In vivo cooperation between introns during pre-mRNA processing

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In higher eukaryotes the large number of introns present in most genes implies that the pre-mRNA processing machinery should be efficient and accurate. Although this could be achieved at the level of each intron, an attractive alternative would be that interactions between introns improve the performance of this machinery. In this study we tested this hypothesis by comparing the processing of transcripts of the tumor necrosis factor β gene, which differ only by their number of introns. We took advantage of the ordered splicing of the three introns present in this gene to design constructs that should generate, as primary transcripts, molecules that are normally produced by splicing. We established that the apparent splicing rate of intron 3 is increased 2.5- and 3.5-fold by the presence of one or two other introns on the primary transcript, respectively. Similarly, the apparent splicing rate of intron 2 is increased by the presence of intron 1. As these effects involve the splice sites of the upstream intron, these observations support the existence of cooperative interactions between introns during pre-mRNA processing.

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RNA biosynthesis constitutes the first step of gene expression. In higher eukaryotes, the primary transcription products generated by RNA polymerase II have to undergo extensive sequence modifications through polyadenylation and splicing, as well as translocation from the nucleus to the cytoplasm before the mRNA can be used for protein synthesis. Because the splicing process affects the coding capacity of transcripts directly, its efficiency and accuracy are obviously critical for cell viability. An initial difficulty comes from the fact that splice sites cannot be identified on the basis of their nucleotide sequence alone, as this does not constitute a stringent enough criteria (Padgett et al. 1986). This is immediately apparent for the acceptor site, characterized by the sequence AG, but it is also true for the donor site, which is characterized by a partial complementarity to the U1 small nuclear RNA (snRNA) 5’ end (Zhuang and Weiner 1986) (for naturally occurring sites, this complementarity frequently involves 6 nucleotides). This has led to the idea that splice sites could be identified by a combination of interactions involving several elements of the splicing machinery and different target sequences within the intron. Thus, identification of the acceptor site could involve three distinct features: the branchpoint, the polypyrimidine tract, and the dinucleotide AG (Smith et al. 1989). These considerations and the fact that the catalytic steps of splicing take place within the intron itself have led to the description of introns as autonomous functional units. However, the sheer number of introns in mammalian genes argues against a model of pre-mRNA processing in which introns would be removed independently of one another. If this were the case and unless splicing occurs with a 100% efficiency, one would predict that the yield of correctly processed transcript should decrease exponentially with the number of introns.

Thus far, the major source of information on the regulation of pre-mRNA processing in vivo has been the analysis of mutations that alter the splicing pattern. One recurrent observation is that mutations located within an intron can affect the processing of an adjacent intron. Specifically, mutations in a donor site frequently lead to the skipping of the exon immediately upstream (Mitchell et al. 1986; Nasim et al. 1990; Talerico and Berget 1990; Tacke and Goridis 1991), and mutations in an acceptor region (i.e., the acceptor site or the polypyrimidine tract) can lead to the skipping of the exon immediately downstream (Mitchell et al. 1986; Horowitz et al. 1989; Chu et al. 1991). Thus, in both cases, a nonfunctional splice site leads to the “fusion” of the defective intron with the nearest intron. Although a 5’ → 3’ scanning model for the identification of splice sites within introns could explain that a mutation in an acceptor site would lead to the selection of the next functional acceptor site, it cannot account simply for the phenotype associated with the mutation of a donor site. These observations, in association with studies on the structure of

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exons, have led S. Berget and co-workers to propose that the splice site recognition involves an interaction between the two sites that flank an exon [Robberson et al. 1990; Talerico and Berget 1990]. In this model a limit of 300 nucleotides, which has been observed as the size of internal exons in vertebrate genes [Hawkins 1988], is interpreted as the steric constraint introduced by the interactions between the complexes assembled on the acceptor and donor sites. This concept of “exon recognition” can account for the aforementioned observations on exon skipping. Moreover, by limiting the search for splice sites to a 300-nucleotide window it provides an elegant way to reject the cryptic sites that may be present in large introns. In summary, these studies have provided strong indications that the intron borders are, in part, defined by genetic information located in the adjacent introns. However, because the normal pattern of splicing is altered in these experiments, it is difficult to analyze whether the efficiency of splicing is also influenced by the presence of adjacent introns.

In this study we have investigated the interactions between introns by comparing the processing of transcripts that differ only by their number of introns. In this case, and at variance with the study of mutations in splice sites, we can monitor both the efficiency of splicing and the appearance of new transcripts. Such a study can be carried out only with a gene for which the nuclear processing events can be analyzed. Previously, we have reported [Weil et al. 1990] that several features of the murine TNFβ gene (tumor necrosis factor β, lymphotoxin) make it an attractive model to study pre-mRNA processing (Fig. 1). First, it associates a small size, which facilitates the characterization of the transcripts, with the presence of three introns [Semon et al. 1987], thus providing an interesting level of complexity. Second, we have established that in CTLL-2, a T-lymphocytic cell line that normally expresses the TNFβ gene, the predominant maturation pathway includes the polyadenylation of the primary transcript followed by the sequential splicing out of the three introns according to a 5' → 3' order. Third, splicing out of intron 3 is incomplete and in competition with the export to the cytoplasm of intron 3-containing transcripts. Therefore, the proportion of cytoplasmic RNA containing intron 3, that is, the level of intron retention, constitutes an indicator of the relative efficiencies of the splicing and transport pathways for these transcripts. The experimental strategy was organized around the idea that it should be possible, by transfecting the appropriate constructs, to generate as primary transcripts, RNAs that are normally produced as processing intermediates. Therefore, splicing of introns 2 and 3 should take place on molecules with identical primary sequences but with differing numbers of previous splicing events.

Results

Observation of intron retention during TNFβ expression in NIH-3T3 cells

To investigate the role of the number of introns in the maturation of TNFβ transcripts, we had to rely on the ectopic expression of TNFβ constructs. We first evaluated the possibility of studying the retention of intron 3 in NIH-3T3 cells. To this end, we designed an expression vector that contained no built-in intron. The plasmid pCM is composed of the simian cytomegalovirus (CMV) immediate early promoter [Jeang et al. 1987], a poly-linker, and a rabbit β-globin polyadenylation signal [Levitt et al. 1989]. The TNFβ constructs (Fig. 2) were obtained by inserting in the pCM vector chimeras between genomic and cDNA sequences derived from the murine TNFβ gene. All of these inserts begin at nucleotide 4 of the first exon and finish at the nucleotide 535 of the fourth exon. Consequently, a putative AT destabilization signal for mRNAs [Shaw and Kamen 1986] and the natural polyadenylation site of TNFβ are truncated from the 3' noncoding region. The three constructs LTI 123, LTI 23, and LTI 3 differ only by the presence of the indicated introns. Thus, LTI 123 contains the three introns of the gene, whereas LTI 3 contains only the third intron. Therefore, except for the modifications of the 5' and 3' ends, the primary transcripts generated by LTI 123, LTI 23, and LTI 3 should be identical to the TNFβ maturation intermediates characterized previously in CTLL-2 cells: N3, N2, and N1 [see Fig. 1]. The GloI 1 plasmid contains a rabbit β-globin minigene composed of the first intron of β-globin and β-globin cDNA sequences inserted in the vector pCM.

Figure 1. Maturation pathway of TNFβ transcripts in CTLL-2 cells. The maturation pathway of TNFβ transcripts was established in CTLL-2 cells by a combination of structural and kinetic studies [Weil et al. 1990]. The organization of the murine TNFβ gene is presented at the top. Exons are represented by boxes, and introns by solid lines labeled i1, i2, and i3.

Cooperation between introns
Expression of these constructs was studied by transient transfection in NIH-3T3 cells. Forty-one hours after transfection by calcium phosphate coprecipitation, RNAs were prepared and the transcripts characterized by RNase mapping. We first investigated the cytoplasmic RNAs using probe A, which encompasses introns 2 and 3 (Fig. 3A). In CTLL-2 RNAs, we observed two fragments that, as established previously (Weil et al. 1990), correspond to two classes of mRNAs (see Fig. 1), C1 (protection of exon 3—intron 3, 332 nucleotides), and C0 (protection of exon 3, 100 nucleotides). No TNFβ transcripts could be detected in untransfected NIH-3T3 cells, whereas the same fragments as for CTLL-2 cells were observed after transfection of LTI 123 (Fig. 3A). Thus, the ectopic expression of this genomic-like TNFβ construct leads to a retention of intron 3, as has been observed for the endogenous gene. Figure 3B presents the analysis of the RNAs generated by the GloI 1 construct using an appropriate probe (probe Glo). The predominant signal (S) corresponds to the expected protection for the spliced mRNA. The smear above the spliced band appears to be the result of an anomalous migration of this protected fragment, which is also observed with RNAs from rabbit erythrocytes (data not shown). Only a very faint band (US) can be observed on long exposures corresponding to the unspliced RNA. Therefore, this experimental approach is well suited for the study of intron 3 retention.

Figure 3A also presents the results obtained with plasmids LTI 23 and LTI 3; and, as with LTI 123, a significant level of intron 3 retention can be observed in the cytoplasmic RNAs. In the case of LTI 3, a contamination of the RNA preparation by the transfected plasmid could generate an artifactual C1 signal. As shown Figure 3A, DNase pretreatment did not change the intensities of the protected fragments, confirming that they were attributable to the transcription products of the transfected plasmids. In conclusion, the retention of intron 3 is observed during the transient expression of appropriate TNFβ constructs in NIH-3T3 cells, and it does not require the presence of introns 1 and 2 in the transfected construct.

Quantitative analysis of intron 3 retention
Although the retention of intron 3 can occur in the absence of introns 1 and 2, Figure 3A indicates that the proportion of mRNA containing intron 3 is higher with
LTI 3 than with the other constructs. To quantify the results of the RNase mapping experiments we used a densitometric analysis of preflashed autoradiographs. Taking into account the number of U residues in each protected fragment, the level of intron retention was defined as the proportion of cytoplasmic RNAs that contain intron 3 [i.e., C1/(C0 + C1)]. This value should be independent of the amount of RNA used in the analysis, and the same level of intron retention was observed with either 4 or 8 μg of RNA. When the same sample was analyzed twice in a given RNase mapping experiment the results differed by <10%. However, if the same sample was analyzed in two distinct RNase mapping experiments the difference could reach 20% (data not shown).

For this reason, all of the samples of a given experiment were always analyzed in the same RNase mapping. When this analysis was applied to the experiment in Figure 3A it yielded the following levels of intron 3 retention: 0.32, 0.36, and 0.58 for LTI 123, LTI 23, and LTI 3, respectively.

To assess the significance of this observation we evaluated the reproducibility of the experiments with the LTI 123 construct. First, a kinetic study was carried out between 24 and 71 hr after transfection. The level of intron retention did not vary during this time course (data not shown), indicating that a steady state had already been reached at 24 hr. As the level of expression was maximal at 41 hr, all of the subsequent experiments were performed under these conditions. We then performed a set of transfections varying the amount of LTI 123 plasmid between 0.67 and 10 μg per 85-mm dish. In spite of a 15-fold variation in the expression level that correlated with the amount of plasmid, the level of intron retention varied only between 0.20 and 0.30 and showed no relation with the expression level (data not shown). The analysis of nine independent transfection experiments (Fig. 4) performed under our standard conditions (10 μg of plasmid and a 41-hr assay) confirmed and extended these results, as no relation between the expression level and the level of intron retention could be observed. It also established that the variability of the results is on the order of 20%, as indicated by the average level of retention (0.24) and the corresponding standard deviation (0.04).

We then considered the reproducibility of the observation with the three LTI constructs by performing three new transfection experiments under the same conditions as in Figure 3A. The results are summarized in Figure 5 with the data of Figure 3A labeled as experiment 1 (exp. 1). In each case, the amount of intron retention is higher with LTI 3 than with LTI 23, and higher with LTI 23 than with LTI 123. In view of this reproducibility and as the level of intron retention increases by 80—120% between LTI 123 and LTI 3, we conclude that the presence of other introns in the primary transcript reduces significantly the level of intron 3 retention. The same conclusion can be reached by simply averaging the results of Figure 5, although each experiment was analyzed by different RNase mapping assays [LTI 123: 0.22 ± 0.03, LTI 23: 0.28 ± 0.05, LTI 3: 0.49 ± 0.07].

The level of intron retention, as we define it, is determined by the cytoplasmic accumulation of C1 and C0. Consequently, this level is controlled by pre-mRNA processing, the cytoplasmic export of N1 and N0, and the stability of C1 and C0 in the cytoplasm. To evaluate whether the cytoplasmic stability of C1 and C0 was involved in the difference in the level of intron retention between LTI 123 and LTI 3, we inhibited transcription with dactinomycin for 0, 3, and 6 hr (Fig. 6). Although the level of intron retention decreased over this time period, indicating that C1 is slightly less stable than C0, it did so with similar rates for the transcripts generated by the two constructs or even slightly more rapidly for those generated by LTI 3. These results therefore eliminate the possibility that an increased stability of C1 or a decreased stability of C0 could account for the variations in the level of intron retention. In conclusion, the transcripts generated by the three LTI constructs differ by their processing or their transport, or both.

Are the differences in intron retention attributable to the use of several maturation pathways?

We investigated whether the transcripts of the different constructs had the expected structure and followed the same maturation pathway as the endogenous TNFβ transcripts. To this end, we characterized the global structure and the polyadenylation of the nuclear transcripts by Northern blot and their intronic content by RNase mapping.

We performed a Northern blot analysis using an exon 4 probe that detects all the transcripts with the same
Figure 5. Variation of the level of intron retention with the number of introns in the TNFβ constructs. Cytoplasmic RNAs from four independent transfection experiments (exp.) with the LTI 123, LTI 23, and LTI 3 plasmids were analyzed by RNase mapping with probe A (Fig. 3). The level of intron 3 retention was determined by densitometry as in Fig. 4. CTLL = the bar representing the level of intron 3 retention in CTLL-2 RNAs; Globin = the bar corresponding to the level of retention of β-globin intron 1 in RNAs from cells transfected with the GloI plasmid. For experiments 1 and 2, the solid bars represent the results of the analysis of the LTI 3 samples following a DNase I pretreatment.

We then analyzed the transcripts generated by the TNFβ constructs by RNase mapping. Two probes were used: probe A (Fig. 8A), which encompasses introns 2 and 3, and probe B (Fig. 8B), which overlaps with introns 1 and 2. Three bands were observed in nuclear RNAs from CTLL-2 cells with probe A (Fig. 8A, right). They correspond to fully spliced RNAs (identified by the protection of exon 3, 100 nucleotides), i2−,i3+ RNAs (exon 3-intron 3, 332 nucleotides), and i2+,i3− RNAs (exon 2-intron 2-exon 3-intron 3, 497 nucleotides). The same pattern of protection was observed in nuclear RNAs generated by the TNFβ constructs (Fig. 8A, left), with the exception of the LTI 3 transcripts which, as expected, did not contain intron 2. Splicing of intron 3 before intron 2 would have generated an i2−,i3− species (exon 2-intron 2-exon 3, 265 nucleotides). Despite the very strong signals corresponding to the other precursors, no such protection could be observed, indicating that the i2−,i3− transcripts accumulate to a level that is ~25-fold lower than that of i2−,i3+ transcripts. With probe B (Fig. 8B), three classes of transcripts were observed in nuclear RNAs from CTLL-2 cells. They correspond to i1−,i2− RNAs (identified by the protection of exons 2 and 3, 105 and 70 nucleotides, respectively), i1−,i2+ RNAs (exon 2-intron 2-exon 3, 258 nucleotides), and i1+,i2+ RNAs (intron 1-exon 2-intron 2-exon 3, 335 nucleotides). The protection of exon 2 was inefficient and generated a set of fragments ranging in size between 105 and 95 nucleotides. A similar artifact was observed with LTI 3 plasmid efficiency. This analysis was carried out before and after oligo(dT)/RNase H digestion of poly(A) tails (Fig. 7). The ethidium bromide staining of the filter (Fig. 7B) shows that the mobility of the rRNAs is not affected by this treatment. In the nucleus, after poly(A) tail digestion, four distinct RNA species can be distinguished according to their migration (Fig. 7A). Their respective sizes agree with those of the nuclear species that we have detected in CTLL-2 cells (Weil et al. 1990). Accordingly, we used the N3, N2, N1, and N0 nomenclature to designate them. The three LTI constructs did generate the expected primary transcripts, N3 for LTI 123, N2 for LTI 23, and N1 for LTI 3. The corresponding bands are highlighted by a dot. The fact that the mobility of all the detected RNAs, including the primary transcripts, was greater after the oligo(dT)/RNase H treatment indicates that as in CTLL-2 cells, they are polyadenylated. In the cytoplasm, after poly(A) tail digestion, two species were observed in agreement with the detection of C1 and C0 by RNase mapping (see Fig. 3A). Moreover, a densitometric analysis of the RNase H-treated samples provided an independent determination of the level of intron retention associated with the different LTI constructs [LTI 123: 0.27, LTI 23: 0.41, LTI 3: 0.51], which confirmed the results presented in Figure 5.

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Figure 6. Role of mRNA stability in the variation of the level of intron retention. Forty-one hours after transfection with either LTI 123 (open bars) or LTI 3 (solid bars), cells were treated with dactinomycin for 0, 3, and 6 hr. The level of intron retention was determined by densitometric analysis of RNase mapping experiments performed on the corresponding cytoplasmic RNA samples with probe A (Fig. 3).
Cooperation between introns

Figure 7. Global structure and polyadenylation of the TNFβ transcripts in NIH-3T3 cells. (A) Northern blot analysis of the TNFβ transcripts. Cytoplasmic (C) or nuclear (N) RNAs (4 μg) from the indicated source were treated (+) or not (-) with oligo(dT)/RNase H and analyzed with probe C (represented at the bottom). N3, N2, N1, and NO indicate the positions of the four nuclear TNFβ transcripts after oligo(dT)/RNase H treatment. Dots indicate the primary transcripts for each of the LTI constructs before and after oligo(dT)/RNase H treatment. The migration of the 18S rRNA is indicated at right. (B) Ethidium bromide staining of the filter. The 28S and 18S rRNAs are identified at left.

mid DNA (lane pLTI 3). A pattern of protection comparable with that of CTLL-2 cells was observed with the RNAs generated by LTI constructs, provided one takes into account the absence of intron 1 or introns 1 and 2 in LTI 23 and LTI 3, respectively. Therefore, we conclude that the nuclear transcripts that can be detected in transfected NIH-3T3 cells have the same structure as the nuclear precursors in CTLL-2 cells.

In the cytoplasm of CTLL-2 cells no signal corresponding to mRNA containing more than intron 3 was observed. Thus, probe A revealed the presence of i2−,i3− and i2−,i3+ RNAs but not i2+,i3+ RNAs (Fig. 8A). Similarly, probe B detected i1−,i2− but not i1−,i2+ RNAs (Fig. 8B). The faint signal visible at the position of i1+,i2+ is an artifactual band that is also detected in RNAs of untransfected NIH-3T3 cells. It would also be detectable in all of the other lanes if comparable exposures were presented (see the legend to Fig. 8B). In transfected NIH-3T3 cells, we could observe a low level of cytoplasmic i1−,i2+,i3− and i1−,i2+,i3+ RNAs. Densi-

Figure 8. Analysis of the intron content of the TNFβ transcripts in NIH-3T3 cells. (A) RNase mapping analysis with probe A, which encompasses introns 2 and 3 as indicated at the bottom. Nuclear (N) or cytoplasmic (C) RNAs (4 μg) from the indicated source were analyzed. P and M designate the undigested probe and the molecular weight markers (MspI-digested pBR322), respectively. The expected protections for the different transcripts are identified at right using + or − to indicate the presence or the absence of the corresponding introns. (B) RNase mapping analysis with probe B, which overlaps with introns 1 and 2 as indicated at the bottom. The methods and the notations are as in A, except that in the lane labeled pLTI 3, 20 ng of LTI 3 plasmid was analyzed. Lanes labeled NIH and CTLL N and C were photographed with a 48-hr exposure of the RNase mapping, whereas all of the other lanes were obtained from a 4 hr exposure.
tometric analysis indicated that their abundance did not exceed 5% of the TNFβ RNAs. This is comparable with the low level of unspliced cytoplasmic β-globin transcripts observed after the transfection of the GloI construct [Fig. 5]. These results suggest that in the transfected cells a small proportion of the partially processed transcripts are leaking out of the nucleus.

In summary, the structure of the transcripts generated by the LTI constructs is fully concordant with that of the endogenous TNFβ transcripts in CTLL-2 cells. Therefore, the expression of LTI 123 closely follows that of the chromosomal TNFβ gene, and the deletion of intron 1 or introns 1 and 2 from the LTI 123 construct simply leads to a correspondingly abridged version of the same maturation pathway.

Is the processing or the transport of TNFβ transcripts modified by the number of introns?

Intron retention in TNFβ mRNAs reflects the existence of two processing pathways for intron 3-containing transcripts [N1]; splicing followed by export to the cytoplasm gives rise to C0, or direct export to the cytoplasm gives rise to C1. If we describe these processes as pseudo-first-order reactions, then the accumulation of C1 is controlled by $\delta C1 = t_{N1}N1 - d_{C1}C1$, where $t_{N1}$ is the transport rate of N1 and $d_{C1}$ is the degradation rate of C1 [Fig. 9A]. Thus, when the steady state is reached, C1/N1 = $t_{N1}/d_{C1}$. Similarly, the accumulation of C0 can be described by a set of two reactions, the first one corresponding to the production of N0: $\delta N0 = s_{i2}N2 - s_{i3}N1 - t_{N0}N0$, where $s_{i3}$ is the apparent splicing rate of intron 3, and the second one to the transport of N0: $\delta C0 = t_{N0}N0 - d_{C0}C0$. The only further assumption introduced here is that there is no significant degradation of N0. This is clearly true in CTLL-2 cells [Weil et al. 1990], and our dactinomycin studies in NIH-3T3 cells support this hypothesis (data not shown). Therefore, at equilibrium, C0/N1 = $s_{i3}/d_{C0}$. As we have established that the degradation rates of C0 and C1 are not modified by the nature of the transfected construct, the values of C0/N1 and C1/N1 can be used to investigate whether intron 3 splicing or transport, or both, are affected by the number of introns. Figures 9, B and C, present the values of C1/N1 and C0/N1 for three of the experiments analyzed in Figure 5. No systematic variation could be observed for the transport pathway [Fig. 9B]. The average values of C1/N1 are 3.4, 3.5, 3.5 for LTI 123, LTI 23, and LTI 3, respectively. In contrast, the efficiency of the splicing pathway varied reproducibly with the number of introns [Fig. 9C]. The average values of C0/N1 are 11.2, 8.2, and 3.2 for LTI 123, LTI 23, and LTI 3, respectively. Therefore, the increase in intron 3 retention associated with the removal of the upstream introns from the transfected constructs results from a decrease in the apparent splicing rate of intron 3.

To assess the generality of this result on splicing, we then analyzed the splicing of intron 2 in the transcripts generated by LTI 123 and LTI 23. Using the same notation as in the preceding section [Fig. 9A], the accumulation of N1 is controlled by $\delta N1 = s_{i2}N2 - s_{i3}N1 - t_{N1}N1$ and, therefore, at equilibrium, N1/N2 = $s_{i2}/(s_{i3} + t_{N1})$. For LTI 23 transcripts the relative abundance of N1 and N2 can be derived directly from the RNase mapping experiment with probe A [Fig. 8A]. For LTI 123 transcripts, the $i2+,i3+$ species detected by probe A includes both N2 and N3. However, as the RNase mapping experiment with probe B [Fig. 8B] indicates that N2 and N3 accumulate to equal levels, half of the $i2+,i3+$ RNAs correspond to N2. This analysis yields values of N1/N2 of 1.0 and 0.3 for the transfection of LTI 123 and LTI 23, respectively. Because we established that $s_{i3}$ decreases in the absence of intron 1 while $t_{N1}$ remains constant, we conclude that $s_{i2}$, the apparent splicing rate of intron 2,
decreases at least threefold between the LTI 123 and LTI 23 constructs. Therefore, we have established that the removal of introns 1 and 2 leads to an increase in intron 3 retention that is attributable to a decrease in the apparent splicing rate of intron 3, si3. Similarly, an effect of intron 1 can be observed on the apparent splicing rate of intron 2, si2.

Role of the exonic and intronic sequences in the regulation of splicing rates

In this study we used the sequential splicing of the three introns present in the TNFβ gene to generate as primary transcripts RNAs that are normally produced through splicing. Our experimental strategy was designed to ensure that the "exogenous" substrates for splicing have primary sequences identical to their "endogenous" counterparts. However, it necessarily follows that the primary transcripts generated by these constructs differ by their organization in exons and introns and their sequence content. We therefore investigated whether these differences significantly influenced the splicing rates. One of the differences between the primary transcripts is the size of the first exon, which varies from 68 nucleotides in LTI 123 to 273 nucleotides in LTI 3. To test for a potential influence of this first exon on the splicing rate of intron 3, a set of deletions was created within the first exon of LTI 3 [Fig. 10A]. ΔXE removes 35 nucleotides within exons 1 and 2 of TNFβ, whereas ΔXN removes 185 nucleotides, deleting exons 1 and 2 completely and exon 3 partially. Finally, the construct E1/E3 was designed to fuse exon 1 of TNFβ to intron 3 to create an LTI 3-like construct with a 5' structure comparable with that of LTI 123 (note, however, that the last 3 nucleotides of exon 1 were replaced by the last 3 of exon 3 to preserve the sequence of the donor site of intron 3; hence, the nomenclature E1/E3). The levels of intron 3 retention observed for these constructs are presented Figure 10A. It is apparent that the splicing of intron 3 is not much affected by these modifications of the first exon and that, if anything, it is reduced in comparison with that of the parental construct LTI 3. This relative insensitivity to the structure of the first exon was confirmed by other studies [H. Neel, D. Weil, and F. Dautry, unpubl.] in which we extended its size up to 582 nucleotides and observed a level of intron retention of 0.64.

Therefore, the presence of other introns rather than differences in the upstream exon leads to an increase in splicing rates. We then investigated whether this "enhancement" of splicing required the actual splicing out of the upstream intron. By focusing on the interaction between introns 2 and 3, we introduced in the LTI 23 construct mutations to inactivate both the donor and the acceptor sites of intron 2 [Fig. 10B]. To preserve as much as possible of the sequence of intron 2, only the two invariant nucleotides of each site, GT and AG, were replaced by TG and GA, respectively. Structural studies by RNase mapping and Northern blotting of the transcripts generated by LTI 2*3 confirmed that in this double mutant, intron 2 was retained in all the transcripts and that no cryptic splice site was activated [data not shown]. As in Figure 10B, expression of this LTI 2*3 construct leads to a level of intron 3 retention (0.62) comparable with that of the LTI 3 construct (0.57) and clearly different from that of LTI 23 (0.29). Analysis of the relative accumulation of the nuclear precursors confirmed that, as in the previous studies, the level of intron 3 retention reflected the splicing rate of intron 3. Thus, in the absence of splicing, the sequences contained within intron 2 were not able to stimulate the splicing of intron 3.

In summary, our studies indicate that the number of introns present within constructs derived from the TNFβ gene influences the processing of the corresponding transcripts. We established that this effect is the result of the interaction between exons and introns. The size and sequence of these introns affect the splicing of the downstream introns. This cooperation between introns is important for the regulation of splicing.
yielded a level of intron retention of 0.24 ± 0.04. It is with our studies indicating that the minimal sequences required for intron 3 retention are contained within introns 1 and 2. Therefore, no information contained in introns 2 and 3 is essential for the retention of intron 3. Although the precise mechanism of intron 3 retention has not been characterized yet, this observation agrees with our studies indicating that the minimal sequences required for intron 3 retention are contained within introns 2 and 3 and the last 3 nucleotides of exon 3 (Fig. 10; C. Giansante, D. Weil, H. Neel and F. Dautry, unpubl.).

The central observation of this study is that the presence of introns 1 and 2 reduces the level of intron 3 retention from 0.49 to 0.22. Because this effect is not the result of a difference in the cytoplasmic stability of the mRNAs, we conclude that the maturation of molecules that contain intron 3 is influenced by the number of introns present in the primary transcript. One explanation could be that the transcripts generated by the three LTI constructs use different maturation pathways. Precisely to avoid this possibility, we designed our constructs according to the predominant maturation pathway of the endogenous TNFβ transcripts in CTLL-2 cells, that is, sequential splicing according to a 5' → 3' order of a polyadenylated primary transcript. To address this issue, we analyzed the structure of the TNFβ nuclear RNAs in NIH-3T3 cells. With LTI 123, we observed the same set of maturation intermediates as in CTLL-2 cells. The same observation was made for LTI 23 and LTI 3, the maturation pathway being simply shortened by the removal of introns from the constructs. Thus, in view of the species that accumulate to a detectable level in the nucleus, the LTI constructs behave as expected. In CTLL-2 cells, we have confirmed that most of the transcripts are processed according to this pathway by kinetic studies of TNFβ expression after IL2 stimulation or dactinomycin treatment (Weil et al. 1990). These studies do not completely exclude the existence of alternative maturation pathways but, rather, put an upper limit of 10–20% on the proportion of molecules that could be processed by these pathways. To evaluate the potential importance of this point, we focused on the comparison of the LTI 3 and LTI 23 constructs. An alternative maturation pathway accessible to the LTI 23 transcripts, but not to the LTI 3 transcripts, would correspond to the splicing out of intron 3 before intron 2. The level of intron retention decreases from 0.49 to 0.27 in the presence of intron 2. Therefore, taking into account the similar half-lives of C1 and C0, LTI 3 generates about as many molecules of C0 as of C1, whereas LTI 23 generates three times as much C0 as C1. Thus, the alternative maturation pathway would account for the production of half of the cytoplasmic molecules, which is incompatible with our results.

As the transcripts of the different constructs predominantly use the same maturation pathway, it follows that the variations in intron retention are the result of quantitative differences in this pathway. To identify which step of the pre-mRNA maturation is influenced by the presence of upstream introns, we used a quantification of product-to-precursor ratios and evaluated reaction rates. According to this analysis, the transport of intron 3-containing transcripts is not affected by the number of introns, whereas the apparent splicing rate of intron 3 is increased 2.5-fold by the presence of intron 2 and 3.5-fold by the presence of both introns 2 and 1. We used the same approach to assess whether this effect of upstream introns could be observed for other TNFβ introns. The splicing rate of intron 2 is also increased, at least threefold, by the presence of intron 1, that is, to a comparable extent as that observed for intron 3.

We have therefore established that the splicing rates of introns 2 and 3 are increased in the presence of upstream introns on the same primary transcript. This effect could result either from the presence of a functional intron or from structural differences between the primary transcripts. For example, the presence or absence of upstream introns could confer different secondary structures to the transcripts (Tomizawa and Itoh 1981; Wong and Polisky 1985) and, thus, regulate splicing (Watakabe et al. 1989; Clouet d’Orval et al. 1991, Domenjoud et al. 1991). Alternatively, it has been observed in other genes that specific sequence elements, distinct from the splice sites, can increase the efficiency of splicing of a nearby intron (Watakabe et al. 1993, Xu et al. 1993). Our studies with deleted versions of the LTI 3 construct indicate that in contrast with the presence of other introns, the size and the sequence content of the upstream exon has little influence on the level of intron retention. Consequently, it is very unlikely that the differences in splicing rates that we have observed were the result of secondary structural effects or steric constraints on the size of the upstream exon. To investigate whether an “enhancer” of
splicing could be present within intron 2, we inactivated both its donor and acceptor splice sites. Analysis of the level of intron 3 retention indicates that under these circumstances no increase in the splicing of intron 3 could be observed. Thus, these studies establish that the increase in the splicing rate of the downstream intron involves the splice sites of the upstream intron, and we conclude that there is cooperation between introns during pre-mRNA processing.

This cooperation between introns could be attributable to direct and indirect interactions between the splicing complexes acting on individual introns. One opportunity for indirect interactions is suggested by the observation that the components of the splicing machinery are not distributed uniformly throughout the nucleus but are clustered in “speckles” [Huang and Spector 1992]. This observation was made for small nuclear ribonucleoprotein particles (snRNPs) [Spector 1990] and some non-snRNP splicing factors such as SC35 [Fu and Maniatis 1990]. One interpretation is that these clusters represent the sites where splicing takes place. This is supported by the colocalization of pre-mRNA with these clusters [Carter et al. 1991; Huang and Spector 1991; Wang et al. 1991] and the observation that newly synthesized transcripts are localized on “tracks” [Lawrence et al. 1989] that come in close contact with these clusters [Huang and Spector 1991; Xing et al. 1993]. In this case, if an intron is the first one to be spliced out from a molecule, the corresponding apparent splicing rate will include the time required for the association with a cluster. Conversely, if other introns have been spliced out previously, no further localization of the transcript should be required. Accordingly, the presence in TNFβ transcripts of introns that are spliced before introns 2 or 3 should lead to an increase in their respective splicing rates. However, because the splicing of introns 1 and 2 occurs much more rapidly than that of intron 3 [Weil et al. 1990; and D. Weil, unpubl.], it is unlikely that kinetic considerations alone could account for our results on intron 3. More relevant would be a model in which intron 3 also constitutes an inefficient localization signal in comparison with introns 1 and 2. The localization of microinjected transcripts requires a functional intron [Wang et al. 1991]. In this case, not only the kinetics but also the efficiency of association with a cluster of splicing factor could be improved by the presence of upstream introns.

Alternatively, this cooperation could require direct interactions between splicing complexes during their assembly on neighboring introns. Because TNFβ expression begins with the synthesis of a complete primary transcript, it is possible that all of the introns are detected simultaneously. In this case, a positive interaction between neighboring pre-spliceosomes should lead to a truly cooperative spliceosome assembly that could impact on both the kinetics and the efficiency of splicing. One implication of this model is that interactions across the exons should take place at some point during spliceosome assembly. This is precisely what S. Berget and co-workers have put forward in their model of exon recognition for splice site selection [Robberson et al. 1990; Talerico and Berget 1990]. As discussed in the introductory section, this concept of exon recognition can account for a wealth of experimental data on the alteration of splicing patterns by mutations. Thus far, the issue of how exon recognition can coexist with spliceosome assembly on individual introns has not been addressed in detail. Exons could be first identified, enabling a stable assembly of complexes on the corresponding acceptor and donor sites, and then a reorganization of these complexes within each intron would lead to a functional spliceosome. Alternatively, exon and intron recognition could coexist, at least transiently, and create a chain of interactions across the introns and the exons. Our results favor the second type of model, as the presence of two introns upstream of intron 3 leads to a greater increase in splicing efficiency than the presence of only one. Another issue related to the concept of exon recognition is the identification of the sites associated with the first and the last exons. Our study bears on the first point and indicates that whatever the mechanism involved in the identification of the first donor site (e.g., an interaction with the 5' end of the transcript), it is less efficient than the presence of an upstream intron.

In mammalian genomes, many genes contain a large number of introns. The ability to identify correctly and efficiently the splice sites is therefore of prime importance for gene expression. It has already been pointed out that recognition of exons with a size limit on the order of 300 nucleotides was an efficient way to reduce the complexity of the sequences to be scanned. In this study we provide evidence that the efficiency of splicing is increased by cooperation between introns. Thus, interactions between introns can increase both the specificity and the efficiency of splicing. Moreover, we found that these interactions are not restricted to the nearest neighbors but can also involve more distant introns. In the case of TNFβ, a full-length primary transcript exists with the possibility that spliceosome assembly occurs on all of the introns at the same time. For larger genes, it is probable that splicing is, at least in part, cotranscriptional [Beyer and Osheim 1988] and, therefore, that not all of the introns are present on the same molecule. Cooperation could then take place either within independent domains containing a few introns or within a domain that slides along the transcript during transcription and at any time encompasses a few introns.

Materials and methods

Transfections

NIH-3T3 cells were maintained routinely at low density in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 5% fetal calf serum (FCS) [IBF Biotechnics, France]. For transfection, 6 × 10^5 cells were seeded on 85-mm dishes, the medium was renewed after 6 hr, and the transfection assay was performed 1 hr later with 10 μg of DNA per dish by a standard calcium phosphate procedure (Sambrook et al. 1989). After 16 hr, the medium was replaced with fresh medium supplemented with 10% FCS. RNAs were extracted 41 hr after the
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initiation of transcription unless otherwise indicated. To determine mRNA stability, cells were treated with 10 μg/ml of dactinomycin (Boehringer Mannheim, France) for the indicated period.

RNA extraction and analysis

Cellular RNAs were extracted by lysis in guanidinium thiocyanate and centrifugation over a cesium chloride cushion [Berger and Kimmel 1987]. Separation of the nuclear and cytoplasmic fractions was achieved as described previously [Weil et al. 1990] by lysis with NP-40 before the addition of guanidinium thiocyanate. On average, the cytoplasmic and nuclear fractions contained 80% and 20% of the cellular RNAs, respectively. When indicated, RNA samples were pretreated with DNase I (RNase-free DNase I, Promega Biotech, Madison, WI) for 30 min at 37°C (1 unit/4 μg sample). Poly(A) tail digestion was achieved by hybridization with an 18-mer oligo(dT) followed by digestion with RNase H (GIBCO BRL Life Technologies, France) as described previously [Weil et al. 1990].

For RNase mapping analysis, 4 μg of RNA was hybridized with 300 pg of RNA probe [labeled with [γ-32P]UTP to asp. act. of 6 x 107 cpm/μg], digested with RNases A and T1, and electrophoresed through 5% urea-polyacrylamide gels as described [Modjtabadi et al. 1992], except that hybridizations were carried out at 60°C. For densitometric analysis, films were preflashed with an Amersham Sensitize [Amersham, France] and scanned with a Chromoscan 3 densitometer [Joyce Loeb, U.K.] and the results were analyzed with CSC3 software.

For Northern blots, samples containing 4 μg of RNA were electrophoresed through 1.5% agarose-formaldehyde gels and transferred onto uncharged nylon membranes [Hybond N, Amersham, France] as described [Dautry et al. 1988]. Hybridizations with 32P-labeled RNA probes [specific activity 3 x 108 cpm/μg] were performed at 60°C in 50% formamide, 5 x SPE, 0.1% SDS, 1 x Denhardt's solution, and 0.1 mg/ml of salmon sperm DNA [Sambrook et al. 1989].

Expression vectors and probes

The pCM vector contains nucleotides 329-1345 of the simian CMV immediate early promoter [Jean et al. 1987] inserted at the SacI site of the plasmid Bluescript II SK (Stratagene Cloning Systems, La Jolla, CA) and a synthetic oligonucleotide containing the rabbit β-globin polyadenylation signal [Levitt et al. 1989] inserted at the KpnI site. The LTI 123 plasmid contains nucleotides 1203-2741 of the murine TNFβ gene [Semon et al. 1987], from exon 2 to exon 4, including introns 2 and 3. The β 2, β 3 protection contains 77 uridines—β 2, β 3 " 57 and β 2, β 3 " 17. Probe B contains nucleotides 1631-1967, from intron 1 to exon 3, including intron 2. The " 1, " 2 protection contains 41 uridines—" 1, " 2 " 36 and " 1, " 2 " 9 uridines for the larger fragments and 12 for the smaller protection. Probe C contains nucleotides 2222-2741, within exon 4. To analyze the expression of the deleted versions of LTI 3 [Fig. 10], a probe encompassing intron 3 and the beginning of exon 4 [nucleotides 1962-2341] was used [cf. probe C in Weil et al. 1990]. Probe Glo was derived from Gloc 1 and contains 6 nucleotides of transcribed plasmid sequences, as well as all the genomic β-globin sequences and 287 nucleotides of β-globin cDNA. The US-protected band contains 98 uridines and the S band 63.

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References

Beger, S.L. and A.L. Kimmel. 1987. Guide to molecular cloning techniques. Academic Press. New York.

Beyer, A.L. and Y.N. Osheim. 1988. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes & Dev. 2: 754-765.

Carter, K.C., K.L. Taneya, and J.B. Lawrence. 1991. Discrete nuclear domains of Poly(A)RNA and their relationship to the functional organization of the nucleus." J. Cell Biol. 115: 1191-1202.

Chu, C.-S., B.C. Trapnell, J.J. Murtagh, J. Moss, W. Dalemans, S. Jallat, A. Mercenier, A. Pavirani, J.P. Lecocq, G.R. Cutting, W.B. Guggino and R.G. Crystal. 1991. Variable deletion of exon 9 coding sequences in cystic fibrosis transmembrane conductance regulator gene mRNA transcripts in normal bronchial epithelium. EMBO J. 10: 1355-1363.

Cloutet d'Orval, B., Y. d'Aubenton Carafa, P. Sirand-Pugnet, M. Gallego, E. Brody, and J. Marie. 1991. RNA secondary structure repression of a muscle-specific exon in HeLa cell nuclear extracts. Science 252: 1823-1828.

Dautry, F., D. Weil, J. Yu, and A. Dautry-Varsat. 1988. Regulation of pim and myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. J. Biol. Chem. 263: 17615-17620.

Domenjoud, L., H. Gallinaro, L. Kister, S. Meyer, and M. Jacob. 1991. Identification of a specific exon sequence that is a cryptic 5’ splice site. Mol. Cell. Biol. 11: 4581-4590.

Efstratiadis, A., F.C. Kafatos, and T. Maniatis. 1977. The primary structure of rabbit β-globin mRNA determined from
Cloned DNA. *Cell* 10: 571–585.

Fu, X.-D. and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localizable to discrete regions in the nucleus. *Nature* 343: 437–441.

Hawkins, J.D. 1988. A survey of intron and exon lengths. *Nucleic Acids Res.* 16: 9893–9908.

Horowitz, J.M., D.W. Yandell, S.-H. Park, S. Canning, P. Whyte, K. Buchkovich, E. Harlow, R.A. Weinberg, and T.P. Dryja. 1989. Point mutational inactivation of the retinoblastoma antioncogene. *Science* 243: 937–940.

Huang, S. and D.L. Spector. 1991. Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes & Dev.* 5: 2288–2302.

——. 1992. Will the real splicing sites please light up? *Curr. Biol.* 2: 188–190.

Jeang, K.-T., D.R. Rawlins, P.J. Rosenfeld, J.H. Sher, T.J. Kelly, and G.S. Hayward. 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell* 57: 493–502.

Lawrence, J.B., R.H. Singer, and L.M. Marselle. 1989. Highly localized tracks of specific transcripts within interphase nuclei visualized by in situ hybridization. *Cell* 57: 493–502.

Levitt, N., D. Briggs, A. Gil, and N.J. Proudfoot. 1989. Definition of an efficient synthetic poly(A) site. *Genes & Dev.* 3: 1019–1025.

Mitchell, P.J., G. Urlaub, and L. Chasin. 1986. Spontaneous splicing mutations at the dihydrofolate reductase locus in Chinese hamster ovary cells. *Mol. Cell. Biol.* 6: 1926–1935.

Modititatedi, N., H. Haddada, T. Lamaronie, E. Lazari, C. Lavialle, and O. Brison. 1992. TGF-α production correlates with tumorigenicity in clones of the SW 613-S human colon carcinoma cell line. *Int. J. Cancer* 52: 483–490.

Nasim, F.-U., P.A. Spears, H.M. Hoffmann, H.-C. Kuo, and P.J. Grabowski. 1990. A sequential splicing mechanism promotes selection of an optional exon by repositioning a downstream 5’ splice site in preprotachykinin pre-mRNA. *Genes & Dev.* 4: 1157–1184.

Padgett, R.A., P.J. Grabowski, M.M. Konarska, S. Seiler, and P.A. Sharp. 1986. Splicing of messenger RNA precursors. *Ann. Rev. Biochem.* 55: 1119–1150.

Robberson, B.L., G.J. Cote, and S.M. Berget. 1990. Exon definition may facilitate splice site selection in RNAs with multiple exons. *Mol. Cell. Biol.* 10: 84–94.

Rohrbaugh, M.L., J.E. Johnson, M.D. James, and R.C. Hardison. 1985. Transcription unit of the rabbit β1 globin gene. *Mol. Cell. Biol.* 5: 147–160.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Semon, D., E. Kawashima, C.V. Jongeneel, A.N. Shakhov, and A. Nedospassov. 1987. Nucleotide sequence of the murine TNF locus, including the TNF-α (tumor necrosis factor and TNF-β [lymphotokinin] genes. *Nucleic Acids Res.* 15: 9083–9084.

Shaw, G. and R. Kamen. 1986. A conserved AU sequence from the 3’ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46: 659–667.

Smith, C.W.J., E.B. Porro, J.G. Patton, and B. Nadal-Ginard. 1989. Scanning from an independently specified branch point defines the 3’ splice site of mammalian introns. *Nature* 342: 243–247.

Spector, D.L. 1990. Higher order nuclear organization: Three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc. Natl. Acad. Sci.* 87: 147–151.

Tacke, R. and C. Goridis. 1991. Alternative splicing in the nuclear cell adhesion molecule pre-mRNA regulation of exon 18 skipping depends on the 5’ splice site. *Genes & Dev.* 5: 1416–1429.

Talerico, M. and S.M. Berget. 1990. Effect of 5’ splice site mutations on splicing of the preceding intron. *Mol. Cell. Biol.* 10: 6299–6305.

Tomizawa, J.-I. and T. Itoh. 1981. Plasmid ColE1 incompatibility determined by interaction of RNA 1 with primer transcript. *Proc. Natl. Acad. Sci.* 78: 6096–6100.

Wang, J., L.G. Cao, Y.L. Wang, and T. Pederson. 1991. Localization of pre-messenger RNA at discrete nuclear sites. *Proc. Natl. Acad. Sci.* 88: 7391–7395.

Watakabe, A., K. Inoue, H. Sakamoto, and Y. Shimura. 1989. A secondary structure at the 3’ splice site affects the in vitro splicing reaction of mouse immunoglobulin μ chain pre-mRNAs. *Nucleic Acids Res.* 17: 8159–8169.

Watakabe, A., K. Tanaka, and Y. Shimura. 1993. The role of exon sequences in splice site selection. *Genes & Dev.* 7: 407–418.

Weil, D. and F. Dautry. 1988. Induction of tumor necrosis factor-α and β interferon-γ mRNA by interleukin 2 in murine lymphocytic cell lines. *Oncogene Res.* 3: 409–414.

Weil, D., S. Brosset, and F. Dautry. 1990. RNA processing is a limiting step for murine tumor necrosis factor β expression in response to interleukin-2. *Mol. Cell. Biol.* 10: 5865–5875.

Wong, E.M. and B. Polisky. 1985. Alternative conformations of the ColE1 replication primer modulate its interaction with RNA I. *Cell* 42: 959–966.

Xing, Y., C.V. Johnson, P.R. Dobner, and J.B. Lawrence 1993. Higher level organization of individual gene transcription and RNA splicing. *Science* 259: 1326–1330.

Xu, R., J. Teng, and T.A. Cooper 1993. The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. *Mol. Cell. Biol.* 13: 3660–3674.

Zhuang, Y. and A.M. Weiner. 1986. A compensatory base change in U1 snRNA suppresses a 5’ splice site mutation. *Cell* 46: 827–835.

**Cooperation between introns**
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