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Requirements for intron-mediated enhancement of gene expression in *Arabidopsis*

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ABSTRACT

To explore possible mechanisms of intron-mediated enhancement of gene expression, the features of *PAT1* intron 1 required to elevate mRNA accumulation were systematically tested in transgenic *Arabidopsis*. This intron is remarkably resilient, retaining some ability to increase mRNA accumulation when splicing was prevented by mutation of 5′ and 3′ splice sites, branchpoint sequences, or when intron U-richness was reduced. Enhancement was abolished by simultaneously eliminating branchpoints and the 5′ splice site, structures involved in the first two steps of spliceosome assembly. Although this suggests that the splicing machinery is required, intron splicing is clearly not enough to enhance mRNA accumulation. Five other introns were all efficiently spliced but varied widely in their ability to increase mRNA levels. Furthermore, *PAT1* intron 1 was spliced but lost the ability to elevate mRNA accumulation when moved to the 3′ UTR. These findings demonstrate that splicing per se is neither necessary nor sufficient for an intron to enhance mRNA accumulation, and suggest a mechanism that requires intron recognition by the splicing machinery but also involves nonconserved intron sequences.

Keywords: intron position; mRNA accumulation; plant; pre-mRNA splicing; splicing efficiency

INTRODUCTION

Approximately 80% of *Arabidopsis* genes contain introns that must be accurately removed from the primary transcripts to create functional mRNAs. Not only do introns facilitate the evolution of new proteins by exon shuffling (Long et al., 1995) and allow multiple proteins to be produced from a single gene through alternative splicing (Maniatis & Tasic, 2002), they often have important functions in gene regulation. Positive and negative regulatory sequences have been identified within specific introns (Rippe et al., 1989; Bruhat et al., 1990; Deyholos & Sieburth, 2000), and introns are generally required for abundant expression of many genes in plants (Callis et al., 1987), mammals (Buchman & Berg, 1988; Huang & Gorman, 1990; Choi et al., 1991), nematodes (Okkema et al., 1993), and insects (Meredith & Storti, 1993).

The frequently observed stimulation of expression caused by plant introns has been termed intron-mediated enhancement (IME) (Mascarenhas et al., 1990). The difference in amount of product derived from an intron-containing gene and an otherwise identical intronless construct is typically between 2- and 10-fold but can be significantly more, especially in monocots (Maas et al., 1991; Zhang et al., 1994). The magnitude of the enhancement varies depending on the promoter, intron, and reporter gene used (Callis et al., 1987; Luehrsen & Walbot, 1991; Rethmeier et al., 1998), the sequences that flank the intron (Maas et al., 1991; Clancy et al., 1994), and the type of cell or tissue in which expression is determined (Gallie & Young, 1994). Even though the high degree of variability makes comparisons between studies difficult, and introns could affect expression in different ways, some common themes have emerged that help to delineate potential mechanisms of IME.

The increase in expression mediated by introns is usually apparent at the level of mRNA accumulation (Callis et al., 1987; Dean et al., 1989; Rethmeier et al., 1997; Rose & Last, 1997; Wang et al., 2002). However, IME is unlike the enhancement caused by transcriptional enhancer elements in that the introns must be located within transcribed sequences and in their normal orientation to boost expression (Callis et al., 1987; Mascarenhas et al., 1990; Clancy et al., 1994). The distinction from transcriptional enhancer elements is further supported by the demonstration that the introns tested increase mRNA accumulation without significantly affecting the rate of transcript initiation, as de-

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termined by nuclear run-on transcription assays (Dean et al., 1989; Rose & Last, 1997; Rose & Beliakoff, 2000). Although introns could elevate steady-state mRNA levels by increasing mRNA stability, the half-life of mRNA from genes with or without an intron has been reported to be the same (Nash & Walbot, 1992; Rethmeier et al., 1997). Thus, the mechanism of IME remains unclear, but presumably operates at a co- or posttranscriptional level.

It is plausible that the enhancement mediated by introns is functionally connected to their recognition and removal by the splicing machinery. Thus, the features that identify a sequence as an intron and/or participate in spliceosome assembly and the splicing reactions might also be involved in IME. In *Arabidopsis*, the only recognizable characteristics of introns are that they have weakly conserved sequences at the 5′ splice site (AG:GUAAGU; ":" indicates cleavage site, underlined residues are highly conserved), branchpoint (YURAY), and 3′ splice site (GCAG:GU; Brown et al., 1996; Lim & Burge, 2001), and are more U rich than the flanking exons (41% U vs. 26% U on average; Goodall & Filipowicz, 1989; Ko et al., 1998; Deutsch & Long, 1999). The U richness is probably important for intron recognition (Ko et al., 1998). The conserved sequences surrounding the 5′ splice site base pair with the U1 RNA of the U1 snRNP in the first step of spliceosome assembly onto an intron (Lorkovic et al., 2000). The U2 snRNP then binds to the sequences around the branchpoint, followed by interactions between the U5 snRNP and the 3′ splice site. Even though it has not been possible to investigate plant splicing in vitro, the assumption that the basic mechanisms of splicing are the same as those established for yeast and mammals (Burge et al., 1999) is supported by the existence of *Arabidopsis* homologs of virtually all of the proteins and RNAs involved (Lorkovic et al., 2000).

One of the most thoroughly characterized examples of IME in *Arabidopsis* involves the first intron from the tryptophan biosynthetic pathway gene *PAT1* (Rose & Last, 1997). This 110-nt intron stimulates a fivefold increase in the steady-state level of mRNA without affecting the transcription of *PAT1* fused to the *GUS* (*β*-glucuronidase) reporter gene in transgenic *Arabidopsis* (Rose & Beliakoff, 2000). All of the sequences between 2 nt downstream of the 5′ splice site and 6 nt upstream of the 3′ splice site were individually deleted without reducing the ability of the intron to stimulate mRNA accumulation (Rose & Beliakoff, 2000). Thus, the sequences that are required to enhance expression are either redundant or form part of the 5′ or 3′ splice site. Derivatives of *PAT1* intron 1 rendered unspliceable either by a point mutation at the 5′ splice site or by making the intron too small to be spliced still enhance mRNA accumulation (Rose & Beliakoff, 2000). The ability to stimulate expression in the absence of splicing rules out models in which the completed act of splicing is required. However, all but one of the seven intron derivatives tested had intact 5′ and 3′ splice sites, all contained potential branchpoints, and all were more U rich than the flanking exons. The exception is an intron with a single nucleotide mutation at the 5′ splice site that may not eliminate base pairing with the U1 snRNP. Therefore, spliceosomes might be able to assemble onto all of the derivatives tested, and IME may depend on an association with the spicing machinery even if splicing cannot be completed.

To differentiate between and refine possible models of IME, the features of *PAT1* intron 1 required to stimulate expression were explored in greater depth. The need for splice sites, branchpoint sequences, and U richness was tested, introns from a variety of sources were compared, and the importance of intron position was examined. *PAT1* intron 1 only lost its ability to stimulate mRNA accumulation when the 5′ splice site and branchpoints were simultaneously destroyed, or when it was moved to the 3′ UTR. Other introns were found to differ widely in their ability to stimulate mRNA accumulation, despite being efficiently spliced. These results show that splicing is neither necessary nor sufficient for IME, and suggest that the intron sequence requirements for splicing and IME are distinct but overlap.

RESULTS

To determine if the intron properties required for IME and splicing are the same, different, or partially overlapping, several derivatives of *PAT1* intron 1 were constructed that lack features normally found in dicot introns. Because many of these alterations would be expected to abolish splicing, each modified intron was engineered to maintain the reading frame of the flanking exons to avoid the possibility of nonsense-mediated decay of unspliced mRNAs (Wilusz et al., 2001). The mutations (Fig. 1A) were introduced into either the 108-nt in-frame *PAT1* intron 1 or Δ41–73, a 75-nt derivative of this intron, both of which are spliced and stimulate mRNA accumulation as much as does the wild-type intron (Rose & Beliakoff, 2000). The modified introns were placed between exons 1 and 2 of a *PAT1:GUS* fusion and introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. Several independent homozygous single-copy transformants containing each fusion were used for each experiment (part C of Figs. 2–5). Expression of the transgene was determined by RNA gel blot analysis, and the splicing efficiency of each intron was estimated by RT-PCR (parts A and B, respectively, of Figs. 2–5). As previously reported for other *PAT1:GUS* fusions (Rose & Last, 1997; Rose & Beliakoff, 2000), variation between single-copy lines containing the same construct was minimal, allowing consistent results to be obtained from relatively few single-copy transgenic lines.

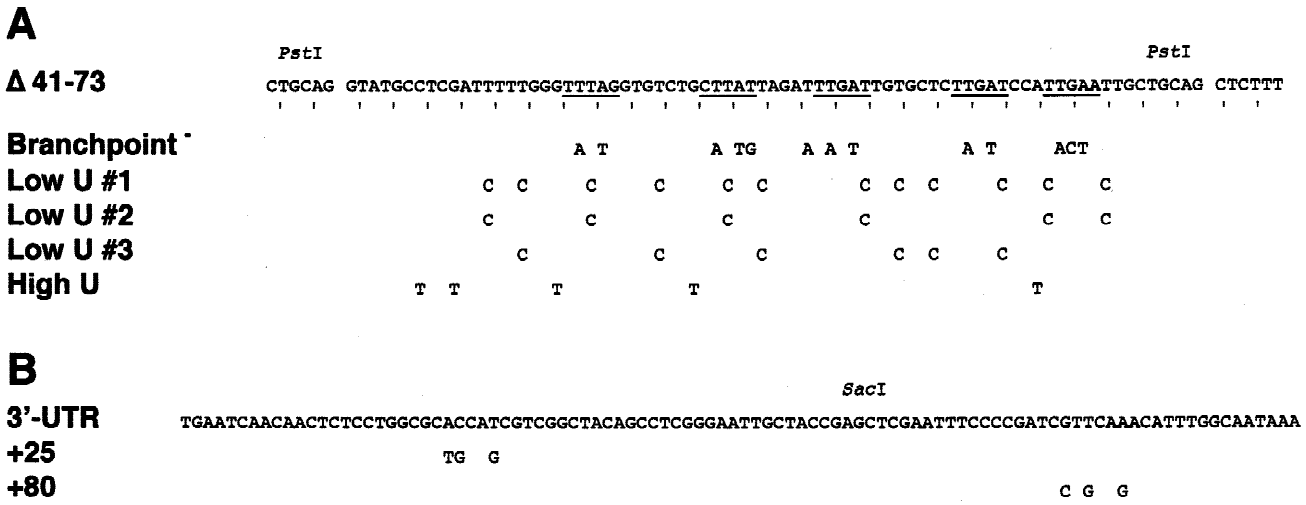

FIGURE 1. Introduced mutations. **A:** Derivatives of *PAT1* intron 1 Δ41–73. The DNA sequence of the starting intron and 6 nt of each flanking exon is shown on the top line, with codons delineated by ticks and potential branchpoint sequences underlined. The changes introduced to generate the mutant introns are detailed below. **B:** Creation of *Pst*I sites in the 3′ UTR of a *PAT1:GUS* fusion. The DNA sequence from the *GUS* stop codon to the first presumed polyadenylation signal is shown. The *Pst*I sites created by the mutations indicated were used to insert an intron after 25 or 80 nt of 3′-UTR sequences.

5′ splice site

A point mutation of the 5′ splice site was previously found to abolish splicing, but did not eliminate the ability of this "intron" to stimulate mRNA accumulation (Rose & Beliakoff, 2000). Although this finding demonstrates that completed splicing per se is not required for IME, it does not rule out the possibility that IME requires an association of the intron with the splicing machinery because a single base mismatch may not prevent U1 snRNP binding to other conserved sequences at the 5′ splice site. To eliminate possible interactions with the U1 snRNP, the first 5 nt of the intron were changed to those that are found least frequently at each position in *Arabidopsis* introns (AACGC; Brown et al., 1996). In both the 108-nt in-frame and 75-nt Δ41–73 versions of *PAT1* intron 1, the 5-nt change had the same effect as the point mutation at the 5′ splice site: It completely prevented splicing and reduced but did not eliminate IME (Table 1; Fig. 2). This result strongly suggests that IME can occur in the absence of an interaction between the U1 snRNP and the 5′ splice site.

Branchpoints

The second step of spliceosome assembly involves an interaction of the U2 snRNP with the sequences that will form the branchpoint of the lariat structure splicing intermediate. The branchpoint sequence YURAY, and particularly the conserved U and branch-forming A residues, was recently shown by mutational analysis to be

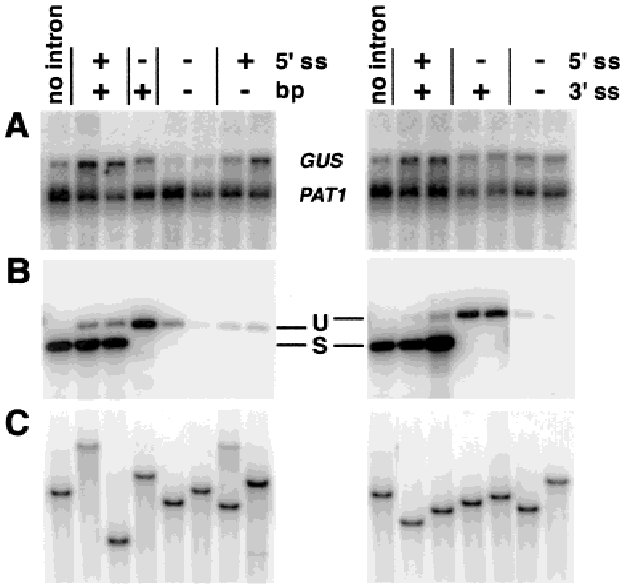

FIGURE 2. The effect of splice site and branchpoint sequence mutations. *Arabidopsis* lines transgenic for *PAT1:GUS* fusions were tested for reporter gene expression (**A**), intron splicing (**B**), and transgene copy number (**C**) with the lanes for each aligned. The introns in each fusion were wild-type (+) or contained mutations (−) as indicated in the 5′ splice site (5′ss), branchpoints (bp), or 3′ splice site (3′ss) of the 108 nt in-frame intron (right panels) or the 75-nt Δ41-73 intron (left panels). **A:** RNA gel blot. The blot was probed with *GUS* to measure transgene expression and exons 4 to 9 of *PAT1* as a loading control. **B:** RT-PCR analysis. The blot was probed with an equimolar mixture of exon 1 and exon 2 probes that flank the intron. The U and S indicate product derived from unspliced and spliced transcripts respectively. **C:** DNA gel blot. Genomic DNA digested with either *Pst*I or *Bam*HI was probed with *GUS*. Each band represents a junction fragment between the transgene and the flanking DNA.

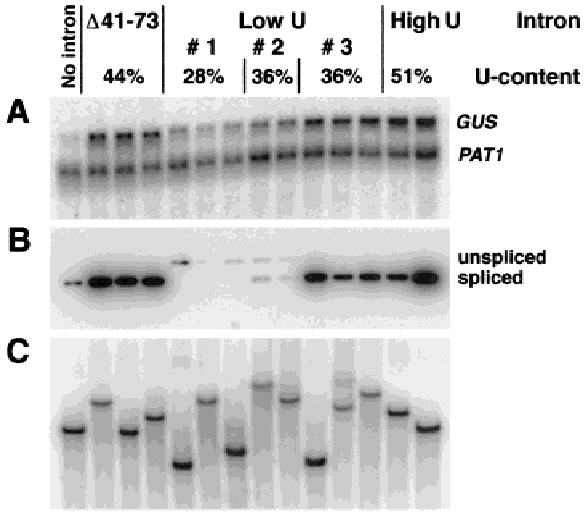

FIGURE 3. The effects of varying intron U richness. Transgenic lines containing *PAT1:GUS* fusions with 75-nt introns altered in the U residues detailed in Figure 1A were analyzed as described in Figure 2.

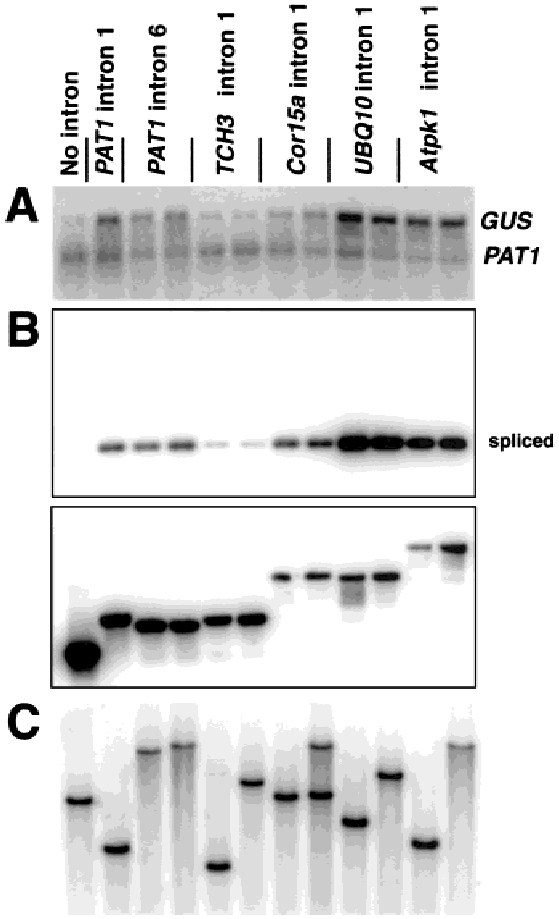

FIGURE 4. Introns differ in their ability to stimulate expression. The indicated introns were inserted between exons one and two of a *PAT1:GUS* fusion and transgenic lines containing these constructs were analyzed as described in Figure 2. The lower part of **B** shows a duplicate blot of products derived from PCR amplification of genomic DNA to illustrate where the RT-PCR products from unspliced mRNA would migrate.

important for splicing in plants (Simpson et al., 2002). To eliminate potential branchpoints without affecting intron nucleotide composition, every occurrence of the sequence UNA in the 75-nt Δ41–73 intron was converted to ANU. In addition, three other nucleotides were changed to prevent the creation of new UNA sequences or stop codons (Fig. 1A). RT-PCR showed that these modifications eliminate splicing (Fig. 2B), confirming that all potential branchpoints have been eliminated. The "intron" lacking branchpoints was able to stimulate *PAT1:GUS* mRNA accumulation relative to the intronless control (Fig. 2A; Table 1), demonstrating that branchpoint sequences are not required for IME. However, an intron simultaneously lacking branchpoints and a functional 5′ splice site was completely unable to elevate mRNA accumulation (Fig. 2A; Table 1). Thus, IME absolutely requires either of the sequence elements involved in the first two steps of spliceosome assembly, even though IME is not eliminated when each is individually mutated.

3′ splice site

To determine the need for the 3′ splice site in IME, the last 3 nt of the 108-nt in-frame intron were changed from CAG to TTC. The effects of destroying the 3′ splice site alone could not be tested because cryptic 3′ splice sites in the downstream exon were efficiently activated, even when the first two potential splice acceptor AG sequences were removed and the first 30 nt of the downstream exon were rendered less intronlike by reducing the U richness (data not shown). Thus, the mutations moved the 3′ splice site but did not eliminate it. However, the need for the normal 3′ splice site in IME could be evaluated by comparing an intron with a mutation in the 5′ splice site to one with mutations in both splice sites. The 3′ splice site mutation further reduced but did not eliminate the ability of an intron with a 5′ splice site mutation to enhance mRNA accumulation (Fig. 2A; Table 1), indicating that the normal 3′ splice site of this intron contributes to but is not essential for IME.

U richness

To test the importance of intron U richness and individual U-rich sequences in IME, four derivatives of the 75-nt Δ41–73 intron (44% U) were constructed in which U content was varied (Fig. 1A). In one (High U), U-content was increased to 51%. Another intron (Low U #1) had 12 U residues converted to C, reducing U content to 28%, less than that of the flanking exons (29% and 33%). The two remaining introns (Low U #2

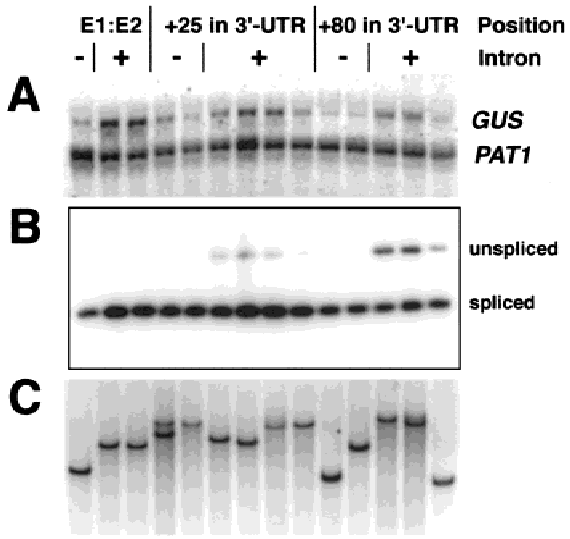

FIGURE 5. Reduced enhancement by *PAT1* intron 1 in the 3′ UTR. Wild-type *PAT1* intron 1 was inserted as a *Pst*I fragment after 25 or 80 nt of 3′-UTR sequences in a *PAT1:GUS* fusion. Also shown are the results from intronless fusions containing only the newly created *Pst*I sites, and constructs with the same intron in its normal position between exons one and two (E1:E2). The panels are as described in Figure 2, except that the probes in **B** were 3′-UTR sequences that flank the intron.

and #3) each had a moderately reduced U content of 36%. In Low U #2, the residues in strings of two or more Us were targeted, whereas Low U #3 contained changes mostly in isolated U residues. The 12 alterations made in Low U #1 are the same as those in Low U #2 and Low U #3 combined (Fig. 1A).

TABLE 1. Intron mutations and their effect on expression.

Starting intron[a]	Mutations	Enhancement relative to starting intron[b]	Splicing efficiency[c]
In-frame		100 ± 5%	>98%
In-frame	5′ SS G to A	36 ± 5%	<2%
In-frame	5′ SS GTATG to AACGC	51 ± 5%	<2%
In-frame	5′ SS GTATG to AACGC and 3′ SS CAG to TTC	25 ± 1%	<2%
Δ41–73		100 ± 2%	>98%
Δ41–73	5′ SS GTATG to AACGC	46%	<2%
Δ41–73	branchpoint⁻	75 ± 57%	<2%
Δ41–73	5′ SS GTATG to AACGC and branchpoint⁻	−9 ± 3%	<2%
Δ41–73	low U-1 (= low U-2 + low U-3)	28 ± 8%	<2%
Δ41–73	low U-2 (reduced oligo U)	44 ± 8%	68 ± 0.2%
Δ41–73	low U-3 (reduced isolated U)	97 ± 17%	98 ± 0.3%
Δ41–73	high U	122 ± 15%	>98%

[a] Mutations were introduced into either the 108-nt in-frame version of *PAT1* intron 1 or the 75-nt derivative lacking bases 41 to 73.

[b] Steady-state *PAT1:GUS* mRNA accumulation (mean ± standard deviation) corrected for loading using *PAT1* mRNA levels, relative to an intronless fusion (0% enhancement) and the appropriate starting intron (100%). The average fold enhancement of the starting introns was 4.7 ± 0.5 (in-frame) and 4.8 ± 0.5 (Δ41–73).

[c] Fraction of total RT-PCR products derived from spliced mRNA.

Generally, the degree of IME correlated with intron U content (Fig. 3; Table 1). Elevating intron U content increased mRNA accumulation modestly compared to the starting intron. The intron with the lowest U content was not spliced and had the least effect on mRNA levels, although it still weakly stimulated *PAT1:GUS* mRNA accumulation relative to an intronless fusion. Surprisingly, the two introns with moderately reduced U content had very different effects on mRNA accumulation and were spliced with different efficiency. The Low U #3 intron was indistinguishable from the 75-nt Δ41–73 intron in splicing efficiency and its ability to stimulate mRNA accumulation. In contrast, the Low U #2 intron was partially spliced and was only slightly better at elevating mRNA levels than the Low U #1 intron. Of the 12 mutations in the Low U #1 intron, the 6 also found in the Low U #2 intron could account for almost all of the reduction in IME, whereas the 6 changes in common with the Low U #3 intron had little effect. Therefore, the specific sequences altered in the Low U #2 and Low U #3 introns were more important than overall U-content in determining the degree of enhancement.

Other introns

The above results show that none of the structural characteristics that *PAT1* intron 1 shares with other dicot introns (conserved splice site and branchpoint sequences, and U richness) are individually essential for IME. As an alternative approach to determine which features of an intron are required, the ability of different introns to boost expression were compared. If introns stimulate mRNA accumulation solely by providing an association with the splicing machinery, then all efficiently spliced introns should enhance expression to a similar degree. Introns have been reported to differ in their effect on gene expression in maize and rice (Callis et al., 1987; Mascarenhas et al., 1990; Luehrsen & Walbot, 1991; Sinibaldi & Mettler, 1992; Jeon et al., 2000). However, it is unclear how much of this divergence is due to the introns themselves versus other variables such as intron position, the sequences that flank each intron in different constructs, or the fluctuations inherent in transient expression assays.

To test several *Arabidopsis* introns in the exact same context, five different introns were flanked with *Pst*I restriction sites as described (Rose & Beliakoff, 2000) and inserted into the *PAT1:GUS* fusion between exons 1 and 2. This technique allowed the introduction of only intron sequences at the same position, so that splicing produced mature mRNAs with identical sequences. Intron 6 from *PAT1* was chosen to determine if another intron from this gene has a different effect on mRNA accumulation. The introns from the *COR15a* and *TCH3* genes were tested because the expression of these genes is apparently not influenced by the introns they contain (Baker et al., 1994; Sistrunk et al.,

1994). The final two introns were previously shown to enhance the expression of their respective genes, *UBQ10* and *atpk1* (Norris et al., 1993; Zhang et al., 1994).

The five introns were found to vary widely in their effect on mRNA accumulation from the *PAT1:GUS* fusion even though all were spliced with greater than 98% efficiency. The *COR15a* and *TCH3* introns stimulated mRNA accumulation less than 3-fold, whereas the *UBQ10* and *atpk1* introns each boosted mRNA accumulation almost 15-fold (Fig. 4; Table 2). *PAT1* intron 6 had an intermediate effect similar to the 5-fold enhancement mediated by intron 1 or 2 of *PAT1* (Rose & Beliakoff, 2000). The observation that the efficiently spliced *TCH3* intron had very little effect on mRNA accumulation demonstrates that splicing is not sufficient for IME.

Intron position

Several groups have shown that introns capable of stimulating expression from near the 5′ end of a gene lose this ability when inserted in the 3′ UTR (Callis et al., 1987; Mascarenhas et al., 1990; Clancy et al., 1994; Snowden et al., 1996; Jeon et al., 2000), suggesting a dependence of IME on intron position. However, the recent finding in yeast and mammals that introns more than 50–55 nt downstream of a stop codon can cause that stop to be recognized as premature, triggering nonsense-mediated RNA decay (Nagy & Maquat, 1998; Sun et al., 2000; Dreyfuss et al., 2002), raises the possibility that a similar system operates in plants. If so, this would provide an alternative explanation for the observed failure of introns to boost expression from the 3′-UTR because in every previous case the intron was more than 60 nt downstream of the stop codon.

To test the importance of position on the ability of *PAT1* intron 1 to stimulate expression, *Pst*I sites were generated to allow insertion of an intron after the 25th or 80th nt downstream of the stop codon in the 3′ UTR of a *PAT1:GUS* fusion (Fig. 1B). As shown in Figure 5 and Table 3, the mRNA accumulation from fusions containing the wild-type *PAT1* intron 1 at either location was not significantly higher than that from intronless fusions containing only the newly created *Pst*I site. The intron was spliced efficiently from the proximal location but with variable efficiency from the distal position. The observation that the ability of *PAT1* intron 1 to enhance mRNA accumulation was greatly diminished when moved to the 3′ UTR, even in a location that should not activate nonsense-mediated decay, confirms and extends the original conclusion that IME depends on intron position.

DISCUSSION

The completed act of splicing is clearly unnecessary for *PAT1* intron 1 to elevate mRNA accumulation. A total of six derivatives of this intron, rendered unspliceable by virtue of small size, mutation of the 5′ splice site, reduction of U content, or elimination of branchpoint sequences, have now been shown to increase the expression of a *PAT1:GUS* fusion (Rose & Beliakoff, 2000; this report). However, each of the alterations that prevented splicing reduced the degree to which *PAT1* intron 1 stimulated mRNA accumulation, and IME was lost completely when both 5′ splice site and branchpoint sequences were mutated. Together these observations suggest that although splicing per se is not essential for IME, intron sequences must interact with the splicing machinery to enhance mRNA accumulation. Each of the singly mutated introns might retain enough of the features by which introns are recognized to allow the spliceosome association needed for IME. For example, spliceosomes may be able to assemble onto an intron at least in part if either of the interactions between the U1 snRNP and the 5′ splice site or the U2 snRNP with branchpoint sequences is possible, but not if both are prevented. The degree to which mutations that block splicing interfere with the

TABLE 2. The ability of a variety of introns to enhance expression.

Intron	Length (nt)	U content (%)	Fold enhancement[a]	Splicing efficiency[b]
PAT1 intron 1	110	40.0	4.5 ± 0.1	>98%
PAT1 intron 6	78	35.9	3.7 ± 0.8	>98%
TCH3 intron 1	100	35.0	1.4 ± 0.3	>98%
COR15a intron 1	306	43.8	2.5 ± 0.2	>98%
UBQ10 intron 1	304	45.7	14.9 ± 0.5	>98%
atpk1 intron 1	517	41.0	12.9 ± 1.1	>98%

[a]Steady-state *PAT1:GUS* mRNA accumulation (mean ± standard deviation), corrected for loading using *PAT1* mRNA levels, relative to an intronless control containing a *Pst*I site at the 3′ end of exon 1.
[b]Fraction of total RT-PCR products derived from spliced mRNA.

TABLE 3. The ability of an intron in the 3′ UTR to enhance expression.

Intron	Location of *Pst*I site[a]	Fold enhancement[b]	Splicing efficiency[c]
PAT1 intron 1	3′ end of exon 1	4.4 ± 0.2	>98%
None	+25 in 3′ UTR	1.4 ± 0.4	
PAT1 intron 1	+25 in 3′ UTR	1.8 ± 0.2	98 ± 1%
None	+80 in 3′ UTR	0.9 ± 0.2	
PAT1 intron 1	+80 in 3′ UTR	1.4 ± 0.4	71 ± 22%

[a]The *Pst*I sites end at the last nucleotide of exon 1 or the 25th or 80th nt past the stop codon in the 3′ UTR. Introns inserted as a *Pst*I fragment begin immediately after the *Pst*I site.
[b]Steady-state *PAT1:GUS* mRNA accumulation (mean ± standard deviation), corrected for loading using *PAT1* mRNA levels, relative to an intronless control containing a *Pst*I site at the 3′ end of exon 1.
[c]Fraction of total RT-PCR products derived from spliced mRNA.

necessary interactions with the spliceosome could vary depending on the intron and organism under study, possibly accounting for the findings by others that splicing is required for IME (Sinibaldi & Mettler, 1992).

Although an association of an intron with the splicing machinery may be necessary for IME, even if splicing is not completed, it is clear that such an association alone is not sufficient to elevate expression. The *TCH3* and *COR15a* introns are efficiently spliced from between exons 1 and 2 of a *PAT1:GUS* fusion, as is the *PAT1* intron from the 3′ UTR, and yet these three constructs had very low levels of mRNA accumulation. Thus, processing by the spliceosome does not automatically increase the accumulation of all spliced mRNAs. Others have previously shown that introns differ in their ability to enhance expression in maize (Callis et al., 1987; Mascarenhas et al., 1990; Luehrsen & Walbot, 1991; Sinibaldi & Mettler, 1992) and a similar pattern was observed with the *Arabidopsis* introns tested here. Thus the features that determine the degree to which an intron will stimulate expression are not the structural components found in all introns (splice sites, branchpoints, and U richness), consistent with the observation that any of these components could be individually mutated in *PAT1* intron 1 without eliminating IME. There is no obvious difference between the *UBQ10* and *COR15a* introns in length (304 nt vs. 306 nt), nucleotide composition (45.7% U vs. 43.8% U, with similar distribution of U residues), or splicing efficiency (both greater than 98%) that could account for their very different effects on mRNA accumulation.

For the five introns tested, the ability of an intron to stimulate mRNA accumulation was most strongly correlated with the type of gene from which the intron was isolated. Both of the introns from genes whose expression is apparently intron-independent (*TCH3* and *COR15a*) had little or no effect on *PAT1:GUS* mRNA accumulation. Both of the introns previously shown to stimulate expression (from the *UBQ10* and *atpk1* genes) elevated *PAT1:GUS* mRNA accumulation more than 10-fold, although the enhancement mediated by the *UBQ10* intron was greater in the *PAT1:GUS* fusion than the 3-fold effect reported for a *UBQ10:GUS* fusion (Norris et al., 1993). The discrepancy may reflect differences between the two promoters, or stable versus transient expression assays. Furthermore, all of the *PAT1* introns that have been tested (introns 1 and 2 previously, and intron 6 reported here) stimulated mRNA accumulation roughly fivefold. However, introns from a single gene can differ, as introns 1, 2, and 6 from the maize *Adh1* gene elevate expression to a much higher degree than do introns 8 or 9 (Callis et al., 1987; Mascarenhas et al., 1990).

The intron structures most likely to be involved in the mechanism of IME are U-rich sequences, as illustrated by the correlation between the degree of IME and the U richness of derivatives of *PAT1* intron 1. The difference in enhancement mediated by the two introns with moderately reduced U-content indicates that the level of mRNA accumulation depends more on individual U-rich sequences than overall intron nucleotide composition, and requires short strings of U residues more than isolated U residues. These U-rich clusters are distributed throughout *PAT1* intron 1, consistent with the observation that any part of the intron can be deleted without reducing IME (Rose & Beliakoff, 2000). The observation that splicing was abolished by drastically reducing *PAT1* intron 1 U-content is in agreement with previous findings that intron U richness strongly influences splicing efficiency and splice site selection in plants (Goodall & Filipowicz, 1989; Luehrsen & Walbot, 1994a, 1994b; Merritt et al., 1997; Ko et al., 1998). The mechanism of IME may depend on proteins similar to UBP1 that bind U-rich intron sequences and can influence splicing and mRNA accumulation (Gniadkowski et al., 1996; Lambermon et al., 2000).

Potential mechanisms

Introns could stimulate mRNA accumulation in several different ways or by a combination of mechanisms. Capping, polyadenylation, and export from the nucleus to the cytoplasm all increase mRNA stability, and a growing number of examples reveal an interconnection between these processes and splicing (Izaurralde et al., 1994; Luo & Reed, 1999; Minvielle-Sebastia & Keller, 1999; Hirose & Manley, 2000; Strasser & Hurt, 2001; Maniatis & Reed, 2002; Orphanides & Reinberg, 2002). For example, mRNAs generated by splicing are exported more rapidly and efficiently than otherwise identical intronless mRNAs (Luo & Reed, 1999). During splicing, several proteins collectively called the exon junction complex are deposited on the mRNA 20 nt or so upstream of the former sites of introns (Dreyfuss et al., 2002). Several of these proteins could affect mRNA stability because they promote nuclear export by interacting directly with an export factor that, in turn, contacts the nuclear pore, and some remain associated with the mRNA in the cytoplasm (Le Hir et al., 2001; Luo et al., 2001; Strasser & Hurt, 2001). However, because some spliced introns have little effect on expression, either these proteins are not involved in IME or the composition or activity of the exon junction complex varies with different introns in a way that affects mRNA accumulation. It remains to be determined whether or not the exon junction complex is deposited on transcripts containing unspliceable introns.

The drastic reduction in the ability of *PAT1* intron 1 to elevate mRNA accumulation when moved to the 3′ UTR is a significant clue about potential mechanisms of IME because expression was altered without changing intron identity or splicing efficiency. One possible explanation for the effect of intron position on IME is that introns stimulate expression during transcription, as in

the following model. Introns could stimulate mRNA accumulation by increasing the processivity of RNA polymerase II via a modification of its carboxy-terminal domain (CTD), thereby promoting transcript elongation without significantly affecting transcript initiation. The transcription of genes containing an intron would therefore be more likely to extend the length of the gene, where 3′ end processing would produce stable transcripts. In this scenario, transcript initiation would be the same in genes containing or lacking introns, aborted transcripts from either gene would be rapidly degraded, and the mRNA produced from full-length transcripts would be equally stable. The presence of an intron would simply increase the probability that full-length stable transcripts will be made, leading to increased mRNA accumulation. Introns in the 3′ UTR of a gene would fail to enhance expression because the polymerases that elongate that far are already quite likely to continue on the short distance to the terminator, generating stable mRNAs. Factors that bind to redundant (U-rich?) intron sequences in conjunction with the splicing machinery could bring about the modifications that affect polymerase processivity, and the degree of stimulation by different introns could vary depending on the number or affinity of binding sites for these factors. In addition, genes could differ in their responsiveness to introns depending on whether other compensatory factors load onto the polymerase.

In support of this model, abundant evidence from yeast and mammals reveals that transcription and pre-mRNA processing are linked (reviewed in Neugebauer & Roth, 1997; Bentley, 1999; Hirose & Manley, 2000; Maniatis & Reed, 2002; Proudfoot et al., 2002). Furthermore, splicing factors have been shown to interact directly with the CTD of RNA polymerase II in a phosphorylation-dependent manner (Kim et al., 1997; Steinmetz, 1997; Hirose et al., 1999). This dependence on the state of the CTD is significant because the phosphorylation of the CTD correlates with the ability of the polymerase to elongate transcription (Dahmus, 1996). The direct connection between splicing factors and polymerase II elongation has been proposed recently to explain why introns can increase transcription in mammalian cell extracts (Fong & Zhou, 2001). Furthermore, the export-promoting components of the exon junction complex in yeast also interact with transcription factors to form the TREX (transcription/export) complex that associates with RNA polymerase II as it travels the length of transcribed genes (Strasser et al., 2002). Thus, the interconnected nature of pre-mRNA synthesis and processing provides multiple opportunities for introns to influence mRNA production.

Although this cotranscriptional model can accommodate most observations about IME in plants, other explanations for the inability of *PAT1* intron 1 to elevate expression from the 3′ UTR are also possible. Introns in the 3′ UTR may retain their stimulatory capacity but simultaneously activate nonsense-mediated decay, resulting in no net increase in mRNA accumulation. Even though an intron 24 nt downstream of the stop codon does not destabilize triosephosphate isomerase mRNA in human cells (Zhang et al., 1998), the minimum distance between the stop codon and downstream intron needed to trigger nonsense-mediated RNA decay in plants has not been established and could be significantly less than the 50–55 nt limit in mammals. Introns can also have a major effect on translational efficiency that depends on intron position. Introns near the 5′ end of some transcripts stimulate translation relative to an intronless mRNA, whereas introns in the 3′ UTR significantly reduce translation in *Xenopus* oocytes (Matsumoto et al., 1998). A similar regulation of translation by plant introns could account for the observed enhancement properties of *PAT1* intron 1 if translation increases mRNA stability.

Because different introns could affect expression by different mechanisms, a complete understanding of IME will require a detailed characterization of the phenomenon using multiple introns, genes, and species. In every case, the findings are likely to be instructive as introns can reveal previously unexpected links between the complicated processes that together constitute gene expression.

MATERIALS AND METHODS

Creating mutations

The desired mutations were introduced by PCR. To eliminate branchpoint sequences or alter the U content, each entire intron was synthesized as two overlapping oligonucleotides that were rendered double stranded by PCR. *PAT1* intron 6 and the first introns from the *Arabidopsis atpk1*, *COR15a*, *TCH3*, and *UBQ10* genes were amplified from cloned genes using primers that introduce a *Pst*I site immediately upstream of each intron and convert the last six intron nucleotides to a *Pst*I site. All introns were sequenced to confirm introduction of only the desired changes and inserted as *Pst*I restriction fragments into a *PAT1:GUS* fusion.

Mutations that destroyed the *Pst*I site at the 3′ splice site of *PAT1* intron 1 were introduced by PCR, the bottom strand of the amplified intron was degraded with lambda exonuclease, and the resulting product was used as a primer to amplify downstream *PAT1* exon sequences. Fragments extending from the *Pst*I site at the 5′ end of the intron to the *Xba*I site just beyond the start of *PAT1* exon 3 (see Fig. 1 of Rose & Beliakoff, 2000) were used to reconstruct *PAT1:GUS* fusions.

Introns were inserted into a *Pst*I restriction site created by PCR at either of two locations in the 3′ UTR (Fig. 1B). A 153-bp *Nru*I–*Sac*I fragment that includes the 3′ end of the *GUS* gene with the proximal *Pst*I site was used to replace the analogous fragment in a *PAT1:GUS* fusion. Similarly, the wild-type terminator sequences of the *PAT1:GUS* fusion were replaced with the version containing the distal *Pst*I site as a 269-bp *Sac*I to *Eco*RI fragment.

Generating and analyzing transgenic lines

The intron-containing *PAT1:GUS* fusions were introduced into wild-type *Arabidopsis thaliana* ecotype Columbia by vacuum infiltration (Clough & Bent, 1998). Single-copy lines were identified by southern blotting from among the transformants whose progeny segregated kanamycin resistance in a 3:1 ratio suggestive of a single locus of transgene insertion. Two to six independent single-copy lines were analyzed for each construct except the derivative of the Δ41–73 intron with the 5-nt mutation of the 5′ splice site, which was represented by a single line.

RNA was isolated from the leaves of 3-week-old homozygous single-copy lines using RNeasy columns (Qiagen, Valencia, California). Transgene expression was determined by phosphorimager quantification of RNA gel blots probed with *GUS* and *PAT1* to correct for slight differences in loading (Rose & Beliakoff, 2000). For each line, the relative mRNA accumulation in two or more independent experiments was averaged, and the expression of each *PAT1:GUS* fusion is presented as the mean ± standard deviation of these averages for all the isolates containing the same construct. The standard deviation for constructs where two lines were identified indicates the range of values obtained.

DNAse-treated total RNA (1 μg) was reverse-transcribed using random hexamer primers, and one sixth of the resulting cDNA was PCR-amplified for 25 cycles using primers 5′-GAAGAAGCAACTTGACCGGAG-3′ and 5′-TAACGCGCTTTCCCACCAACG-3′. Splicing efficiency was estimated from the phosphorimager counts of RT-PCR products derived from spliced and unspliced transcripts that had been blotted and hybridized with an equimolar mixture of exon probes that flanked the intron (Rose & Beliakoff, 2000). The RT-PCR products were sequenced to confirm splicing fidelity and transgene identity.

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REFERENCES

Baker SS, Wilhelm KS, Thomashow MF. 1994. The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol Biol 24*:701–713.

Bentley D. 1999. Coupling RNA polymerase II transcription with pre-mRNA processing. *Curr Opin Cell Biol 11*:347–351.

Brown JWS, Smith P, Simpson CG. 1996. *Arabidopsis* consensus intron sequences. *Plant Mol Biol 32*:533–535.

Bruhat A, Tourmente S, Chapel S, Sobrier ML, Couderc JL, Dastugue B. 1990. Regulatory elements in the first intron contribute to transcriptional regulation of the β_3 tubulin gene by 20-hydroxyecdysone in *Drosophila* Kc cells. *Nucleic Acids Res 18*:2863–2867.

Buchman AR, Berg P. 1988. Comparison of intron-dependent and intron-independent gene expression. *Mol Cell Biol 8*:4395–4405.

Burge CB, Tuschl T, Sharp PA. 1999. Splicing of precursors to mRNAs by the spliceosomes. In: Gesteland RF, Cech TR, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.

Callis J, Fromm M, Walbot V. 1987. Introns increase gene expression in cultured maize cells. *Genes & Dev 1*:1183–1200.

Choi T, Huang M, Gorman C, Jaenisch R. 1991. A generic intron increases gene expression in transgenic mice. *Mol Cell Biol 11*:3070–3074.

Clancy M, Vasil V, Hannah LC, Vasil IK. 1994. Maize *Shrunken-1* intron and exon regions increase gene expression in maize protoplasts. *Plant Sci 98*:153–161.

Clough SJ, Bent AF. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J 16*:735–743.

Dahmus ME. 1996. Reversible phosphorylation of the C-terminal domain of RNA polymerase II. *J Biol Chem 271*:19009–19012.

Dean C, Favreau M, Bond-Nutter D, Bedbrook J, Dunsmuir P. 1989. Sequences downstream of translation start regulate quantitative expression of two petunia *rbcS* genes. *Plant Cell 1*:203–208.

Deutsch M, Long M. 1999. Intron-exon structures of eukaryotic model organisms. *Nucleic Acids Res 27*:3219–3228.

Deyholos MK, Sieburth LE. 2000. Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. *Plant Cell 12*:1799–1810.

Dreyfuss G, Kim VN, Kataoka N. 2002. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol 3*:195–205.

Fong YW, Zhou Q. 2001. Stimulatory effect of splicing factors on transcriptional elongation. *Nature 414*:929–933.

Gallie DR, Young TE. 1994. The regulation of gene expression in transformed maize aleurone and endosperm protoplasts. *Plant Physiol 106*:929–939.

Gniadkowski M, Hemmings-Mieszczak M, Klahre U, Liu H-X, Filipowicz W. 1996. Characterisation of intronic uridine-rich sequence elements acting as possible targets for nuclear proteins during pre-mRNA splicing in *Nicotiana plumbaginifolia*. *Nucleic Acids Res 24*:619–627.

Goodall GJ, Filipowicz W. 1989. The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. *Cell 58*:473–483.

Hirose Y, Manley JL. 2000. RNA polymerase II and the integration of nuclear events. *Genes & Dev 14*:1415–1429.

Hirose Y, Tacke R, Manley JL. 1999. Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. *Genes & Dev 13*:1234–1239.

Huang MTF, Gorman CM. 1990. Intervening sequences increase efficiency of RNA 3′ processing and accumulation of cytoplasmic RNA. *Nucleic Acids Res 18*:937–947.

Izaurralde E, Lewis J, McGuigan C, Jankowska M, Darzynkiewicz E, Mattaj IW. 1994. A nuclear cap binding protein complex involved in pre-mRNA splicing. *Cell 78*:657–668.

Jeon J-S, Lee S, Jung K-H, Jun S-H, Kim C, An G. 2000. Tissue-preferential expression of a rice α-tubulin gene, *OsTubA1*, mediated by the first intron. *Plant Physiol 123*:1005–1014.

Kim E, Du L, Bregman DB, Warren SL. 1997. Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. *J Cell Biol 136*:19–28.

Ko CH, Brendel V, Taylor RD, Walbot V. 1998. U-richness is a defining feature of plant introns and may function as an intron recognition signal in maize. *Plant Mol Biol 36*:573–583.

Lambermon MH, Simpson GG, Wieczorek Kirk DA, Hemmings-Mieszczak M, Klahre U, Filipowicz W. 2000. UBP1, a novel hnRNP-like protein that functions at multiple steps of higher plant nuclear pre-mRNA maturation. *EMBO J 19*:1638–1649.

Le Hir H, Gatfield D, Izaurralde E, Moore MJ. 2001. The exon–exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. *EMBO J 20*:4987–4997.

Lim LP, Burge CB. 2001. A computational analysis of sequence features involved in recognition of short introns. *Proc Natl Acad Sci USA 98*:11193–11198.

Long M, de Souza SJ, Gilbert W. 1995. Evolution of the intron-exon structure of eukaryotic genes. *Curr Opin Gen Dev 5*:774–778.

Lorkovic ZJ, Wieczorek Kirk DA, Lambermon MH, Filipowicz W. 2000. Pre-mRNA splicing in higher plants. *Trends Plant Sci 5*:160–167.

Luehrsen KR, Walbot V. 1991. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. *Mol Gen Genet 225*:83–93.

Luehrsen KR, Walbot V. 1994a. Addition of A- and U-rich sequence increases the splicing efficiency of a deleted form of a maize intron. *Plant Mol Biol 24*:449–463.

Luehrsen KR, Walbot V. 1994b. Intron creation and polyadenylation in maize are directed by AU-rich RNA. *Genes & Dev 8*:1117–1130.

Luo M-J, Reed R. 1999. Splicing is required for rapid and efficient mRNA export in metazoans. *Proc Natl Acad Sci USA 96*:14937–14942.

Luo M-J, Zhou Z, Magni K, Christoforides C, Rappsilber J, Mann M, Reed R. 2001. Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. *Nature 413*:644–647.

Maas C, Laufs J, Grant S, Korfhage C, Werr W. 1991. The combination of a novel stimulatory element in the first exon of the maize *Shrunken-1* gene with the following intron 1 enhances reporter gene expression up to 1000-fold. *Plant Mol Biol 16*:199–207.

Maniatis T, Reed R. 2002. An extensive network of coupling among gene expression machines. *Nature 416*:499–506.

Maniatis T, Tasic B. 2002. Alternative pre-mRNA splicing and proteome expansion in metazoans. *Nature 418*:236–243.

Mascarenhas D, Mettler IJ, Pierce DA, Lowe HW. 1990. Intron-mediated enhancement of heterologous gene expression in maize. *Plant Mol Biol 15*:913–920.

Matsumoto K, Wassarman KM, Wolffe AP. 1998. Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J 17*:2107–2121.

Meredith J, Storti RV. 1993. Developmental regulation of the *Drosophila* tropomyosin II gene in different muscles is controlled by muscle-type-specific intron enhancer elements and distal and proximal promoter control elements. *Dev Biol 159*:500–512.

Merritt H, McCullough AJ, Schuler MA. 1997. Internal AU-rich elements modulate activity of two competing 3′ splice sites in plant nuclei. *Plant J 12*:937–943.

Minvielle-Sebastia L, Keller W. 1999. mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. *Curr Opin Cell Biol 11*:352–357.

Nagy E, Maquat LE. 1998. A rule for termination-codon position within intron-containing genes: When nonsense affects RNA abundance. *Trends Biochem Sci 23*:198–199.

Nash J, Walbot V. 1992. *Bronze-2* gene expression and intron splicing patterns in cells and tissues of *Zea mays* L. *Plant Physiol 100*:464–471.

Neugebauer KM, Roth MB. 1997. Transcription units as RNA processing units. *Genes & Dev 11*:3279–3285.

Norris SR, Meyer SE, Callis J. 1993. The intron of *Arabidopsis thaliana* polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression. *Plant Mol Biol 21*:895–906.

Okkema PG, Harrison SW, Plunger V, Aryana A, Fire A. 1993. Sequence requirements for myosin gene expression and regulation in *Caenorhabditis elegans*. *Genetics 135*:385–404.

Orphanides G, Reinberg D. 2002. A unified theory of gene expression. *Cell 108*:439–451.

Proudfoot NJ, Furger A, Dye MJ. 2002. Integrating mRNA processing with transcription. *Cell 108*:503–512.

Rethmeier N, Kramer E, van Montagu M, Cornelissen M. 1998. Identification of *cat* sequences required for intron-dependent gene expression in maize cells. *Plant J 13*:833–835.

Rethmeier N, Seurinck J, van Montagu M, Cornelissen M. 1997. Intron-mediated enhancement of transgene expression in maize is a nuclear, gene-dependent process. *Plant J 12*:895–899.

Rippe RA, Lorenzen SI, Brenner DA, Breindl M. 1989. Regulatory elements in the 5′-flanking region and the first intron contribute to transcriptional control of the mouse alpha 1 type I collagen gene. *Mol Cell Biol 9*:2224–2227.

Rose AB, Beliakoff JA. 2000. Intron-mediated enhancement of gene expression independent of unique intron sequences and splicing. *Plant Physiol 122*:535–542.

Rose AB, Last RL. 1997. Introns act post-transcriptionally to increase expression of the *Arabidopsis thaliana* tryptophan pathway gene *PAT1*. *Plant J 11*:455–464.

Simpson CG, Thow G, Clark GP, Jennings SN, Watters JA, Brown JWS. 2002. Mutational analysis of a plant branchpoint and polypyrimidine tract required for constitutive splicing of a mini-exon. *RNA 8*:47–56.

Sinibaldi RM, Mettler IJ. 1992. Intron splicing and intron-mediated enhanced expression in monocots. In: Cohn WE, Moldave K, eds. *Progress in nucleic acid research and molecular biology*. New York: Academic Press. pp 229–257.

Sistrunk ML, Antosiewicz DM, Purugganan MM, Braam J. 1994. *Arabidopsis TCH3* encodes a novel Ca^{2+} binding protein and shows environmentally induced and tissue-specific regulation. *Plant Cell 6*:1553–1565.

Snowden KC, Buchholz WG, Hall TC. 1996. Intron position affects expression from the *tpi* promoter in rice. *Plant Mol Biol 31*:689–692.

Steinmetz EJ. 1997. Pre-mRNA processing and the CTD of RNA polymerase II: The tail that wags the dog? *Cell 89*:493–494.

Strasser K, Hurt E. 2001. Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. *Nature 413*:648–652.

Strasser K, Masuda S, Mason P, Pfannstiel J, Oppizzi M, Rodriguez-Navarro S, Rondon AG, Aguilera A, Struhl K, Reed R, Hurt E. 2002. TREX is a conserved complex coupling transcription with messenger RNA export. *Nature 417*:304–308.

Sun X, Moriarty PM, Maquat LE. 2000. Nonsense-mediated decay of glutathione peroxidase 1 mRNA in the cytoplasm depends on intron position. *EMBO J 19*:4734–4744.

Wang H, Lee MM, Schiefelbein JW. 2002. Regulation of the cell expansion gene *RHD3* during *Arabidopsis* development. *Plant Physiol 129*:638–649.

Wilusz CJ, Wang W, Peltz SW. 2001. Curbing the nonsense: The activation and regulation of mRNA surveillance. *Genes & Dev 15*:2783–2785.

Zhang J, Sun X, Qian Y, LaDuca JP, Maquat LE. 1998. At least one intron is required for the nonsense-mediated decay of triosephosphate isomerase mRNA: A possible link between nuclear splicing and cytoplasmic translation. *Mol Cell Biol 18*:5272–5283.

Zhang S-H, Lawton MA, Hunter T, Lamb CJ. 1994. *atpk1*, a novel ribosomal protein kinase gene from *Arabidopsis*. I. Isolation, characterization, and expression. *J Biol Chem 269*:17586–17592.