Regulation of pre-mRNA processing by *src*

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Background: Changes in gene expression in response to external signals provide a key mechanism for the regulation of higher eukaryotic cell functions. The importance of transcriptional control in the response of cells to growth factors and cytokines has been extensively documented, but gene expression has also been shown to be controlled at other levels, such as the stability of mRNA in the cytoplasm, its localization and translation. By contrast to transcriptional control, little is known of the contribution of pre-mRNA nuclear processing to the regulation of gene expression, as most of our knowledge of pre-mRNA processing *in vivo* is indirect, being inferred from comparisons of transcription rates and levels of mRNA accumulation.

Results: In this study, we have used as a model the well-characterized maturation pathway of transcripts of the cytokine, tumour necrosis factor β (TNFβ). We have used the murine TNFβ gene as a reporter for pre-mRNA processing, using a co-transfection approach to investigate whether overproduction of proteins involved in signal transduction influences the processing of TNFβ transcripts. Although transfection of both activated *ras* and *src* genes led to an increase in RNA accumulation in the nuclear and cytoplasmic compartments, as expected from their transactivation of the TNFβ expression vector, only *src* induced a modification of RNA processing. Comparison of several modes of *src* activation indicated that two distinct effects of *src* on pre-mRNA processing can be uncoupled: one involves slowing down splicing and the other allows the export of partially spliced transcripts. These effects can be observed not only on the three introns of TNFβ but also on transcripts from a β globin expression vector .

Discussion: We have characterized how the processing of transcripts of TNFβ and β globin is regulated by the signal transduction pathway that includes the Src protein, establishing that external signals have the capacity to regulate gene expression at a post-transcriptional level within the nucleus. Src seems to act on a general mechanism of splicing and/or mRNA transport, but its biologically relevant targets are likely to be restricted to genes for which either alternative processing pathways are in competition, or the kinetics of splicing is critical. This regulation could reflect a modulation by Src of the activity of components of the splicing and transport machineries, but could also involve RNA-binding proteins, which have been shown to interact with Src.

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Background

In higher eukaryotes, the genesis of a functional mRNA requires a complex set of post-transcriptional processes, and it has long been suggested that each of these steps could be involved in the regulation of gene expression [1]. The stability of mRNA in the cytoplasm was the first level of post-transcriptional regulation to be studied in detail, and its importance in the control of gene expression, in particular in response to external stimuli, is well documented [2–4]. More recently, control by growth factors of the localization of mRNA for β actin has been reported [5,6], suggesting the possibility of another cytoplasmic level of regulation. By contrast, little is known about the involvement of nuclear pre-mRNA processing, although it could impinge on three major aspects of gene expression: the production of different protein isoforms, through alternative splicing [7]; the kinetics of expression, by altering the duration of processing [8]; and the level of final product, through the efficiency of processing [9].

A major limitation in evaluating the importance of nuclear pre-mRNA processing is the difficulty of obtaining relevant experimental data. Indeed, with the possible exception of alternative splicing, the products of which can be studied in the cytoplasm, most of our knowledge on nuclear processing is indirect. Usually, the contribution of nuclear processing is estimated by comparing the transcription rate, as assessed by nuclear run-on, with the rate of cytoplasmic mRNA accumulation, taking into account mRNA half-life. Although many studies have concluded on the basis of this approach that nuclear processing plays an important role in controlling gene expression in response to external signals [10–12], the precise level of regulation cannot usually be specified because of the indirect nature of the analysis.

One possible approach to the study of nuclear pre-mRNA processing is to follow the incorporation of labelled nucleotides into different RNA species [8,9]. Although this technique does provide true kinetic information, it is limited in both time resolution and sensitivity. We have previously shown that transcripts of the murine gene for the cytokine, tumour necrosis factor β (TNFβ), provide an interesting alternative to the labelling approach, because of the possibility of measuring the accumulation in the nucleus of processing intermediates [13]. The product-to-precursor ratios can then be used to estimate pseudo-first-order reaction rates, provided firstly that a steady state has been reached, and secondly that all the quantitatively important reactions are taken into account.

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We have documented, both in lymphocytes that express the endogenous TNFβ gene [13] and in NIH 3T3 fibroblasts transfected with a TNFβ expression vector [14], that a majority of TNFβ transcripts are processed according to a simple maturation pathway (Fig. 1a), which begins with a polyadenylated primary transcript and involves ordered splicing of the three introns. In these studies, we found no indication of significant nuclear degradation of the TNFβ transcripts; for instance, the total number of nuclear transcripts remains constant during a chase with the transcriptional inhibitor actinomycin D ([13]; and our unpublished observations).

The TNFβ mRNA maturation pathway is therefore simple, with only one branch-point, at the level of intron 3; this provides easy access to measures of the relative efficiency of the splicing and transport pathways by looking at the level of retention of intron 3 in the cytoplasm. In this study we have used the TNFβ gene as a reporter to investigate whether signal transduction could regulate pre-mRNA processing. We find that *src* can modify processing rates, in particular by altering the rates of splicing and of the export of partially spliced transcripts. Thus there are multiple levels of post-transcriptional control of gene expression.

Results

Experimental approach

As a detailed analysis of pre-mRNA processing can only be performed for a system at equilibrium, we have used a co-transfection assay to investigate the interactions between pre-mRNA processing and signal transduction. In this approach, a TNFβ reporter construct was introduced into NIH 3T3 cells along with expression vectors for *src* or *ras* and, at 41 hours after transfection, nuclear and cytoplasmic RNAs were extracted and analysed by northern hybridisation and RNase mapping (see Materials and methods). The structure of the TNFβ construct is presented in Figure 1b; it encodes the simian cytomegalovirus (CMV) immediate early promoter [15] and a β globin polyadenylation signal [16]. This TNFβ construct differs from that previously used [14] in that it has a longer 3′ untranslated region which includes the AU-rich destabilization signal. Inclusion of this sequence leads to a shorter half-life for the cytoplasmic species ($t_{½}$ = 100 minutes for both C0 and C1, instead of about 10 hours, for the previously used construct); and the lower level of cytoplasmic RNA accumulation renders determination of the reaction rates less sensitive to cytoplasmic contamination of the nuclear fractions.

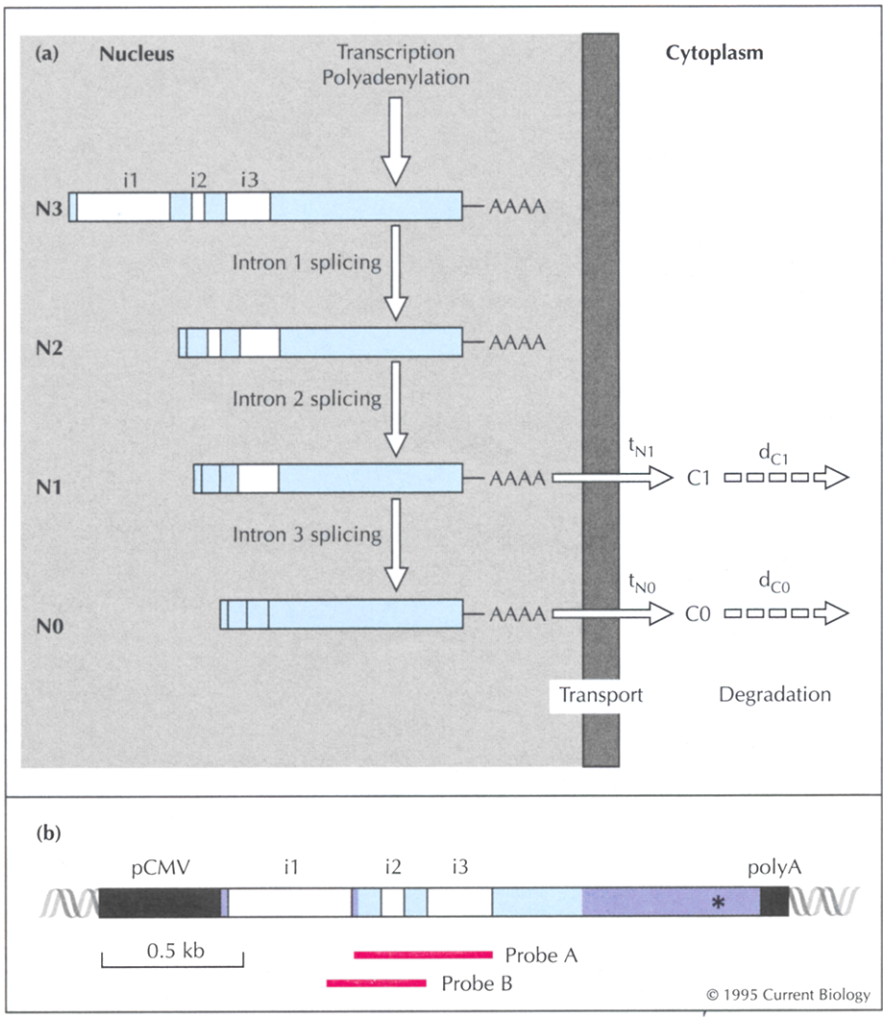

Fig. 1. Expression of the murine TNFβ gene. **(a)** Schematic representation of the maturation pathway of the TNFβ transcripts. This scheme was obtained by combining the results of structural studies on the nuclear and cytoplasmic RNA species with the kinetic data obtained using actinomycin D or following induction of the TNFβ promoter by interleukin 2. The same maturation pathway was observed for transcripts of the endogenous TNFβ gene in CTLL-2 cells or following the transfection of a genomic TNFβ expression vector in NIH 3T3 cells. **(b)** Representation of the TNFβ expression vector used in this study. The simian CMV promoter and a polyadenylation signal derived from the rabbit β globin gene are indicated by black boxes. The non-coding regions of the exons within the TNFβ insert are represented by mauve boxes and the introns by white boxes; the coding regions are shaded light blue; * indicates the location of an AU-type destabilization signal.

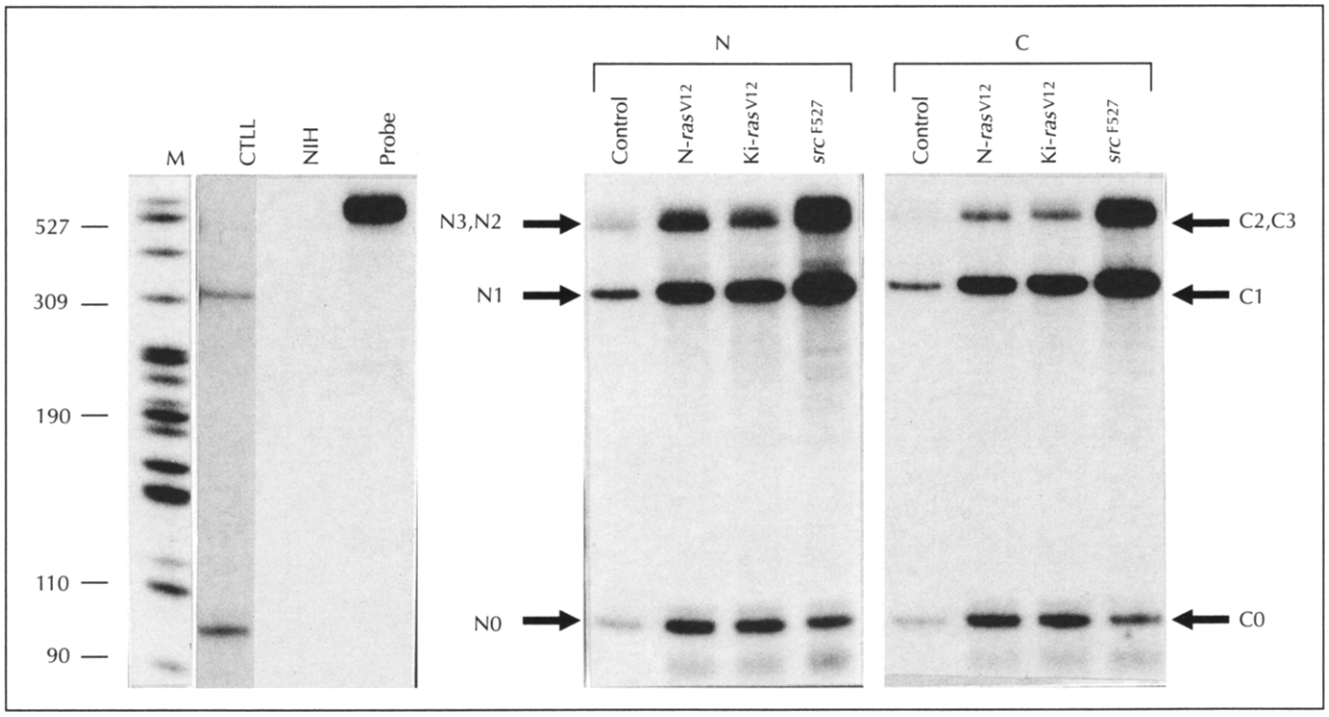

Fig. 2. RNase mapping of TNFβ transcripts in the presence of an activated *ras* or *src* gene. RNase mapping was carried out on 4 μg nuclear (N) or cytoplasmic (C) RNA isolated from a co-transfection of NIH 3T3 cells with the control vector (control), an N-ras^{V12}, Ki-ras^{V12} or a src^{F527} expression vector. RNAs were analyzed with probe A (shown in Fig. 1b) which spans introns 2 and 3. The different species identified according to the maturation pathway shown in Fig. 1a are indicated with arrows. In the left panel, samples from CTLL-2 cells (cytoplasmic RNA) and untransfected NIH 3T3 cells (total RNA) were included as controls. The molecular weight markers were the end-labelled *MspI* fragments of pBR322 DNA.

Irrespective of the expression level, this construct leads to higher levels of retention of intron 3 than the previous construct, reflecting the role in *cis* of the sequences present in the transcript (P.G. and E.D., unpublished observations).

In Figure 2 we present an RNase mapping analysis (using probe A; see Fig. 1b) of a transfection experiment performed using an expression vector containing either no cDNA (control lane), human cDNAs encoding N-Ras and Kirsten-Ras carrying the Val 12 mutation (lanes labelled N-ras^{V12} and Ki-ras^{V12}, respectively), or a chicken *src* cDNA encoding a Src protein carrying the Phe 527 mutation [17] (lane labelled src^{F527}). The presence of an activated *ras* or *src* gene induced increased accumulation of TNFβ transcripts in both the nuclear and cytoplasmic compartments. Such a result was expected, as these genes are known to increase the activity of the transcription factor NF-κB in transfected cells [18,19], and so to transactivate the CMV promoter [20]. Quantification of the signals indicated that the presence of N-ras^{V12} or Ki-ras^{V12} induced a three-fold increase in the accumulation of all the RNA species compared to their levels both in the nucleus and in the cytoplasm in the control; this is consistent with an increase in the synthesis of the primary transcript without any modification of its processing. This result was confirmed by two other separate transfection experiments (data not shown). By contrast, src^{F527} altered the RNA profile in the nucleus as well as in the cytoplasm, with an increased accumulation of partially spliced transcripts in both compartments. Indeed, in the presence of src^{F527}, species containing several introns accumulate in the cytoplasm. Analysis by northern blot and RNase mapping (data not shown) confirmed that these cytoplasmic RNAs were identical in structure to the N2 and N3 nuclear precursors, so they are designated C2 and C3. Thus, although both *ras* and *src* genes can induce an increase in the expression of the TNFβ reporter construct, only *src* induces a change in the pattern of RNA accumulation in the nucleus as well as in the cytoplasm.

Processing of intron 3 of the TNFβ transcript in the presence of *src* and the polyoma virus early region, *Py*

To investigate the effect of *src* on the processing of the TNFβ transcripts, we performed a set of four independent transfection experiments with: the control vector; the polyoma virus early region, *Py*, which is a known activator of the endogenous Src protein [21,22]; an expression vector carrying a normal *src* cDNA (*src*); or one encoding the Phe 527 Src mutant (src^{F527}). These experiments were first analyzed by RNase mapping with probe A (see Fig. 1b), which spans exon 2, intron 2, exon 3 and intron 3. The relative accumulation of the different transcripts (mean values ± standard error of means, SEM) are presented Table 1; these numbers were derived from the quantification of the radioactivity present in each protected fragment, taking into account the number of

Table 1. Quantification of nuclear and cytoplasmic TNFβ transcript accumulation in *Py* and *src* transfection experiments.

	Transcripts					
	Nuclear			Cytoplasmic		
	N3 + N2	N1	N0	C3 + C2	C1	C0
Control	25 ± 3	100	126 ± 14	8 ± 1	50 ± 10	84 ± 22
Py	28 ± 6	100	55 ± 15	4 ± 1.5	30 ± 7	39 ± 9
src	30 ± 4	100	46 ± 5	10 ± 4.5	41 ± 9	27 ± 5.5
***src*^{F527}**	27 ± 2	100	33 ± 4	15 ± 3	52 ± 11	18 ± 4

Results are presented as means ± SEM for a set of four independent experiments. Quantification was derived from RNase mapping with probe A (see Fig. 1) and takes into account the number of labelled residues in each protected fragment. To facilitate comparison between effector genes, the accumulation of transcript has been normalized to 100 for N1 in each set of transfections; the relative levels of expression are indicated in Fig. 3b.

labelled residues (see Materials and methods). To facilitate comparison of the transcript profiles, each set of values was normalized with respect to N1, which is the most abundant species in the *Py*, *src* and *src*^{F527} experiments. Note that in Table 1, the values given correspond to equal amounts of nuclear and cytoplasmic RNAs, but that in the subsequent analysis shown in Figure 3, the partition of RNAs between the nucleus and cytoplasm was taken into account (Figs 3c and 3d) as was the level of expression (Fig. 3b). Two major modifications of the patterns of transcript accumulation are apparent in the results shown in Table 1. First, in the cytoplasm, a higher proportion of intron-containing transcripts accumulate in the presence of *src* and *src*^{F527} and, to a lesser extent, in the presence of *Py*. Secondly, in the nucleus, a marked decrease in the accumulation of fully spliced transcripts can be observed in the presence of *Py*, *src* and *src*^{F527}. These observations suggest that *Py* and *src* have differential effects on nuclear and cytoplasmic accumulation of RNA.

If we look first at the cytoplasmic compartment, the relative abundance of transcripts containing intron 3 can be described by the level of intron 3 retention — C1 / (C0 + C1) [14] — and the corresponding values (means ± SEM) are presented in Figure 3a. For simplicity, we do not take into account at this stage the presence of

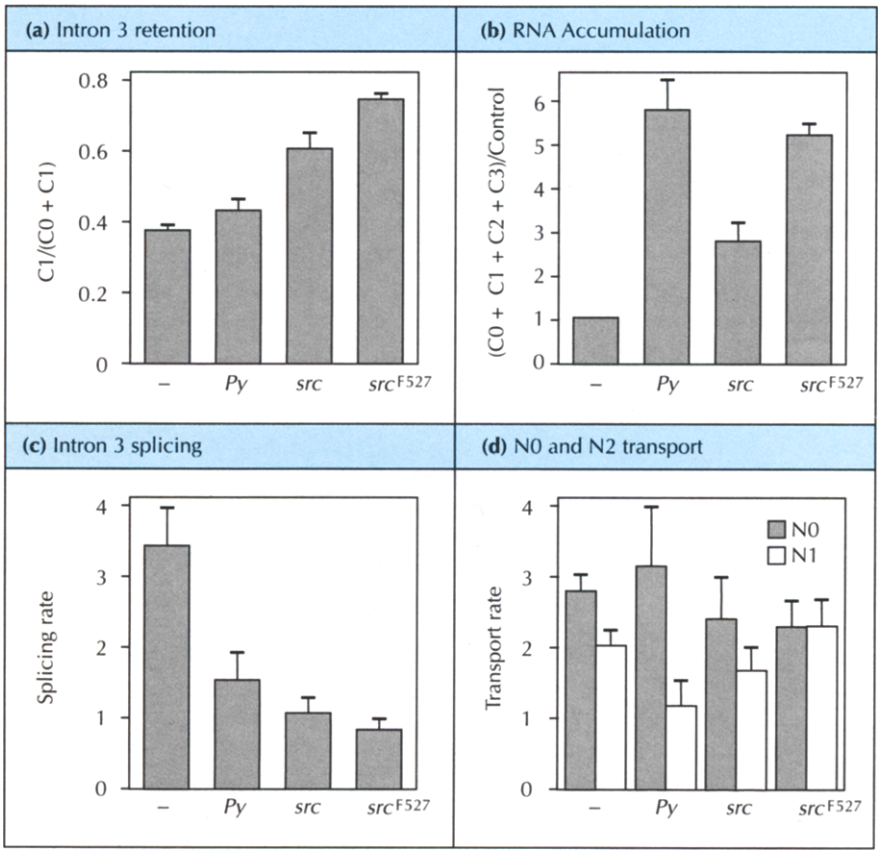

Fig. 3. Quantitative analysis of TNFβ expression in the presence of *Py*, *src* or *src*^{F527}. Nuclear and cytoplasmic RNAs from a set of four independent experiments were analyzed by RNase mapping to assess the accumulation of the different TNFβ transcripts. The amount of radioactivity was quantified on a Fuji Bio-imaging Analyzer and the values corrected for the number of labelled residues in each protected fragment. Results are presented as mean values ± SEM for the transfections with the control vector (–), *Py*, *src* or *src*^{F527}. **(a)** Retention of intron 3 in the cytoplasm is expressed as C1 divided by C0 + C1. **(b)** Cytoplasmic RNA accumulation (C0 + C1 + C2 + C3); for each transfection, the control value was taken to be an expression level of 1. **(c)** The splicing rate of intron 3 as determined by the value of C0 / N1. **(d)** Transport rates of the N0 and N1 transcripts as determined by the values C0 / N0 and C1 / N1, respectively.

C3 and C2, which accumulate to significant levels only in the presence of *src* and *src*^{F527} (see below). As already observed in the experiment shown in Figure 2, *src*^{F527} induced a major increase in intron 3 retention (from 38 to 74 %). By contrast, *Py* had only a small effect on intron 3 retention (44 %), and a normal *src* cDNA yielded an intermediate response (61 %). Co-transfection with either *Py* or *src*^{F527} led to an increase in RNA accumulation, as illustrated in Figure 3b by the relative levels of accumulation of the cytoplasmic RNAs (C0 + C1 + C2 + C3). In addition to the transactivation of the CMV promoter, changes in mRNA stability could also contribute to RNA accumulation, but experiments with the transcription blocker actinomycin D indicated that none of *Py*, *src*, or *src*^{F527} had a significant effect on the half-life of C0 or C1 (data not shown). This precluded an effect of *src*^{F527} on mRNA stability contributing significantly to the level of intron retention, as a five- fold difference in the relative stability of C0 and C1 would be required for a change from 38 % to 74 % retention. In conclusion, activation of the Src signalling pathway by overexpression of either a normal or a mutated *src* cDNA leads to an increase in intron 3 retention which is due to a modification of pre-mRNA processing .

To investigate which aspect of pre-mRNA processing was modified in the presence of *src*, we used the accumulation of the nuclear precursors to quantitate the reaction rates (see Materials and methods). The apparent splicing rate of intron 3 (s_{i3}) can be derived from the product-to- precursor ratio according to the equation C0 / N1 = s_{i3}/d_{C0}, where d_{C0} is the degradation rate of the C0 species. Figure 3c presents the values of s_{i3}/d_{C0}. As we have found no evidence that the degradation rates of C0 and C1 vary in the presence of *src*, we will treat s_{i3}/d_{C0} as a dimensionless splicing rate. Note that to derive the values shown in Figure 3, the level of intron retention, or the reaction rates, were first determined for each independent experiment and then averaged. Although the mean values obtained are comparable to those that could be derived from the results shown in Table 1 (taking into account a nucleocytoplasmic ratio of 1:4), the standard errors shown in Figure 3 are smaller, as expected for non-independent variables. The presence of *src* or *src*^{F527} induced a three- to four-fold reduction in s_{i3}, whereas *Py* also showed a significant effect on s_{i3}, with a 2.5-fold reduction. Consequently, the lack of effect of *Py* on intron 3 retention implied that another aspect of pre-mRNA processing was differentially affected by *Py* and *src*^{F527}.

The data shown in Figure 3d present the transport rates for the mature and the intron-3-containing messages, given by t_{N0} / d_{C0} = C0 / N0 and t_{N1} / d_{C1} = C1 / N1, respectively. In the control experiments, the apparent transport rates for N0 and N1 were almost equal. In the presence of *Py*, the relative efficiency of transport of N0 and N1 was increased two-fold in favour of the mature RNA, whereas in the presence of *src*^{F527}, both species were transported with the same apparent rates. In summary, although the three effectors — *Py*, *src* and *src*^{F527} — lead to a reduction of the intron 3 splicing rate, their effect on the retention of intron 3 in the cytoplasm depends upon the relative transport rates of N0 and N1. Thus, the 2.5-fold slowing down of splicing that is induced by *Py* is almost entirely compensated for by a 2-fold increase in the transport rate of N0 with respect to that of N1.

Processing of introns 1 and 2 in the presence of *src* and *Py*

We analyzed the accumulation of the N3, N2, C3 and C2 species in order to investigate the processing of introns 1 and 2 of the TNFβ transcript. In order to determine the relative contributions of N3 and N2, C3 and C2 to the accumulation of transcripts detected by probe A, we performed an RNase mapping with probe B, which overlaps intron 1, exon 2 and intron 2 (see Fig. 1b). The mean values of N3 / N2 and C3 / C2 are shown in Table 2. The splicing rates for introns 1 and 2 (see Materials and methods for their derivation) are presented in Figure 4a for a set of three experiments. In the control experiments, the values of s_{i1} and s_{i2} (42 and 50, respectively) are about 15-fold higher than those for s_{i3} (Fig. 3c), in agreement with the kinetic data which indicate that the splicing of intron 3 occurs much more slowly than that of introns 1 or 2 (D.W. and F.D., unpublished observations; see [13] for the kinetics of splicing in the presence of actinomycin D).

Co-transfection with *Py*, *src* or *src*^{F527} led to an approximately three-fold reduction of both s_{i1} and s_{i2}, indicating that splicing of the three introns present within the TNFβ transcript was affected to a similar extent by the presence of *Py* or *src*. In the presence of *src*^{F527}, a significant amount of transcripts containing multiple introns accumulated in the cytoplasm. This export of 'higher order' precursors was specific to the *src* and *src*^{F527} transfections (increasing from a base level of 5 % up to 13 % and 17 %, respectively) and was not observed in the presence of *Py* (5 %). The basis of this differential effect of *Py* and *src* on the retention of these introns is analyzed in Figure 4b, which presents the ratios of the transport rates for the nuclear precursors (N3, N2 and N1) to that of the fully spliced transcript (N0). Although *Py* decreased

Table 2. Quantification of accumulation of introns 1 and 2 of TNFβ in *Py* and *src* transfection experiments.

	N3 / N2	C3 /C2
Control	1.22 ± 0.06	0.89 ± 0.05
Py	1.63 ± 0.10	1.04 ± 0.08
src	1.13 ± 0.07	0.92 ± 0.06
***src*^{F527}**	1.22 ± 0.05	1.38 ± 0.11

To supplement the analysis shown in Table 1, which did not consider intron 1, RNase mapping experiments were done using probe B, which spans introns 1 and 2 (see Fig. 1). The ratios N3 / N2 and C3 /C2 are presented as means ± SEM and were derived as indicated in Materials and methods.

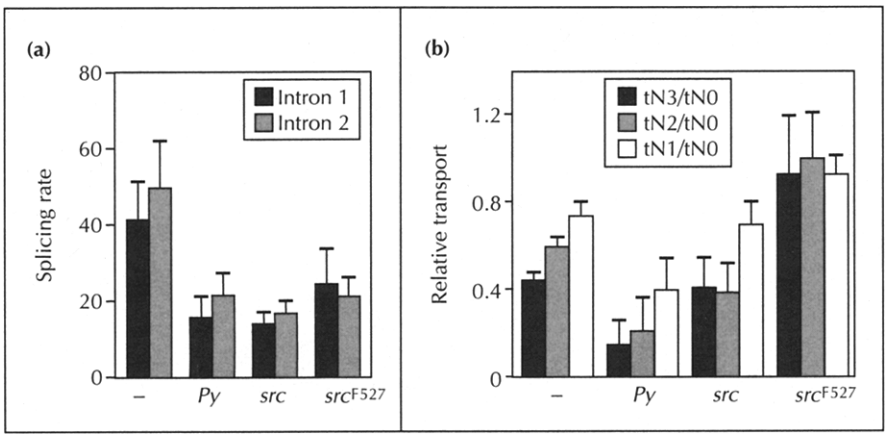

Fig. 4. Quantitative analysis of the processing of introns 1 and 2 of TNFβ in the presence of *Py*, *src* or src^{F527}. The accumulation of TNFβ transcripts was determined as in Fig. 3 and the results (mean ± SEM) are presented for a set of three transfection experiments with the control vector (–), *Py*, *src* and src^{F527}. **(a)** Splicing rates of introns 1 and 2, as determined by the value (CO + C1 + C2) / N3 and (C0 + C1) / N2, respectively. **(b)** Ratios of the transport rates of N1, N2 and N3 to that of N0.

the export of the three presursors and maintained the hierarchy of the transport rates ($t_{N3} < t_{N2} < t_{N1} < t_{N0}$), src^{F527} led to equivalent transport rates for all the transcripts ($t_{N3} = t_{N2} = t_{N1} = t_{N0}$). Therefore, in the presence of *Py*, *src* or src^{F527}, the processing of introns 1 and 2 closely follows that of intron 3 in terms of both splicing and transport rates.

Processing of β globin transcripts in the presence of *src* and *Py*

The results discussed above indicated that the actions of *Py*, *src* and src^{F527} were not restricted to the processing of the TNFβ intron 3. To assess whether they could be observed during the processing of an unrelated transcript, we replaced the TNFβ insert in the reporter construct with a genomic rabbit β globin insert. As can be seen from the northern blot analysis following transfection experiments (Fig. 5), β globin precursors were virtually undetectable under standard conditions but accumulated in the nucleus and in the cytoplasm in the presence of *src* or src^{F527}. Quantification by RNase mapping following a set of three independent experiments indicated that the proportion of cytoplasmic transcripts containing intron 2 increased from a base level of 0.4 % to 1.6 % and 2.5 % in the presence of *src* and src^{F527}, respectively.

Using the same derivation as used for the TNFβ transcript, splicing and transport rates can be derived for β globin from the results of RNase mapping experiments. Figure 6a presents the splicing rate of the β globin intron 2 in these experiments: *Py* had almost no effect, whereas *src* and src^{F527} induced a two- to three-fold reduction of this splicing rate. Figure 6b presents the ratio of the transport rates for transcripts containing intron 2 and mature transcripts: t_{i2+} / t_{i2-}. A pattern similar to that seen in Figure 3d can be observed, with the highest relative transport of precursors in the presence of src^{F527} and the lowest in the presence of *Py*. Thus, the action of *src* on pre-mRNA processing is not restricted to TNFβ transcripts but can be also be observed with an efficiently spliced reporter gene, such as β globin. In the case of β globin, however, the absolute levels of intron retention remained very low, indicating that, although src^{F527} might be acting on the general processing machinery, the consequences for gene expression depend upon the specific features of each gene.

Discussion

We describe here an increased accumulation of partially spliced TNFβ transcripts in the presence of an activated *src* gene. According to our simple description of the reaction rates, which is based on the product-to-precursor ratios, the accumulation of nuclear precursors is due to a slowing down of the splicing reactions for each of the three TNFβ introns. As one of the consequences of activating the Src signal transduction pathway is transactivation of the CMV promoter of the reporter gene, saturation of the splicing machinery could be in part responsible for this accumulation of precursors. Indeed,

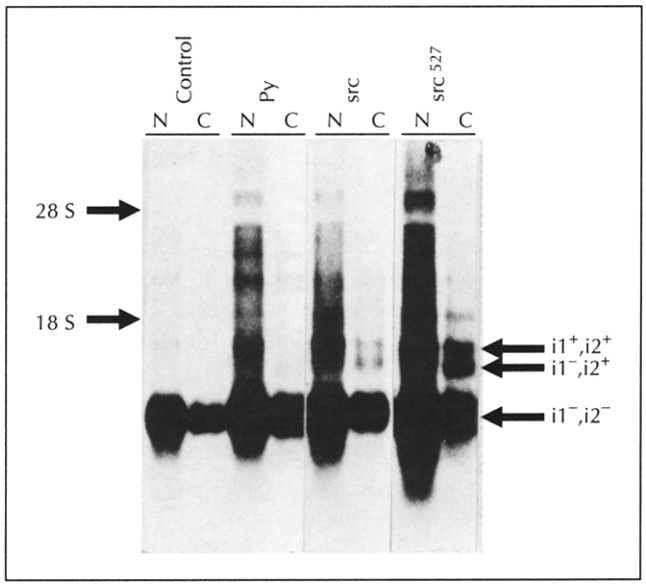

Fig. 5. Northern blot analysis of β globin expression in the presence of *Py*, *src* or src^{F527}. RNAs from an experiment in which a β globin expression vector was co-transfected with either a control vector, *Py*, *src* or src^{F527}, were analyzed by northern blot; 4 μg nuclear (N) or cytoplasmic (C) RNAs were electrophoresed through a 1.5 % agarose–formaldehyde gel, transfered onto an uncharged nylon membrane and hybridized with β globin cDNA. The migration of the 28 S and 18 S rRNAs is indicated, as well as that of the different β globin transcripts. The low abundance $i1^+,i2^-$ species cannot be distinguished from the fully spliced species because of the small size of intron 1 (126 nucleotides). The blot was overexposed to facilitate the visualization of the intron-containing species.

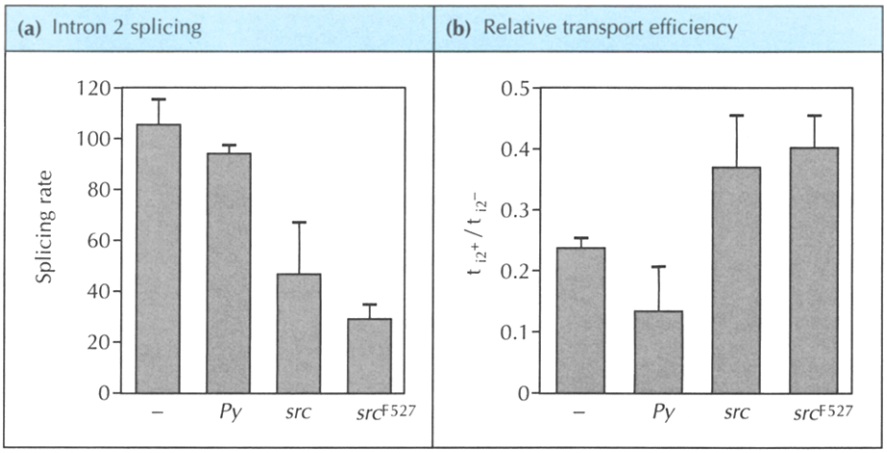

Fig. 6. Quantitative analysis of β globin expression in the presence of *Py*, *src* or src^{F527}. Nuclear and cytoplasmic RNAs from a set of three independent experiments were analyzed by RNase mapping for the accumulation of the different β globin transcripts as shown in Fig. 3. Results are presented as mean ± SEM for a set of three transfections experiments with the control vector (–), *Py*, *src* or src^{F527}. **(a)** The splicing rate of intron 2 as determined by $C(i1^-,i2^-) / N(i1^-,i2^+)$. **(b)** The ratio of transport rates for the transcripts containing intron 2 and the fully spliced versions.

titration of some components of the splicing machinery has been invoked to explain the transition from the early to the late splicing pattern of adenoviral transcripts [23].

Two observations, however, indicate that saturation of the splicing machinery is unlikely to play a major role in the effects of *src*. Firstly, ras^{V12} and src^{F527} induce comparable accumulations of nuclear TNFβ transcripts, but only src^{F527} has an effect on splicing. Secondly, there is no correlation between the level of accumulation of transcripts and splicing rates in the presence of *Py*, *src* or src^{F527} (Figs 3b and 3c). In particular, overexpression of a normal *src* cDNA has a strong effect on splicing, although it induces the smallest increase in RNA accumulation. Finally, the fact that ras^{V12}, src^{F527} and *Py* induce comparable levels of transactivation of the TNFβ construct but exert different effects on splicing and transport establishes that the regulation of pre-mRNA processing can be uncoupled from that of transcription. Furthermore, there is no correlation between the transforming potential of the genes and their activity on pre-mRNA processing, activated *ras* genes being potent transforming genes in NIH 3T3 cells [24] whereas the overexpression of a normal chicken *src* cDNA has almost no transforming capacity [17]. Thus, activation of the Src signalling pathway leads to a specific modification of the processing of the TNFβ transcripts that does not simply reflect the level of expression or an early event in cellular transformation. This last point also suggests that the cytotoxic activity of TNFβ towards transformed cells is not required for the effect on pre-mRNA processing that is observed in the presence of *src*. This was further confirmed by the use of the 'neutral' reporter gene, β globin.

The difference in the levels of intron retention in the presence of *Py* and src^{F527} is due to a differential effect on the transport rates of N0 and N1. Our analysis of product-to-precursor ratios defines apparent reaction rates which can include other steps besides the reaction itself, such as the proper localization and formation of the splicing committment complex. Consequently, variations in reactions rates could reflect changes in these other processes, as well as in the reaction itself. This issue is particularly important for the N1 transport rate, as N1 transcripts are the precursors of both N0 and C1 and, in this analysis, we cannot determine whether some of these transcripts are already committed to either transport or splicing. If we assume that, once they are committed to export, N1 and N0 are transported by the same machinery and with the same kinetics, then relative changes in t_{N1} and t_{N0} are most easily interpreted as reflecting differences in the proportion of N1 transcripts that are available for export.

It is often considered that the export of transcripts from the nucleus is a default pathway (albeit an active transport process) and that any transcript not associated with a nuclear structure will automatically be transported. In this model of the nucleus, the main factor underlying nuclear retention of pre-mRNA is the splicing machinery itself, which limits the export of partially spliced transcripts not only by removing the introns but also by masking them from the transport machinery [25,26]. Our observations, including the export of transcripts that contain multiple introns, suggest that src^{F527} can either prevent formation of the comittment complex, or facilitate the dissociation of transcripts from the splicing machinery.

In contrast to *src*, *Py* induces the accumulation of precursors that are not efficiently transported, suggesting that splicing is slowed down at a post-committment step. Thus, in the model of the nucleus in which splicing is 'dominant' over transport, regulation of splicing at different steps by *Py* and *src* is sufficient to account for their differential effects on pre-mRNA processing. There are, however, indications from viral systems that the transport of RNA from the nucleus can be regulated by both general and specific mechanisms [27–29], and that transport can be dominant over splicing [29]. If we assume that similar regulations also operate in uninfected cells, we should consider alternative models in which *Py* and *src* could differentially regulate the transport machinery.

Signal transduction by membrane-associated tyrosine kinases, such as Src, generates multiple signals, many of which can reach the nucleus. Accordingly, several models could account for the regulation of pre-mRNA processing by Src. Firstly, the genes encoding some of the

constituents of the processing machinery could be among the targets of transcriptional regulation by Src. It has recently been established, by co-transfection experiments, that the relative expression levels of the splicing factor ASF/SF2 and the hnRNP A1 can modulate the alternative processing of several transcripts [30]. Secondly, the activity of the processing machinery could be controlled at a post-transcriptional level; splicing, for example, is dependent upon a cycle of phosphorylation and dephosphorylation [31,32], and the enzymatic activities involved could be regulated by the Src signalling pathway.

In the case of Src, a third and even more direct regulation of pre-mRNA processing could be operating, as suggested by the observation of an interaction between the Src homology 3 (SH3) domain of Src and a set of RNA-binding proteins [33–35]. The p62 protein was characterized initially as a major substrate of the tyrosine kinase v–Src and as a protein that interacts with the Ras GTPase activating protein (GAP) [36]; a related protein, p68, is phosphorylated by c-Src during mitosis [33,34]. These proteins belong to the hnRNP K family of RNA-binding proteins [37] and they bind to RNA *in vitro* [36], but their function is at present unknown. The hnRNP K protein itself is also able to interact with Src [33–35], although in this case it is unclear whether or not the interaction takes place *in vivo*. However, the presence of hnRNP K in both the cytoplasm and the nucleus [38], the shuttling of some hnRNP proteins between nucleus and cytoplasm [39], and the translocation of Src to a perinuclear location following activation by growth factors [40], could provide the opportunity for such an interaction. These observations raise the possibility of a direct link between Src and mRNA metabolism, and we are currently investigating this possibility.

The observation that *Py* does not induce the same response as src^{F527} was somewhat unexpected, as the polyoma virus middle T antigen activates members of the Src family of kinases [21,22]. This discrepancy could reflect a threshold effect in the response to Src activation, as a comparison of the results observed in the presence of *src* and src^{F527} cDNAs suggests that efficient export of partially spliced transcripts is associated with maximal activation of Src. Although the fact that *Py* induces the highest level of RNA accumulation testifies to the efficiency of the co-transfection, it is also clear that *Py* can generate other signals beside the activation of the endogenous Src protein. For instance, both the polyoma small t antigen and middle T–Src complexes associate with protein phosphatase 2A [41]. Remarkably, in splicing reactions *in vitro*, inhibition of phosphatase 2A leads to a blockade of the second step of splicing [42], reminiscent of the slowing down of splicing in the absence of export of partially spliced transcripts that is observed in the presence of *Py*.

As the processing of both TNFβ and β globin transcripts is affected by the presence of Src, it is possible that Src acts on some general aspects of pre-mRNA processing. In order to determine the spectrum of genes that are post-transcriptional targets of the Src signalling pathway, it will be necessary to delineate more precisely the range of processes that can be regulated by Src. The examples of TNFβ and β globin suggest that Src will have a minimal impact on genes such as β globin which are processed efficiently and constitutively (without alternative processing pathways). By contrast, Src induces a major shift in the proportion of alternatively processed messages for TNFβ. Alternative splicing often generates functionally different protein isoforms [43]; intron retention, because of the frequent occurence of stop codons within introns, tends to yield partly disabled proteins which, in some cases, can act as dominant-negative inhibitory mutants [44]. If we assume that the modifications of splicing and transport rates that are observed in co-transfection experiments also apply to the transcripts of the endogenous TNFβ gene in T lymphocytes, the level of intron 3 retention would change from 15 % to 45 %. Such a shift from a low level of intron retention to levels of the order of 50 % could be critical for some cellular phenomena. More generally, there is growing awareness that modification of the processing machinery could contribute in an essential manner to pathophysiological situations such as tumorigenesis [12,45,46]. Further studies will be required in order to evaluate the contribution to the response of mammalian cells to growth factors and tumorigenesis of the link between Src activation and pre-mRNA processing that we have described.

Conclusion

We report that, in co-transfection experiments, a *src* but not a *ras* gene can modify the splicing rates of TNFβ and β globin transcripts. Moreover, the differential effects of polyoma virus early region and *src* on the export of partially spliced transcripts indicate the existence of multiple levels of regulation. Thus, we have established that specific signal transduction pathways can control nuclear pre-mRNA processing. Further studies will be required in order to identify biologically relevant target genes that are regulated in this way. The availability of an experimental model will make it possible to analyse the nature of such regulation and, in particular, to investigate the role of RNA-binding proteins that are known to interact with Src.

Materials and methods

Transfections

NIH 3T3 cells were routinely maintained at low density in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5 % fetal calf serum (IBF Biotechnics, France). For transfection, 6 x 10^5 cells were seeded per 85 mm dish, the medium was renewed after 6 h and the transfection performed 1 h later with 10 μg of DNA per dish (5 μg of each plasmid) by a standard calcium phosphate procedure [47]. After 16 h, the medium was replaced with fresh medium supplemented with 10 % fetal calf serum. RNAs were extracted 41 h after the initiation of transfection.

RNA extraction and analysis

Separation of the nuclear and cytoplasmic fractions was achieved by NP40 lysis as in [13], except that cells were scraped in phosphate-buffered saline containing 8 mM EGTA to chelate the Ca^{2+} introduced by transfection. RNA was purified by addition of guanidium thiocyanate and centrifugation over a cesium chloride cushion [48]. On average, the cytoplasmic and nuclear fractions contained 80 % and 20 % of cellular RNA, respectively.

For RNase mapping analysis, 4 μg RNA was hybridized with 300 pg RNA probe (labelled with [α-^{32}P] UTP to a specific activity of 6×10^7 cpm μg^{-1}), digested with RNases A and T1 and electrophoresed through 5 % urea–polyacrylamide gels as described [14]. Quantification of the results was performed with a Fuji Bio-imaging Analyzer, taking into account the number of labelled residues in each protected fragment and the relative sizes of the nuclear and cytoplasmic fractions.

For northern hybridization blots, samples containing 4 μg RNA were electrophoresed through 1.5 % agarose–formaldehyde gels and transferred onto uncharged nylon membranes (Hybond N, Amersham, France) as described [49]. Hybridizations with ^{32}P-labelled RNA probes (specific activity 3×10^8 cpm μg^{-1}) were performed at 60 °C in 50 % formamide, five-fold concentrated SPE, 0.1 % SDS, Denhardt's and 0.1 mg ml^{-1} salmon sperm DNA [47].

Expression vectors and probes

The pCM vector [14] contains nucleotides 329–1345 of the simian cytomegalovirus immediate early promoter [15] inserted at the *Sac*I site of the plasmid Bluescript II SK (Stratagene cloning systems, La Jolla, USA) and a synthetic oligonucleotide containing the rabbit β-globin polyadenylation signal [16] inserted at the *Kpn*I site. The TNFβ expression vector contains the nucleotides 1203–3100 of the murine TNFβ gene [50] inserted between the *Xba*I and *Sac*I sites of pCM. The β globin expression vector contains the *Pvu*II–*Xho*I fragment of the rabbit β globin gene in lieu of the TNFβ gene. The *ras* and *src* expression vectors contain, in addition to the respective cDNAs [24,51] and located 5′ to them, intron 1 of the rabbit β globin gene introduced with 10 nucleotides of β globin flanking sequences on each side. The polyoma early region *Py* is the *Hinc*II–*Bam*HI fragment of the viral genome as present in the DOL$^-$ vector [52].

Probe A contains nucleotides 1733–2226 of the murine TNFβ gene [50], from exon 2 to exon 4 and including introns 2 and 3. The $i2^+$,$i3^+$ protection contains 77 uridines, the $i2^-$,$i3^+$ 57 and the $i2^-$,$i3^-$ 17. Probe B contains nucleotides 1631–1967, from intron 1 to exon 3, including intron 2. The $i1^+$,$i2^+$ protection contains 41 uridines, the $i1^-$,$i2^+$ 36 and the $i1^-$,$i2^-$ 9.

Modelling the splicing and transport reactions

Our derivation of reaction rates from transcript accumulation is as outlined previously [14]. It relies on a description of transport and splicing reactions as pseudo-first-order processes and uses the fact that, at equilibrium, the product-to-precursor ratios are directly related to reaction rates. Specifically, we take into account three splicing rates (s_{i1}, s_{i2}, s_{i3}), four transport rates (t_{N0}, t_{N1}, t_{N2}, t_{N3}), and four cytoplasmic degradation rates (d_{C0}, d_{C1}, d_{C2}, d_{C3}). For any nuclear transcript Nn, an apparent transport rate can be derived by supposing that the flux of exported molecules ($t_{Nn} \times Nn$) is equal to that of cytoplasmic degradation ($d_{Cn} \times Cn$), hence $t_{Nn} = Cn / Nn \times d_{Cn}$. Similarly, splicing rates can be obtained by equating the flux of molecules generated by splicing (for example, $s_{i3} \times N1$, for intron 3 removal) to that of all the final products ($t_{N0} \times N0$, in this case), hence $s_{i3} = t_{N0} \times N0 / N1 = d_{C0} \times C0 / N1$. For higher-order precursors, the same approach leads to $s_{i2} = (d_{C0} \times C0 + d_{C1} \times C1) / N2$, and $s_{i1} = (d_{C0} \times C0 + d_{C1} \times C1 + d_{C2} \times C2) / N3$.

Intuitively, one would expect the ratios of nuclear precursors (such as N2 / N3) to be more directly related to splicing rates than the ratios of the cytoplasmic end products to their nuclear precursors. However, there are two limitations to the use of only nuclear information: the first occurs in cases where the export of transcript contributes in an essential way to the nuclear ratios (for example $N0 / N1 = s_{i3} / t_{N0}$); the use of cytoplasmic information is then required to determine the transport rate ($t_{N0} = d_{C0} \times C0 / N0$). The second occurs when export can be neglected; the ratio of nuclear precursors then reflects a dimensionless quantity which is only partly informative (for example $N2 / N3 = si1 / (s_{i2} + t_{N2})$ as in this case $s_{i2} >> t_{N2}$, $N2 / N3 = s_{i1} / s_{i2}$), and in our case, as these two splicing rates are equally affected by the presence of either Src or polyoma, N2 / N3 does not significantly vary.

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