its Own Translation

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