Regulation of splicing at an intermediate step in the formation of the spliceosome

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In vivo experiments have demonstrated that the ribosomal protein L32 of *Saccharomyces cerevisiae* brings about the inhibition of splicing of the transcript of its own gene through an RNA structure comprised largely of the first exon. We now show that L32, itself, binds specifically to this RNA. Splicing of the *RPL32* transcript in vitro is blocked by the presence of L32. Furthermore, addition of the 75-nucleotide RNA representing the 5' end of the *RPL32* transcript stimulates specifically the splicing of the *RPL32* substrate, presumably by competing for L32 present in the extract. Use of RNAs carrying mutations shown to abolish the regulation of splicing, either as substrates or as competitors, confirmed that the in vitro reaction is a faithful representation of the situation in vivo. We conclude that the regulation of splicing occurs through the specific binding of L32 to an RNA structure within the first 75 nucleotides of the *RPL32* transcript. The *RPL32* substrate, bound to L32, forms a complex with U1 snRNP, the first step in spliceosome assembly. The presence of L32 prevents the ATP-dependent association of the U2 snRNP necessary to form a complete spliceosome.

[Key Words: RNA structure; ribosome protein; yeast; U1 snRNP; spliceosome]

Received October 12, 1993; revised version accepted November 29, 1993.

It is now clear that regulated splicing is a major element of the control of gene expression in eukaryotes. Such regulation occurs by a number of mechanisms (for review, see Smith et al. 1989; Nadal-Ginard et al. 1991; McKeown 1992; Rio 1992). The widely studied sex determination pathway of *Drosophila melanogaster* uses at least two (for review, see Baker 1989). The sex lethal protein (Sxl) competes with the general splicing factor U2 accessory factor, (U2AF), for binding to a sequence between the lariat site and the 3' acceptor site in a constitutively spliced second intron of the *Sxl* transcript (Valcarcel et al. 1993) and probably of the *tra* transcript. The prevention of those splicing events each leads to the avoidance of a termination codon and to the female-specific production of the *sxl* and the *tra* proteins. The *tra* protein and the constitutive *tra-2* protein bind to an exon of the *dsx* transcript serving as an enhancer for the formation of a splicing complex at an unfavorable upstream 3' site (Tian and Maniatis 1992, 1993). These provide examples of both positive and negative regulation of splicing based on the binding of specific proteins.

Tissue-specific splicing can also be regulated in a positive or negative way. The splice leading to the P-element transposase of *Drosophila* is restricted to the germ-line cells because somatic cells contain proteins that bind to the 5' exon, leading to the formation of a non-productive splicing complex at a pseudo-5' splice site (Siebel et al. 1992). In *Saccharomyces cerevisiae*, Mer2p,

a protein essential for meiosis, is produced only in the presence of Mer1p, which helps the *MER2* transcript to associate with the splicing apparatus (Nandabalan et al. 1993). In other cases, the relative concentration of general splicing factors seems to determine which alternative 3' splice sites will be selected (Ge and Manley 1990; Krainer et al. 1990).

Finally, the regulation of splicing can modulate the amount of a gene product. In *S. cerevisiae*, ribosomal protein L32 appears to control its own synthesis through a feedback inhibition of the splicing of the *RPL32* transcript (Dabeva et al. 1986). The synthesis of ribosomes requires an equimolar amount of the >75 ribosomal proteins. The fact that most ribosomal protein genes contain an intron (Woolford 1989), otherwise rare in yeast genes, suggested that this might be a general mechanism for maintaining the balance of ribosomal proteins.

The regulation of splicing of the *RPL32* transcript is mediated by sequences including the first exon and the first few nucleotides of the intron. Sequence comparison between the genes of *S. cerevisiae* and *Kluyveromyces lactis*, computer analysis of RNA folding, and the effects of mutations on the regulation of splicing in vivo led us to propose a model of the RNA secondary structure with which L32 could interact (Fig. 1A). The 5' splice site is within a stem region of this structure, suggesting that the binding of L32 would prevent the interaction of U1 RNA with the *RPL32* transcript (Eng and Warner 1991).

We have now carried out an in vitro analysis of this regulation of splicing. We find that purified L32, in the

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Figure 1. (A) Proposed secondary structure of the first 70 nucleotides of the *RPL32* transcript (Eng and Warner 1991). The region between nucleotides 18 and 48 appears not to be involved in the regulation of splicing and is shown as a loop. Dots show those mutations that abolish the regulation of splicing. Positions 62–64 were tested only for MBP:L32 binding (see Fig. 3). The position of an AAG insertion is indicated by an arrowhead. (B) Scheme of the MBP:L32 protein. (C) RNA probes used in the *in vitro* experiments. Numbers in parentheses indicate the pre-mRNA sequences contained in the probe, referred to the start of transcription (Dabeva et al. 1986).

form of a fusion with the maltose-binding protein of *Escherichia coli* (MBP:L32), binds specifically to a transcript containing the first 75 nucleotides of the *RPL32* gene. The splicing of an *RPL32* transcript in a cell extract is detectable but inefficient. It is abolished specifically by the addition of MBP:L32, and it is enhanced specifically by addition of the RNA fragment that binds L32, presumably by titrating the L32 protein present in the extract. Surprisingly, we find that a substrate bound to MBP:L32 can undergo the initial step of spliceosome assembly, the association with U1 small nuclear ribonucleoprotein (snRNP). L32 blocks subsequent assembly steps, namely the ATP dependent interaction with U2 snRNP.

Results

L32 can be replaced in vivo by a MBP:L32 fusion protein

To study the regulation of splicing of the *RPL32* transcript *in vitro*, it was essential to have a supply of L32 in an active form. However, purification of a ribosomal protein from ribosomes entails extensive denaturation, and there is no assay for the return of the protein to the proper conformation at the end of the process. Attempts to synthesize L32 in *E. coli* yielded only an insoluble product; therefore, a gene was constructed that encodes a fusion between the MBP from *E. coli* (Maina et al. 1988) and L32 (MBP:L32; Fig. 1B). The MBP:L32 obtained from *E. coli* is highly soluble and can be readily purified.

To ask whether MBP:L32 has biological activity, its gene, driven by the *GAL10* promoter, was inserted into a multicopy plasmid that was introduced into a diploid yeast strain in which one *RPL32* gene has been disrupted by the *HIS3* marker (JV1; see Materials and methods). Sporulation yielded haploid cells (JV2) that were His⁺ and dependent on galactose. Analysis of ribosomal proteins in those cells showed that the entire MBP:L32 is incorporated into the ribosomes, apparently in equimolar amounts with the other ribosomal proteins (B-J. Li, J. Vilardell, and J.R. Warner, in prep.). No free MBP was detected in Western blot analysis of protein extracts of strain JV2. Furthermore, overproduction of the MBP:L32 in a wild-type strain leads to an accumulation of the *RPL32* unspliced transcript (Fig. 2A, lanes 2,3). The degree of inhibition is comparable to that observed when L32 itself is overproduced (Eng and Warner 1991). It is interesting that the intact MBP:L32, of 53 kD, can substitute for ribosomal protein L32, of 11 kD, both in the formation of a ribosome and in the regulation of splicing of the *RPL32* transcript.

The MBP:L32 protein binds specifically the RPL32 transcript

Genetic analysis showed that the first 75 nucleotides of the *RPL32* transcript, comprising the first exon and 14 bases of the intron, are sufficient for the regulation of splicing. Comparative sequence analysis and the phenotype of numerous site-directed mutations suggested that

Figure 2. Northern analysis of total RNA extracted from yeast cells (strain W303) expressing the MBP:L32 protein. (Lanes 1) RNA from wild-type W303 strain; (lanes 2,3) RNA from two independent transformants of plasmid pRDKFP carrying the MBP:L32 gene. In each lane, 5 μ g of total RNA was analyzed (Eng and Warner 1991). (A) Filter probed with an oligonucleotide complementary to nucleotides +3 to -19 in the first exon of *RPL32*. Positions of the precursor unspliced RNA (P) and the mature spliced RNA (M) are indicated. (B) The same filter hybridized with an oligonucleotide complementary to the *male* gene from *E. coli*, for specific detection of the MBP:L32 transcript.

the region can fold in a stem-loop structure as depicted in Figure 1A (Eng and Warner 1991). Using the in vitro transcripts shown in Figure 1C, we ask whether the MBP:L32 protein can bind to this regulatory sequence.

A radioactive copy of this RNA molecule (RNA-I) was subjected to bandshift analysis with purified MBP:L32 (Fig. 3). It is evident (lane 2) that a strong interaction occurs between the two. The binding is competed both by an excess of unlabeled RNA-I (lane 3) and by a longer transcript of *RPL32* that includes the entire intron and part of the second exon (RNA-II; lane 4). This result shows that a substrate for splicing can also fold into a conformation that binds L32. RNA molecules derived from other genes, *ACT1* (lane 5) or *RPS10A* (lane 12), do not compete. More important, transcripts of several mutant forms of *RPL32*, not subject to the regulation of splicing by L32 in vivo (Eng and Warner 1991), do not compete for binding (lanes 6–8). This is true of mutations that weaken either the lower stem ($C_9 \rightarrow U$; lane 6) or the upper stem ($U_{14} \rightarrow A$; lane 7).

The in vitro assay enabled us to test the effect of mutations that were inaccessible to the in vivo assay, such as those at the 5' splice site that prevent splicing. Mutations $G_{61} \rightarrow A$ or $G_{62} \rightarrow A$ abolish binding of the MBP:L32 (lanes 9,10); $U_{63} \rightarrow C$ weakens it substantially (lane 11). These results strengthen the experimental ba-

sis of the structure proposed previously (see Fig. 1A) (Eng and Warner 1991) and suggest that the regulation of splicing is attributable directly to the binding of L32 to the structure diagrammed in Figure 1A.

Binding of MBP:L32 inhibits splicing in vitro

The model of autoregulation specifies that binding of L32 prevents the splicing of the *RPL32* transcript. This prediction was tested by assaying the effect of MBP:L32 on splicing in vitro. As shown in Figure 4A, splicing of the *ACT1* transcript is efficient (lane 3) and is not affected by the presence of MBP:L32 in the extracts (lane 4). In contrast, the *RPL32* transcript is spliced poorly in vitro under the same conditions (lane 8). Furthermore, addition of MBP:L32 (lane 9), but not of MBP alone (lane 10), significantly inhibits its splicing. There is a clear relationship between the amount of added MBP:L32 and the inhibition of splicing (Fig. 5A).

The model predicts that the MBP:L32 would not affect the splicing of the mutant transcripts of *RPL32* to which it does not bind. This was confirmed in the case of the mutant $C_9 \rightarrow U$ (Fig. 4B). Its transcript is spliced effectively in vitro (lane 3), even in the presence of 2 μ g of MBP:L32 in the reaction (lane 4). The mutant $U_{14} \rightarrow A$ has been tested with identical results (data not shown).

One explanation of the low splicing efficiency of the wild-type *RPL32* transcript is that the L32 protein could be present in the splicing extracts. If so, it should be feasible to increase the splicing specifically of an *RPL32* transcript by removing L32. To this end, cold RNA-I, the first 75 nucleotides of the *RPL32* transcript, was added to the splicing extract before the substrate. Because RNA-I has only the 5' splice site, it will not enter into the splicing pathway (Ruby and Abelson 1988; Séraphin and Rosbash 1989). Yet it binds L32 (see Fig. 3, lane 2); thus, any

Figure 3. Mobility-shift analysis. 32 P-labeled RNA-I was mixed with tRNA and tested for binding in the presence of the following components: (Lane 1) blank; (lane 2) MBP:L32; (lanes 3–12) MBP:L32 and the indicated competitors in 50- to 100-fold molar excess; (lane 13) MBP alone.

Figure 4. In vitro *RPL32* splicing. The high background is attributable, at least in part, to the high specific activity of the probes [see Materials and methods]. The MBP and MBP:L32 experiments were done adding 1 μ g (A) or 2 μ g (B) of MBP:L32 or 2 μ g of MBP to the extracts before they were mixed with substrates. (A) In vitro splicing of the *RPL32* substrate (RNA-II, lanes 6–10) compared with that of *ACT1* (RNA-III, lanes 1–5). (Lanes 2, 7) Splicing extracts but no ATP, conditions in which no splicing activity should be observed. Free exon 1 from RNA-II is not detected in this gel. The multiple bands representing the spliced product have been attributed to polyadenylation in the extract (Sraphin and Rosbash 1990). (B) In vitro splicing of a mutant *RPL32* substrate ($C_9 \rightarrow U$). Splicing extracts were supplemented with 2 μ g of MBP:L32 (lane 4) or preincubated with RNA-I (lane 5) or mutant RNA-I $C_9 \rightarrow U$ (lane 6). (C) In vitro splicing of the *RPL32* substrate (RNA-II) in the presence of RNA-I. Splicing extracts were incubated with 25 ng of cold RNA-I (wild type in lane 4, $C_9 \rightarrow U$ mutant in lane 5) for 5–10 min at room temperature before being used in the reaction.

L32 in the extract could be titrated out. Comparing lanes 3 and 4 of Figure 4C shows that the splicing of *RPL32* RNA is improved when the extracts are preincubated with RNA-I. The increase in splicing activity is correlated with the amount of RNA-I added (Fig. 5B).

The treatment of splicing extracts with RNA-I has no effect on their splicing activity toward a mutant *RPL32* transcript whose splicing is not regulated in vivo (Fig. 4B, lane 5). Moreover, preincubation with a mutant RNA-I that does not bind the L32 protein [e.g., $C_9 \rightarrow U$ (Fig. 3, lane 6)] does not lead to improved splicing either of the *RPL32* wild-type substrate (Fig. 4C, lane 5) or of the $C_9 \rightarrow U$ mutant substrate (Fig. 4B, lane 6).

These experiments demonstrate that splicing of the *RPL32* transcript is inhibited through binding of the L32 protein to the 5' exon/intron sequences.

MBP:L32 inhibits spliceosome formation

Because no lariat intermediates were observed either in

the original in vivo experiments demonstrating the inhibition of splicing by L32 (Dabeva et al. 1986; Eng and Warner 1991) or in the in vitro experiments where splicing was abolished by MBP:L32 (Fig. 4A, lane 9), it seemed likely that inhibition occurs at an early stage in the splicing process. Much progress has been made recently in the analysis of the early steps of splicing (for review, see Woolford 1989; Rymond and Rosbash 1992). The first step appears to be the binding of the U1 snRNP to the transcript, probably at the 5' splice site, to form a commitment complex (CC) (Sraphin and Rosbash 1989; Ruby and Abelson 1988). Subsequently, in an ATP-dependent process, several splicing factors as well as the U2 snRNP bind, followed by the assembly of other components to form the complete spliceosome (Liao et al. 1992).

At what step does L32 block the splicing machinery? This point was addressed using native gel electrophoresis (Pikielny et al. 1986) (Fig. 6). When the *RPL32* splicing substrate (RNA-II) is added to an extract in the ab-

Figure 5. Regulation of *RPL32* splicing. Only the top portion of the gels are shown. *A* was exposed to film for a much longer time. (*A*) Inhibition of in vitro splicing by increasing amounts of MBP:L32. (Lane 1) Free probe (RNA-II); (lane 2) probe incubated with splicing extracts and ATP; (lanes 3–7) splicing buffer with indicated amounts of MBP:L32. (*B*) Stimulation of in vitro splicing of the *RPL32* transcript by incubation of the splicing extracts with RNA-I. (Lane 1) Free probe; (lane 2) probe with splicing extracts and without ATP; (lane 3) as in lane 2 but with ATP; (lanes 4–8) splicing activity after addition to the splicing extracts of the indicated amounts of RNA-I, as described in Fig. 4C.

sence of ATP, the RNA forms heterogeneous complexes (lane 2). A significant band is present at a position that appears to correspond to the CC (Pikielny et al. 1986). Addition of ATP to the intact extract permits assembly of a limited amount of more slowly migrating complexes, characteristic of intermediate and mature spliceosomes (lane 3). The presence of cold RNA-I, which stimulates splicing (see Fig. 4A), also stimulates the formation of the slowly migrating spliceosome complexes at

the expense of the CC (Fig. 6; lane 4), suggesting that L32 inhibits the formation of mature *RPL32* spliceosomes. On the other hand, MBP:L32 abolishes completely the formation of the spliceosome complexes (Fig. 6, lane 5). The presence of MBP:L32 leads to a band migrating slightly behind the CC (Fig. 6, lanes 5–8) that cannot be a true spliceosome, as it is seen in the absence either of ATP (Fig. 6, lanes 6,7) or of U1 RNA (lanes 7,8). The specificity of the effect of the MBP:L32 on the formation of spliceosomes was explored using the $C_9 \rightarrow U$ mutant substrate. Complexes involving this mutant transcript (Fig. 6, lanes 11,12) are unaffected by the presence of the MBP:L32 (lane 13).

These results demonstrate that the binding of L32 to its transcript blocks splicing at a step before the association with U2 snRNP. The presence of U1 snRNP is inferred by the formation of an apparent CC (Fig. 6, lanes 5,6) but a more positive identification was desirable.

The nonspliceable complex contains U1 snRNP

The structure predicted for the stem-loop in the *RPL32* transcript (see Fig. 1A), on the basis of both experimental results (Eng and Warner 1991; Fig. 3) and computer analysis, would preclude the base-pairing of the U1 snRNP to the 5' splice site, which is thought to initiate the splicing reaction.

Yet even in the presence of MBP:L32 an apparent CC is seen in Figure 6 (lanes 5,6). We asked whether this had the characteristics of a true CC by depleting the extracts of U1 snRNA (Kretzner et al. 1987) (Fig. 6, lanes 7–9).

Figure 6. Formation of commitment complexes and mature spliceosomes by the *RPL32* transcript. Labeled RNA-II, either wild type (lanes 1–9) or the $C_9 \rightarrow U$ mutant (lanes 10–13), was incubated for 20 min at 23°C and loaded on a native gel as described by Séraphin and Rosbash (1989). The CCs and spliceosomes (SP) are named according to (Séraphin and Rosbash 1989). (–U1) Extracts depleted of U1 RNA (see Materials and methods).

Comparison of lane 9 with lanes 2 and 3 shows that depletion of U1 from the extract prevents the formation both of spliceosomes and of the putative CC. Similarly, comparison of lanes 7 and 8 with lanes 5 and 6 shows that the apparent CC formed in the presence of MBP:L32 cannot form after the depletion of U1 snRNA. Our preliminary conclusion is that the *RPL32* transcript, even when bound to L32, can form a CC by interacting with U1 snRNP and presumably other factors.

The possibility remains that in the presence of MBP:L32 the formation of the apparent CC of the *RPL32* transcript requires U1 snRNP only in a temporary manner and does not involve a stable association of U1 snRNA with the substrate. To demonstrate directly the association of U1 snRNA with the *RPL32* transcript, we have used immunoaffinity purification of the MBP:L32-*RPL32* pre-mRNA complex using anti-MBP antibody (Arenas and Abelson 1993). The affinity-purified complex was then assayed for the presence of U1 RNA. The results show clearly (Fig. 7, lane 4) that a ternary complex between the *RPL32* substrate MBP:L32 and U1 RNA can be formed in the extract and is sufficiently stable to withstand the affinity purification protocol, including the presence of a strong nonspecific competitor

Figure 7. Presence of U1 RNA in the blocked CC. Extracts were supplemented with either MBP (lanes 1,3,5) or MBP:L32 (lanes 2,4,6). The substrates [(lanes 1,2) none; (lanes 3,4) RNA-II; (lanes 5,6) mutant RNA-II ($C_9 \rightarrow U$)] were added in the presence of ATP for 20 min at 23°C. Complexes were immunoprecipitated with anti-MBP antibody as described in Materials and methods. RNA was prepared from the immunoprecipitation, analyzed on a Northern blot that was hybridized with a probe complementary to U1 RNA. The location of U1 RNA was verified from an adjacent lane of total RNA.

such as heparin. Omission of the *RPL32* transcript (lane 2), the use of MBP rather than MBP:L32 (lanes 1,3,5), or the use of the $C_9 \rightarrow U$ mutant substrate (lanes 5,6) yields little if any U1 snRNA. As expected, as no spliceosomes were formed in the presence of MBP:L32 (Fig. 6, lane 6), U2 RNA was not detected in complexes formed between RNA-II and MBP:L32 (data not shown).

We conclude that even when bound to L32, the *RPL32* transcript can associate with U1 RNA to form what appears to be a CC. The presence of L32 blocks splicing by preventing the next step, the ATP-dependent association of the U2 snRNP and/or other components of the splicing apparatus.

Discussion

On the basis of a variety of experiments carried out in vivo, we had postulated that ribosomal protein L32 would bind to its transcript to inhibit its splicing (Dabeva et al. 1986). The results described above have confirmed this prediction entirely. The binding of L32 to the transcript requires no auxiliary factors. Furthermore, the identification of the key nucleotides involved in the regulation has also been confirmed. The details of the interactions, however, are somewhat different than we had predicted, leading to the development of a new understanding of the mechanism by which regulation takes place.

The regulation of splicing of the *RPL32* transcript shares some features with other regulation systems and differs in others. Many of the proteins involved in the regulation of splicing have one or both of the characteristics in common: (1) a stretch rich in arginine and serine, and (2) a consensus RNA-binding site, first identified in proteins binding to heterogeneous RNA (hnRNA) and mRNA (Dreyfuss et al. 1988). L32 has neither, nor do any of the other ribosomal proteins. We presume that L32 binds to its transcript using the same motif with which it binds to the rRNA.

In many cases, negative regulation occurs at a splice site that is somewhat unfavorable. An unusually long stretch between the branchpoint and the 3' splice site is involved in the regulated splicing of the rat α -tropomyosin transcript (Smith and Nadal-Ginard 1989; Mullen et al. 1991) and of the autoregulated splice of the *Drosophila su(w^a)* transcript (Spikes and Bingham 1992), among others. The transcript encoding ribosomal protein L1 of *Xenopus*, whose splicing is subject to regulation, has a suboptimal 3' acceptor site (Caffarelli et al. 1992). In the other instance of regulated splicing described in *S. cerevisiae*, the *MER2* transcript has a non-canonical 5' splice site, G/GUUCGU. . . , that should form one less base pair with U1 RNA. Splicing occurs naturally only when the *MER1* gene product is present, but the need for Mer1p is relieved if *MER2* is mutated to improve the interaction with U1, even outside the usual site of pairing (Nandabalan et al. 1993). Although the 5' splice site of the *RPL32* transcript is G/GUCAGU. . . rather than the canonical G/GUAUGU. . . , it can form the same number of base pairs with U1 RNA. Under

optimal growth conditions, where there is presumably little free L32, the transcript is spliced effectively in vivo with a ratio of mRNA to pre-mRNA of >50:1 (Dabeva et al. 1986). Because the half-life of *RPL32* mRNA is 16 min (Kim and Warner 1983), the half-time for splicing must be ~15 sec. Introduction of the canonical 5' splice site, together with a compensatory mutation at position 6 (Fig. 1A) to maintain base-pairing, has little effect on regulation of splicing in vivo (Eng and Warner 1991) or in vitro (data not shown). Thus, the regulation of splicing of the *RPL32* transcript is not the result of a noncanonical splice site but of the presence of L32.

In several instances, the RNA sequences responsible for the regulation of splicing have been identified. Thus, a series of repeats in the downstream exon of the *dsx* transcript bind the *tra* and *tra-2* proteins (Nagoshi and Baker 1990; Hedley and Maniatis 1991; Inoue et al. 1992; Tian and Maniatis 1993). A sequence in the 5' exon of the P-element transposase binds proteins not yet unidentified (Siebel et al. 1992). *Sxl* binds to a polypyrimidine tract specific to both the *Sxl* and the *tra* sex-specific 3' splice site (Valcarcel et al. 1993). However, only in rare instances has an RNA secondary structure been implicated in the regulation of splicing (for review, see Balvay et al. 1993). In *K. lactis*, a distal 3' splice site is used apparently because the proximal one is involved in a secondary structure (Deshler and Rossi 1991). Similar situations occur for regulated splicing of adenovirus transcripts (Chebli et al. 1989; Domenjoud et al. 1993) and of an immunoglobulin transcript (Watakabe et al. 1989). Exon 6A of the chicken tropomyosin gene is used preferentially to exon 6B in myoblasts because the 3' acceptor site of the latter is within a stem. Mutations that weaken the stem permit utilization of exon 6B; compensatory mutations restore the regulation (Libri et al. 1991). Presumably, as yet undescribed tissue-specific proteins permit the use of exon 6B in differentiated skeletal muscle.

The situation for L32 is clearer. Not only have we shown that L32 binds to the transcript whose splicing is regulated, but we have considerable evidence about the structure of the key regions of the transcript. The binding results in Figure 3 match exactly the data obtained previously on the regulation of splicing in vivo (Eng and Warner 1991). The change of a single base >50 nucleotides distant from the 5' splice site will abolish both the binding of L32 to its transcript in vitro and the regulation of splicing in vivo.

The structure depicted in Figure 1A was derived from comparing the sequences of the 5' exons of *RPL32* from *S. cerevisiae* and *K. lactis* and from computer predictions (Zuker 1991). It was supported by the effects of numerous mutations on the regulation of splicing (Eng and Warner 1991). However, confirmation of the lower stem was less secure. Several mutations could not be examined because they would have abolished splicing altogether. In testing mutant transcripts for the binding of L32 in vitro (Fig. 3), we confirm the importance of G₆₁ and G₆₂ on either side of the 5' splice site. In any case, the structure of the lower stem is probably more com-

plex than shown in Figure 1A. For instance, the mutant U₆₃ → C retains some binding activity (Fig. 3, lane 11). Also, the mutant G₁₀ → A, which should strengthen the stem, actually abolishes regulation of splicing (Eng and Warner 1991). Although it is clear that the upper stem is essential for the regulation of splicing, and that the loop from nucleotides 18 to 49 plays little role, understanding the three-dimensional arrangement of the complex will have to wait for better methods.

The predicted stability of the structure shown in Figure 1A is rather low, with a ΔG of <9 kcal/mole (Zuker 1991). This is far less than necessary to block splicing (Solnick and Lee 1987). Originally, we hypothesized that L32 stabilized this structure, thereby preventing access of U1 RNA (Eng and Warner 1991). However, Figures 6 and 7 show that U1 RNA is associated with the *RPL32* transcript even in the presence of MBP:L32. Thus, there is a ternary complex in which U1 RNA can associate with the substrate in a presplicing complex, whereas L32 remains bound. The presence of L32 prevents the next steps of splicing, namely the ATP-dependent association of U2 RNA and the subsequent formation of the complete spliceosome (Ruby et al. 1993). The detailed interactions of the RNAs remain speculative. Although we cannot exclude the possibility that the lower stem remains intact during the interaction of the 5' splice site with U1 RNA, it seems more likely that U1 RNA displaces nucleotides 6–9 (GACC) from their base-pairing with the 5' splice site without eliminating the binding of L32. What is the role of the purine-rich bulges connecting the two stems?

The fact that L32 blocks the splicing of its transcript at a step after the association of the substrate with U1 RNA and before its association with U2 RNA may provide a new experimental tool to dissect this key step in splicing.

The structure described in Figure 1A is composed almost entirely of the first exon. The spliced transcript can form the same structure, except that the lower stem can form only 3 bp instead of 4. The mutant U₆₃ → C (Fig. 3, lane 11) has the equivalent sequence. L32 binds to this structure, but only weakly. Nevertheless, the presence of L32 has a substantial effect on the efficiency of translation of the spliced mRNA (Dabeva and Warner 1993).

The in vitro splicing assay used above has the advantage that it can be manipulated in both directions. Addition of MBP:L32 inhibits specifically the splicing of the *RPL32* transcript; addition of RNA-I stimulates specifically the splicing of the *RPL32* transcript, presumably by competing for the L32 that is in the extract. It is not clear whether the L32 in the extract represents molecules not yet assembled into ribosomes, or molecules that have been stripped from ribosomes during the preparation of the extract. The loss of L32 from a very small proportion of the ribosomes would be sufficient.

The original observation that L32 could regulate the splicing of its own transcript provided a rationale for the presence of introns in two-thirds of the ribosomal protein genes, whereas they are rare in other genes of *S. cerevisiae* (Woolford 1989). However, assays for the reg-

ulation of splicing of several other ribosomal protein transcripts have failed in that no regulated accumulation of unspliced precursor molecules was observed. We now suggest an explanation. The formation of a CC with U1 RNA may lead to stabilization of the *RPL32* transcript. In the case of the numerous *prp* mutations that have been identified because of the accumulation of unspliced transcripts, the blockage at the nonpermissive temperature invariably occurs at a step subsequent to the formation of a CC (Woolford 1989). Perhaps, then, the splicing of other ribosomal protein transcripts is regulated, but in such a way that the unspliced transcript is degraded so rapidly that it is undetectable. For example, the regulated splicing of the ribosomal protein L1 transcript of *Xenopus* leads to a U1 RNA-dependent degradation of the transcript (Fragapane et al. 1993). Using the in vitro splicing assay described above may permit us to detect the regulation of splicing of other ribosomal protein transcripts using decoy transcripts to compete for free ribosomal proteins within the extract. Our use of RNA-I in the splicing experiments of Figures 4, 5, and 6 was sufficient to demonstrate the regulation of splicing of the *RPL32* transcript.

The finding of a more general regulation of splicing of ribosomal protein transcripts would not only provide a satisfying explanation for the presence of introns in ribosomal protein genes, but would also extend the parallel between *E. coli* and *S. cerevisiae*. In the former, the balance between rRNA and ribosomal proteins is maintained because certain ribosomal proteins, when in excess over the rRNA, bind to their mRNA to prevent translation of an entire operon. In a eukaryote, as translation and ribosome assembly occur in different compartments of the cell, a more economical way of maintaining the balance between rRNA and ribosomal proteins would be to regulate a nuclear event, such as splicing.

Materials and methods

Fusion protein and strains

The MBP:L32 fusion protein gene was constructed as follows: The L32 fragment was obtained by PCR of exon II (nucleotides 234–554, taking as position +1 the A of the initiation codon in exon I) of the *RPL32* gene and cloned between the *StuI* and *SalI* sites of the vector pMALc (BioLabs). Factor Xa digestion of the product would yield the L32 protein as it is in vivo. Purification of the fusion protein by absorption to and elution from an amylose column followed the manufacturer's instructions (ProFusion kit, BioLabs).

To express the MBP:L32 in yeast cells its gene was modified to put an *XhoI* site at the 5' end and the 3'-untranslated region of the *RPL32* gene at the 3' end, and then used to replace the *SEP1* gene of pRDK249 (Johnson and Kolodner 1991) to yield plasmid pRDKFP.

The diploid strain JV1 (*RPL32/rpl32::HIS3*) was constructed by disruption of one *RPL32* gene in the homozygous diploid W303 (*Mata/α*, *ade2-1*, *his3-11*, *leu2-3,112*, *trp1-1*, *ura3-1*, *can1-100*) using the one-step gene disruption technique (Rothstein 1983). Haploid *Mata* and *Mata* strains were derived from spores of W303. Strains were subjected to transformation by the lithium acetate procedure (Ito et al. 1983).

RNA probes and substrates

For the transcripts of *RPL32* and its mutants, a sequence corresponding to the entire transcript of *RPL32* was amplified by PCR and cloned in pGEM3Z (Promega). For preparation of RNA-I (nucleotides 1–75, taking as +1 the start of transcription; see Fig. 1C), the plasmid was digested with *NlaIII*, then treated with Klenow and transcribed with T7 polymerase. For preparation of RNA-II (nucleotides 1–360), the plasmid was cut with *DdeI*. pACT was constructed by cloning the PCR fragment representing nucleotides 606–1052 of the yeast actin gene (GenBank accession no. 100026) (Ng and Abelson 1980) in the *SacI* and *EcoRI* sites of pKS (Stratagene). For the actin probe pACT was digested with *EcoRI* and transcribed with T7 polymerase.

The S10 transcript was made by T7 transcription of pS10A-1 digested with *DraI*. pS10A-1 is a pT7/T3-18 clone of the *RPS10* transcript (Johnson and Warner 1987). For probing Northern blots, U1 antisense RNA was synthesized from a pSK subclone of pT7U1 plasmid (kindly provided by Dr. J. Arenas, Caltech, Pasadena, CA).

In vitro transcription

In vitro transcriptions were performed in 20 μ l of 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 500 μ M all NTPs except UTP, 200 ng of DNA (digested, proteinase K-treated and phenol-chloroform extracted), 20 units of RNasin (Promega), and 10–20 units of T7 or SP6 RNA polymerase (Promega, Biolabs). For mobility-shift substrates the cold UTP was 500 μ M with 100 μ Ci of [α -³²P]UTP (800 Ci/mmol, NEN). For in vitro splicing substrates cold UTP was 20 μ M with 160 μ Ci of [α -³²P]UTP. Riboprobes for hybridization were synthesized using 200–250 μ Ci of [α -³²P]UTP. After 1 hr at 37°C, 1 unit of RQ1 DNase (Promega) was added and the reaction was incubated 15 min more. When the transcript was to be gel purified (as for the splicing reactions), this step was omitted. Cold RNA competitors were synthesized in 100 μ l reactions, in the same buffer, containing 0.5 mM of all nucleotides, 100 units of RNasin, 0.5–2 μ g of DNA, and 20–40 units of RNA polymerase. The DNA template was digested with 2 units of RQ1 DNase. RNA was purified by phenol-chloroform extraction, precipitated with ammonium acetate, and checked by gel electrophoresis.

Site-directed mutagenesis

The in vitro mutagenesis was done by cloning the product of a PCR performed using mutant oligonucleotides. For some mutations a circular PCR method was used (Imai et al. 1991). In all cases, the products were sequenced. The *RPL32* mutant with an AAG insertion after the first AUG is described elsewhere (Eng and Warner 1991).

Mobility-shift assays

RNA probes were dissolved in renaturation buffer (RB), containing 30 mM Tris-HCl (pH 7.5), 350 mM KCl, and 10 mM DTT, heated at 65°C for 10 min and then slowly cooled to binding temperature. This step could be omitted for the short RNAs. Typical binding reactions were performed in 20 μ l of 30 mM Tris-HCl (pH 7.5), 75 mM KCl, 2 mM MgCl₂, 1 mM DTT, 50 ng/ μ l of BSA (Promega), 40 ng/ μ l of tRNA (wheat germ), 5–50 fmol of probe, 50 ng of MBP:L32 and, when indicated, 25–50 ng of cold RNA competitor. Reactions were incubated at 25°C for 20 min and loaded immediately in a running acrylamide gel. For short probes (RNA-I; Fig. 1C) gels were 6% acrylamide (acry-

lamide/bisacrylamide ratio of 30:0.5), 0.5% agarose, 0.25× TBE, with recirculation.

In vitro splicing

Because of the poor *in vitro* splicing efficiency of the *RPL32* transcript, we used RNA probes of higher specific activity than usual. Although these probes are degraded rapidly (Yisraeli and Melton 1989), they allow us to detect the reaction on *RPL32* substrates with more sensitivity. For consistency, actin substrates were labeled in the same way, although the background in splicing gels is substantially increased.

Splicing extracts were prepared (Lin et al. 1985; Cheng et al. 1990) from strain W303. Splicing reactions were performed following standard procedures (Cheng et al. 1990) but using 0.2–0.5 nmoles of RNA substrate. At the end of the reaction the sample was treated with proteinase K, with phenol–CHCl₃, and the RNA precipitated with ethanol, washed, redissolved in loading buffer, and the radioactivity determined. The proportion of CPM recovered was independent of whether the substrate was spliced or not. The same number of counts per minute were loaded in each gel lane.

U1 snRNP depletion was carried out using the oligonucleotide-directed RNase H method, as described (Kretzner et al. 1987). The oligonucleotide sequence was CTTAAGGTAAG-TAT, complementary to the first 14 bases of U1 RNA.

Native gel analysis was according to Séraphin and Rosbash (1989), but omitting the final addition of loading buffer. In these experiments the extracts were depleted of ATP (Liao et al. 1992).

Immunoprecipitations

Splicing complexes containing the MBP:L32 were isolated by immunoprecipitation (Arenas and Abelson 1993). Splicing reactions (20 µl) were set up with 50–100 fmoles of RNA under different conditions (see Results). After 20 min at 23°C, 80 µl of SPL buffer [50 mM K₂HPO₄ (pH 7.2), 2.5 mM MgCl₂, 1 mM DTT], with 5 µg/µl of heparin (Sigma), was added and the reaction was incubated in ice for 10 min. To the reaction mixture 20 µl of anti-MBP–protein A–Sepharose beads was added, prepared by mixing 200 µl of rabbit anti-MBP antiserum (BioLabs) and ~300 µl of protein A–Sepharose (PAS, Zymed). After 1 hr of gentle rocking at 4°C, beads were washed three times (20 min at 4°C) with 200 µl of SPL-150 mM KCl, resuspended in 90 µl of NET buffer [50 mM Tris-HCl (pH 7.5), 0.1% NP-40, 1 mM EDTA], transferred to a new tube, brought to a final concentration of 5 mM EDTA, 0.1% SDS, 10 ng/µl of *E. coli* tRNA, and 10 ng/µl of proteinase K, and digested for 15 min at 37°C. The RNA was extracted with phenol–chloroform, precipitated, and washed.

Acknowledgments

We are grateful to Dr. S.P. Johnson for carrying out earlier experiments, to Drs. J. Woolford, J. Arenas, H. Colot, and D.S. McPheeters for useful discussions, to Dr. R. Raz for critical reading of the manuscript, to M. Studeny for technical assistance, and to A. Greaney for assistance with the manuscript. This research was supported by American Cancer Society grant NP-793 (to JRW) and National Institutes of Health grant CA13330, which supports the Cancer Center of the Albert Einstein College of Medicine. J.V. has been the holder of a Fulbright Fellowship from the Spanish Ministry of Education.

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Genes Dev. 1994, **8**:

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