CHAPTER 11

Coupling Transcription and Alternative Splicing

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Abstract

Alternative splicing regulation not only depends on the interaction of splicing factors with splicing enhancers and silencers in the pre-mRNA, but also on the coupling between transcription and splicing. This coupling is possible because splicing is often cotranscriptional and promoter identity and occupation may affect alternative splicing. We discuss here the different mechanisms by which transcription regulates alternative splicing. These include the recruitment of splicing factors to the transcribing polymerase and "kinetic coupling", which involves changes in the rate of transcriptional elongation that in turn affect the timing in which splice sites are presented to the splicing machinery. The recruitment mechanism may depend on the particular features of the carboxyl terminal domain of RNA polymerase II, whereas kinetic coupling seems to be linked to how changes in chromatin structure and other factors affect transcription elongation.

Introduction

For decades RNA polymerase II (RNAPII) transcription and pre-mRNA processing have been thought to be independent steps in the pathway of eukaryotic gene expression until a series of biochemical, cytological and functional experiments demonstrated that capping, splicing and cleavage/polyadenylation are coupled to transcription.[1-7] This requires that splicing occurs cotranscriptionally. However, the existence of cotranscriptionality per se does not necessarily imply a mechanistic coupling. Indeed, splicing often occurs cotranscriptionally. Electron microscopy visualization of *Drosophila* embryo nascent transcripts (Fig. 1A) has clearly demonstrated that splicing occurs cotranscriptionally with a reasonable frequency and that splice site selection precedes polyadenylation.[8] Cotranscriptional splicing was also demonstrated in the *dystrophin* gene.[9] Since transcription of this 2.4 Mb-gene, the largest in the human genome, would take approximately 16 hours to be completed, cotranscriptional splicing of its pre-mRNA appears as a very intuitive concept. In fact, it would be very difficult to conceive that the splicing of the dozens of *dystrophin* introns would "wait" until the entire *dystrophin* pre-mRNA is synthesized. Nevertheless most biology (and even molecular biology) textbooks continue to show figures in which a fully transcribed primary transcript, with all its introns, appears as the substrate for splicing (Fig. 1B, left). A more realistic view would depict splicing as taking place while the pre-mRNA is still attached to the template DNA by RNAPII (Fig. 1B, right).

It is worth noting that cotranscriptional splicing is not obligatory. The time it takes RNAPII to synthesize each intron defines the minimal time in which splicing factors can be recruited to

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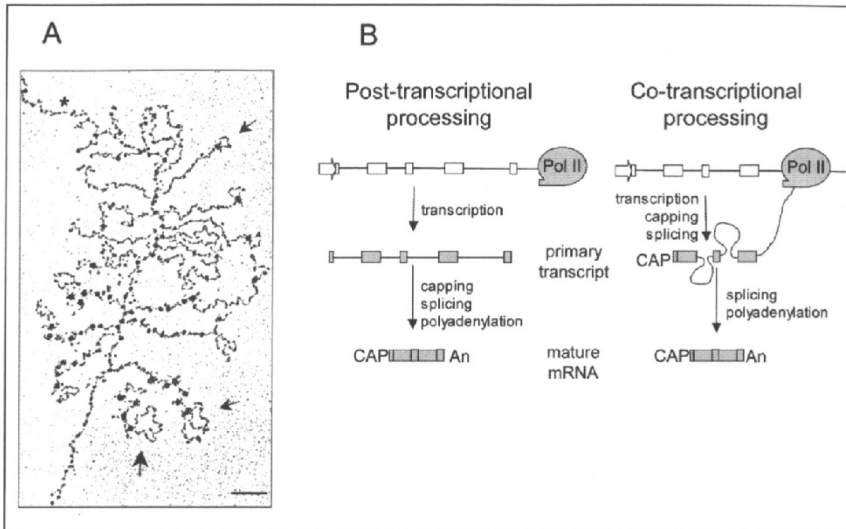

Figure 1. Splicing occurs cotranscriptionally. A) Electron micrograph of an actively transcribing *Drosophila* embryo gene shows that pre-mRNA splicing occurs on the nascent transcript. Asterisk: promoter region. Arrows: intron loops (lariats). Taken with permission from: Beyer AL, Osheim YN. Genes Dev 1988; 2(6):754-765.[8] B) Left: classical textbook picture where all pre-mRNA processing reactions are depicted as posttranscriptional. Right: pre-mRNA processing is cotranscriptional. In the depicted pre-mRNA molecule splicing of intron 1 has already occurred, introns 2 and 3 are being processed and exon 4 has not been transcribed yet.

and spliceosomes assembled upon an intron, whereas the time that it takes RNAPII to reach the end of the transcription unit and release the nascent pre-mRNA defines the maximal time in which splicing could occur cotranscriptionally.[6] For long genes, for example, some introns could be cotranscriptionally spliced whereas others could be processed well after transcription has been completed. This is indeed what happens in the Balbiani ring 1 (BR1) gene where intron 3, located 3 kb from the 5' end of the 40 kb-pre-mRNA, is mostly excised cotranscriptionally, but intron 4, located 0.6 kb from the poly A site, is excised cotranscriptionally in only 10% of the molecules.[10] For most genes, we do not know when each intron is spliced. We do not even know if a particular intron always follows the same pattern of processing. It is worth noting that if splicing were strictly cotranscriptional and that the splicing of one intron must be completed before the following intron is transcribed, alternative splicing would not exist.

Only recently has the cotranscriptional assembly of splicing factors been examined directly by chromatin immunoprecipitation (ChIP). In the budding yeast *Saccharomyces cerevisiae* it was observed that the small nuclear ribonucleoprotein particles (snRNPs) accumulate at positions along intron-containing genes that coincide with the appearance of their target splicing sequences in nascent pre-mRNA.[11-13] For instance, U1 snRNP becomes associated with the pre-mRNA shortly after the 5' splice site is transcribed, while U2 snRNP becomes associated later, after the 3' splice site has been synthesized. Studies in mammalian cells have confirmed these conclusions and have extended them by showing that in addition to general splicing factors, regulatory factors like hnRNPA1 accumulate cotranscriptionally on intron-containing genes but not on intronless ones.[14] The use of efficient in vitro transcription/splicing assays has corroborated the results obtained in living cells: nascent pre-mRNA synthesized by RNAPII is stabilized and efficiently spliced[15] apparently because it is immediately and quantitatively directed into the spliceosome assembly pathway. In contrast, nascent pre-mRNA synthesized by T7 phage

RNA polymerase is quantitatively assembled into the nonspecific hnRNP complexes which are inhibitory to spliceosome assembly, indicating that RNAPII mediates the functional coupling of transcription and splicing by directing the nascent pre-mRNA into spliceosome assembly.[16] Moreover, only genes transcribed by RNAPII encode pre-mRNAs and are efficiently recognized by the spliceosome—pre-mRNAs transcribed by RNAPI, RNAPIII or T7 RNA polymerase are poorly spliced or not at all.[17-20]

This chapter will focus on the evidence, gathered mainly during the last decade, supporting the existence of mechanisms that couple RNAPII transcription to alternative splicing and this evidence will be discussed in light of knowledge of the functional links between transcription and splicing in general. Both transcription and splicing are extremely complex processes because they involve thousands of protein factors, RNA molecules and DNA sequences. This complexity hinders any attempt at generalization and simplification. The reader should bear in mind that certain molecular interactions or kinetic constraints might be relevant for a particular gene or set of genes but not for others.

Promoters Affect Alternative Splicing

The idea that promoter regulation affects only the quantity and not the quality of the gene transcript has dominated our conception of gene expression in the past. However, the finding that promoter identity and occupation by transcription factors modulates alternative splicing[4,21,22] not only strengthened the concept of a physical and functional coupling between transcription and splicing but directed our attention towards how this coupling might affect protein expression patterns. The original observation of the promoter effect involved transient transfection of mammalian cells with reporter minigenes for the alternatively spliced extra domain I (EDI) cassette exon of *fibronectin* (*FN*) under the control of different RNAPII promoters. EDI is 270 bp long and contains an exonic splicing enhancer (ESE) that is recognized by the SR proteins SF2/ASF and 9G8. When transcription of the minigene is driven by the *α-globin* promoter EDI inclusion levels in the mature mRNA are about 10 times lower than when transcription is driven by the *FN* or *cytomegalovirus* (*CMV*) promoters (Fig. 2). 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. This observation is consistent with microarray studies indicating that although, like global transcription profiles, global alternative splicing profiles reflect tissue identity, transcription (evaluated as promoter usage and strength) and alternative splicing act largely independently on different sets of genes to define tissue-specific expression profiles.[23]

Promoter identity has been shown independently to affect the alternative splicing of pre-mRNAs transcribed from several other genes. Reporter minigenes containing alternative exons and flanking intron regions from the *CD44* and the *calcitonin gene-related product* genes, were put under the control of steroid-sensitive promoters or promoters that do not respond to steroid hormones. Steroid hormones affected splice site selection only of pre-mRNAs produced by the steroid-sensitive promoters. As in the case of the *FN* EDI exon, promoter-dependent hormonal effects on splicing were not a consequence of an increase in transcription rate or of a saturation of the splicing machinery.[24] Promoter-dependent alternative splicing patterns have been also found in the *cystic fibrosis transmembrane regulator*[25] and in the *fibroblast growth factor receptor 2* genes.[26]

The finding that promoter structure is important for alternative splicing predicts that factors that regulate alternative splicing could be acting in part through promoters and that cell-specific alternative splicing may not only result from the differential abundance of various splicing factors, but also 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, would be the differential occupation of promoters by transcription or splicing factors of different natures and/or mechanistic properties. Accordingly, it has been found that transcriptional activators and

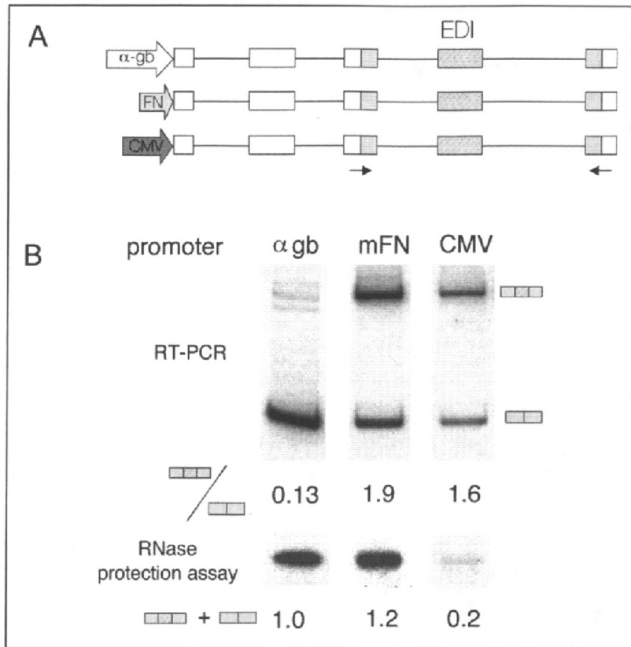

Figure 2. Promoters affect alternative splicing. A) α-*globin* (white)/*fibronectin* (gray) hybrid minigenes under the control of three different promoters, used in transient transfections of mammalian cells in culture to assess inclusion levels of the alternatively spliced EDI cassette exon (dashed) Arrows mark the positions of the primers used for RT-PCR. B. RT-PCR (top) show that inclusion levels with the *FN* and *CMV* promoters are more than 10-fold higher compared with inclusion levels with the α-*globin* promoter. RNase protection assays (bottom) show that expression levels are higher with the α-*globin* and *FN* promoters compared to the *CMV* promoter. Based on Cramer et al.[21,22]

coactivators with different actions on RNAPII initiation and elongation affect alternative splicing differentially[27,28] (see below). Alternative promoter usage is frequent in mammalian genes and could impact the usage of downstream alternative splice sites. However, the the link between alternative promoter usage and splice site selection might not be the result of transcription/splicing coupling but the consequence of deep changes in pre-mRNA secondary structure due to different first exon sequences in the pre-mRNA.

Two non-exclusive mechanisms have been proposed to explain promoter effects on alternative splicing. On the one hand, the promoter might recruit splicing factors or bifunctional factors acting on both transcription and splicing to the transcribing gene. On the other hand, the promoter might alter the rate of RNAPII elongation, affecting in turn the timing of cotranscriptional splicing. I will discuss these two modes in depth, but first will describe the features and roles of what is likely to be a central participant in these process, the carboxy terminal domain (CTD) of the large subunit of RNAPII.

RNAPII CTD and Coupling

The CTD plays a role in the nuclear distribution of components of the transcription and splicing machineries. In fact, transcriptional activation of RNAPII genes increases the association of splicing factors at sites of transcription, but this relocalization does not occur if RNAPII lacks the

CTD.[29] This is consistent with findings that overexpression of CTD-containing large subunits of RNAPII in mammalian cells induces selective nuclear reorganization of splicing factors.[30] Also consistent with the above, stimulation of transcription by strong activators is associated with increased splicing efficiency and this property of activators depends on the CTD.[51,72]

The CTD is composed of 52 tandem heptapeptide repeats in mammals (26 in yeast), with the consensus sequence YSPTSPS. The serines at positions 2 and 5 of this repeat are subject to regulatory phosphorylation. Phosphorylation of Ser5 by TFIIH is linked with transcriptional initiation, whereas phosphorylation of Ser2 by P-TEFb is associated to transcriptional elongation.[31,32] Coupling of transcription and pre-mRNA processing may in part be due to the ability of RNAPII to bind and "piggyback" some of the processing factors in a complex referred to as an "mRNA factory".[2] This concept arose from the observation by McCracken et al[33] that deletion of the CTD causes defects in capping, cleavage/polyadenylation and splicing. These authors showed that deletion of the CTD inhibits splicing of the β-*globin* gene, which is consistent with the findings that isolated CTD fragments[34] as well as purified phosphorylated RNAPII[35] are able to activate splicing in vitro. Nevertheless, isolated CTD fragments cannot duplicate the effect of the RNAPII holoenzyme unless the pre-mRNA is recognized via exon definition, i.e., it contains at least one complete internal exon with 3' and 5' splice sites. In other words, the CTD does not appear to activate splicing of pairs of splice sites across an intron. These findings support a direct role for the CTD in exon recognition and have led to speculation that the CTD functions to bring consecutive exons in proximity, thereby facilitating spliceosome assembly. Consistent with this model, Dye and Proudfoot[36] showed that exons flanking an intron that had been engineered to be cotranscriptionally cleaved by inserting a ribozyme in the middle are accurately and efficiently spliced together. These data suggest that a continuous intron transcript is not required for pre-mRNA splicing in vivo and provide evidence for a molecular tether connecting emergent splice sites in the pre-mRNA to an elongating transcription complex.

Dynamic changes in the CTD structure and phosphorylation may play significant roles in RNA processing. For instance, the peptidyl-prolyl isomerase Pin 1 stimulates CTD phosphorylation by cdc2/cyclin B and inhibits RNAPII-dependent splicing in vitro.[37] Inhibition of P-TEFb-mediated CTD phosphorylation prevents cotranscriptional splicing and 3'-end formation in *Xenopus* oocytes. In contrast, processing of injected pre-mRNA is unaffected by P-TEFb kinase inhibition, which strongly indicates that RNAPII does not participate directly in posttranscriptional processing, but phosphorylation of its CTD is required for efficient cotranscriptional processing.[38] New insights into the mechanism by which the CTD functions in splicing come from in vitro experiments with a fusion protein consisting of the CTD fused to the C-terminus of the splicing factor SF2/ASF (ASF-CTD). Compared to SF2/ASF alone, ASF-CTD increased the reaction rate during the early stages of splicing and this required both RNA-binding activity and phosphorylation of CTD in the fusion protein.[39]

It is worth mentioning that the roles of CTD in splicing may be gene-specific. For example, the absence of the CTD can affect alternative splicing of the *FN* EDI cassette exon[40] (see below) and mRNA editing of the *ADAR2* gene[41] without inhibiting general splicing.

Factor Recruitment

One model that could explain the promoter effect on alternative splicing is that the promoter itself recruits splicing factors to the site of transcription, possibly through transcription factors that bind to the promoter or to transcriptional enhancers. Some proteins, such as the transcriptional activator of the human papilloma virus,[42] and the thermogenic coactivator PGC-1, naturally function in both transcription and splicing. Interestingly, PGC-1 affects alternative splicing, but only when it is recruited to complexes associated with gene promoters[43] (Fig. 3). Another example of a bifunctional factor is the transcription factor Spi-1, required for myeloid and B lymphoid differentiation. Spi-1 is able to regulate alternative splicing of a pre-mRNA for a gene whose transcription it regulates. Guillouf et al[44] demonstrated that, similar to PGC-1, Spi-1 must bind and transactivate its cognate promoter to favor the use of a proximal 5' alternative site.

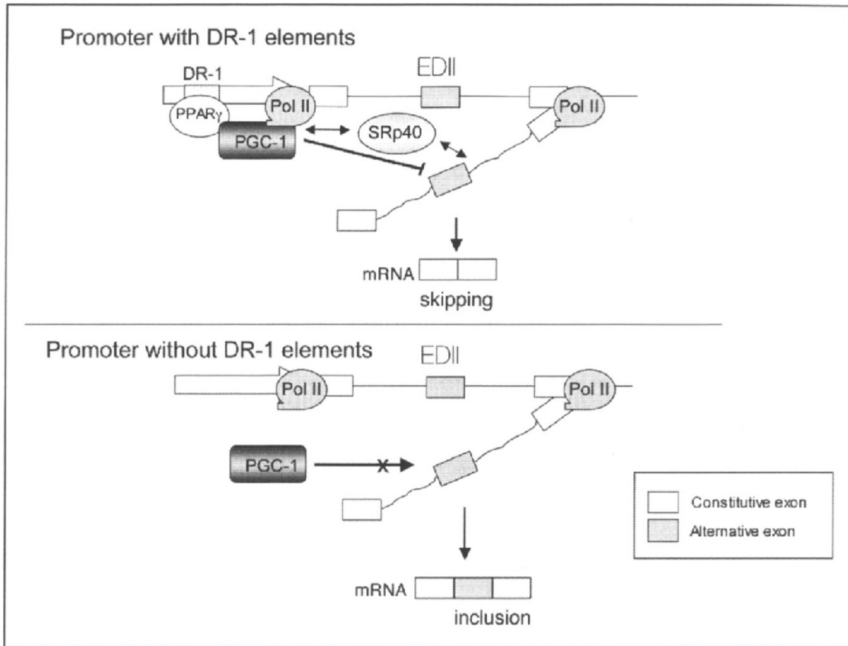

Figure 3. Example of how promoters may affect alternative splicing through recruitment of factors with dual function in transcription and splicing. A promoter with a DR-1 element binds the transcription factor PPARγ, which in turn recruits the transcriptional coactivator PGC-1. PGC-1 interacts with RNAPII and other proteins of the pre-initiation complex as well as with the splicing factor SRp40, which controls inclusion of the *fibronectin* EDII alternative exon. PGC-1 inhibits inclusion of EDII into the mature mRNA, only when targeted to a promoter. Based on Monsalve et al.[43]

Other mammalian proteins that appear to act as bifunctional factors include the product of the *WT-1* gene, which is essential for normal kidney development,[45] SAF-B, which mediates chromatin attachment to the nuclear matrix,[46] CA150, a human nuclear factor with characteristic WW and FF domains implicated in transcriptional elongation[47,48] and a group of proteins known as SCAFs (SR-like CTD associated factors) which interact with the CTD and, similarly to SR proteins, contain an RS domain and an RNA binding domain.[49] However there is no formal evidence that SCAFs function in splicing.

Transcriptional coregulators have also been implicated in the control of alternative splicing. Several coregulators of steroid hormone nuclear receptors have shown to have differential effects on alternative splicing in a promoter-dependent manner.[50] Some coregulators, such as CoAA (coactivator activator), act by recruiting coactivators. CoAA interacts with the transcriptional coregulator TRBP, which is in turn recruited to promoters through interactions with activated nuclear receptors. CoAA regulates alternative splicing in a promoter-dependent manner. It similarly enhances transcription of steroid-sensitive or -insensitive promoters, but only affects alternative splicing of transcripts synthesized from the progesterone-activated MMTV promoter.[28] In addition, transcriptional activators seem to not only modulate alternative but also constitutive splicing in a RNAPII CTD-dependent manner.[51]

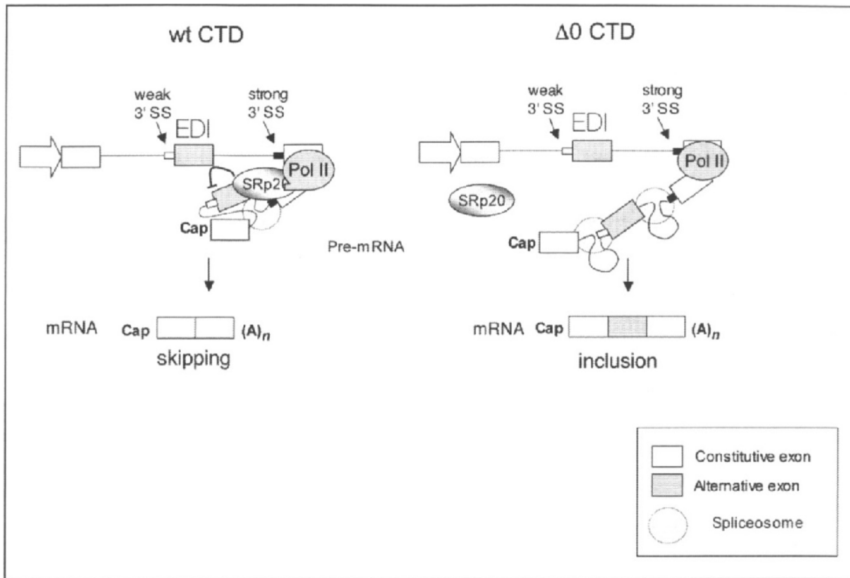

Figure 4. The carboxy terminal domain (CTD) of RNA polymerase II mediates the inhibitory effect of the SR protein SRp20 on the inclusion of the alternatively spliced *fibronectin* EDI exon. Transcription by a WT RNAPII (left) allows recruitment of SRp20 to the transcription machinery which stimulates EDI skipping. Transcription by a mutated RNAPII lacking the CTD (ΔCTD, right) causes higher EDI inclusion because SRp20 is not recruited. Based on de la Mata et al.[40]

Recruitment of SRp20, the CTD and Alternative Splicing

Transcription by an RNAPII mutant lacking the CTD provokes a dramatic enhancement in the inclusion levels of the *FN* EDI alternative cassette exon without affecting the efficiency of general splicing. Interestingly, the CTD influences alternative splicing in a way that is independent of capping and 3' end processing. Experiments using RNAPII CTD variants with different numbers of repeats revealed that the length of the CTD correlates inversely with EDI inclusion levels, with 19 heptads being the minimum number of repeats necessary to sustain normal EDI splicing. This finding is in agreement with reports showing that 22 tandem repeats are sufficient to support wild-type levels of splicing of pre-mRNAs containing constitutively spliced introns or enhancer-dependent introns.[52] Using siRNA knockdown strategies we found that whereas activation of EDI inclusion by the SR protein SF2/ASF is not affected by the absence of the CTD, inhibition of EDI inclusion by another SR protein, SRp20, is completely abolished when transcription is carried out by a ΔCTD RNAPII, suggesting that SRp20 requires the CTD to be recruited to the transcription/splicing machinery[40] (Fig. 4). We were not able to demonstrate direct physical interactions between SRp20 and any portion of the RNAPII large subunit. However, we believe that such an interaction, perhaps weak or indirect, must exist because SRp20 has been found in a transcription complex known as "mediator" together with the large subunit of RNAPII[53] and because immunocytochemical studies have shown that SRp20 preferentially associates with sites of RNAPII transcription[54] and is efficiently recruited to the *tau* gene when one of its alternative exons is included, but not when it is excluded.[55]

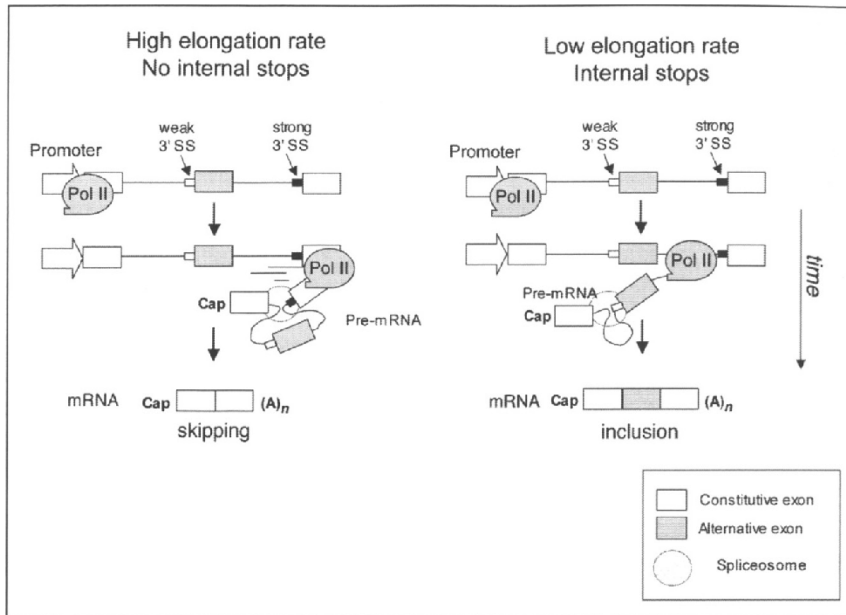

Figure 5. Kinetic coupling model for the regulation of alternative splicing by RNAPII elongation. The 3' splice site (SS) by the alternative cassette exon (white) is weaker than the 3' SS of the downstream intron (black). Low transcriptional elongation rates (right) favor exon inclusion, whereas high elongation rates (left) favor skipping.

Transcription Elongation and Alternative Splicing

Promoters can also control alternative splicing by regulating the rates of RNAPII transcription elongation. Low elongation rates or transcriptional pausing would favor the inclusion of alternative exons governed by an exon skipping mechanism, whereas rapid elongation rates or the absence of transcriptional pausing would favor exclusion of these exons. The mechanism by which elongation rates 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 weaker than the 3' splice site of the downstream intron. If the polymerase pauses between these two splice sites, the upstream intron will be spliced. Once the transcription complex resumes elongation, the downstream intron will be removed and the exon will be included. In contrast, a rapidly elongating transcription complex will transcribe both introns before the 5' splice site of the upstream intron can be used. As a result, the 5' splice site will be preferentially spliced to the strong downstream 3' splice site, rather than the weak upstream 3' splice site, resulting in exon skipping (Fig. 5). When a weak 3' splice site is followed by a strong one, as is the case in many alternative splicing events, the transcription elongation rate can affect the relative amounts of the mRNA isoforms. However, when two consecutive strong 3' splice sites occur, as in constitutive splicing, transcription elongation rates are less relevant.

A kinetic role for transcription in alternative splicing was originally suggested by Eperon et al[56] who found that the rate of RNA synthesis can affect the secondary structure of a nascent transcript surrounding a 5' splice site, which affects splicing. A similar mechanism involving a kinetic link was suggested from experiments in which transcription pause sites were found to affect alternative splicing by delaying the transcription of an essential splicing inhibitory element (DRE) required for regulation of tropomyosin exon 3.[57]

Several additional experiments indirectly support a role for transcriptipon elongation in alternative splicing:

- Transcription factors that primarily stimulate transcriptional initiation, such as Sp1 and CTF/NF1, have little effect on alternative splicing, whereas factors that stimulate elongation, such as VP16, promote skipping of the EDI exon.[27,58]
- Phosphorylation of the RNAPII CTD at Ser2 by the elongation factor P-TEFb converts the polymerase from a nonprocessive to a processive form. Inhibitors of this kinase such as DRB (dichlororibofuranosylbenzimidazole) inhibit RNAPII elongation. 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.[27]
- Changes in chromatin structure also affect splicing. Treatment of cells with trichostatin A, a potent inhibitor of histone deacetylation, favors EDI skipping.[27] This finding supports the hypothesis that acetylation of the core histones would facilitate the passage of the transcribing polymerase, which is in turn consistent with the model of chromatin opening being mediated by a RNAPII transcription elongation complex piggybacking a histone acetyltransferase activity tracking along the DNA.[59] Moreover, replication of transfected minigene reporters, which compacts the chromatin structure and slows the passage of the polymerase, causes a 10 to 30-fold increase in EDI exon inclusion levels in the transcript.[58] Interestingly, it has recently been shown that DNA methylation at internal regions of a gene provokes a closed chromatin structure and reduces the efficiency of transcription elongation.[60] This suggests that alternative splicing could be indirectly modulated by the DNA methylation status not only at the promoter but also internally.
- Transcriptional regulatory elements that activate transcription elongation, such as the SV40 enhancer, promote skipping of the EDI exon.[61]
- Chromatin immunoprecipitation experiments have shown that stalled transcription elongation complexes exist more frequently upstream of the alternative EDI exon on minigenes with promoters that favor EDI inclusion (i.e., the *FN* promoter) than on minigenes with promoters that favor EDI skipping (i.e., the α-*globin* promoter).[61]
- Mutation analysis shows that the better the EDI alternative exon is recognized by the splicing machinery, the less its degree of inclusion is affected by factors that modulate transcriptional elongation.[62]
- Although dealing with general and not alternative splicing, two recent reports provide strong evidence for a kinetic link between transcription, splicing factor recruitment and splicing catalysis. Using chromatin-RNA immunoprecipitation (ChRIP), Listerman et al[14] showed that while *fos* pre-mRNA can be spliced in vivo both co- and posttranscriptionally, the topoisomerase inhibitor camptothecin, which stalls RNAPII elongation, increased cotranscriptional splicing factor accumulation and splicing in parallel. The second report by the Rosbash lab[63] elegantly shows that cleavage of an intron by a hammerhead ribozyme competes with the splicing of that intron. If splicing of this pre-mRNA is prevented by mutating the 5' splice site, the ribozyme is able to cleave the intron, while for a wild-type pre-mRNA cotranscriptional splicing occurs prior to ribozyme cleavage. These results strongly suggest that introns are recognized cotranscriptionally. Furthermore, the *DST1* gene, which encodes the transcription elongation factor TFIIS, was identified in a screen for genes required to prevent cleavage of the intronic ribozyme in a normal splicing reporter. This again provides a link between transcription elongation and pre-mRNA splicing.

Slow Polymerases and Alternative Splicing

A more direct demonstration that transcription elongation affects alternative splicing in human cells was provided by the use of a mutant form of RNAPII (called C4) that possesses a reduced elongation rate.[64] The slow polymerase stimulates the inclusion of the *fibronectin* EDI exon by 4-fold, confirming the inverse correlation between elongation rate and inclusion of this alternative exon. The C4 mutation also affected the splicing of the adenovirus *E1a* pre-mRNA,

by favoring the use of the most upstream of the three alternative 5' splice sites that compete for a common 3' splice site. Most importantly and of physiological relevance, *Drosophila* flies carrying the C4 mutation show changes in the alternative splicing profile of transcripts encoded by the large *ultrabithorax* (*Ubx*) endogenous gene.[64] The observed changes are consistent with a kinetic mechanism which allows more time for early splicing events. Most interestingly, *C4* heterozygous flies display a phenotype, known as the "Ubx effect", where the halteres present a morphology that resembles the one of the *Ultrabithorax* mutation.[72]

Similar effects of RNAPII elongation rates on splicing were found in yeast. Alternative splicing is a very rare event in yeast. Mutating the branchpoint upstream of the constitutive internal exon of the *DYN2* gene creates an artificial alternatively spliced cassette exon. Skipping of this exon is prevented when expressed in a yeast mutant carrying a slow RNAPII or in the presence elongation inhibitors.[65] This supports the hypothesis that what is important to the balance between exon skipping and exon inclusion are relative rates of spliceosome formation and RNAPII processivity.

Chromatin, Elongation and Alternative Splicing

Batsché et al[66] revealed a new role in alternative splicing for the chromatin remodeling factor SWI/SNF, whose mechanism of action involves the regulation of RNAPII elongation. SWI/SNF is known to interact with RNAPII, splicing factors and spliceosome-associated proteins. Overexpression of Brahma (Brm), the key subunit of SWI/SNF, favors inclusion of a block of consecutive alternative exons in the *CD44* gene, which is a target for SWI/SNF transcriptional activation. As expected for a splicing regulator, Brm interacts with complexes containing U1 and U5 snRNAs, which are present in spliceosomes, but not with U3 snRNA, which is involved in ribosomal RNA processing. Brm also interacts with Sam68, a nuclear RNA-binding protein that in turn binds splicing regulatory elements present in the *CD44* alternative exons and stimulates their inclusion upon activation of the ERK MAP kinases. How does Brm use these multiple interactions to control alternative splicing? *CD44* contains a cluster of ten consecutive alternative exons (v1 to v10) located between constitutive exons 5 and 16. ChIP experiments have shown that Brm is not only present at the gene promoter but appears to be distributed along the whole transcriptional unit with levels that decrease gradually towards the 3' end. Although also concentrated at the promoter region, RNAPII displays a different distribution inside the gene, with a clear accumulation within the variable region peaking on exon v4. This peak disappears when Brm is knocked down by RNAi, but is higher when cells are treated with phorbol esthers that activate ERKs.

These findings strongly suggest that activation of Sam68 by ERK triggers the formation of macromolecular complexes containing Sam68, RNAPII and Brm at the central block of variable exons. This results in the stalling of RNAPII and the inclusion of the variable exons into mature mRNA, in agreement with the kinetic coupling model (Fig. 5). Interestingly, there is a dramatic change in the phosphorylation status of RNAPII at the pause site.[66] Successive ChIPs (ChIP-reChIPs) using first anti-Brm and then anti-phospho-CTD antibodies specific for either phospho-Ser5 or phospho-Ser2 revealed that within the *CD44* constant region Brm associates with phospho-Ser2 CTD RNAPII. However, Brm associates with phospho-Ser5 CTD RNAPII species at the *CD44* alternative exons. The return of the RNAPII phosphorylation status to that typical of promoters at specific sites within genes could generate internal "road blocks" to elongation (Fig. 6). In any case it is now clear that internal road blocks exist in vivo, can be regulated by external signals and are very important for alternative splicing.

Coordination Between and Polarity in Multiple Alternative Splicing Events

Soon after the discovery of splicing it became evident that many genes contained more than one region that is alternatively spliced, a feature that significantly expands the protein-encoding potential of a genome. The *fibronectin* gene is a paradigmatic example,[67] as it contains three regions of alternative splicing that display cell type- and developmental stage-specific regulation. This organization can give rise to up to 20 mRNA isoforms in humans, 12 in rodents and 8 in

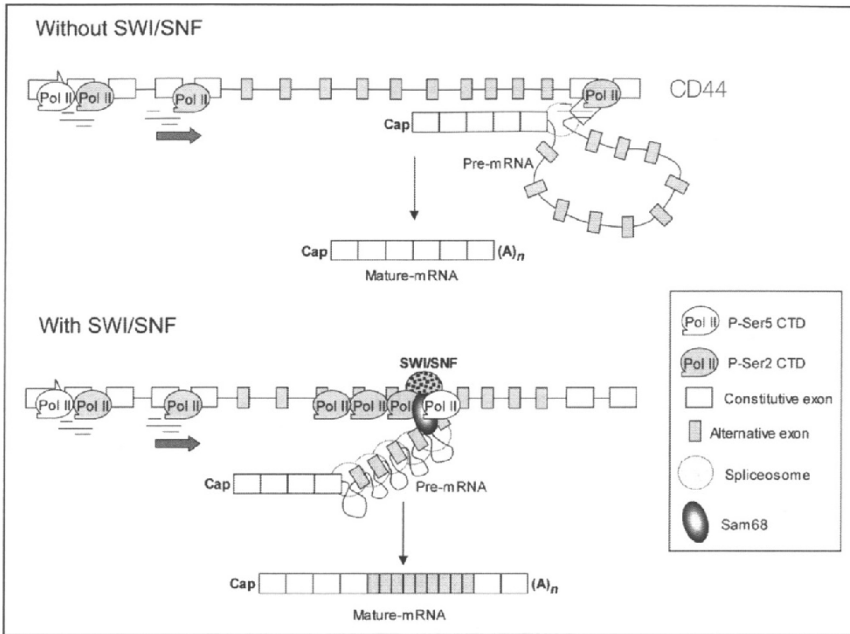

Figure 6. SWI/SNF stimulates inclusion of alternative exons in the *CD44* gene by creating a "road block" to RNAPII elongation at the variable region. The pause is the consequence of multiple protein interactions involving SWI/SNF, RNAPII, the splicing regulator Sam68 and spliceosomal components. The phosphorylation pattern of RNAPII CTD associated to Brm is changed from phospho-Ser2 to phospho-Ser5. This might cause the stalling of RNAPII molecules coming behind, even if they are phosphorylated at the elongation-competent Ser2. Based on Batsché et al.[56]

chickens.[68] Although other genes with multiple regions of alternative splicing have been characterized individually, the general prevalence of this phenomenon has been only recently examined by bioinformatic approaches which indicate that a significant fraction (25%) of human genes have such an organization.[69] This organization also raises the question of whether the different alternatively spliced regions of a gene are coordinately regulated. This has been studied by transfecting human cells with minigenes carrying two alternative EDI regions in tandem, separated by 3,400 bp spanning three constitutive exons and the corresponding introns. Mutations at splice sites or regulatory elements of the proximal (with respect to the promoter) EDI exon that either stimulate or inhibit its inclusion cause parallel effects in the inclusion levels of the distal EDI. In contrast, the same mutations introduced in the distal EDI have much smaller effects on the inclusion levels of the proximal exon.[69] Although the molecular mechanism for the coordinating effect remains to be elucidated, it is clear that coordination displays gene polarity. Most interestingly, coordination persists but polarity disappears when the rate of transcriptional elongation is high (Fig. 7) but is reestablished when elongation is inhibited by DRB. Thus, the rate of transcription elongation is not only important for splice site selection at a single alternative splicing event but also for long distance effects in splicing regulation. Other examples of long distance regulation of splice site selection have been reported in the equine *β-casein* intron 1,[70] and in the human *thrombopoietin* gene.[71] However, coordination and polarity of multiple alternative splicing events do not appear to occur in every gene as splicing of the *Drosophila Dscam* gene does not appear to be governed by such rules.[74]

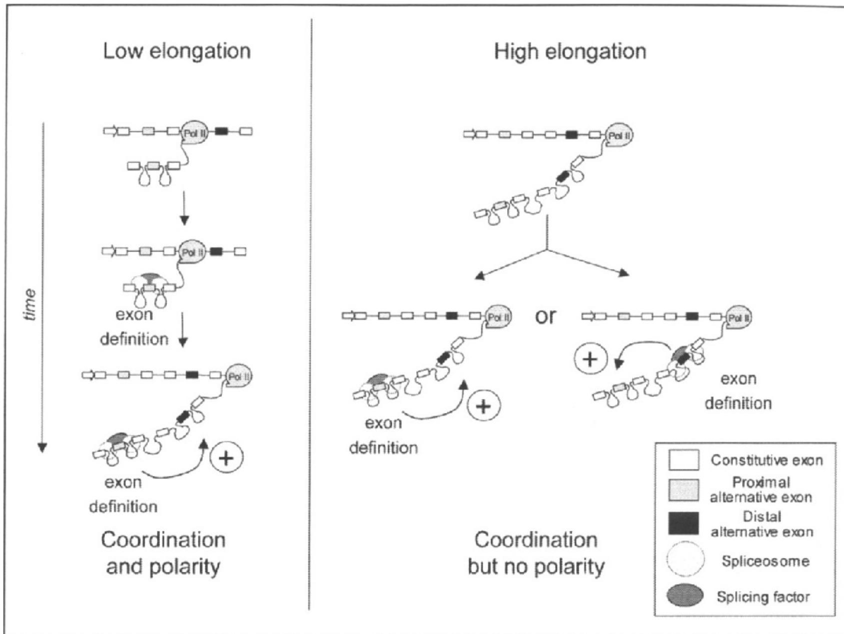

Figure 7. Model for the role of RNAPII elongation on alternative splicing polarity. Low elongation rates or internal pauses (left) allow a temporal window of opportunity for splicing complexes to assemble at the proximal alternative exon before the distal alternative exon is transcribed. As RNAPII proceeds, the exon definition complexes at the proximal alternative exon stimulate distal alternative exon inclusion in a polar way. High elongation rate or lack of internal pauses allows both proximal and distal alternative exons to be exposed simultaneously to the splicing machinery which results in the absence of polarity. Based on Fededa et al.[69]

Conclusions and Perspectives

Transcription elongation and transcription factor recruitment may contribute independently or in a concerted way to the mechanisms by which transcription controls alternative splicing. Some years ago we proposed the idea that changes in the "pausing architecture" of a gene would provoke changes in the alternative splicing pattern of its transcript. In this context, perhaps the contribution of different promoters or differential occupation of a single promoter is not crucial in the cell, but experiments of promoter swapping were important to investigate the real determinants in kinetic and recruitment coupling. Several lines of evidence point to changes in the chromatin structure in internal regions of genes as elicitors of changes in RNAPII elongation and stalling. The use of ChIP methodology has come of age to depict the "orography" of RNAPII, as well as that of proteins involved in transcription, splicingand chromatin structure along genes during cotranscriptional mRNA processing. The roles of posttranslational modifications, such as acetylation and methylation of core histones, should also be investigated. One could imagine that, in the not so distant future, a detailed map will be available of the peaks and valleys corresponding to the distributions of regulatory proteins and modifications on each gene in the genome and under different physiological or pathological conditions. Such information will likely be extremely informative for predicting the corresponding patterns of transcription and processing.

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