Upstream introns influence the efficiency of final intron removal and RNA 3'-end formation

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For all intron-containing pre-mRNAs of higher eukaryotes that have been examined using either living cells or cell-free extracts, a functional 3' splice site within the 3'-terminal intron is required for efficient RNA 3'-end formation. The mechanism by which intron sequences facilitate RNA 3'-end formation, which is achieved by endonucleolytic cleavage and polyadenylation, is not understood. We report here that in intact cells the efficiency of RNA 3'-end formation correlates with the efficiency of final intron removal, even when the intron is normally a 5'-terminal or internal intron. Therefore, the influence of the 3'-terminal intron on 3'-end formation is likely to be attributable to the determinants of splicing efficiency, which include but are not limited to the 3' splice site. Quantitative RNase mapping and methods that couple reverse transcription and the polymerase chain reaction were used to assess the consequence to RNA 3'-end formation of intron deletions within the human gene for triosephosphate isomerase (TPI). Results indicate that the formation of TPI RNA 3' ends requires TPI gene introns in addition to the last intron, intron 6, to proceed efficiently. These additional TPI gene introns are also required for the efficient removal of intron 6. When introns 1 and 5 were engineered to be the final intron, they were found, as was intron 6, to function in RNA 3'-end formation with an efficiency that correlated with their efficiency of removal. The simultaneous deletion of the 5' and 3' splice sites of intron 6 reduced the efficiencies of both RNA 3'-end formation and the removal of intron 5, which constituted the 3'-most functional intron. Deletion of only the 3' splice site of intron 6 precluded RNA 3'-end formation but had no effect on the efficiency of intron 5 removal. Deletion of only the 5' splice site of intron 6, which resulted in exon 6 skipping (i.e., the removal of intron 5, exon 6, and intron 6 as a single unit), had no effect on the efficiencies of either RNA 3'-end formation or the removal of intron 5–exon 6–intron 6. These results indicate that sequences within the 3'-terminal intron are functionally coupled to both RNA 3'-end formation and removal of the penultimate intron via a network of interactions that form across the last two exons and, most likely, between RNA processing factors.

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Most pre-mRNAs of higher eukaryotes are processed in the nucleus by a series of steps that include capping at the 5' end, intron removal by splicing, and polyadenylation at the 3' end. The pre-mRNA sequences and *trans*-acting factors that are required for splicing (Green 1991; Guthrie 1991; Wassarman and Steitz 1992) and polyadenylation (Wahle and Keller 1992) have been studied extensively. Splicing is directed by conserved sequences at the 5' and 3' ends of introns that interact either directly or indirectly with small nuclear ribonucleoproteins (snRNPs), including U1, U2, U5, and U4/U6, as well as a multitude of protein factors. During splicing, an ordered series of noncovalent bonds are formed and broken between interactive molecules. As a consequence, the pre-mRNA sequentially enters configurations that are

ultimately conducive to each of the two phosphodiester transesterifications that result in the removal of each intron and the ligation of adjacent exons. Polyadenylation is directed by the conserved hexanucleotide AAUAAA, located 10–30 nucleotides 5' to the cleavage and polyadenylation site, the cleavage and polyadenylation site itself, as well as several less conserved sequence elements that reside either 5' or 3' to the site. Multiple factors have been identified as being necessary for RNA 3'-end formation in cell-free extracts.

Despite the finding that RNA splicing and 3'-end formation can proceed independently of one another in cell-free extracts (Krainer et al. 1984; Moore and Sharp 1984, 1985), evidence indicates that the two processes are likely to be coupled in intact cells. First, there are no examples of mRNAs that are spliced but not polyadenylated, and the presence of an intron within a normally

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intronless RNA targets that RNA toward the polyadenylation pathway in intact cells (Huang and Gorman 1990; Pandey et al. 1990). Second, under the appropriate cell-free conditions, polyadenylation can be stimulated by the presence of an upstream 3' splice site (Niwa et al. 1990; Niwa and Berget 1991a), and mutation of the conserved AAUAAA hexanucleotide can inhibit splicing of the upstream 3'-terminal intron (Niwa and Berget 1991b). Third, insertion of a 5' splice site within the 3'-terminal exon of synthetic adenovirus pre-mRNAs results in product RNAs that are inefficiently polyadenylated and inefficiently cross-linked to the CstF polyadenylation factor in nuclear extracts (Niwa et al. 1992). This finding suggests that RNA 3'-end formation is negatively affected by the partial assembly of a spliceosome at the 5' but not the 3' splice site, the failure to assemble a spliceosome at the 3' splice site, or both. Although both possibilities are probably realistic, the requirement of a functional 3' splice site within the last intron for efficient 3'-end formation is consistent with studies using cultured cells of pre-mRNAs that consisted of chloramphenicol acetyltransferase (CAT) sequences flanked by the SV40 late gene promoter-leader and polyadenylation signals (Chiou et al. 1991).

The requirement of a functional 3'-terminal intron for efficient 3'-end formation is also supported by studies using cultured cells of transcripts for the human glycolytic enzyme triosephosphate isomerase (TPI) (Nesic et al. 1993). The transcripts were generated in mouse L cells by the transient expression of TPI genes in which the mouse metallothionein (MT)-1 promoter was substituted for the TPI promoter. The co-expression of a reference globin (G1) gene that was similarly driven by the MT-1 promoter was used to control for variations in the efficiencies of cell transfection and RNA recovery. A sensitive RNase protection assay permitted the detection of MT-TPI RNA with either an unprocessed or a processed 3' end as well as MT-G1 transcripts, the latter of which ensured a quantitative analysis. Therefore, the effects of pre-mRNA sequences on 3'-end formation could be evaluated directly as an increase in the level of RNA having an unprocessed 3' end, rather than only indirectly as a decrease in the level of RNA having a processed 3' end. Deletion within the final intron, intron 6, of the lariat and acceptor splice sites (hereafter referred to as the 3' splice site) or the 5' and 3' splice sites reduced the level of product mRNA to <1 or 27% of normal, respectively, whereas deletion of the entire intron 6 reduced the level of product mRNA to 51% of normal. Each decrease in the level of mRNA was accompanied by an increase in the level of nuclear pre-mRNA having an unprocessed 3' end, that is, one that was neither cleaved nor polyadenylated. In all instances, a lower level of processed 3' ends was accompanied by a higher level of unprocessed 3' ends. Taken together, these studies indicate that the final intron of a pre-mRNA plays a critical role in RNA 3'-end formation. A favored model to explain this role has been that definition of the 5' end of the final exon by spliceosome assembly at the 3' splice site of the upstream intron is required for efficient RNA 3'-end for-

mation (Niwa et al. 1990; Chiou et al. 1991; Niwa and Berget 1991a; Niwa et al. 1992; Nesic et al. 1993).

The aim of the present study was to determine whether the 3'-terminal intron functions independently of other introns and could be substituted for what are normally non-terminal introns in the process of RNA 3'-end formation. We present evidence that the 3'-terminal intron, intron 6, of the pre-mRNA for human TPI functions in RNA 3'-end formation in a manner that is facilitated by one or more of the upstream introns. These introns also enhance the rate at which intron 6 is removed, raising the possibility that the efficiency with which intron 6 functions in RNA 3'-end formation correlates with the efficiency of intron 6 removal by splicing. This correlation was confirmed and extended to introns that are not normally 3'-terminal introns by the analysis of TPI pre-mRNAs that derived from constructs harboring either intron 1 or intron 5 as the final intron. The involvement of intron 6 in pre-mRNA metabolism was extended to the process of intron 5 removal by demonstrating that deletion of either all of intron 6 or the 5' and 3' splice sites of intron 6 retarded the removal of intron 5. The concomitant decrease in the efficiency of RNA 3'-end formation by these deletions suggests that intron 5 functions in the deletion-bearing pre-mRNA as the 3'-terminal intron. These results support the concept that the processes of splicing and 3'-end formation involve collaborations across internal and the 3'-terminal exons, presumably via processing factors that are bound to the pre-mRNA. These results also clarify the role of the 3' splice site of the 3'-terminal intron in 3'-end formation by demonstrating the additional role of other sequences that function in 3'-terminal intron removal, including sequences that reside in one or more of the upstream introns.

Results

Efficient RNA 3'-end formation requires TPI gene introns in addition to intron 6

It was shown previously that the deletion of TPI gene introns either individually or in combinations, with the exception of the sole deletion of intron 1, reduced the cellular abundance of product mRNA (Nesic et al. 1993). The best characterized intron, intron 6, does not function in transcriptional initiation (data not shown) but clearly, although not necessarily exclusively, plays a role in RNA 3'-end formation (Nesic et al. 1993). In experiments that examined this role, the intron 6 lariat and acceptor splice sites, which will hereafter be referred to collectively as the 3' splice site, were found to be necessary for efficient 3'-end formation (Nesic et al. 1993).

To determine whether intron 6 alone is sufficient for efficient 3'-end formation, a pMT-TPI plasmid in which TPI gene introns 1–5 had been deleted was transiently expressed in mouse L cells together with the reference pMT-G1 plasmid. The level of MT-TPI transcripts that had either processed or unprocessed 3' ends was quantitated in nuclear and cytoplasmic cell fractions by RNase

mapping. The probe consisted of a uniformly ^{32}P -labeled, antisense transcript of 699 nucleotides, 642 of which consisted of TPI gene sequences that included 365 nucleotides of the 3'-untranslated region and 277 nucleotides of 3'-flanking DNA (Fig. 1B; Nesic et al. 1993). RNase mapping was made quantitative by concomitantly assaying for RNA that derived from the reference pMT-GI plasmid using another uniformly ^{32}P -labeled, antisense transcript of 298 nucleotides, 235 of which consisted of mouse β^{major} -globin cDNA that included 19 nucleotides of exon 2 and 216 nucleotides of exon 3 (Fig. 1B; Nesic et al. 1993). Each protected fragment was quantitated by radiographic imaging, and band intensities were corrected for the number of radioactive nucleotides. The effect of each intron deletion on the efficiency of 3'-end formation was determined by first normalizing the quantity of each product of the TPI probe to the product of the globin probe and subsequently calcu-

lating each normalized value for each deletion construct as a percentage of the corresponding normalized value for the normal construct.

RNA from the normal MT-TPI construct, like RNA from the endogenous TPI gene of the human melanoma cell line Malme-3, protected several regions of the TPI probe from RNase digestion. The most abundant protected region that was detected with both nuclear and cytoplasmic RNA migrated at 365 nucleotides (Fig. 1A, lanes 1,2,11,12) and was the result of hybridization to MT-TPI RNA or human TPI RNA that had been cleaved and polyadenylated (C^+/A^+) at the major polyadenylation (pA) site [Nesic et al. 1993]. Other protected regions of the TPI probe migrated at ~ 642 and 446 nucleotides (Fig. 1A, lanes 1,2,11,12). The 642-nucleotide fragment, which was detected with nuclear but not cytoplasmic RNA, was the result of hybridization to transcripts having 3' ends that extended beyond the limits of the probe

Figure 1. Intron 6 is necessary, but not sufficient, for efficient RNA 3'-end formation. (A) Nuclear (N) and cytoplasmic (C) RNAs were isolated from untransfected L cells, Malme-3 cells, and L cells cotransfected with the specified pMT-TPI construct and pMT-GI DNA. RNA that was synthesized in vitro was analyzed in parallel (lanes 13–15). This RNA derived from pSPTPI/c, which generates intron-less TPI RNA that terminates with a synthetic tract of A_{60-80} at the pA site, pSPTPI/c+3', which generates intron-less TPI RNA that terminates at the PvuII site residing 277 nucleotides downstream of the pA site, or pGEM4Z/GI, which generates β -globin RNA that consists of 9 nucleotides of exon 2, 216 nucleotides of exon 3, and 39 bp of the pGEM4Z polylinker sequence. Transcripts were mapped by hybridization to antisense RNA probes. The 699-nucleotide TPI probe spanned the TPI gene NdeI site in exon 7 to the PvuII site in 3'-flanking DNA and included sequences transcribed from the pGEM3Z expression vector on either side. The 298-nucleotide globin probe spanned the mouse β^{major} -globin cDNA from the BamHI site in exon 2 to the MboII site in exon 3 and also included sequences transcribed from the pGEM4Z expression vector on either side. Before electrophoresis, samples were treated with a mixture of RNases A and T_1 . As a control, one-tenth of each of the input RNA probes was electrophoresed without exposure to RNases in lanes 17 and 19. C^+/A^+ specifies the 365-nucleotide region of the TPI probe that was protected by TPI transcripts that had been cleaved and polyadenylated at the pA site. The 642- and 446-nucleotide regions of the TPI probe were protected by C^-/A^- transcripts that were apparently neither cleaved nor polyadenylated, i.e., extended beyond the PvuII site of the probe and, in the case of those that protected the 446-nucleotide region, were cleaved by RNases A and T_1 . The 446-nucleotide region also was protected by $\text{C}_2^+/\text{A}_2^+$ transcripts that were cleaved and polyadenylated at the pA2 site. Two regions of the globin probe that consisted of 235 and 250 nucleotides were protected by MT-GI transcripts. (B) The probes and the protected fragments are diagrammed. Diagonal lines through a restriction enzyme indicate the destruction of the enzyme cleavage site during the cloning process.

and were neither cleaved nor polyadenylated (C^-/A^-) (Nesic et al. 1993). The 446-nucleotide fragment that was detected with nuclear RNA resulted from hybridization to a mixture of transcripts. Some of these transcripts had been cleaved and polyadenylated (C_2^+/A_2^+) at the minor polyadenylation site, referred to as pA2, which resides 83 nucleotides downstream of the pA site; other transcripts were C^-/A^- and the consequence of RNase cleavage during the protection assay within the stretch of 13 A residues that resides 83 nucleotides downstream of the pA site (Nesic et al. 1993). Because only cleaved and polyadenylated RNA is thought to be transported to the cytoplasm (Eckner et al. 1991), the small amount of 446-nucleotide fragment that is detected with cytoplasmic RNA after a sufficiently long exposure to X-ray film is apt to result from hybridization to transcripts that are mostly, if not exclusively, C_2^+/A_2^+ (Nesic et al. 1993).

The deletion of introns 1–5 [Δ (int 1–5)] reduced the levels of C^+/A^+ RNA in the nuclear and cytoplasmic cell fractions to 22% and 9% of normal, respectively (Fig. 1A, lanes 5,6; Table 1). The higher level in the nuclear fraction, relative to the cytoplasmic fraction, indicated that this RNA may manifest a below-normal efficiency of export to the cytoplasm, a below-normal half-life in the cytoplasm, or both. These metabolic abnormalities could exist in addition to or as a consequence of the metabolic abnormalities that brought about the reduction in the abundance of nuclear C^+/A^+ RNA. As expected, the level of cytoplasmic C^+/A^+ RNA approximated the level of MT–TPI mRNA in unfractionated cells, which was 5% of normal as measured by Northern blot hybridization (J. Cheng and L.E. Maquat, unpubl.). The deletion of introns 1–5 also increased the level of C^-/A^- RNA in the nuclear fraction to 275% of normal

(Fig. 1A, lane 5; Table 1). Therefore, intron 6 is not sufficient for efficient RNA 3'-end formation in the absence of the rest of the TPI gene introns. It appears that sequences residing within one or a combination of the deleted introns normally augment the efficiency of intron 6 function in 3'-end formation.

Intron 1 probably does not normally function in 3'-end formation, as its deletion has no effect on mRNA abundance (Nesic et al. 1993). To determine whether intron 1 could contribute to 3'-end formation as the sole TPI gene intron, a pMT–TPI plasmid in which TPI introns 2–6 had been deleted [Δ (int 2–6)] was analyzed. These deletions reduced mRNA abundance in unfractionated cells to 3% of normal as measured by Northern blot hybridization (Nesic et al. 1993). As measured by RNase mapping, the deletions reduced the levels of C^+/A^+ RNA in the nuclear and cytoplasmic cell fractions to 19% and 0.7% of normal, respectively (Fig. 1A, lanes 3,4; Table 1). These deletions also increased the level of C^-/A^- RNA in the nuclear fraction to 422% of normal (Fig. 1A, lane 3; Table 1). Therefore, compared with the transcripts that derive from either the normal MT–TPI construct or the Δ (int 1–5) construct, the efficiency of 3'-end formation for transcripts that derive from the Δ (int 2–6) construct is inferior. In summary, the deletion of all introns but either intron 1 or intron 6 results in an abnormally low level of mRNA, which is partly due to an inefficiency in RNA 3'-end formation. The finding that the deletion of all TPI gene introns resulted in levels of C^+/A^+ and C^-/A^- RNAs that were $\leq 1\%$ of normal (data not shown; Table 1) suggests that at least one intron is required to prevent RNA degradation prior to 3'-end formation. These findings reinforce the idea that introns contribute to the process of mRNA generation in multiple ways.

Table 1. TPI RNA levels

TPI RNA	Nuclear RNA				Cytoplasmic RNA (percentage of norm)
	percentage of [C ⁻ /A ⁻ + C ⁺ /A ⁺]		percentage of norm		
	C ⁻ /A ⁻	C ⁺ /A ⁺	C ⁻ /A ⁻	C ⁺ /A ⁺	
MT-TPI					
Norm	25 ± 2	75 ± 2	100	100	100
Δ (int 2-6)	88 ± 1	12 ± 1	422 ± 10	19 ± 1	0.7 ± 0
Δ (int 1-5)	77 ± 5	23 ± 5	275 ± 30	22 ± 3	9 ± 2
Δ (int 1-6)	≤1	≤1	≤1	≤1	≤1
Δ (int 6)	37 ± 4	63 ± 6	179 ± 20	76 ± 7	57 ± 9
TPI of Malme-3 cells	15 ± 3	85 ± 3	—	—	—

The level of each MT–TPI RNA and the TPI RNA of Malme-3 cells was quantitated by PhosphorImaging. The PhosphorImaging intensity of each RNase-resistant portion of the probe was normalized by correcting for the number of radioactive nucleotides. The level of each MT–TPI RNA was additionally normalized to the level of MT–G1 mRNA to control for variations in the efficiencies of transfection, cell fractionation, and RNA recovery. In determining the percentage of [$C^-/A^- + C^+/A^+$], the sum of the normalized values for the C^-/A^- (i.e., 642- and 446-nucleotide fragments) and C^+/A^+ species that derive from each MT–TPI construct or the TPI gene of Malme-3 cells was defined as 100%. In determining the percentage of norm, the normalized values for the C^-/A^- and C^+/A^+ transcripts were calculated as a percentage of the normalized values, respectively, for the C^-/A^- or C^+/A^+ transcripts that derived from the normal MT–TPI construct, each of which was defined as 100. The numbers represent the average of at least two independently performed experiments. Data for Δ (int 1–6) are not shown.

The efficiency of intron 6 removal is augmented by other TPI gene introns

Our data suggest the possibility that sequences residing within one or a combination of introns 1–5 augment intron 6 function in RNA 3'-end formation. This possibility was examined using a quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay to compare the efficiency of intron 6 removal from transcripts that derived from the normal MT-TPI construct with transcripts that derived from the construct that harbored the deletions of introns 1–5 (Cheng and Maquat 1993). In the assay, cDNA was made from nuclear RNA by using random hexamers. The cDNA was then used as a template for PCR. One primer pair amplified sequences that spanned exon 6 and exon 7 of TPI RNA, and another primer pair amplified part of intron 1 plus part of exon 2 of MT-Gl pre-mRNA.

Conditions were empirically established to provide a linear relationship between the amount of each RT-PCR product and the amount of input nuclear RNA (Fig. 2A). For each RNA region analyzed, the size of the RT-PCR product was as expected. Products of MT-TPI RNA consisted of 276 and 151 bp, which derived from intron 6-containing and intron 6-less RNAs, respectively, and the product of MT-Gl RNA consisted of 250 bp (Fig. 2A). None of the products were synthesized from either untransfected L-cell RNA (Fig. 2A) or transfected L-cell RNA that had been pretreated with RNase A (data not shown). Although all RNA preparations had been purified by cesium chloride centrifugation, which should eliminate most if not all DNA, samples were treated with RNase-free DNase I prior to cDNA synthesis to eliminate the possibility of amplifying any residual plasmid DNA. Each RT-PCR product was quantitated by radiographic imaging. The effect of deleting introns 1–5 on the efficiency of intron 6 removal was determined by first normalizing the quantity of the 276- and 151-bp products of MT-TPI RNA to the quantity of the 250-bp product of MT-Gl pre-mRNA after considering the number of ^{32}P -labeled nucleotides in each product and subsequently calculating each normalized value for the deletion construct as a percentage of the corresponding normalized value for the normal construct. Results indicated that the deletion of introns 1–5 resulted in a 1.5-fold increase in the level of intron 6-containing transcripts and a 2.5-fold decrease in the level of intron 6-less transcripts. Therefore, in the case of the $\Delta(\text{int } 1-5)$ construct, the abnormally high level of transcripts that contain intron 6 (Fig. 2A) is accompanied by an abnormally high level of transcripts having an unprocessed 3' end (Fig. 1A, lane 5). These data suggest that the inefficiency in 3'-end formation is accompanied by an inefficiency in intron 6 removal.

Inefficient removal of the 3'-most functional intron, regardless of its source, correlates with inefficient RNA 3'-end formation

It seemed reasonable to generalize for any 3'-terminal intron, regardless of its context, a correlation between

the efficiency of its removal and the efficiency of RNA 3'-end formation. This generalization was tested by comparing for several intron-less variants of the MT-TPI gene the level of nuclear MT-TPI RNA that contained the most 3' functional intron to the level of nuclear MT-TPI RNA having an unprocessed 3' end. The variants consisted of the construct lacking introns 2–6 (Fig. 2B), the construct lacking all of intron 6 [$\Delta(\text{int } 6)$], and the constructs lacking either the intron 6 5' and 3' splice sites [$\Delta(\text{int } 6 \text{ 5'ss, 3'ss})$], the intron 6 3' splice site [$\Delta(\text{int } 6 \text{ 3'ss})$] (Fig. 2C), or the intron 6 5' splice site [$\Delta(\text{int } 6 \text{ 5'ss})$] (Fig. 2D). The normal MT-TPI construct and the endogenous TPI gene of Malme-3 cells were analyzed in parallel. As usual, the MT-Gl gene was coexpressed with each MT-TPI construct to control for variations in the efficiencies of cell transfection and RNA recovery.

For the $\Delta(\text{int } 2-6)$ construct, the efficiency of intron 1 removal from product RNA was measured by using RT-PCR and a primer pair that amplified a 151-nucleotide stretch of sequences that spanned the distal (3') end of intron 1 and the proximal (5') end of exon 2. Notably, this primer pair specifically amplifies intron 1-containing RNA and was chosen for use over a primer pair that spanned exons 1 and 2 because intron 1 (1433 nucleotides) is too large to be amplified efficiently in its entirety. Thus, whereas assessment of the ratio of intron 1-containing to intron 1-less RNA was not possible, the efficiency of intron 1 removal could be determined solely on the basis of the level of intron 1-containing RNA. The level of intron 1-containing transcripts that derived from the $\Delta(\text{int } 2-6)$ construct was ninefold higher than the level of intron 1-containing RNA that derived from the normal construct (Fig. 2B). Therefore, sequences residing within one or more introns downstream of intron 1 normally facilitate intron 1 removal. This finding is reminiscent of the finding that sequences within one or more introns that reside upstream of intron 6 normally facilitate intron 6 removal (see Discussion). More to the point with regard to RNA 3'-end formation, the abnormally high level of intron 1-containing transcripts that derived from the $\Delta(\text{int } 2-6)$ construct (Fig. 2B) was accompanied by an abnormally high level of transcripts with an unprocessed 3' end (Fig. 1A, lane 3).

To compare the efficiency with which the most 3' functional intron, intron 5, is removed from transcripts that derive from the $\Delta(\text{int } 6)$, $\Delta(\text{int } 6 \text{ 5'ss, 3'ss})$, and $\Delta(\text{int } 6 \text{ 3'ss})$ constructs, RT-PCR and a pair of primers that spanned exon 5 and exon 6 were used. Transcripts that contained intron 5 generated a 354-bp product, and transcripts that lacked intron 5 generated an 83-bp product (Fig. 2C). Complete deletion of intron 6 resulted in a 1.4-fold increase in the level of intron 5-containing transcripts and a 2.5-fold decrease in the level of intron 5-less transcripts (Fig. 2C), indicating that the presence of intron 6 is required for efficient intron 5 removal and mRNA generation. Deletion of both the 5' and 3' splice sites of intron 6 resulted in a 2.2-fold increase in the level of intron 5-containing transcripts and a 2.9-fold decrease in the level of intron 5-less transcripts (Fig. 2C). The finding that each intron 6 deletion results in an abnor-

Figure 2. The efficiency of intron removal is facilitated by the presence of adjacent introns. Specified amounts of nuclear RNA (*A,B,C*) or nuclear (N) and cytoplasmic (C) RNA (*D*) from Malme-3 cells or L cells that were either untransfected (–) or transfected with the designated pMT–TPI construct and pMT–GI were amplified by RT–PCR. The region of amplified TPI RNA is indicated at the top of each panel. As an internal control, the level of intron 1 within MT–GI pre-mRNA was simultaneously amplified. PCR products were electrophoresed in a 5% polyacrylamide gel and quantitated by PhosphorImaging. The conditions used for each RT–PCR were demonstrated to be quantitative using RNA standards (Std) that consisted of serial dilutions of a nuclear preparation of transfected L-cell RNA. Quantitations of TPI RNAs were corrected for the number of radioactive nucleotides and normalized to the level of MT–GI RNA. Normalized values are specified below the lanes. Arrowheads denote the electrophoretic positions of each product, the structures and sizes of which are provided to the right of the autoradiographic image. The structures consist of numbered boxes and lines, which represent exons and introns, respectively.

mally high level of transcripts containing intron 5, together with our previous findings that each intron 6 deletion results in an abnormally high level of transcripts having an unprocessed 3' end (Nesic et al. 1993), indicate that an inefficiency in intron 5 removal is accompanied by an inefficiency in 3'-end formation. The lower the efficiency of intron 5 removal, the lower the efficiency of 3'-end formation. Deletion of only the 3' splice site of intron 6 had no effect on intron 5 removal (Fig. 2C). This result is not unexpected given data demonstrating that the presence of a 5' splice site affects recognition and removal of the upstream intron (Robberson et al. 1990; Talerico and Berget 1990). As reported earlier, the deletion of the 3' splice site of intron 6 precluded RNA 3'-end formation, indicating that the deletion precluded the function of both intron 6 and intron 5 in 3'-end formation (Nesic et al. 1993).

The effects of deleting the 5' splice site of intron 6 on mRNA generation, intron 5 removal, and RNA 3'-end formation were then examined. Northern blot hybridization of total cell RNA revealed that MT-TPI mRNA was smaller than normal and produced at 78% of the normal level (Fig. 3, lane 2). As determined by using RT-PCR and a pair of primers that spanned exons 5 and 7, the

Figure 3. Deletion of the intron 6 5' splice site results in exon 6 skipping. Mouse L cells were cotransfected with the specified pMT-TPI construct and pMT-GI DNA. Total cellular RNA (25 μ g) from these cells, untransfected L cells, and human Malme-3 cells was denatured, electrophoresed in agarose, transferred to a nylon membrane, and cohybridized with the 32 P-labeled 300-bp *NdeI*-*NcoI* fragment of the TPI gene and the 32 P-labeled 170-bp *BalI*-*DraI* fragment of the mouse β^{major} -globin gene. The level of each MT-TPI mRNA was normalized to the level of MT-GI mRNA and calculated as a percentage of the normalized value of MT-TPI^{norm} mRNA (% of norm), which was defined as 100. Each represents the average of two independently performed experiments. mRNA that derives from the $\Delta(\text{int6 } 5'\text{ss})$ construct is abnormally small as a consequence of exon 6 skipping (see text). The positions of 18S rRNA and 2.37- and 1.35-kb molecular size standards are provided.

Figure 4. Two different splicing pathways are evident for TPI transcripts that harbor a deletion of the 5' splice site of intron 6. Boxes specify TPI gene exons, and the lines with numbers specify the intervening introns. ($\blacktriangle$) Deletion of the 5' splice site of intron 6. The levels of the RT-PCR products that derived from nuclear (N) or cytoplasmic (C) transcripts, from which solely intron 5 or both introns 5 and 6 had been removed (Fig. 2D), were normalized by correcting for the number of radioactive nucleotides and presented as a percentage of the sum of the normalized values for all possible intron 5-intron 6 combinations that were present in the same cell fraction, which was defined as 100%.

reduced size of the mRNA was the consequence of exon 6 skipping, that is, the splicing of intron 5-exon 6 intron 6 as a unit (Fig. 2D). Exon skipping has also been reported for other RNAs that harbor multiple introns, one of which harbors a deletion of the 5' splice site (Robberson et al. 1990; Dominski and Kole 1991; Chen and Chasin 1993, and references therein). RT-PCR, in being more sensitive than Northern blot hybridization, revealed that exon 6 skipping took place most frequently but not to the exclusion of an alternative splicing pathway that allowed for the removal of intron 5 but not intron 6. Three species of nuclear MT-TPI RNA were evident by RT-PCR. As a percentage of 100, 80% had undergone exon 6 skipping, 5% had undergone intron 5 removal but not intron 6 removal, and 15% had undergone neither intron 5 nor intron 6 removal (Figs. 2D and 4). A similar analysis of cytoplasmic RNA indicated that 93% had undergone exon 6 skipping, 5% had undergone intron 5 removal but not intron 6 removal, and 2% had undergone neither intron 5 nor intron 6 removal (Figs. 2D and 4). The detection in the cytoplasm of the small amount of intron 6-containing transcripts was unexpected given the results of previous studies demonstrating that partial spliceosome assembly on a pre-mRNA that harbors a partially deleted intron can impair pre-mRNA export to the cytoplasm (Green and Zapp 1989; Legrain and Rosbash 1989; Ryu and Mertz 1989; Kopczynski and Muskavitch 1992). The presence of intron 6-containing transcripts in the cytoplasm could be attributable to the failure to assemble spliceosomes on the 3' splice site of intron 6 and the consequential function of intron 5 as the 3'-terminal intron.

The ratio of intron 5-exon 6-intron 6-containing transcripts to intron 5-exon 6-intron 6-less transcripts that derived from the $\Delta(\text{int6 } 5'\text{ss})$ construct was comparable

to the ratio of intron 6-containing to intron 6-less transcripts that derived from the normal construct. On the basis of our previous findings, the efficiency of 3'-end formation for $\Delta(\text{int6 } 5'\text{ss})$ RNA would be predicted to be near normal. This was found to be the case, because the levels of C^-/A^- RNA and C^+/A^+ RNA as determined by RNase mapping were $102 \pm 5\%$ and $83 \pm 3\%$ of normal, respectively (data not shown).

All data suggest that inefficient removal of the most 3' intron correlates with inefficient RNA 3'-end formation. To compare the level of transcripts having an unprocessed 3'-terminal intron with those having an unprocessed 3' end for the different deletion constructs, the annealing, extension, and amplification efficiencies of each primer pair used in the intron quantitations must be similar. Similar efficiencies were evident as judged using the primer pairs and PCR to quantitate to levels of introns 1, 5, and 6 in pMT-TPI DNA. The ratio of the levels obtained was 1.0:0.6:1.3 (data not shown), which approximates the actual 1:to:1:1 ratio. For the transcripts that derived from each of the deletion constructs, the levels of the different 3'-terminal introns were then compared with the levels of unprocessed RNA 3' ends (Fig. 5). Results indicated that the higher the level of the most 3' functional intron, the higher the level of unprocessed 3' ends. Therefore, the efficiency with which the 3'-terminal intron is removed appears to correlate with the efficiency of RNA 3'-end formation.

Figure 5. The level of the most 3' functional intron correlates with the level of unprocessed RNA 3' ends. The pMT-TPI constructs that were analyzed are specified below the x-axis, and the levels of the most 3' functional intron (solid bars) and unprocessed 3' ends (open bars) of transcripts produced by each construct are specified on the y-axis. The levels were calculated relative to the corresponding levels produced by the normal construct, which were defined as one. The most 3' functional intron in each construct is specified above the appropriate solid bar.

The order of intron 6 removal and 3'-end formation is not fixed

Because 3'-end formation appears to be linked to removal of the 3'-terminal intron, the temporal relationship between the two processes was examined. RNase mapping was used to analyze the intramolecular status of intron 6 and the 3' end. The probe consisted of a uniformly ^{32}P -labeled, antisense transcript of 896 nucleotides, 842 of which included 77 nucleotides of exon 6, all (128 nucleotides) of intron 6, all (565 nucleotides) of exon 7, and 72 nucleotides of 3'-flanking DNA (Fig. 6B). Notably, all 72 nucleotides of 3'-flanking DNA resides upstream of the oligo(A)₁₃ tract. Thus, nonpolyadenylated RNA and RNA that has been polyadenylated at the pA2 site will be detected as a single RNase-resistant portion of the probe. Nuclear and cytoplasmic fractions of Malme-3 cells and L cells that had been transfected with the normal MT-TPI construct were analyzed either before or after separation into poly(A)⁺ and poly(A)[−] constituents. In theory, six RNA structures are possible: (1) S^-/A^- , which has been neither spliced nor cleaved and polyadenylated yet harbors the internal oligo(A) tract that results in partial copurification with poly(A)⁺ RNA; (2) S^-/A_2^+ , which has not been spliced but has undergone 3'-end formation at the pA2 site; (3) S^-/A^+ , which has not been spliced but has been cleaved and polyadenylated at the pA site; (4) S^+/A^- , which has been spliced but has not undergone 3'-end formation; (5) S^+/A_2^+ , which has been spliced and polyadenylated at the pA2 site; and (6) S^+/A^+ , which has been both spliced and cleaved and polyadenylated at the pA site (Fig. 6B). Because these experiments were not designed to assay introns 1-5, the splicing notation refers specifically and exclusively to intron 6.

All six RNA structures were detected in the nuclear RNA fraction of both Malme-3 cells and the transfected L cells (Fig. 6). Although S^-/A^- and S^-/A_2^+ transcripts were detected as the same 842-nucleotide fragment, detection of this fragment in poly(A)[−] RNA is indicative of the presence of S^-/A^- RNA, and detection of this fragment in poly(A)⁺ is indicative of the presence of S^-/A_2^+ transcripts. Similarly, whereas S^+/A^- and S^+/A_2^+ transcripts were detected as the same 637-nucleotide fragment, detection of this fragment in poly(A)[−] as well as poly(A)⁺ RNA is indicative of the presence of S^+/A^- and S^+/A_2^+ transcripts, respectively. The detection of S^-/A^+ and S^-/A_2^+ transcripts indicates that 3'-end cleavage and polyadenylation can precede the removal of intron 6. Similarly, the detection of S^+/A^- transcripts indicates that the removal of intron 6 can precede 3'-end formation. The abundance of each transcript (Table 2) necessarily reflects the rates of RNA synthesis, further processing to S^+/A^+ RNA, degradation in the nucleus, and export to the cytoplasm. Because no information is currently available on any of these rates in intact cells, it is possible to conclude that the order of intron 6 removal and 3'-end formation is not fixed, but it is difficult to conclude that one pathway is used more often than the other. At least in theory, either of the two partially pro-

Figure 6. The order of intron 6 removal and 3'-end formation is not fixed. (A) RNA from nuclear (N) and cytoplasmic (C) fractions either before (T) or after separation into poly(A)⁺ or poly(A)⁻ constituents was isolated from Malme-3 cells, L cells that had been transfected with the normal pMT-TPI construct, or untransfected L cells. The probe consisted of a uniformly ³²P-labeled, antisense RNA that was synthesized from recombinant pGEM3Z that contained the 896 nucleotide RsaI-BclI of human TPI DNA. This fragment consists of 77 nucleotides of exon 6, all 128 nucleotides of intron 6, all 565 nucleotides of exon 7, and 72 nucleotides of 3' flanking sequences. Protected fragments are specified in terms of the RNA to which they hybridized as follows: (S⁻/A⁻), neither spliced nor polyadenylated; (S⁻/A₂⁺), not spliced but polyadenylated at the pA2 site; (S⁺/A₂⁺), not spliced but polyadenylated at the pA site; (S⁺/A⁻) spliced but not polyadenylated; (S⁺/A₂⁺) spliced and polyadenylated at the pA2 site; (S⁺/A⁺) spliced and polyadenylated at the pA site. (S) The status of intron 6 alone. Horizontal arrows indicate that the RNA extends beyond the limits of the probe and includes the internal oligo(A) tract that allows for partial retention on oligo(dT)-cellulose and, therefore, partial fractionation with poly(A)⁺ RNA. Lanes 15, 16, and 17 are results obtained using in vitro-synthesized pSPTPI/c, pSPTPI/c + 3', and pSPTPI/g RNAs, respectively. One-fifth of the input probe was electrophoresed in lane 19. The probe and protected fragments are diagrammed. (B) Diagonal lines through a restriction enzyme indicate the destruction of the enzyme cleavage site during the cloning process.

cessed products could be processed further to fully processed mRNA.

Discussion

We have shown previously that sequences within the last intron, intron 6, of TPI pre-mRNA are necessary for efficient RNA 3'-end formation. The present study was undertaken to understand better how intron 6 contributes to 3'-end formation. We demonstrate here that intron 6 in the absence of introns 1–5 is insufficient for efficient 3'-end formation. It appears that the efficiency

of RNA 3'-end formation correlates with the efficiency with which intron 6 is removed, and both processes are activated by upstream introns. The finding that the removal of one intron is influenced by the presence of another is not limited to intron 6 but applies also to the other intron tested, intron 1. Relative to intron 6 in the absence of introns 1–5, intron 1 in the absence of introns 2–6 was found to function inefficiently as the 3'-terminal intron in RNA 3'-end formation. This inefficiency could be attributable to differences between intron 1 and intron 6 sequences, the abnormally large distance between intron 1 and the polyadenylation signals within

Table 2. TPI RNA levels

TPI RNA	Nuclear RNA				Cytoplasmic RNA			
	S ⁻ /A ⁻ and S ⁻ /A ₂ ⁺	S ⁻ /A ⁺	S ⁺ /A ⁻ and S ⁺ /A ₂ ⁺	S ⁺ /A ⁺	S ⁻ /A ⁻ and S ⁻ /A ₂ ⁺	S ⁻ /A ⁺	S ⁺ /A ⁻ and S ⁺ /A ₂ ⁺	S ⁺ /A ⁺
MT-TPI norm	7 ± 3	2 ± 0.5	15 ± 2	76 ± 7	—	—	6 ± 2	94 ± 2
TPI Malme-3 cells	7 ± 1	3 ± 1	11 ± 2	79 ± 1	—	—	4 ± 2	96 ± 2

The PhosphorImaging intensity of each RNase-resistant position of the probe was normalized by correcting for the number of radioactive nucleotides. The quantities of each RNA in each cell fraction were calculated to sum to 100. Because cleavage and polyadenylation appear to be prerequisites for RNA export from the nucleus to the cytoplasm (Eckner et al. 1991), it is probable, although not certain, that all of the cytoplasmic RNA that is assayed to be both S⁺/A⁻ and S⁺/A₂⁺ is likely to be only S⁺/A₂⁺. The numbers represent the average of four independently performed experiments.

the pre-mRNA that derives from the construct deleted for introns 2–6, or both. Regardless, the relative inefficiency of intron 1 function in RNA 3'-end formation correlates with the relative inefficiency of intron 1 removal. The pervasive theme of these studies is that the efficiency of removal of the 3'-terminal intron is predictive of the use of the cleavage and polyadenylation signal. This theme applies also when intron 6 is substituted precisely for a different intron. For example, intron 2 in the place of intron 6 is removed with an efficiency that is comparable to that of intron 6, and 3'-end formation proceeds with an efficiency that is comparable to the efficiency of the normal pre-mRNA (data not shown). We also demonstrate that the removal of intron 5 is normally influenced by intron 6 because deletion of either the 5' and 3' splice sites within intron 6 or all of intron 6 decreases the efficiency of intron 5 removal. Because ~84% of the time intron 6 is removed before intron 5 (Fig. 2D), either spliceosome assembly on pre-mRNA, that is, the commitment to splice intron 6, or intron 6 removal, per se, could facilitate the efficiency with which the adjacent intron is removed. Similarly, because there is no fixed order of intron 6 removal and 3'-end formation (Fig. 6), either the commitment to splice intron 6 or intron 6 splicing, per se, could facilitate the efficiency of 3'-end formation. Our data have broad implications to the mechanisms that accomplish RNA 3'-end formation and constitutive splicing. In particular, 3'-end formation appears to be linked to splicing, and the splicing of one intron appears to be linked to the splicing of other introns. Cooperativity between introns during the process of intron removal has also been reported for tumor necrosis factor β pre-mRNA (Neel et al. 1993). The link between 3'-end formation and splicing is likely to be attributable to the determinants of splicing efficiency, which include both the 5' and 3' splice sites of the 3'-terminal intron as well as sequences within one or more upstream introns. Conceivably, the link may be most evident for splice sites of 3'-terminal introns and sites of cleavage and polyadenylation that are suboptimal. For example, a very efficiently used site of cleavage and polyadenylation may function essentially independently of a very inefficiently spliced 3'-terminal intron.

The finding for some transcripts that spliceosome assembly and intron removal are cotranscriptional (Beyer and Osheim 1988; LeMaire and Thummel 1990) cannot be generalized to all transcripts but, together with evidence that 3'-end formation takes place on nascent transcripts (Whitelaw and Proudfoot 1986; Logan and Shenk 1987; Connelly and Manley 1988; Edwalds-Gilbert et al. 1993; Tantravahi et al. 1993), suggests that the opportunity exists for processing factors to bind pre-mRNA with 5' $\rightarrow$ 3' polarity. Although this may be true, data for both yeast and mammals indicate that productive factor binding, that is, binding that leads to RNA processing, is influenced by RNA sequences that reside both upstream and downstream of the processing site. (1) Usage of a 5' splice site can be dependent on the source of the 3' splice site and vice versa, indicating that definitive 5' splice site choice may not be made until the 3' splice site is

transcribed (Wassarman and Steitz 1992; Goguel and Rosbash 1993; Parker and Siliciano 1993). By inference, factors must interact along the entire length of an intron. (2) Competition may exist between 3' splice sites that reside in *cis*, depending on the proximity of each to the branchpoint, the presence of secondary structures upstream of or involving the 3' splice sites, and sequences flanking the 3' splice sites (Deshler and Rossi 1991; Smith et al. 1993). (3) The efficient removal of certain introns that harbor suboptimal splice sites may require sequences within another intron, as exemplified by the R1 RNA of the minute virus of mouse (Naeger et al. 1992). (4) Factors associated with adjacent introns may interact across the intermediate exon and, in so doing, guard against exon skipping (Robberson et al. 1990). For example, U2AF65 binding to a 3' splice site of prepro-tachykinin pre-mRNA is facilitated by a downstream 5' splice site and U1 snRNP particles (Hoffman and Grabowski 1992). (5) Exon sequences may stimulate the splicing of an upstream intron that naturally harbors a suboptimal 3' splice site. Examples include 3' splice site activation within the mouse immunoglobulin pre-mRNA by the adjacent 5' portion of the M2 exon (Watakabe et al. 1993) or within the *Drosophila doublesex* pre-mRNA by the *tra* and *tra-2* proteins, which bind selectively to repeated sequence elements in the adjacent female-specific exon (Hedley and Maniatis 1991; Ryner and Baker 1991; Inoue et al. 1992). Interestingly, female-specific polyadenylation immediately downstream of the female-specific exon of the *doublesex* pre-mRNA is also activated by the *tra* and *tra-2* proteins, suggesting that female-specific splicing and polyadenylation may be linked (Ryner and Baker 1991). (6) Selection of the RNA 3'-end cleavage and polyadenylation site is facilitated by a functional 3' splice site within the upstream intron, and, under certain circumstances in vitro, vice versa (Collis et al. 1990; Niwa et al. 1990; Chiou et al. 1991; Niwa and Berget 1991a, 1991b; Nesic et al. 1993; Wassarman and Steitz 1993). In particular, Wassarman and Steitz (1993), using psoralen and HeLa cell nuclear extracts, detected sites of U1 cross-linking located upstream of yet different distances from the AAUAAA hexanucleotide of SV40 late and adenovirus L3 pre-mRNAs, each of which contained an unprocessed 3' end. U1 cross-linking was not dependent on the presence of an upstream intron but was enhanced by the addition of an upstream 3' splice site, which also enhanced polyadenylation. Although there appeared to be no consistent effect on U1 cross-linking of mutations within the AAUAAA hexanucleotide that decrease polyadenylation, the data suggest that U1 snRNP may coordinate splicing and 3'-end formation. Cross-linking studies using late SV40 pre-mRNA have also implicated an involvement of the U1 snRNP in polyadenylation, albeit through interaction of the A protein component of the RNP with the three functionally additive AUUUGURA motifs that reside upstream of the AAUAAA sequence (Schek et al. 1992; C. Lutz and J.C. Alwine, pers. comm.). The idea that 3'-end formation can be facilitated by the 3' splice site of the 3'-terminal intron is compatible with

the findings presented here, which implicate not only this site but other determinants of 3'-terminal intron removal.

To summarize all of these data, usage of a particular splice site may be coupled to the reactivity of other splice sites as well as other intron or exon sequences that reside within the same pre-mRNA, and usage of the cleavage and polyadenylation site may be coupled to the splice site reactivity of the upstream intron(s). Notably, the rate of the consequential processing reactions would not necessarily reflect the rate at which the network of committed complexes forms but, rather, the rate of the subsequent intermolecular and intramolecular rearrangements that take place within and between the pre-mRNA substrate and the *trans*-acting processing factors.

Materials and methods

Cell culture, transfections, and RNA purification

The human melanoma cell line Malme-3 was grown in RPMI 1640 medium containing 10% bovine calf serum. Mouse Ltk⁻ cells were grown in minimal essential medium containing 10% fetal calf serum and 5% bovine calf serum. Transfections were as described in Nesic et al. (1993). Ltk⁻ cells were transfected at 60–80% confluency with DEAE-dextran and a 3.15 M excess of a specific pMT-TPI test plasmid relative to the pMT-GI reference plasmid (12 µg of each/15-cm dish). Total, nuclear, and cytoplasmic RNAs were isolated 36 hr after transfection. Poly(A)⁺ and poly(A)⁻ RNAs were isolated by four passages through a column of oligo(dT)-cellulose (Aviv and Leder 1972).

RNAse mapping

Uniformly ³²P-labeled antisense RNA probes for the mapping of TPI and β-globin transcripts were synthesized from linearized pGEM vectors using T7 RNA polymerase (Promega) as described previously (Nesic et al. 1993). Antisense RNA to the 642-bp *NdeI*-*PvuII* fragment of the TPI gene, which includes 365 bp of the 3'-untranslated region and 277 bp of 3'-flanking DNA, was used to map the cleavage and polyadenylation sites of TPI RNA. Antisense RNA to the 896 *RsaI*-*BclI* fragment of the TPI gene, which includes 77 bp of exon 6, all (128 bp) of intron 6, all (565 bp) of exon 7, and 72 bp of 3'-flanking DNA, was used to analyze concomitantly the processes of intron 6 removal and 3'-end formation. Antisense RNA to the 235-bp *BamHI*-*MboII* fragment from mouse β^{major}-globin cDNA, which includes 19 bp of exon 2 and 216 bp of exon 3, was used to control for experimental variations. The resulting transcripts were mixed in hybridization buffer (Ambion) with either L-cell RNA, Malme-3 cellular RNA, or sense RNA that had been synthesized in vitro from pSPTPI/c and pSPTPI/c + 3' (Nesic et al. 1993). pSPTPI/g was constructed by replacing the 1.1-kbp *NcoI*-*NcoI* fragment of pSPTPI/c with the corresponding 3.1-kbp *NcoI*-*NcoI* fragment of the TPI gene. After denaturation, the hybridization temperature was allowed to drop from 85°C to 55°C over a period of 12–16 hr. Hybrids were exposed to RNase A and RNase T₁ (Ambion) for 30 min at 37°C, concentrated by precipitation, denatured, and electrophoresed in an 8.3 M urea–5% polyacrylamide gel. Quantitative analysis was performed by PhosphorImaging (Molecular Dynamics).

RT-PCR analyses

L-cell RNA was treated with RQ1 RNase-free DNase (Promega) to eliminate any residual plasmid and cellular DNA. cDNA was

made using 0.11–3 µg of nuclear RNA from untransfected or transfected L cells or Malme-3 cells and 0.4 µg of random hexamer (Promega) by using Moloney murine leukemia virus reverse transcriptase (Superscript, BRL). PCR was then used to amplify MT-TPI cDNA and MT-GI cDNA. For each PCR sample, 8 µl of the 20-µl reaction was brought to 50 µl and contained 140 µM of each of the four deoxynucleoside triphosphates, 100 pmoles of each of the sets of primers, and 3.25 units of *Taq* DNA polymerase (Promega). After 19 amplification cycles, one-tenth of the PCR reaction was electrophoresed in a 5% native polyacrylamide gel, and the PCR products were quantitated by PhosphorImaging.

PCR primer pairs

TPI cDNA primers used are as follows: Intron 1–exon 2: 5'-GTCTCATCTGTGGGGTACCATC-3' (sense); 5'-CTCCCCAGTAAAAGCCCC-3' (antisense); PCR product, 151 bp. Exon 5–exon 6: 5'-AGACTGCAACACCCCAAC-3' (sense); 5'-AGCCACCGCATCAGAGCGTTGGACTTCAGCCA-3' (antisense); PCR products with intron 5, 354 bp; PCR products without intron 5, 83 bp. Exon 5–exon 7: 5'-AGACTGCAACACCCCAAC-3' (sense); 5'-AATTCGGGCTTGAGGGAAG-3' (antisense); PCR products with introns 5 and 6, 597 bp; PCR products with intron 5 but without intron 6, 471 bp; PCR products with intron 6 but without intron 5, 326 bp; PCR products without introns 5 and 6, 200 bp; PCR products without intron 5–exon 6–intron 6, 112 bp. Exon 6–exon 7: 5'-GAAGTCAACGTCTCT-3' (sense); 5'-AATTCGGGCTTGAGGGAAG-3' (antisense); PCR product with intron 6, 276 bp; PCR product without intron 6, 150 bp. β-globin cDNA primers are as follows: Intron 1–exon 2: 5'-GCCTATTGGTCTATTTTCCC-3' (sense); 5'-CCTGAAGTTCTCAGGATCC-3' (antisense); PCR product, 251 bp.

RNA blotting

Total RNA (25 µg) was denatured with glyoxal, electrophoresed in a 1.5% agarose gel, and transferred to a nylon membrane (Zeta-bind). Blot hybridization was performed with two DNA fragments that had been ³²P labeled by random priming. MT-TPI RNA was detected with a 300-bp *NcoI*-*NdeI* fragment that derived from the 3'-untranslated region of human TPI cDNA. MT-GI RNA was detected with a 170-bp *BalI*-*DraI* fragment that derived from exon 3 of the mouse β^{major}-globin gene. Hybridization and washing conditions were such that human, but not mouse, TPI RNA was detected (Cheng et al. 1990).

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Upstream introns influence the efficiency of final intron removal and RNA 3'-end formation.

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