A *trans*-acting suppressor restores splicing of a yeast intron with a branch point mutation

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Splicing of introns from *Saccharomyces cerevisiae* pre-mRNA requires the conserved sequence TACTAAC; the 3'-most A residue is utilized as the site of branch formation. We showed previously that the transcript from an actin-*HIS4* gene fusion containing the mutation TACTAAC to TACTACC (designated C259) is spliced inefficiently, thereby preventing growth on the histidine precursor histidinol. By selecting for growth on histidinol, we have identified a mutant in which the splicing of the C259 transcript is increased fourfold; splicing of other mutated introns is not significantly improved. The mutant locus encodes a *trans*-acting suppressor. A single mutation, *rna16-1*, is sufficient for suppression; however, suppression is maximized in heterozygous diploids containing both *rna16-1* and the wild-type allele *RNA16*. In addition, wild-type pre-mRNAs (and lariat intermediates) accumulate in *rna16-1* cells. We propose that the *RNA16* locus encodes a component of the splicing machinery.

[Key Words: RNA splicing; *trans*-acting suppressor; branch point; yeast]

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The removal of group I, group II, and nuclear pre-mRNA introns is accomplished by mechanisms that share certain fundamental principles (Cech and Bass 1986). For example, each splicing process appears to occur via two sequential transesterification reactions. The most noteworthy distinctions among these splicing substrates involve the strategies used to fold the intron into a functionally active geometry. At one extreme is the autocatalytic splicing of the prototype group I intron; at least in vitro, splicing of the *Tetrahymena* pre-rRNA depends solely on the presence of highly conserved structural elements within the intron. In contrast, the only conserved features of nuclear mRNA introns appear to be restricted to short regions at or near the splice junctions; in metazoans, moreover, these sequences are often degenerate (Mount 1982; Green 1986). Presumably the low information content within this class of introns is compensated by the extensive participation of *trans*-acting factors. A major challenge in understanding nuclear pre-mRNA splicing is thus the elucidation of the nature and the role of these *trans*-acting factors.

The first nucleolytic step in the splicing of nuclear pre-mRNA involves cleavage at the 5' splice junction

and concomitant formation of a lariat intermediate (Padgett et al. 1984; Ruskin et al. 1984). In yeast, the lariats form at the 3'-most A in the sequence TACTAAC (Domdey et al. 1984; Rodriguez et al. 1984); this invariant sequence seems to be absolutely required for efficient splicing (Langford and Gallwitz 1983; Pikielny et al. 1983; Langford et al. 1984; Fouser and Friesen 1986; Vijayraghavan et al. 1986). In mammals, sequences adjacent to the site of lariat formation exhibit degeneracy; however, branching invariably occurs at an adenosine residue. Splicing is completed by cleavage at the 3' splice junction and concomitant exon ligation, to release the intron in a lariat form and mature mRNA.

The earliest events in the pathway involve the assembly of the precursor into a large multicomponent complex referred to as the spliceosome (Brody and Abelson 1985; Frendewey and Keller 1985; Grabowski et al. 1985). Data from mammalian systems suggest that the abundant class of small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U5, and U4-U6 (Maniatis and Reed 1987) play a major role in organizing the spliceosome via various snRNP : intron (Mount et al. 1983; Black et al. 1985; Chabot et al. 1985; Zhuang and Weiner 1986) and (presumably) snRNP : snRNP interactions (Chabot and Steitz 1987); the participation of at least one heteronuclear (hn) RNP protein is indicated (Choi et al. 1986) but currently less well understood.

Genetics is a powerful ally to the biochemical dissec-

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tion of complex cellular processes, particularly in cases involving multicomponent assemblies (for review, see Botstein and Maurer 1982). A successful route to the identification of interacting components has been to isolate extragenic suppressors of mutations that affect the process of interest. With this rationale in mind, several years ago we constructed a genetically manipulable splicing substrate; yeast cells containing an actin-*HIS4* gene fusion can grow on the histidine precursor histidinol only if the 309-nucleotide actin intron is efficiently and accurately spliced (Parker and Guthrie 1985). The effects of point mutations introduced into the fusion intron can be scored by both biological and biochemical analyses. In this way, a number of mutations that inhibit splicing have been identified (Parker and Guthrie 1985; Vijayraghavan et al. 1986); these mutations cause growth defects on histidinol, which correlate with the accumulation of unspliced fusion transcripts. Thus, we are now in the position to search for extragenic suppressors that restore splicing of these altered fusion transcripts.

Here we describe a reversion study of the C259 mutation (Vijayraghavan et al. 1986), which alters the branch point nucleotide within the TACTAAC box (TACTAAC to TACTACC). Among cells that had spontaneously regained the ability to grow on histidinol, we identified a mutant that fulfilled a set of criteria demanded for the possession of an authentic *trans*-acting suppressor of the splicing defect. On the basis of the properties of this suppressor, we suggest that we have identified a structural gene encoding a component of the splicing machinery. This component might interact with the branch nucleotide.

Results

Isolation of *trans*-acting suppressors

An A → C transversion at the branch point of the actin-*HIS4* gene fusion (designated C259) causes the accumulation of 80% or more of the fusion transcript as unspliced pre-mRNA *in vivo* (Vijayraghavan et al. 1986). A 20% level of mature mRNA is insufficient to allow growth on histidinol, thus, restoration of growth on histidinol provides a powerful selection for suppressors. Some extragenic revertants are expected to arise by alterations in *trans*-acting factors that improve splicing of the C259 transcript.

We employed diploids in the selection process for two reasons. First, to allow the recovery of recessive-lethal suppressors, and second, because dominant suppressors, which are more likely to arise in diploids, generally confer a gain of function. The relevant genotype of the diploid parent used to select revertants is shown in Table 1. The strain is homozygous for the C259 actin-*HIS4* fusion, integrated at the substantially deleted *HIS4* locus.

To facilitate the identification of revertants that are good candidates for further analysis, we developed a multistep protocol (explained in detail in Materials and methods). Briefly, in the first step, independent domi-

Table 1. Genotype of C259 parent

Genotype	Purpose
<i>his4-401/his4-401</i>	deletes <i>HIS4</i> fusion integration site
<i>ura3-52/ura3-52</i>	maintenance of the C259-actin- <i>HIS4</i> integrated fusion
<i>trp1-1/trp1-1</i>	maintenance of the actin- <i>lacZ</i> plasmids
<i>HOL1/HOL1</i>	import of histidinol
<i>URA3 C259-actin-HIS4/URA3 C259-actin-HIS4</i>	selection of revertants
<i>pYAHB-2 (C259 actin-lacZ CENIII TRP1 ARS1)</i>	screen for <i>trans</i> -acting suppressors
<i>pΔIVS (ΔIVS actin-lacZ CENIII TRP1 ARS1)</i>	screen for splicing-specific suppressors

nant revertants of the C259 parent were selected by their ability to grow on histidinol media. This requires the conversion of histidinol to histidine, which is the function of the *HIS4C* gene product (Keesey et al. 1979). In the second step, extragenic revertants were identified by assaying the initial revertants for their ability to suppress the phenotype of a second independent C259 mutation in the intron of an actin-*lacZ* gene fusion carried on the centromere plasmid pYAHB-2 (Vijayraghavan et al. 1986). Because splicing of the *lacZ* fusion transcript occurs at low efficiency in the C259 parent, this strain is faint blue on X-Gal plates; revertants of interest are substantially darker in color.

Finally, to restrict our attention to *trans*-acting suppressors implicated in splicing per se, we included a third screen. The centromere plasmid (pYAHB-2) was replaced with a nearly identical construct (pΔIVS) in which the actin intron has been deleted precisely (Larson et al. 1983); thus, β-galactosidase activity does not require splicing of the fusion transcript from pΔIVS. Any suppressor whose function is specific for intron removal should not affect the expression of an RNA lacking an intron. Thus, mutants that enhanced *lacZ* production from non-intervening sequence (IVS)-containing RNA were discarded.

Using this protocol we selected 521 independent revertants. Of these, only one, R8, satisfied both of the preceding criteria for a *trans*-acting, splicing-specific suppressor of the defect caused by the C259 mutation.

Splicing of the C259 pre-mRNA substrate is enhanced in the R8 revertant

Biochemical analyses were required to verify that the biological phenotypes reflected the restoration of efficient splicing of the C259 substrate. We prepared poly(A)⁺ RNA from the revertant R8, the C259 parent, and a control strain (WT) that carries a wild-type actin-*HIS4* fusion. Figure 1A shows a primer extension analysis using an oligonucleotide primer that is specific for fusion transcripts and allows the detection of pre-mRNA, mRNA, and the lariat intermediate. In WT (lane 3), pre-mRNA is essentially undetectable, while in the C259 parent (lane

Figure 1. RNA analysis of R8. Poly(A)⁺ RNA was extracted from the strains indicated (grown in YEPD) and analyzed by primer extension (A and C) or Northern hybridization (B). The following oligonucleotides were used (see Materials and methods): (A) the 19'-mer, which hybridizes the *HIS4* sequences at the actin-*HIS4* junction, including 2 nucleotides of actin, (B) the 21'-mer, which hybridizes to the actin 3' exon, and (C) the 24'-mer which hybridizes to the 3' end of the actin intron. Size markers are 5'-end-labeled, *Hpa*II-cut pBR325. (WT) 39/59 containing a wild-type actin-*HIS4* fusion; (A1) FC8-24D with an A1 actin-*HIS4* fusion (used here to mark the branch point); (No fusion) 39/59, a strain with no actin-*HIS4* gene fusions. In the diagrams (not drawn to scale) the position of the oligonucleotide annealing is indicated by *; actin exons are open boxes, *HIS4* sequences are slashed boxes, the actin intron is a solid line, and cDNAs are wavy lines. The numbers indicate the length of the cDNAs. The first band migrating above the lariat band in lane 2 (A) is not reproducible in other RNA preparations. The band immediately above it is most likely caused by a stable hairpin in pre-mRNA (Parker and Patterson 1987). In the Northern (B), fusion lariat intermediates run just above mRNA and are not clearly resolved. In lane 2 (C), lariat and pre-mRNA cDNA products are detected in the absence of the fusion due to the presence of the actin gene. In lanes 4, 5, and 6 (C), the band at approximately 70 nucleotides is most likely the same stop caused by the stable hairpin noted in A.

1) more than 90% of the fusion transcript accumulates as mRNA precursor. In contrast, R8 (lane 2) accumulates approximately 40% pre-mRNA with a concomitant increase in the levels of mRNA and lariat intermediate. To

confirm these results, we performed the Northern analysis shown in Figure 1B; again we see that more than half the transcripts in R8 are spliced RNA. Note that suppression is indicated by a change in the *ratio* of pre-

mRNA to mRNA (and intermediates); the absolute amounts of RNA are not germane to the suppressing effect because they depend on the number of gene fusions present in the particular strain. Specifically, R8 shows a decrease in the relative amount of pre-mRNA ($[\text{pre-mRNA}/(\text{pre-mRNA} + \text{ariat} + \text{mRNA})]$).

In Figure 1A, the band corresponding to the lariat intermediate is significantly darker in R8 than in the C259 parent. This is a reproducible finding (Figs. 2, 3, and 5, and other data not shown). To demonstrate that the primer extension block characteristic of the lariat species occurs at the same nucleotide in R8 as in the C259 parent, we performed a primer extension analysis using an oligonucleotide primer whose 3' terminus is 43 nucleotides downstream of the branch point. As shown in Figure 1C, the R8 block coincides precisely with that of the parent; as we demonstrated previously (Vijayraghavan et al. 1986), the lariat forms at the position of the mutated C259 residue (TACTACC). In principle, these lariats may be dead-end products, while the functional intermediates branch at an alternative site and are converted rapidly to mRNA. We consider this possibility unlikely, however. As shown in lane 3, lariat intermediates are detectable by this assay even in wild-type substrates. Almost certainly, a lariat formed at an alternative branch point should accumulate at least as much, if not more, than do wild-type species, and thus be detected in our analysis. We conclude that R8 suppression does not occur by selection of an alternative branch point, but rather by more efficient processing using the mutated site.

The suppressor is encoded by a single genetic locus

If the suppressor in R8 is due to a mutation at a single genetic locus, then it should determine a phenotype that segregates 2 : 2 in tetrads. It is possible to follow the phenotype of suppression itself in tetrads because R8 diploids yield four viable meiotic products; i.e., the suppressor is not lethal. The analysis of suppression is most easily done by scoring growth on histidinol medium. This has been done for eight tetrads (see Materials and methods) and allows us to conclude that growth on histidinol segregates 2 : 2. To confirm that suppression occurs at the level of splicing, we analyzed RNA patterns directly. Total RNA was extracted from two tetrads grown in medium lacking uracil (– Ura) and analyzed by primer extension, using the primer employed in Figure 1A. The analysis of tetrad JJ1-1 is shown in Figure 2. Spores A and C grow on histidinol and show an increase in the splicing efficiency, as measured by the relative amounts of each RNA species (see Fig. 2 legend for densitometric scanning values). Note, however, that splicing is considerably less efficient in either spore A or spore C than in the parent R8 diploid (see Fig. 1A). An explanation for the observation that suppression is submaximal in haploid cells is the subject of a later section. The analysis of the second tetrad (JJ1-2) gave similar results (not shown). We conclude that suppression of the splicing defect exhibits a 2 : 2 segregation, and is therefore determined by a mutation at a single genetic locus.

Figure 2. Primer extension analysis of an R8 tetrad. R8 was sporulated and tetrads were dissected. Tetrad JJ1-1 was grown in synthetic medium lacking uracil (– Ura) and total RNA was extracted from each haploid. The RNAs were analyzed by primer extension using the oligonucleotide 19'-mer (see Fig. 1A). The film was traced to determine the percentages of pre-mRNA, mRNA, and lariat. They are, respectively: lane A (48, 34, 18), lane B (79, 19, 2), lane C (59, 30, 11), and D (75, 21, 4).

The suppressor is extragenic

The preceding section does not address the possibility that suppression is caused by a second site mutation within the actin–*HIS4* gene fusion. To demonstrate that the suppressor is extragenic, we first mated a haploid that exhibited biochemical suppression (JJ1-2D) to a wild-type haploid that does not contain an actin–*HIS4* gene fusion (JJ5-31B) (see Materials and methods); the resulting diploid was sporulated and dissected. The segregation of the actin–*HIS4* fusion was scored by assaying the ability of the spores to grow on synthetic media lacking uracil (Table 1). As shown in Figure 3, haploids that grow are scored as “+” for the fusion. Since only half the spores are expected to receive the fusion, and since the fusion is necessary to determine the segregation of the suppressor, we crossed each of the meiotic products to an otherwise wild-type strain that carries the C259 actin–*HIS4* fusion (see legend to Fig. 3).

Poly(A)⁺ RNA was extracted from each of the resulting diploids and assayed by primer extension. If a diploid contains the suppressor it accumulates approximately 40% of the fusion transcript as precursor and is scored as “+” in Figure 3; diploids lacking the suppressor accumulate mainly (80%) full-length fusion precursor and are scored as “–”. As in Figure 2, we see a 2 : 2 segregation of biochemical suppression. Most importantly, these results demonstrate that suppression of the C259 splicing defect segregates independently of the actin–*HIS4* gene fusion derived from R8 and is, therefore, extragenic. We have designated the suppressor mutation *ma16-1*. This nomenclature was chosen because other mutations affecting RNA splicing also have the

Figure 3. Suppressor linkage analysis. The two tetrads shown here were obtained as described in the text. To score for the presence of the fusion we tested the haploids on synthetic medium lacking uracil (– Ura plates) and scored growth as indicated after “Fusion”; the scores were confirmed with Southern analysis (not shown). The haploids were then mated to either YJC39 or YJC59, depending on the mating type; both mating strains contained C259 actin–*HIS4* fusions. Poly(A)⁺ RNA from the resulting diploids (grown in YEPD) was analyzed by primer extension using the oligonucleotide 19′-mer (see Fig. 1A). Suppression was scored on the basis of the pre-mRNA/(pre-mRNA + mRNA + lariat) ratio. “No Fusion” is 39/59, a strain with no actin–*HIS4* fusions. Differences in the total RNA levels in different lanes are attributable to differences in the number of gene fusions.

RNA designation (Rosbash et al. 1981; Larkin and Woolford 1983; Teem et al. 1983; Miller 1984; Lustig et al. 1986).

The suppressor is specific for the C259 mutation

It is important to determine if the suppressor interacts with a specific region of the intron, i.e., does it suppress only the intron mutation that it was selected for, or can it also restore the splicing of various mutated introns? A specific suppressor will potentially provide more information about the mechanism of splicing. Thus, we asked whether *ma16-1* could suppress additional mutations in the intron of the actin–*HIS4* fusion (Vijayraghavan et al. 1986). The mutations tested include alterations at two other positions within the TACTAAC box. TAATAAC (A256) and TACAAC (A257), shown in Figure 4; two mutations at the 5′ junction, /ATATGT (A1) and /CTATGT (C1) (not shown); and a mutation at the 3′ splice junction AC/ac (C303/305) (not shown). With the possible exception of the A256 case, the presence of the suppressor has no appreciable effect on the relative amounts of pre-mRNA, lariat, and mRNA. It is difficult to rule out completely suppression in the case of A256 because this mutation already allows a considerable amount of pre-mRNA (50–70%) to be processed completely. Nonetheless, it is clear that the level of A256 mRNA observed in the presence of the suppressor is not increased appreciably.

We can thus conclude that the only dramatic effect observed in our tests is the suppression of the defect caused by the C259 mutation. Therefore, the suppressor exhibits specificity with respect to functionally important sites within the actin intron. It will be of interest to determine whether the phenotypes of branch point mutations other than C259 can also be suppressed.

The wild-type RNA16 allele increases the efficiency of suppression

We observed (Table 2) that when RNA for primer extension analysis is prepared from strains cultured in rich medium (YEPD), suppression is observed in the R8 diploid and in diploids derived from it, but not in its haploid meiotic products. (The data shown in Fig. 2 were ob-

Figure 4. Suppressor specificity. Primer extensions were performed on total RNA prepared from the diploids described below (grown in – Ura synthetic medium) using the 19′-mer oligonucleotide. Parent diploids (odd lanes) are *RNA16/RNA16* diploids that were made as described for C259 parent (see Materials and methods) except that appropriately mutated pYAH-I2 replaced C259-mutated pYAH-I2. Suppressor diploids (even lanes) are *RNA16/ma16-1* diploids that were obtained by first mating haploids JJ16-3D and JJ5-31B (see Materials and methods), and then transforming with appropriately mutated pYAH-I2.

Table 2. Relative amounts of RNA species

	Rich medium ^a			Synthetic medium ^a		
	%P	%M	%L	%P	%M	%L
<i>RNA16</i>	92	7	1	77	20	3
<i>RNA16/RNA16</i>	83	14	3	82	14	4
<i>rna16-1</i>	84	13	3	53	32	15
<i>rna16-1/rna16-1</i>	87	8	5	45	39	16
<i>RNA16/rna16-1</i>	41	34	25	22	58	20

These values were obtained by quantitating densitometric tracings of typical primer-extension analyses (including those shown in Figs. 2 and 5B).

^a(P) pre-mRNA; (M) mRNA; (L) lariat.

tained from cells grown in synthetic media; see below.) To test the hypothesis that, in cells grown in rich medium, suppression requires heterozygosity at the *RNA16* locus, we constructed and analyzed by primer extension a series of heterozygous and homozygous diploids, as described in Figure 5A and summarized in Table 2. Homozygous wild-type diploids (lanes 1 and 2), as expected, accumulate primarily unspliced C259 fusion transcript (approximately 85%). Three independent *rna16-1/RNA16* heterozygotes (lanes 3, 4, and 5) show the expected increase in mRNA splicing and accumulate only 40% pre-mRNA. In contrast, each of three diploids homozygous for *rna16-1* (lanes 6, 7, and 8) show a splicing pattern essentially indistinguishable from that of the homozygous wild-type. These results were confirmed by Northern analysis (not shown). We conclude that, during growth in rich medium, measurable suppression of the splicing defect requires both the *RNA16* and *rna16-1* alleles.

We then asked if the presence of the *RNA16* allele affected the degree of suppression when strains are cultured in synthetic media. RNA prepared from each of the three diploid types was analyzed by primer extension, and the results compared to those shown in Figure 5A by densitometric tracing of the autoradiograms. As shown in Figure 5B, and in Table 2, splicing is almost undetectable in *rna16-1/rna16-1* cells grown in rich medium (Fig. 5B, panel F), but is very efficient when these cells are cultured in synthetic medium (panel C). In fact, the relative amounts of pre-mRNA, mRNA, and lariat in homozygotes grown in synthetic medium (panel C) are similar to those of *rna16-1/RNA16* heterozygotes grown in rich medium (panel E). Thus, under certain growth conditions, efficient suppression does not require both alleles. On the other hand, the efficiency of splicing in *rna16-1/RNA16* heterozygotes grown in synthetic medium (panel B) is even greater than that seen for the *rna16-1/rna16-1* homozygotes grown under the same conditions (panel C). The relative amounts of mature fusion RNA from cells cultured in synthetic medium (panels A, B, and C) correlate with the relative growth rates of the same cells on histidinol plates (which are synthetic); *RNA16/rna16-1* diploids grow well, *rna16-1/rna16-1* diploids grow poorly, and *RNA16/RNA16* diploids do not grow (not shown). We conclude that the presence of the *RNA16* allele significantly increases the efficiency of suppression in all growth conditions.

Cells homozygous for *rna16-1* accumulate precursors of wild-type transcripts

If *RNA16* encodes a spliceosome component, it is reasonable to expect that wild-type transcripts would be spliced less efficiently in *rna16-1* mutants. To test this hypothesis, we first analyzed the splicing of two such transcripts in *RNA16/RNA16*, *RNA16/rna16-1*, and *rna16-1/rna16-1* diploids. As shown in Figure 6A, the splicing of the *MATa1* transcript is significantly affected in mutant homozygous diploids (lanes 6, 7, and 8); full-length precursor and partially spliced intermediates accumulate dramatically at the expense of mature RNA. Splicing efficiency in the heterozygous diploid (lanes 3, 4, and 5) is essentially indistinguishable from that in the homozygous wild-type strain (lanes 1 and 2). Analysis of a second spliced transcript, *rp51A* (Fig. 6B), shows that unspliced pre-mRNA, which is undetectable in wild-type (lanes 1 and 2) or heterozygous (lanes 3–5) strains also accumulates in *rna16-1/rna16-1* cells (lanes 6–8).

To extend these observations to haploids, we performed primer extension analysis of these wild-type transcripts in the two tetrads examined previously (JJ1-1, JJ1-2). As predicted, *rp51A* pre-mRNA accumulates in each of the two spores containing the *rna16-1* allele (data not shown). Similarly, as shown in Figure 7, the *rna16-1* spores containing the *MATa* mating type allele accumulate unspliced *MATa1* pre-mRNA and intermediates.

We conclude that *rna16-1* determines two distinct phenotypes: (1) the dominant suppression of the splicing defect caused by the C259 mutation; and (2) the recessive decrease in the splicing efficiency of pre-mRNAs containing wild-type introns.

Discussion

The suppressor has the properties expected for an altered component of the splicing machinery

We have described the isolation in diploids of a suppressor that enhances the splicing of the mutant C259–actin–*HIS4* transcript fourfold in vivo (see Table 2). The suppressor mutation, designated *rna16-1*, is genetically unlinked to the parental C259 gene fusion, and segregates 2 : 2 in tetrads. Therefore, suppression occurs because of a modification in a single, *trans*-acting factor. It is unlikely that this *trans*-acting factor affects either the transcription or the stability of the fusion RNA since the revertant R8 shows no difference in actin–*lacZ* expression, relative to the parent strain, when this fusion lacks the actin IVS. Moreover, the suppressor is dominant, which makes it unlikely to affect RNA stability. In addition to suppressing the splicing defect of C259 actin–*HIS4* mutant transcripts, the *trans*-acting factor causes an increase in the amount of unspliced wild-type pre-mRNAs; this phenotype is recessive. These two independent splicing phenotypes argue strongly that the *trans*-acting factor is directly involved in the splicing of nuclear pre-mRNAs.

Figure 5. (A) Primer extension analysis of diploids grown in rich medium (YEPA). Poly(A)⁺ RNAs were analyzed by primer extension using the oligonucleotide 19'-mer (see Fig. 1A). The different kinds of diploids were obtained as follows. First, an *rna16-1* haploid (JJ1-2D) containing the C259 gene fusion was mated to a wild-type haploid (JJ5-31B). The heterozygous diploids were then sporulated and dissected, and two tetrads (1 and 2) were analyzed for the presence of the fusion and for the segregation of *rna16-1*. This analysis is the subject of Fig. 3. The two *RNA16/RNA16* diploids were obtained by mating 1A × YJC59 and 2A × YJC39, respectively. The *rna16-1/RNA16* diploids were obtained by mating haploids 1C × YJC39, 2B × YJC59, and 2D × YJC39. Both YJC39 and YJC59 contained C259 actin-*HIS4* gene fusions. The *rna16-1/rna16-1* diploids were obtained by crossing the haploids 1B × 1C, 1C × 2B, and 2B × 2D, respectively. (B) The effect of the growth media upon suppression. (D, E, and F) Densitometric tracings of a lighter exposure of lanes 1, 3, and 6 of Fig. 5A. These same diploids were grown in synthetic medium lacking uracil (–Ura); total RNA was extracted and analyzed by primer extension using the oligonucleotide 19'-mer. Scans of light exposures of these primer extensions are shown in A, B, and C.

Figure 6. Primer extension analysis of wild-type mRNAs in homozygous diploids. The strains analyzed here are the same as those shown in Fig. 5A; all cells were grown in rich medium (YEPA). Poly(A)⁺ RNA was analyzed using either the *MATa1* oligonucleotide (A) or an rp51A oligonucleotide, RB1 (B). The RNA species were identified on the basis of electrophoretic mobility. The doublets in A are reproducible and probably represent two transcription initiation sites. In B, the two mRNA bands represent two different RNA polymerase starts. Overexposure of the gel in B did not reveal an appreciable accumulation of rp51A pre-mRNA in lanes 1–5. Conversely an underexposure still allowed detection of pre-mRNA in lanes 6–8. In the diagrams (not drawn to scale), exons are open boxes and introns are solid lines.

The suppressor acts early in the splicing pathway

Certain mutations at the 5' and 3' splice sites [such as A1 and C303/305 (Vijayraghavan et al. 1986)] result in the accumulation of lariats. In contrast, the C259 mutation causes the accumulation of pre-mRNA but not of lariat intermediates. Therefore, C259 must disrupt one or more steps in the splicing pathway that precede lariat formation. Since the *rna16-1* gene product suppresses the C259 defect, resulting in a decreased ratio of pre-mRNA to mRNA, it must also (at least) act prior to lariat formation.

Concomitantly with the decrease in the relative level of C259 pre-mRNA, the relative level of lariat increases in the presence of the suppressor. Possibly, then, the *rna16-1* gene product also inhibits the conversion of lariat to mRNA. In support of this hypothesis, the relative levels of *MATa1* and rp51A lariats also seem to increase in *rna16-1/rna16-1* diploids (this is less apparent in haploids). One explanation for these observations is that while the presence of the *rna16-1* gene product in the splicing complex mainly affects the conversion of pre-mRNA to lariat, it might also affect other steps indi-

rectly, by causing a physical distortion of the spliceosome.

The RNA16 factor is likely to recognize the TACTAAC region

The suppressor has no apparent effect on the splicing of A1, C1, A257, or C303/305 transcripts and has little or no effect on the splicing of the A256 transcript (cf. Vijayraghavan et al. 1986). Since the C1, A257, and C303/305 mutations are phenotypically tight, a slight effect of the suppressor on the splicing of these substrates might not be detectable. In contrast, the A1 and the A256 mutations, like C259, both block the conversion of pre-mRNA to lariat, and both are phenotypically leaky; yet, the A1 mutation is not suppressed at all, and the A256 mutation is, at best, suppressed only marginally. We conclude, therefore, that the suppressor is likely to be allele specific. This preference for the site of branch formation is indicative of a physical contact between the suppressor molecule and the TACTAAC box.

Our laboratory has recently demonstrated a base-pairing interaction between a yeast U2-like RNA (Ares

Figure 7. Primer extension analysis of wild-type mRNAs in haploids. Total RNA was extracted from *MATa* haploids of tetrads JJ1-1 and JJ1-2 and analyzed by primer extension using the *MATa1* oligonucleotide.

et al. 1986) and the TACTAAC box (Parker et al. 1987). It is likely that this complementarity mediates an early association between the U2-like snRNP and the site of subsequent branch formation. Interestingly, in the proposed scheme, the branch point nucleotide is not itself paired, and it is suggested that recognition of the branch nucleotide occurs subsequent to this Watson-Crick interaction. The *RNA16* gene product is obviously an excellent candidate for this function. Recent results make it unlikely that *RNA16* is this snRNA (snR20 in our nomenclature; Riedel et al. 1986), since *snR20*, when encoded in a centromere plasmid and transformed into an *rna16-1* haploid, was unable to relieve the *rna16⁻* recessive defect on the splicing of the *MATa1* transcript.

Cloning of the *RNA16* and *rna16-1* alleles is in progress. The recessive defect conferred by *rna16-1* on splicing of wild-type transcripts suggests to us that a null allele will be lethal. If this is the case, it should then be possible to identify gene products that interact with the *RNA16* gene product by the isolation of suppressors of *rna16* mutations with conditional phenotypes. In particular, these experiments should reveal the relationship between *RNA16*—and the gene products with which it interacts—and the *ma2-rna11* mutations, which produce splicing defects both in vivo (Rosbash et al. 1981; Larkin and Woolford 1983; Teem et al. 1983; Miller 1984) and (at least in some cases) in vitro (Lustig et al. 1986).

The clones will also help in our understanding of the following two observations that make the *rna16-1* suppressor unique. Suppression of the C259 defect is most efficient in heterozygous diploids, i.e., when both the

wild-type and mutant alleles are present. In addition, suppression is enhanced when cells are grown in synthetic medium (see Table 2). One model that addresses both observations is that, in *rna16-1* haploids, wild-type pre-mRNAs act as competitive inhibitors of suppression, since the *rna16-1* gene product must splice both the C259 and the wild-type transcripts. In heterozygous diploids, the participation of the *RNA16* gene product in the splicing of wild-type pre-mRNAs would allow a higher effective concentration of the *rna16-1* gene product to splice the C259 transcripts. The media effect would be explained if the concentration of wild-type pre-mRNAs is disproportionately increased in rich medium. If this model is correct, then the expression of the *rna16-1* clone by a multicopy plasmid in *rna16-1* haploids should improve suppression, especially in rich medium.

Materials and methods

Plasmids

The plasmid pYAH-I2 contains the actin-*HIS4* gene fusion and was constructed by Parker and Guthrie (1985). The TACTAAC sequence, 14 nucleotides upstream of the TACTAAC box, is deleted from the actin intron except in A256 (see below). The TACTAAC box is present either in its wild-type form or mutated as follows: C259-TACTACC, A256-TAATAAC, and A257-TACAAAC (Vijayraghavan et al. 1986). Likewise, the 5' (/GTATGT) and 3' (AG/) consensus sequences are mutated in the appropriate versions of the plasmid: A1-/ATATGT, C1-/CTATGT, and C303/305-AC/ac (Vijayraghavan et al. 1986). The plasmid pYAHB-2 contains an actin-*lacZ* gene fusion and is described in Vijayraghavan et al. (1986); pADIVS is similar to pYAHB-2 but it has a precise deletion of the actin intron (Larson et al. 1983).

Strains

YJC39: (*MATa RNA16 ade5 trp1-1 his4-401 leu2 ura3-52 can1 HOL1*). YJC59: (*MATa RNA16 ade2 trp1-1 his4-401 leu2 ura3-52 can1 HOL1*). JJ5-25C: (*MATa RNA16 ade5 his4-401 leu2 ura3-52 lys2 HOL1*). JJ5-31B: (*MATa RNA16 ade5 his4-401 leu2 ura3-52 lys2 HOL1*). FC8-24D containing the A1 actin-*HIS4* fusion was used to map the branch point as described in Vijayraghavan et al. (1986). The diploid 39/59 was made by mating YJC39 with YJC59. The diploid "C259 Parent" was made as follows: YJC39 and YJC59 were each transformed with *XbaI*-cut C259-mutated pYAH-I2; YJC39 and YJC59 transformants were selected on -Ura plates and subsequently mated one to the other; correct integration of the plasmid into the *his4-401* locus was confirmed by Southern analysis of the resulting diploids; they were then transformed with C259-mutated pYAHB-2. JJ1-2D is a haploid from an R8 tetrad. Its genotype is *MATa rna16-1 ade2 trp1-1 his4-401 leu2 ura3-52 HOL1*, and it contains the C259 actin-*HIS4* fusion (with *URA3*) integrated at the *his4-401* locus. JJ16-3D is a haploid with the following genotype: *MATa rna16-1 his4-401 trp1-1 leu2 ura3-52 HOL1 ade-*.

Media

YEPD, -Ura, and -Trp (synthetic media lacking uracil or tryptophan) are described in Sherman et al. (1974). Histidinol

medium is described in Parker and Guthrie (1985); X-Gal plates are described in Rose et al. (1981).

Yeast genetic methods

All yeast genetic methods, including matings, sporulation, tetrad dissection, phenotypic analysis, replica-plating with velvets and with a 48-point inoculator, and lithium acetate transformations were performed as described in Sherman et al. (1974).

Isolation of the R8 revertant

Ten thousand colonies of the C259 parent growing on YEPD plates (about 200/plate) were replica-plated onto histidinol plates and incubated at 30°C. A total of 521 spontaneous papillae, each arising from an independent colony, were picked after 5 days. Each of these isolates and the C259 parent were transferred with a 48-point inoculator to X-Gal plates to monitor expression of the C259-mutated actin-*lacZ* fusion from pYAHB-2. After incubating 3–4 days at 30°C, the color intensity of the revertants was compared with that of the parent. Sixteen clones exhibited a darker blue color than the parent and were chosen for further study. The 16 clones were cured of their *CEN-ARS* plasmid (pYAHB-2) by growing them overnight in YEPD. Plasmid loss was monitored by scoring growth on YEPD and –Trp plates. Cells that had lost pYAHB-2 were retransformed with the plasmid pAΔIVS. Transformants were tested on X-Gal as before and their color intensity was compared with that of the C259 parent (without pYAHB-2) transformed with pAΔIVS. Only one revertant, R8, was equal in color to the C259 control. The remaining 15 were darker and were discarded.

Phenotypic analysis of R8 tetrads

The diploid revertant R8 was sporulated and tetrads were dissected. Eight complete tetrads were analyzed for the presence of the suppressor by scoring growth on histidinol plates. Four growth phenotypes were observed. Very strong growth and normal growth were scored +. Leaky growth was very slow growth that was not reproducible, i.e., sometimes the same haploid would grow poorly and sometimes not at all. This phenotype and reproducible nongrowth were both scored –. Seven of the eight tetrads showed a 2 : 2, + : – segregation and one a 3 : 1, + : – segregation; 20/39 spores from additional dissections (which included incomplete tetrads) scored +.

We believe the reason for the differences in growth rates is as follows: R8 has acquired an extra copy of actin-*HIS4* gene fusion so that one chromosome contains two gene fusions and the other contains one. This was confirmed by Southern analysis and probably resulted from recombination between flanking direct repeats in different chromatids. The segregation of the gene fusions among R8 tetrads is then 2 : 2 : 1 : 1. This was also confirmed by Southern analysis across three tetrads (JJ1-1, -2, and -4). Very strong growth is observed when a spore has two gene fusions and the suppressor. Normal growth is observed when a spore has one fusion and the suppressor. Leaky growth may occur if the spore has two gene fusions and no suppressor.

RNA analysis

Total RNA was prepared as described by Domdey et al. (1984). Poly(A)⁺ RNA was prepared as follows. Total RNA was dissolved in 4.5 ml of low-salt buffer [10 mM Tris-HCl (pH 7.4), 0.2% SDS, 1 mM EDTA], incubated at 65°C for 3 min, and quickly cooled on ice. NaCl was added to a concentration of 0.5 M and the RNA was mixed with 100 mg of oligo(dT)-cellulose,

type 3 (Collaborative Research Inc.), previously equilibrated in high-salt buffer [0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.2% SDS, 5 mM EDTA]. The mixture was gently rocked for 40 min in a 15-ml tube at 23°C. Following centrifugation, the supernatant, containing poly(A)[–] RNA, was removed. The resin was washed five times with 5 ml of high-salt buffer, at 23°C, each time rocking for 3 min and pelleting the resin by centrifugation. Poly(A)⁺ RNA was eluted in two successive 5-min washes each with 2.5 ml of low-salt buffer at 23°C. The eluates were combined and ethanol-precipitated. The RNA was resuspended in 50 μl of water.

Northern analysis: RNA was resolved on glyoxal gels as described in McMaster and Carmichael (1977), blotted to Gene-Screen, and probed with end-labeled oligonucleotides.

Primer and primer extensions: The oligonucleotides 19'-mer, and 24'-mer are described in Vijayraghavan et al. (1986); the 21'-mer [CCAGAACCGTTATCAATAACC] hybridizes to exon 2 of actin. RB1 is described in Teem and Rosbash (1983) and was a generous gift from Dr. Michael Rosbash. The *MATa1* oligonucleotide [GAATTTATTTAGATCTCATACGTTT] hybridizes to exon 3 of the *MATa1* transcript. Primer extensions were performed as described by Domdey et al. (1984).

Southern analyses were performed basically as described in Sherman et al. (1974). Genomic DNA was digested with *Pst*I, and the fragments were resolved on 0.8% agarose gels, transferred to nitrocellulose, and probed with a nick-translated *HIS4* clone.

Densitometric tracings: Light exposures on Kodak Diagnostic Film, X-Omat AR, were scanned with a Zeineh Soft Laser scanning densitometer (LKB) using the laser beam. Quantitation was done by cutting and weighing photocopies of the tracings.

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