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Review Article

Control of Alternative Pre-mRNA Splicing by RNA Pol II Elongation: Faster is Not Always Better

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Summary

The realization that the mammalian proteomic complexity is achieved with a limited number of genes demands a better understanding of alternative splicing regulation. Promoter control of alternative splicing was originally described by our group in studies performed on the fibronectin gene. Recently, other labs extended our findings to the cystic fibrosis, CD44 and CGRP genes strongly supporting a coupling between transcription and pre-mRNA splicing. A possible mechanism that would fit in these results is that the promoter itself is responsible for recruiting splicing factors, such as SR proteins, to the site of transcription, possibly through transcription factors that bind the promoter or the transcriptional enhancers. An alternative model, discussed more extensively in this review, involves modulation of RNA pol II (pol II) elongation rate. The model is supported by findings that cis- and trans- acting factors that modulate pol II elongation on a particular template also provoke changes in the alternative splicing balance of the encoded mRNAs.

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Keywords Alternative splicing; mRNA processing; transcription; RNA polymerase II; elongation.

INTRODUCTION

For years gene transcription and pre-mRNA processing have been thought to be independent events until a series of biochemical, cytological and functional experiments demonstrated that all three processing reactions (capping, splicing and cleavage/polyadenylation) are tightly coupled to RNA polymerase II (pol II) transcription. Coupling is in part due to the ability of pol II to bind and 'piggyback' some of the processing factors in a complex known as the 'mRNA factory' (for reviews see *1–5*). The carboxy terminal domain (CTD) of pol II plays a central role in the coupling process: truncation of the CTD causes defects in capping, cleavage/polyadenylation and splicing (*6*). The CTD comprises 52 heptad repeats. Fong and Bentley found that the CTD carboxyl terminus including heptads 27–52 supported all three processing reactions but the amino terminus supported only capping, concluding that different CTD regions can display different functions in pre-mRNA processing (*7*). Therefore, CTD phosphorylation/dephosphorylation at specific serines influences the recruitment of capping enzymes and cleavage/polyadenylation factors to the mRNA factory during transcript elongation (*8, 9*). Furthermore, transcriptional activation of pol II genes provokes association of splicing factors to sites of transcription (see below). This relocalization does not occur if pol II has a truncated C-terminal domain (CTD) (*10*).

Alternative splicing appears as a widespread mean for producing polypeptide diversity from a single gene (*11*). In human fibronectin (FN), for example, up to 20 different polypeptide variants arise from alternative splicing in three regions of a single gene (*12*). However, this figure remains modest when compared with that of Drosophila dscam gene where an extremely complex array of alternative exons could potentially give rise to 38,016 DSCAM proteins, of which 49 mRNA species have been already identified (*13*). In spite of the estimation that 35% of human genes are expressed through alternative splicing (*14*) (41% in the mouse, ref. *15*) and the sophisticated functional, cell-type and developmental specificities documented in many cases, the mechanisms

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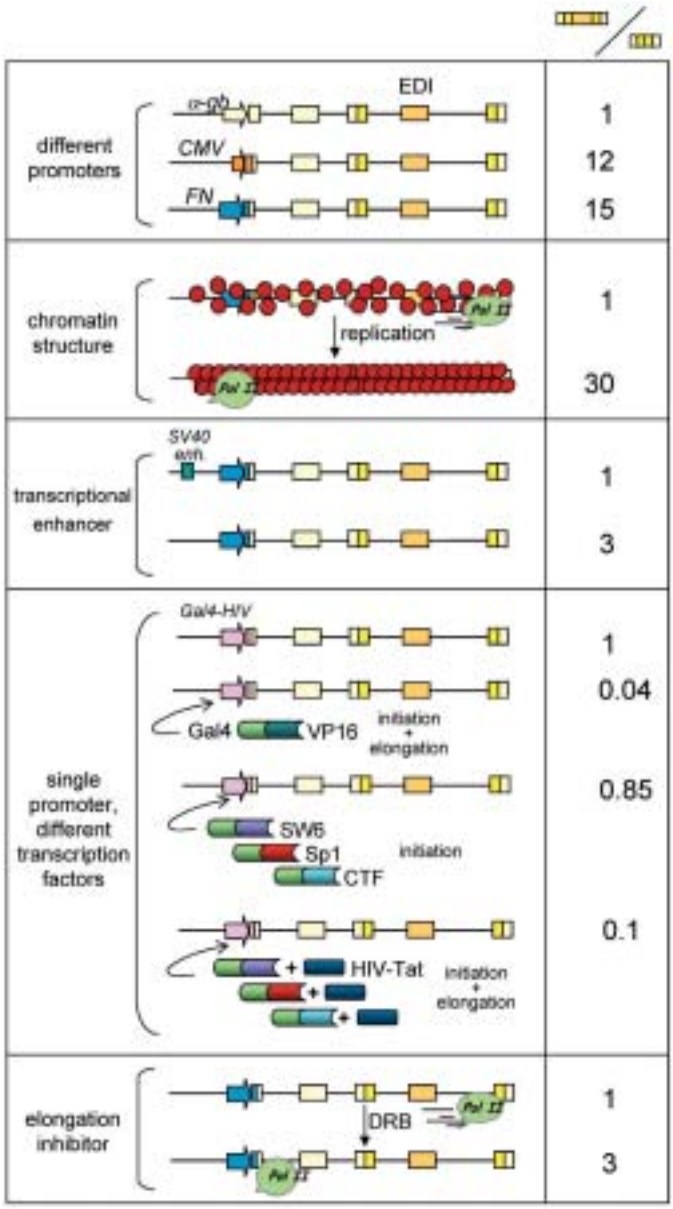

Figure 1. Effects of several cis- and trans- acting factors that affect pol II elongation on the alternative splicing of the fibronectin EDI (extra domain I) exon. Promoters (*20*), enhancers (*28*) and chromatin structure changes caused by template replication (*22*) act in cis. Transcription factors (*26, 28*) and drugs such as DRB (dichlororibofuranosylbenzimidazole) (*26*) act in trans. The right column displays the ratios of the amounts of mRNA isoforms containing vs. lacking the EDI exon. Ratio standardizations are valid only within each condition analyzed.

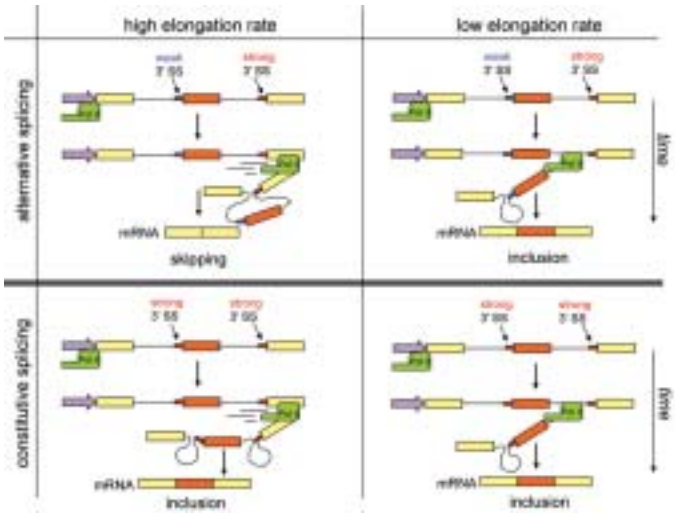

Figure 2. Influence of RNA polymerase II elongation rate on alternative splicing by 'exon skipping'. Alternative splicing (top): when the 3′ splice site (SS) by the alternative exon is weaker than the 3′ SS of the downstream intron, low transcriptional elongation rates (right) favor exon inclusion, whereas high elongation rates (left) favor skipping. Constitutive splicing (bottom): when both 3′ SS are strong, the exon is included constitutively independently of the elongation rate.

of alternative splicing regulation are poorly understood. A key role in splice site choice regulation is played by members of the SR (Ser/Arg-rich) family of proteins. These proteins participate in constitutive and alternative splicing, by binding to exonic splicing enhancers (ESE) and enhancing or repressing spliceosome assembly at adjacent splice sites. It is conceivable that alternative splicing in different cell types or different points in time is regulated by variation in relative abundance of SR proteins. However, although relative proportions of SR proteins and their antagonistic splicing factors (namely heterogeneous nuclear ribonucleoprotein A1) vary naturally in several rat tissues and cell lines in culture (*16*), SR proteins do not seem to have a highly specific tissue distribution, which suggests the existence of more complex regulatory mechanisms. SR proteins are present and highly mobile in the nucleoplasm where they accumulate at active sites of transcription (*17–19*).

We have demonstrated that differences in promoter structure lead to differences in alternative splicing of the transcript (*20*). The system analyzed involved transient transfection of mammalian cells with minigenes carrying the EDI exon, which encodes a facultative repeat of FN. EDI contains an exonic splicing enhancer (ESE), which is targeted by the SR proteins SF2/ASF and 9G8. Overexpression of SF2/ASF and 9G8 markedly stimulates EDI inclusion, but the effect of these proteins is modulated by the promoter (*21*) (Fig. 1). These effects are not the trivial consequence of different mRNA levels produced by each promoter (promoter strength) but depend on some qualitative properties conferred by

promoters to the transcription/RNA processing machinery. The promoter effect is also observed in cell lines stably transfected with the same minigenes used as episomal templates, indicating that a physiological chromatin assembly of the integrated minigenes is compatible with the promoter mechanism (*22*).

The promoter effect is not restricted to the FN EDI exon. Similar effects have been found independently in other genes. Reporter minigenes whose products are subject to alternative splicing decisions in the CD44 and the calcitonin gene related product (CGRP) genes were put under the control of steroid sensitive promoters (mouse mammary tumor virus and synthetic promoters containing either the progesterone or the estrogen response elements) or promoters that do not respond to steroid hormones (CMV and thymidine kinase). Steroid hormones affected splice site selection only of pre-mRNAs produced by the first type of promoters. As in the case of FN EDI, promoter-dependent hormonal effects on splicing were not a consequence of an increase in transcription rate or of a saturation of the splicing machinery (*23*). Promoter dependent alternative splicing patterns have been also found when reporter minigenes for the cystic fibrosis transmembrane regulator (CFTR) exon 9, transiently expressed in mammalian cells (*24*). In this case, overexpression of the SR protein SF2/ASF stimulates exon 9 skipping and this effect is also modulated by the promoter.

The finding that promoter structure is important for alternative splicing predicts that factors that regulate alternative splicing could be acting through promoters and that cell-specific alternative splicing may not simply result from the differential abundance of ubiquitous SR proteins, but from a more complex process involving cell-specific promoter occupation. However, promoters are not swapped in nature and since most genes have a single promoter, the only conceivable way by which promoter architecture could control alternative splicing *in vivo*, should be the differential occupation of promoters by transcription factors of different natures and/or mechanistical properties. Accordingly, it has been found that transcriptional activators affect alternative splicing. Three functional classes of transcriptional activation domains have been defined according to their abilities to stimulate the initiation and elongation steps of pol II transcription *in vivo* (*25*). Class I activators, such as SW6, Sp1 and CTF/NF1, stimulate predominantly initiation; class IIA activators, such as HIV-1 Tat, stimulate elongation; and class IIB activators, which include VP16, p53 and E2F1, stimulate both initiation and elongation.

Independently of the nature of their activations domain, class I activators like SW6 (acidic), Sp1 (glutamine-rich) and CTF/NF1 (proline-rich) that promote predominantly initiation have little effect on EDI splicing. On the other hand, VP16 (acidic), which promotes both initiation and elongation, stimulates EDI exon skipping. HIV-1 Tat, which has little effect on transcription in the absence of other activators, has no effect on EDI splicing. Stimulation of both initiation and elongation can be reconstituted by the synergistic activities of Tat with class I activators (*25*). In agreement with these observations, we found that Tat synergizes with SW6, Sp1 and CTF, but not with VP16, in promoting transcriptional elongation and therefore in inhibiting EDI inclusion (*26*) (Fig. 1).

Promoters and enhancers are cis-acting elements that control gene transcription via complex networks of protein-DNA and protein-protein interactions. While promoters deal with putting in place the RNA polymerase, both enhancers and promoters can control transcriptional initiation and elongation. The presence of the SV40 transcriptional enhancer near a promoter stimulates pol II elongation (*27*). Consistently, deletion of the SV40 enhancer provokes a 3–10 fold reduction in exon skipping, independently of the promoter (*28*) (Fig. 1).

Transcriptional co-regulators have been also implicated in the control of alternative splicing. Steroid hormones affect the processing of pre-mRNA synthesized from steroid-sensitive promoters, but not from steroid-unresponsive promoters, in a steroid-receptor dependent and receptor-selective manner. Several co-regulators of these nuclear receptors showed differential effects on alternative splicing (*23*). These results not only confirm the promoter effect in a system different from the one used in our studies, but pointed out its physiological relevance.

Recruitment Model

A possible mechanism that would fit in these results is that the promoter itself is responsible for recruiting splicing factors, such as SR proteins, to the site of transcription, possibly through transcription factors that bind the promoter or the transcriptional enhancers. The finding that p52, a transcriptional coactivator, directly interacts with SF2/ASF stimulating pre-mRNA splicing is consistent with this model (*29*). Furthermore, some proteins could display a dual function; acting in both processes as is the case of a transcriptional activator of the human papilloma virus (*30*), or the thermogenic coactivator PGC-1. Interestingly, PGC-1 can affect alternative splicing, but only when it is recruited to complexes that interact with gene promoters (*31*). The product of the WT-1 gene, which is essential for normal kidney development, could also be involved in both transcription and splicing. Although generally considered a transcription factor, WT1 isoforms that include three amino acids, KTS, interact with the essential splicing factor U2AF65 *in vitro* (*32*). Other proteins, such as SAF-B, which mediate chromatin attachment to the nuclear matrix, have been implicated in the coupling of transcription and pre-mRNA splicing (*33*). The RNA polymerase itself could be responsible for recruiting these proteins, perhaps through its CTD. Three proteins carrying WW and/or FF domains, and whose activities might be related to the coupling between transcription and splicing, were found to

bind specifically to phosphorylated CTD (P-CTD): (i) The yeast splicing factor Prp40 (*34*) (ii) Ess1, a yeast peptidyl prolyl isomerase, proposed to act in cis/trans protein isomerizations that could play a crucial role in the recognition of CTD by other proteins (*35*), and (iii) CA150, a human nuclear factor implicated in transcriptional elongation (*36*). Other candidates to function in the coupling of splicing and transcription are a group of proteins known as SCAFs (SR-like CTD associated factors). These are CTD-interacting proteins which, similarly to SR proteins, contain an RS domain and an RNA binding domain (*37*). Nevertheless, binding of these splicing regulators to the CTD has not been correlated experimentally to the promoter effect on alternative splicing. While evidence of CTD recruitment of processing factors explains satisfactorily the coupling of transcription with capping and cleavage/polyadenylation, evidence for a link between recruitment and splicing is still circumstantial and needs further investigation.

Pol II Elongation Model

An alternative, but not exclusive, model suggests that promoters might control alternative splicing via the regulation of pol II elongation rate or processivity. Low pol II elongation rate or internal pauses for elongation would favor the inclusion of alternative exons governed by an exon skipping mechanism, whereas a highly elongating pol II, or the absence of internal pauses, would favor exclusion of these kinds of exons. The mechanism by which the elongation rate would affect EDI splicing is a consequence of EDI pre-mRNA sequence. EDI exon skipping occurs because the 3′ splice site of the upstream intron is suboptimal compared to the 3′ splice site of the downstream intron. If the polymerase pauses anywhere between these two sites, only elimination of the upstream intron can take place. Once the pause is passed or the polymerase proceeds, there is no option for the splicing machinery but to eliminate the downstream intron, which leads to exon inclusion. A highly processive elongating pol II, or the absence of internal pauses, would favor the simultaneous presentation of both introns to the splicing machinery, a situation in which the stronger 3′ splice site of the downstream intron outcompetes the weaker 3′ splice site of the upstream intron, resulting in exon skipping. Figure 2 shows how when a weak 3′ splice site is followed by a strong one, as in many alternative splicing examples, pol II elongation rates affect the relative amounts of splicing isoforms. On the contrary, when two consecutive strong 3′ splice sites occur, as in constitutive splicing, pol II elongation rates are irrelevant.

Evidence Supporting the Elongation Model

A kinetic role for transcription on splicing was originally suggested by Eperon et al. (*38*), who found that the rate of RNA synthesis may affect its secondary structure, which may affect splicing. A similar mechanism involving a kinetic link between transcription and splicing was suggested from experiments in which RNA pol II pause sites affect alternative splicing by delaying the transcription of an essential splicing inhibitory element (DRE) required for regulation of tropomyosin exon 3 (*39*).

The elongation factor P-TEFb converts the polymerase from a nonprocessive to a processive form, which is consistent with the fact that inhibitors of this kinase such as DRB (dichlororibofuranosylbenzimidazole) inhibit pol II elongation (*40*). Cells transfected with EDI splicing reporters and treated with DRB displayed a 3-fold increase in EDI inclusion into mature mRNA compared to untreated cells (*26*) (Fig. 1).

Recent evidence, of independent nature, highlights the importance of coupling between splicing and transcriptional elongation. Fong and Zhou (*41*) found that spliceosomal U small nuclear ribonucleoproteins (UsnRNPs) interact with the human transcription elongation factor TAT-SF1 and strongly stimulate pol II elongation, probably via the binding of TAT-SF1 to the elongation factor P-TEFb. Because the TAT-SF1-UsnRNP complex also stimulates splicing *in vitro* (*41*), these results not only reveal that splicing factors function directly to promote transcriptional elongation but that reciprocal interactions exist in the coupling process. The existence of reciprocal interactions between transcription and splicing is also supported by findings that removal of promoter proximal splice signals or elimination of introns markedly reduce nascent transcription (*42*), which indicates that factors controlling intron removal are important for normal levels of transcription.

Transcriptional processivity is defined as the ability to elongate through sites where polymerase (in our case pol II) is prone to pause or terminate prematurely. Promoters have been implicated in the control of pol II elongation in several cases (*25, 27*). Consistently, we found that constructs carrying different promoters elicit different transcriptional processivities which correlate inversely with their ability to promote EDI exon inclusion. The FN promoter, for instance, which provokes EDI inclusion levels 15-fold higher than those of the α-gb promoter, promotes a less processive transcription as evidenced by proximal/distal mRNA levels that are two-fold higher than those elicited by the α-gb promoter. A more direct way to determine differences in RNA pol II processivities is to measure pol II densities along the transfected minigenes using the chromatin immunoprecipitation (ChIP) technique with an antibody to pol II. In agreement with steady state mRNA level results, conditions that favor EDI inclusion display higher pol II densities in regions upstream of the alternative exon, reflecting stalling of the polymerase (*28*).

Further evidence that pol II elongation rate is involved in the promoter effect comes from the finding that minigenes impelled to replicate within the transfected mammalian cell elicit higher EDI inclusion levels. Replication was provoked by overexpression of the simian virus 40 large T antigen (SV40 T-Ag) which activates replication of templates

containing the SV40 origin of replication by only two-fold. Plasmids maintained as episomes in mammalian cells are known to acquire nucleosomal organization. Replication of these plasmids provokes a more specific and compact nucleosomal assembly (*43*). Simultaneously, transcription of replicated templates is activated by 25-fold (T-Ag is also a transcriptional activator). Paradoxically, replication-mediated transcriptional activation provokes an important decrease in RNA pol II processivity, evidenced by the generation of shorter transcripts (*44*). In these conditions, T-Ag provokes a 10–30-fold increase in the inclusion of the alternative EDI exon and this effect is almost entirely due to the ability of T-Ag to promote replication and not to promote cell transformation (*22*) (Fig. 1).

Changes in chromatin structure provoked by histone acetylation also affect splicing. In fact, trichostatin A (TSA), a potent inhibitor of histone deacetylation, inhibits EDI inclusion by two-fold (*26*). This effect is observed only when the minigene template is allowed to replicate, a condition that favors a more compact chromatin organization. EDI splicing elicited by plasmid templates which have not replicated within the mammalian cell is not affected by TSA. The effect of TSA favors the hypothesis that acetylation of the core histones would facilitate the passage of the transcribing polymerase, which is in turn consistent with the proposal of chromatin opening mediated by DNA tracking by a transcribing pol II complex piggybacking a histone acetyltransferase activity (*2*).

Peaceful Coexistence of the Two Models

Findings that splicing factors increase transcriptional elongation (*41*) and that introns are necessary for efficient pol II transcription (*42*) suggest that the strong connection between transcription and splicing might be the consequence of a combination of the two models discussed above. Although evidence for the elongation mechanism is stronger in terms of the variety of molecular approaches that support it, certain data allow us to speculate that recruitment and elongation might be interconnected. For instance, the CTD is preferentially phosphorylated at Ser-5 when pol II is recruited at promoter sites but becomes phosphorylated at Ser-2 when located at the coding region (*45*). This change in phosphorylation quality might be relevant for the recruitment of splicing factors. Simultaneously, it would be important to determine whether a pausing pol II has the same phosphorylation status and recruitment properties of a fast elongating pol II. Chromatin immunoprecipitation experiments with antibodies to different kinds of phospho-pol II and characterization of protein complexes throughout different segments of transcribed regions should help to test the combined hypothesis.

Studies on the yeast Spt5 factor also suggest a combined mechanism. This factor has been proposed to regulate pol II elongation through nucleosomes. General elongation factors such as TFIIF and TFIIS coimmunopurify with Spt5, which in turn is able to interact with capping enzymes. Lindstrom et al. (*46*) found that spt5 mutations lead to accumulation of unspliced pre-mRNAs. One possibility is that inhibition of splicing occurs because splicing factors fail to interact with the transcription machinery in the absence of Spt5. Another possibility, less exciting but important to rule out, is that splicing inhibition is the consequence of the generation of uncapped pre-mRNAs.

Results reviewed here add a novel component to the various and complex network that controls gene expression. Several growth factors have been implicated in alternative splicing regulation (*47*). We recently added a new actor to the cast by finding that a basement membrane extracellular matrix, rich in laminin and collagen IV, stimulates EDI exon skipping. The effect correlates with a decrease in cell proliferation, consistently with high EDI inclusion levels observed in many physiological and pathological proliferative processes (*48*). It will be interesting to define to what extent the elongation mechanism mediates control of alternative splicing by the transduction cascades triggered by external signals.

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