Coupling Between Transcription and Alternative Splicing

Ignacio E. Schor, Luciana I. Gómez Acuña and Alberto R. Kornblihtt

Abstract

The scenario of alternative splicing regulation is far more complex than the classical picture of a pre-mRNA being processed post-transcriptionally in more than one way. Introns are efficiently removed while transcripts are still being synthesized, supporting the idea of a co-transcriptional regulation of alternative splicing. Evidence of a functional coupling between splicing and transcription has recently emerged as it was observed that properties of one process may affect the outcome of the other. Co-transcriptionality is thought to improve splicing efficiency and kinetics by directing the nascent pre-mRNA into proper spliceosome assembly and favoring splicing factor recruitment. Two models have been proposed to explain the coupling of transcription and alternative splicing: in the recruitment model, promoters and pol II status affect the recruitment to the transcribing gene of splicing factors or bifunctional factors acting on both transcription and splicing; in the kinetic model, differences in the elongation rate of pol II would determine the timing in which splicing sites are presented, and thus the outcome of alternative splicing decisions. In the later model, chromatin structure has emerged as a key regulator. Although definitive evidence for transcriptionally coupled alternative splicing alterations in tumor development or cancer pathogenesis is still missing, many alternative splicing events altered in cancer might be subject to transcription-splicing coupling regulation.

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J. Y. Wu (ed.), *RNA and Cancer*, Cancer Treatment and Research,
DOI: 10.1007/978-3-642-31659-3_1, © Springer-Verlag Berlin Heidelberg 2013

Keywords
Alternative splicing · Co-transcriptional splicing · Transcription-splicing coupling · RNA polymerase II-CTD · Kinetic model · Chromatin

Contents

1 Introduction

Transcription and splicing are two processes that occur simultaneously in the eukaryotic nucleus and that can functionally influence each other, due to coupling mechanisms.

Several reports in which alterations of either of the two processes are associated with cancer progression can be found. In the case of alternative splicing, examples of cancer associated-mutations that change splicing patterns [1] include tumor suppresor genes like Klf6, where a mutation that originates a binding site for the regulatory protein SRSF5 (formerly SRp40) originates isoforms that presumably act as dominant-negative mutants. Another example is the Brca1 gene, where mutations are thought to eliminate a splicing enhancer on exon 18. In other cases, mutations might not affect the alternatively spliced gene product itself, but can alter the levels and activity of trans-acting splicing regulators instead. This is probably the case of the Ron alternative isoform ΔRon, an inductor of invasive phenotype in gastric carcinomas, whose splicing is regulated by the binding of the SR protein SRSF1.

As it will be shown in this chapter, the transcriptional status can modulate the recruitment of splicing regulators to splice sites. Also, kinetic considerations of transcription must be taken into account, especially in cases of long genes (for example, Brca1 gene is 81kbp-long).

Although we do not have definitive evidence of transcriptionally coupled alternative splicing alterations responsible for tumor development or cancer pathogenesis, recent evidence suggests that the influence of transcription on alternative splicing might determine cell fate decision between apoptosis and cell cycle progression. DNA damage results in changes in RNA polymerase II elongation rate, which ultimately affects the alternative splicing patterns of the *Bcl-*

X and *Caspase9* genes toward the proaptoptotic isoforms [2]. In this context, alterations in the DNA damage response that lead to changes in transcription and, as a consequence, in alternative splicing, might provoke cell cycle progression, and oncogenic transformation.

Another intriguing issue is the existence of genes with multiple alternative promoters in addition to alternative splicing events. In these cases, such as the gene coding for p53 tumor-suppresor protein, interaction between altered promoter choice and altered alternative splicing pattern in cancer is a suggestive possibility deduced from the coupling between transcription and splicing.

In the following pages, we will summarize the evidence for coupling accumulated mainly during the last decade.

2 Alternative Splicing and Its Regulation

We will review here some concepts that will apply to understand the coupling between transcription and alternative splicing that is covered in detail in Chap. 3 of this book.

Understanding alternative splicing regulation requires first a description of the way the splicing machinery identifies exons and introns. Constitutive splice sites are defined by consensus sequences (3′ splice donor, 5′ splice acceptor, polypyrimidine tract, and branching point) that recruit constitutive splicing factors, primarily the small nuclear ribonucleoprotein particles (snRNPs) and several auxiliary factors that mark the boundaries between introns and exons. In order to get accurate splicing, these intron–exon boundaries have to be paired. This pairing can occur across an intron (marking the intron that has to be removed) or across an exon (specifying the exon that must be joined to the adjacent exon). In the first case, we are in the presence of splicing via intron definition, typical of yeast where introns are short; in the second one, splicing takes place via exon definition as can be seen in higher eukaryotes that have small exons separated by much longer introns [3].

Both circumstances (recognition of intron–exon boundaries and pairing of the splicing complexes) are prerequisites for the spliceosome to carry out the splicing reaction. Any interference with this recognition (for example, due to mutation of the consensus sequences or increase in the length of an exon in exon definition) can affect splicing efficiency leading to complete inhibition of the splicing event, or, in milder cases, to alternative splicing. In these cases, strong splices sites would compete against weaker ones leading to a less frequent use of the inefficient splice site.

Using high-throughput sequencing approaches, it is now estimated that more than 90 % of the human genes with multiple exons have alternatively spliced mRNA isoforms [4, 5] and that nearly 86 % of all human genes undergo alternative splicing to generate appreciable levels of two or more mRNA isoforms [4]. Why are not these mutations that decrease splicing efficiency of a given splice site eliminated by negative selection? One possible answer is that new splicing events are created as alternative ones and go through a “test” period before they are

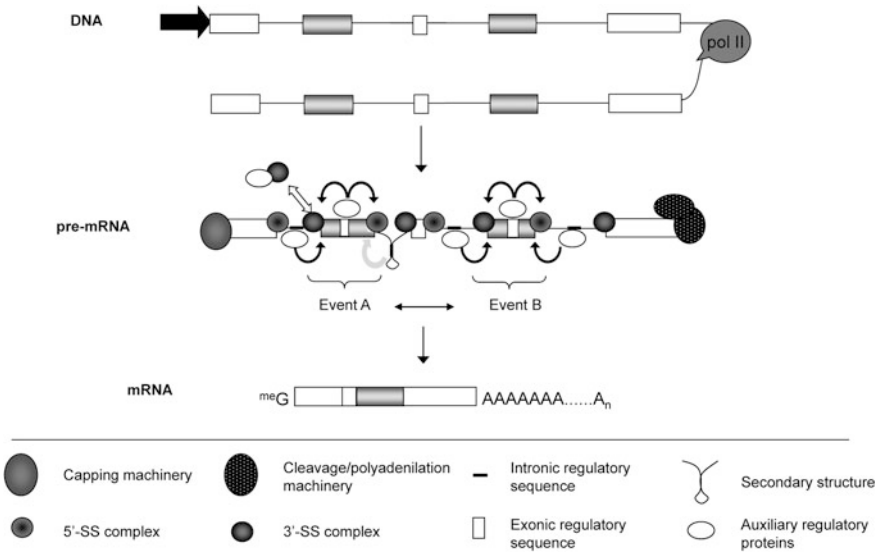

Fig. 1 Combinatorial regulation of alternative splicing, considering post-transcriptional RNA processing. An example of a pre-mRNA with two alternative exon cassettes is presented. Auxiliary proteins can help to recruit basal splicing factors (*light blue arrow*). Auxiliary proteins binding to exonic or intronic sequences modulate the use of the suboptimal splice sites of alternative exons (*black arrows*). Secondary structures of pre-mRNA can inhibit binding of factors to some of these sequences (*gray arrow*). The interaction can either promote of inhibit inclusion of the exons. In addition, splicing outcome of one event (e.g. Event A) can affect splicing of the other (Event B) and viceversa. The final mRNA results from the interaction of all these factors

eliminated by negative selection or stabilized as constitutive [6]. Another explanation would be that the result of these apparently harmful mutations (alternative splicing) is sometimes very useful. The most obvious favorable consequence is that if the exon was part of the coding region of the gene, and the proteins produced by the different mRNAs generated by alternative splicing were stable, that cell would be able to increase its proteomic diversity by means of a very simple and economic way. But perhaps the most important advantage of this process is the ability to control and regulate the relative amounts of the different protein products through alternative splicing regulation. Accordingly to the latter hypothesis, it was estimated that nearly 60 % of the human alternative splicing events have tissue specific regulation [4]. This observation also supports the hypothesis that phenotypic complexity in mammals can be explained, at least partially, by alternative splicing, and that such a mechanism clearly contributes to cell lineage and tissue identity.

If we think in the classical picture of a precursor messenger RNA (pre-mRNA) that has just been transcribed and is waiting to be processed (capped, spliced, and polyadenylated), there should be several ways in which splicing efficiency or recognition of poor splicing targets can be modulated: [7–9].

1. Auxiliary proteins (that would recognize *cis*-acting elements in the RNA molecule) can help or inhibit recruitment of basal splicing factors.
2. Auxiliary proteins interacting with the 3′ and 5′ splicing complexes of the spliceosome can help or inhibit pairing between contiguous exon–intron boundaries.
3. A secondary structure of the pre-mRNA can block the access of the splicing machinery to certain important sequence element, so it would be necessary to recruit additional factors that change the secondary structure.

The picture is even more complex if we consider the existence of pre-mRNAs with more than one event of alternative splicing (it is estimated that this occurs in 25 % of the human genes). The fibronectin (FN) gene is a paradigmatic example [10] as it contains three regions of alternative splicing that display cell type- and development-specific regulation. The combination of splicing events can eventually give rise up to 20 mRNA isoforms in humans, 12 in rodents, and 8 in chicken [11]. In that situation, it is expected that the outcome of one alternative splicing choice can affect the other/s, and that is indeed what is seen within this gene [12]. This effect (known as coordination) is likely to have influence on the processing of several transcripts, as bioinformatics searches detect a strong bias in the splicing isoforms of ESTs (sequences derived from mRNAs) annotated in genomic databases. Other examples of long distance regulation of splice site selection have been reported in equine ß-casein intron 1, [13] and in the human thrombopoietin gene [14].

These levels of modulation can exert their effects alone or in combination with each other, to give some degree of regulation complexity (Fig. 1).

3 Splicing Co-Transcriptionality and Coupling

The classical view of the pre-mRNA as a full length molecule that will be processed (capped, spliced, and polyadenylated) only after transcription is completed has been long known to be incorrect. It is becoming clearer that the nucleus is a highly organized organelle and that nuclear processes occur in coordinated complexes of different proteins and ribonucleoproteins acting in the same region. The concept of co-transcriptional processing has been widely accepted in the case of capping. However, it is now clear that it applies also to splicing and polyadenylation [15–21].

Electron microscopy visualization of *Drosophila* embryo nascent transcripts clearly demonstrated that splicing occurs co-transcriptionally with a reasonable frequency and that splice site selection precedes polyadenylation [22]. Co-transcriptional splicing was also demonstrated for the dystrophin gene [23]. Since transcription of this 2,400 kb-gene, the largest in the human genome, would take approximately 16 h to be completed, co-transcriptional splicing of its pre-mRNA appears as a very intuitive concept. In fact, it is very difficult to conceive that the splicing of the dozens of dystrophin introns would “wait” until the synthesis of a huge 2,400 kb pre-mRNA substrate molecule is finished.

More recently, nascent RNA associated with actively transcribed chromatin was analyzed in comparison with RNA found in nucleoplasm, no longer associated with the transcribing machinery [24]. As predicted for co-transcriptional splicing, introns are efficiently removed within the chromatin-associated RNA fraction suggesting that they are excised while still associated with a transcribing RNA polymerase II (pol II). Moreover, the presence of introns in the pre-mRNA increases toward the 5′end of the two genes analyzed, *c-Src,* and *FN,* suggesting that they are removed as they are transcribed. Exceptions can be found, however, as the 3′terminal *FN* intron is excised, co-transcriptionally, with higher efficiency that the upstream ones.

It should be noted, then, that co-transcriptionality of splicing is not strict, in the sense that introns are not necessarily removed in the exact order they are transcribed. If that was the case, the competition among splicing sites that leads to alternative splicing would be impossible. What really happens is that introns in a transcript can be eliminated in different orders, and some can be processed co-transcriptionally while others are processed post-transcriptionally. This is exemplified by studies with the Balbiani ring 1 (BR1) gene where intron 3, located 3 kb from the 5′ end of the 40-kb-pre-mRNA, is excised mostly co-transcriptionally, but intron 4, located 0.6 kb from the poly A site, is excised co-transcriptionally in only 10 % of the molecules [25]. As in the case of constitutive splicing, alternative splicing was also found to be co-transcriptional, in the sense that it occurs mainly when the transcript is still being synthesized, but the order of the flanking intron removal is not strict [24]. When analyzing alternative splicing events that differ in their regulation mechanisms, flanking introns were removed in different orders [24, 26], indicating the existence of diverse splicing pathways. In the case of the *FN* alternative exon E33, the downstream intron is removed prior to the upstream one and the rate of intron removal is altered by *cis* acting mutations of splicing factors binding sites or SR proteins abundance changes that affect the alternative exon inclusion levels [26]. Overall, this evidence suggests that intron order removal of an alternative splicing event is a reflection of the underlying regulation mechanism.

All the evidence mentioned above suggest that the reaction of splicing occurs while transcripts are still being synthesized, i.e., co-transcriptionally. However, this does not imply, *per se*, that transcription and splicing are two processes that are functionally coupled in terms that one alters the properties or the outcome of the other [27, 28]. On the other hand, the fact that these processes occur at the same time and in the same place seems to be a prerequisite for their machineries to functionally interact in a coordinated manner.

Recently, two reports showed some evidence that strongly suggest coupling between constitutive splicing and transcription in yeast. In one of them [29], inducible reporter minigenes with or without an intron were stably integrated into *S. cereviciae* genome. Upon induction, and at the time point when spliced mRNA was first detected, a transient pol II accumulation, indicating transcriptional pausing, was observed around the 3′ splice site (3′SS) of the intron bearing minigene. When mutating the 3′SS or the 5′SS, pol II signal became lower at the promoter, suggesting reduced transcriptional activity, and its accumulation around the 3′SS was no longer detected. Moreover, in the case of the 3′SS mutation, pol II

accumulation was observed around the 5′SS. Overall, this work indicates that the dynamics of pol II elongation along a gene depend on the presence of a splicing event. In agreement, several splicing factors were found to affect transcription. SC35, for example, stimulates pol II elongation by interacting with P-TEFb, a complex that phosphorilates Ser2 of the CTD, engaging pol II to active elongation [30]. In the second study [31], a genome wide approach was used to provide evidence of a functional association between splicing and transcription. Total RNAs purified from the chromatin associated fraction (i.e. nascent pre-mRNAs) were analyzed with high density tilling microarrays, looking for intronic under, or overrepresentation. The majority of intron bearing genes (taking into account that in yeast most intron containing genes have only one intron) showed co-transcriptional intron removal. In silico simulations, assuming no coupling, predicts a positive correlation between co-transcriptionallity and terminal exon length. However, this did not correlate with the experimental observations as genes with higher efficiency in co-transcriptional splicing than predicted bear shorter terminal exons. Accordingly, this group of genes showed a significant increase in pol II density within the gene body, downstream the intron, in opposition to genes that display lower efficiency than predicted in co-transcriptional splicing [31].

This evidence suggests a selective pressure toward co-transcriptionality in splicing, perhaps to allow the functional coupling between both mechanisms. This raises the question of the possible benefits on nuclear metabolism and gene expression fine tuning. Currently, transcription and processing of mRNAs is thought to be carried out by coordinated complexes of proteins and ribonucleoproteins that link the two steps in the genesis of mature mRNAs, so the efficiency of the general process is greatly improved [15]. As a general principle that can be deduced intuitively, the transcriptional elongation complex might provide a scaffold over which the splicing machinery is recruited or assembled, so that the sequence of events is established in order and there is less chance for mistakes [28]. The general result could be both improved efficiency and enhanced splicing kinetics.

4 Molecular Mechanisms of Coupling

The coordination between transcription and processing seems to be a specific feature of RNA polymerase II. Indeed, when protein-encoding genes are placed under the control of either pol I, pol III, or T7 RNA polymerase promoters, transcription takes place, but pre-mRNA processing is deeply affected, and in particular the resulting transcripts are poorly spliced [32–35]. In vitro assays show that nascent pre-mRNA synthesized by pol II is stabilized and efficiently spliced [36] 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 nonspecific hnRNP complexes which are inhibitory for spliceosome assembly, indicating that pol II mediates the functional coupling of transcription to splicing by directing the nascent pre-mRNA into proper spliceosome assembly [37]. However, other in

vitro assays reported no changes in splicing efficiency or kinetics between pol II synthesized RNA and presynthesized pre-mRNA [27]. This apparent contradiction may be due to the fact that RNA stability is not properly measured and taken into account as a possible cause of the splicing efficiency improvement [27]. This brings us back to the notion that a concurrent splicing reaction along with transcription, which appears to be the case in these assays, does not necessarily imply coupling. We must consider, nevertheless, the limitations of in vitro systems, consisting mainly on nuclear extracts that do not reproduce nuclear organization and architecture and in which DNA is necessarily short and not packed into chromatin [27]. More recently, the Reed lab demonstrated that a large group of proteins interacts with RNA pol II in vivo. This includes almost all known SR proteins and U1snRNP, but not other splicing factors or hnRNPs. Futhermore, using nuclear extracts that perform both transcription and splicing in vitro, they showed that SR proteins are necessary for pol II-mediated coupling, as efficient splicing of extracts depleted of SR proteins is restored if these are added before but not after transcription [38].

A key player in the coupling appears to be the carboxy-terminal domain (CTD) of the catalytic subunit of RNA pol II. In fact, association of splicing factors to sites of transcription is dependent on pol II CTD [39] and deletion of CTD causes defects in capping, cleavage/polyadenylation, and splicing of the β-globin transcript [40].Besides, isolated CTD fragments [41] as well as the entire purified phosphorylated pol II [42] are able to activate splicing in vitro. Nevertheless, isolated CTD fragments cannot duplicate the whole pol II effect unless the precursor RNA is recognized via exon definition. These findings support a direct role for the CTD in exon recognition and lead to the speculation that the CTD would bring consecutive exons closer, which would then facilitate spliceosome assembly. Consistently, Dye and Proudfoot [43] showed that exons flanking an intron that has been engineered to be co-transcriptionally cleaved by inserting a ribozyme in the middle are accurately and efficiently spliced together. These data suggest that a continuous 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 transcribing pol II.

The structure of the CTD is quite unique: it is composed of 52 tandem repeats in mammals (26 in yeasts) of the consensus heptad YSPTSPS. CTD serines at positions 2 and 5 within each heptad are subject to regulatory phosphorylations. Phosphorylation of Ser5 by TFIIH is linked to transcriptional initiation, whereas phosphorylation of Ser2 by P-TEFb is associated to transcriptional elongation [44, 45]. However, it is becoming clearer now that this might be an oversimplification as the mammalian CTD bear 46 Ser2 and 51 Ser1 residues and it can be subjected to other post translational modifications [46]. In this scenario, post-translational modifications are likely to act in a combinatory way to exert different effects to the properties of the pol II in terms of elongation and protein recruitment.

It has been shown that changes in the structure and phosphorylation pattern of CTD modulate co-transcriptional but not post-transcriptional processing [47, 48]. It is worth mentioning that the roles of CTD in splicing may vary depending on the

gene. Transcription by a pol II mutant lacking the CTD causes a dramatic enhancement in the inclusion levels of the FN E33 alternative cassette exon without affecting the efficiency of general splicing [49, 50]. The use of pol II CTD variants with different numbers of repeats revealed that the length of the CTD correlates inversely with E33 inclusion levels, with 19 heptads being the minimum number of repeats necessary to sustain normal E33 splicing. This 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 introns that depend on an exonic splicing enhancer for efficient splicing [51].

Another feature that should be considered as a link between transcription and alternative splicing is chromatin, since some splicing factors are known to be recruited to the transcription site by interacting with specific histone post-translational modifications. Histone H3 lysine 4 tri-methylation (H3K4me3), for example, was shown to associate to major spliceosomal snRNPs, with U2 snRNP being the strongest interaction, through binding to the CHD1 adaptor protein. Downregulation of either CHD1 or H3K4me3 exerted a reduction of the IRF1 pre-mRNA splicing efficiency [52]. A similar adaptor system seems to be relevant in genes whose alternative splicing regulation is dependent on the polypyrimidine tract binding protein (PTB) splicing factor. In such genes, tri-methylated H3 lysine 36 (H3K36me3) recruits PTB via binding the adaptor protein MRG15 [53]. H3K36me3 is a mark associated with transcription as the H3K36 methyltransferase Set1 is known to bind pol II CTD [54, 55].

All this evidence points to a temporal and functional coupling between pol II mediated transcription and mRNA processing, with a central role for the CTD. However, while this is a pre-requisite for an influence of transcription quality on splicing choices, it does not imply this influence *per se*.

5 Evidence of Functional Coupling Between Transcription and Alternative Splicing

Although the results mentioned above strongly suggest that the quality of transcription would influence alternative splicing choices, they do not provide a direct evidence of this "functionally-coupled regulation". The first direct evidence came from experiments of promoter swapping using a minigene containing the extra domain I alternative exon (E33, also known as EDI or EDA) of the FN gene as a splicing reporter [56, 57]. E33 is 270 bp-long and contains an exonic splicing enhancer (ESE) with the core sequence GAAGAAGA, which is targeted by the SR proteins SRSF1 and SRSF7. When transcription of the minigene is driven by the α-globin promoter; for example, E33 inclusion levels in the mature mRNA are about 10 times lower than when transcription is driven by the FN or cytomegalovirus (CMV) promoters. In other genes, similar effects can be observed. Alternative splicing reporter minigenes corresponding to the CD44 and the calcitonin gene-related product (CGRP) genes were placed 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 first type of promoters [58]. However, the 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. Promoter dependent alternative splicing patterns have been also found in the cystic fibrosis transmembrane regulator [59] and in the fibroblast growth factor receptor 2 genes [60].

Promoter swapping is not likely to be found in nature (although alternative promoter usage might resemble it), but this artificial situation may reflect differences in the transcriptional machinery that physiologically are modulated via transcription factor and co-regulator recruitment. In agreement with this view, targeting of different transcription factors to a minigene promoter, using a Gal4-fusion system, differentially affects splicing choices [61]. Constitutive splicing can be modulated also by transcriptional activators, in a pol II CTD-dependent manner [62]. Transcriptional co-regulators have been also implicated in the control of alternative splicing. Several co-regulators of steroid hormone nuclear receptors showed differential effects on alternative splicing in a promoter-dependent manner [63]. Transcriptional co-regulators are proteins recruited to gene promoters through protein–protein interactions with transcription factors that bind directly to DNA. They can act as transcriptional co-activators or co-repressors or both, depending on the promoter context on which they are recruited. For example CoAA (co-activator activator), recruited by TRBP, which is, in turn, recruited to promoters through direct interactions with activated nuclear receptor, can regulate alternative splicing in a promoter-dependent manner. It similarly enhances transcriptional activities fired by the steroid sensitive or insensitive promoters, but only affects alternative splicing of transcripts synthesized from the progesterone-activated MMTV promoter [64].

6 Consequence of Co-Transcriptionality in Splicing

Regulation of splicing, as summarized in Fig. 1, becomes even more complex as we add the dimension of time.

Splicing complexes must be recruited to all introns and exons in a time window that begins when the target sequence is transcribed and extends to the moment of transcription termination (or even after, if we take into account post-transcriptional processing) [20]. Thanks to the powerful chromatin immunoprecipitation technique, co-transcriptional assembly of splicing factors can be examined in vivo. In both yeast and mammalian cells it has been observed that snRNPs and other processing factors accumulate at positions along intron-containing genes, coincidently with the appearance of their target splicing sequences in nascent pre-mRNA [65–68]. As said before, analysis of RNA associated to actively transcribed chromatin showed that both spliced and unspliced forms are detected, which tells us that splicing has already undergone in some of the RNA molecules [24, 68]. Furthermore, the proportion of spliced mRNA associated with chromatin is increased when transcription is paused

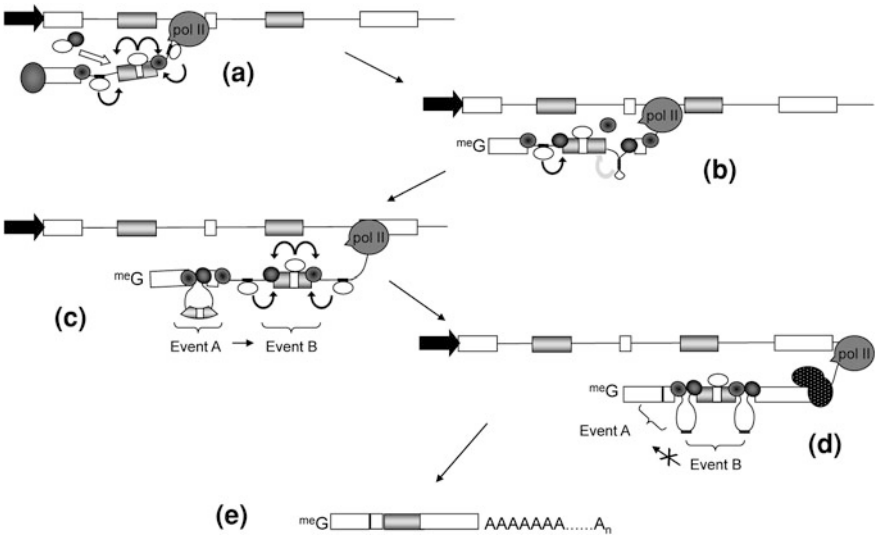

Fig. 2 Example of regulation of alternative splicing in time, considering co-transcriptional RNA processing. (**a**). First alternative exon has recently been transcribed and its fate is being determined by a combination of *cis*-acting sequences and *trans*-acting factors, as described for Fig. 1. The downstream intronic sequence can be targeted by a regulatory factor that is able to regulate 5′ SS use. (**b**). As the downstream intron continues being transcribed, a secondary structure forms that inhibits binding of factors to the intronic regulator, preventing its participation in splicing regulation if the alternative exon fate has not been determined yet. Also, the downstream constitutive exon is now transcribed, enabling competition for the upstream splicing donor. (**c**). The first alternative splicing event (Event A) is already commited for exon exclusion while the second alternative splicing event (Event B) is in an earlier step. In this situation, the outcome of the first event is likely to influence the second. (**d**). When the Event B is commited to exon inclusion, is not possible for it to influence the outcome of Event A, since the later is already spliced. This situation explains the polarity observed in coordination phenomena. (**e**). The final mRNA is the result of the combination of the different regulation steps and the kinetics of transcription

by application of the drug camptothecin (an inhibitor of DNA topoisomerase I) [68]. Therefore, splicing sequences exist that are transcribed in time following a predetermined order, splicing complexes are recruited to these sequences in a different order and splicing catalysis occurs in another order, depending on the quality of splicing complexes and the interaction between them. Processing complexes are also interacting with the transcription machinery (mainly RNA pol II CTD), which might affect co-transcriptional splicing.

The relevance of this situation to alternative splicing is obvious: the timing of these different steps would either favor or disfavor the competition between splicing sites, altering the outcome of alternative splicing. At the same time, exonic and intronic splicing enhancer or silencers are being transcribed and secondary structures in the precursor RNA (that could inhibit or enhance splicing) are changing, further influencing the result of alternative splicing choices. To add an extra level of

complexity, the coordination of different alternative splicing events within a same transcript (the influence of one event in the outcome of the other) would be influenced by the degree of co-transcriptionality.

Bearing these new considerations in mind, we surely need to upgrade our model of alternative splicing regulation by including co-transcriptionality as a key factor (Fig. 2). In the following sections, we will present evidence for the different models of functional coupling between transcription and alternative splicing regulation.

7 Models for Co-Transcriptional Regulation of Alternative Splicing

Two different models have been proposed to deal with the promoter influence on alternative splicing [18]. On the one hand, the promoter might affect the recruitment to the transcribing gene of splicing factors or bifunctional factors acting on both transcription and splicing. On the other hand, the promoter might affect the rate of pol II elongation, affecting, in turn, the timing of co-transcriptional splicing. It must be taken into account that these models do not exclude each other, as they can both act physiologically at different times, in different exons or even together on the same alternative splicing event. However, for the sake of simplicity, we discuss in depth the evidence for the two models separately.

7.1 The Recruitment Model

There are several examples of transcription factors with effects in alternative splicing, such as the transcriptional activator of the human papilloma virus [69], and the thermogenic coactivator PGC-1. Interestingly, PGC-1 affects alternative splicing, but only when it is recruited to complexes that interact with gene promoters [70]. Another example 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. [71]. demonstrated that, similarly to PGC-1, Spi-1 must bind and transactivate its cognate promoter to favor the use of a proximal 5′ alternative site. Other mammalian cell candidates to act as bifunctional factors include the product of the WT-1 gene, which is essential for normal kidney development, [72] SAF-B, which mediates chromatin attachment to the nuclear matrix [73], CA150, a human nuclear factor with characteristic WW and FF domains implicated in transcriptional elongation [74, 75] 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 [76].

There are other DNA-binding proteins, with function in transcriptional regulation, which can exert some effects on splicing regulation. One example is the methyl-CpG-binding protein 2 (MeCP2), originally reported to participate in

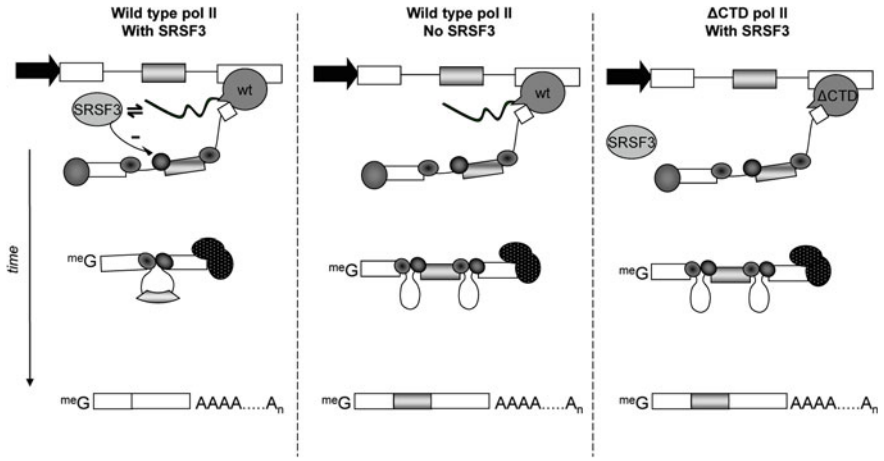

Fig. 3 An example of how the transcription complex can affect alternative splicing by recruitment of splicing factors. The carboxy terminal domain (CTD) of RNA polymerase II mediates the inhibitory effect of the SR protein SRp20 on the inclusion of the alternatively spliced fibronectin E33 exon. Transcription by a WT pol II (*left*) allows recruitment of SRSF3 to the transcription machinery which stimulates E33 skipping. Either absence of SRSF3 or transcription by a mutated pol II lacking the CTD (ΔCTD, right) causes higher E33 inclusion because in both cases SRSF3 is not recruited

transcriptional repression of methylated genes in part via recruitment of histone deacetylases. Young et al. showed that MeCP2 is able to promote the inclusion of a CD44 minigene reporter. Interestingly, this effect does not depend on binding to the DNA, but on interaction with the YB-1 splicing regulator [77].

Based on the experiments that determined the important role of RNA pol II CTD for efficient mRNA processing, some lines of research pointed to the possibility that splicing factors were "loaded" on the CTD and delivered to the splicing sites by the pol II holoenzyme. New insights into this possible mechanism came from in vitro experiments in which the CTD was fused at the C terminus of the splicing factor SRSF1 (formerly called SF2/ASF). Compared to SRSF1 alone, SRSF1-CTD increased the reaction rate during the early stages of splicing. Both the RNA-targeting domain of SRSF1 and phosphorylation of the CTD moiety were necessary for the stimulation of splicing by the chimerical protein [78].

Using a combination of siRNA knockdown, inducible reporter systems and transfection of different RNA polymerases, de la Mata et al. determined that the requirement of CTD coupling differs among splicing factors. Whereas activation of E33 inclusion by the SR protein SRSF1 is not affected by the absence of the CTD, inhibition of E33 inclusion by another SR protein, SRSF3 (formerly SRp20), is completely abolished when transcription is carried out by a ΔCTD pol II, indicating that SRSF3 needs the CTD to be recruited to the transcription/splicing machinery (Fig. 3) [48]. Interestingly, the CTD influences alternative splicing in a way that is independent of capping and 3′ end processing. Although no direct

physical interaction between SRSF3 and the CTD or the whole pol II large subunit could be detected, it should probably exist, perhaps weak or indirect, because SRSF3 has been found in a transcription complex known as "mediator", together with the large subunit of RNA pol II [79].

7.2 The Kinetic Model

As we stated, when we first considered the consequences of co-transcriptionality in alternative splicing, differences in the elongation rate of pol II would determine the timing in which splicing sites are presented, and thus the outcome of alternative splicing decisions. Accordingly, it is expected that regulators of pol II elongation would also act as alternative splicing regulators. A kinetic role for transcription on alternative splicing was originally suggested by Eperon et al. [80], who found that the rate of RNA synthesis affects its secondary structure, which in turn affects splicing. A similar mechanism involving a kinetic link was suggested from experiments in which 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 [81].

7.2.1 Control of RNA pol II Elongation

Further evidences for a role of pol II elongation rates in regulating alternative splicing came from a series of experiments using fibronectin E33 exon as a model. For example, it was showed that transcription factors that stimulate mostly transcriptional initiation, such as Sp1 and CTF/NF1, have little effect on alternative splicing, whereas factors (such as VP16) and transcriptional regulatory elements (such as the SV40 enhancer) that activate pol II elongation provoke skipping of the E33 [61, 82, 83]. Also, the use of a pharmacological inhibitor of the kinase that promotes pol II elongation phosphorylating ser 2 on CTD (p-TEFb) increased E33 inclusion by threefold [61, 84].

Given that, E33 (and most alternative exon cassettes) is alternatively spliced due to a suboptimal 3′ splice site that competes with the stronger 3′ splice site of the downstream exon, a highly processively 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. On the contrary, in the case of a slow polymerase or the presence of 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. This dependence on competing splice sites for splicing response to elongation was supported by mutation analysis showing that the better the E33 alternative exon is recognized by the splicing machinery, the less its degree of inclusion is affected by factors that modulate transcriptional elongation [84].

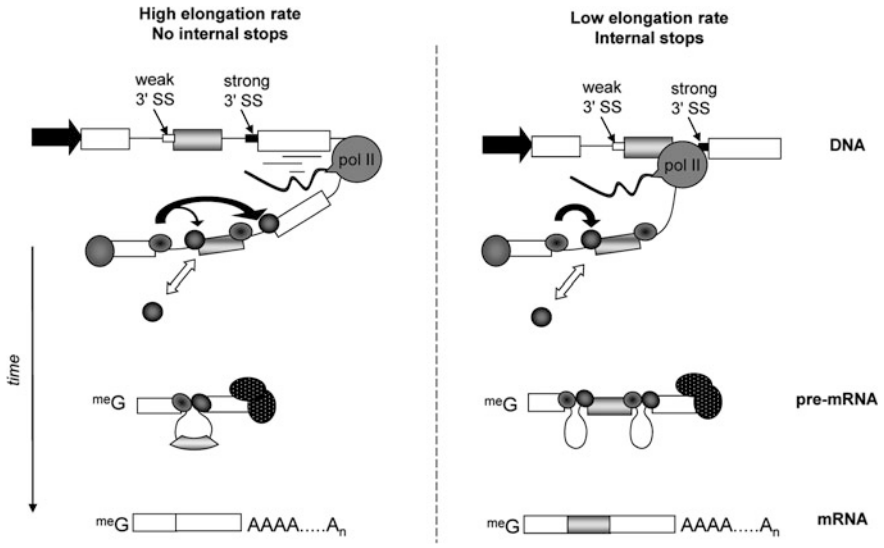

Fig. 4 Kinetic coupling model for the regulation of alternative splicing by pol II elongation. The 3′ splice site (SS) by the alternative cassette exon (*white*) is weaker than the 3′ SS of the downstream intron (*black*). Low transcriptional elongation rates (*right*) favor exon inclusion because it gives time for the weaker 3′ SS to be used, whereas high elongation rates (*left*) favor skipping as it results in almost simultaneous presentation of both 3′ SS

Therefore, in theory, low pol II elongation rates or internal pauses for elongation would favor the inclusion of alternative exons governed by an exon skipping mechanism (such as E33), whereas a highly elongating pol II, or the absence of internal pauses, would favor exclusion of these kinds of exons (Fig. 4). This hypothesis was tested directly using a mutant form of RNA pol II (called C4) with lower elongation rates [85]. The result supported the hypothesis: when a E33 reporter minigene is transcribed by the C4 pol II, the inclusion is 4-fold higher than the same reporter transcribed by a wild-type pol II. Most importantly and of physiological relevance, *Drosophila* flies carrying the C4 mutation show changes in the alternative splicing profile of the large ultrabithorax (Ubx) endogenous gene. The observed changes are consistent with a kinetic mechanism which allows more time for early splicing events. Interestingly, *Drosophila* with the C4 allele in heterozygosis but being wild-type for both Ubx alleles show a mutant phenotype called "Ubx effect" that resembles the one seen in flies haploinsufficient for the Ubx protein. Similar effects of pol II elongation rates on splicing were found in yeast. Alternative splicing is a very rare event in yeast. By mutating the branch point upstream of the constitutive internal exon of the DYN2 gene, an artificial cassette exon that becomes alternatively spliced was created. Skipping of this exon is prevented when expressed in a yeast mutant carrying a slow pol II or in the presence elongation inhibitors [86]. This supports the hypothesis that relative rates of spliceosome formation and pol II processivity are important to the balance between exon skipping and exon inclusion.

More recent evidence called for a revision of the original kinetic model. Using the E33 reporter minigen, it was observed that, when the alternative exon is included, the downstream intron is removed prior to the upstream one. Alterations on splicing factors recruitment that changed E33 inclusion levels, affects the relative rate of intron removal according to the respective splicing factors mechanism of action, as discussed above. However, changes in pol II elongation, caused either by DRB (an inhibitor of the elongation factor pTEFb) treatment or by transcribing the minigene with the C4 mutant, did not alter the rate of intron removal [26]. Therefore, reducing pol II elongation might not necessarily affect the kinetics of intron removal itself, but the early recruitment of splicing factors and commitment to splice.

The kinetics of transcription can also affect the coordination between different splicing events in the same pre-mRNA molecule. According to the model, when the elongation rate is high, both events are processed almost simultaneously so they can influence each other equaly. However, if pol II elongation is slower, polarity is expected as the proximal event can modulate the distal one, but no viceversa. This is because when the second event is transcribed, the first one will already be commited to splice or even spliced. In fact, when coordination and polarity were tested transfecting human cells with minigenes carrying two alternative E33 regions in tandem, separated by 3,400 bp spanning three constitutive exons and the corresponding introns, coordination was observed with transcription driven by different promoters, but polarity was abolished when using a promoter known to transcribe at higher elongation rates. More strikingly, polarity was restored with this promoter if the cells were treated with an inhibitor of pTEF-b that disfavor pol II elongation [12].

Recently, changes in the alternative splicing outcome due to alterations of pol II elongation properties were reported in a more physiologically relevant cellular context. In Muñoz et al. study [2], UV treatment caused an increase in the inclusion levels of E33 into the endogenous FN mRNA, independently of DNA damage in *cis*. Such increase is explained by pol II CTD hyper phosphorylation of Ser2 and Ser5 residues and subsequent transcription elongation inhibition, in agreement with the kinetic model. The same inclusion level increase was observed, without UV treatment, using a mutant pol II that mimics the hyperphosphorylated state (Ser2 and Ser5 are replaced by a negatively charged glutamate). By a similar mechanism, UV treatment also leads to an increase of the Bcl-x and C9 proapoptotic isoforms. Interestingly, this UV effect is independent of p53, a factor shown to be a key player in the apoptotic response. Overexpression of the Bcl-x antiapoptotic long isoform abolished the UV response toward apoptosis, suggesting that modulation on Bcl-x alternative splicing through this mechanism is a key part of the p53-independent apoptotic response.

7.2.2 Chromatin Structure

Modification of the transcription machinery is not the only way to alter elongation rates. The characteristics of the transcription template can be very important as well. In particular, chromatin structure is known to affect elongation. Being

dynamically regulated in vivo (by chromatin remodeling factors or post-transcriptional modifications of histones—such as acetylation and methylation), the modification of chromatin can be an important step of regulation of transcription and transcription-coupled alternative splicing. Accordingly, Trichostatin A, a potent inhibitor of histone deacetylation, favors E33 skipping [61]. This is consistent with the idea that acetylation of the core histones would facilitate the passage of the transcribing polymerase leading to a situation of higher elongation rates. On the other hand, replication of the transfected minigene reporters, after which these template plasmids adopt a more compact chromatin structure which resists the pass of the polymerase, causes a 10 to 30-fold increase in E33 exon inclusion levels in the transcript [82]. It is worth noting that intragenic DNA methylation provokes a close chromatin structure and subsequently reduces the efficiency of pol II elongation, [87] suggesting an additional role of DNA methylation in alternative splicing regulation as it might not only be involved in transcriptional silencing when located at promoter regions.

Batsché et al. [88]. revealed a new role in alternative splicing for the chromatin remodeling factor SWI/SNF whose mechanism of action involves the regulation of pol II elongation. SWI/SNF is known to interact with pol II, 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 (v1 to v10) in the CD44 gene, which is a target for SWI/SNF transcriptional activation. Brm interacts with Sam68, a nuclear RNA-binding protein that, in turn, binds splicing regulatory elements present in the CD44 variable exons and to stimulate their inclusion upon activation of the ERK MAP kinases. ChIP experiments showed that Brm is not only present at the gene promoter but appears distributed along the whole transcription unit with levels that decrease gradually toward the 3′ end. Although also concentrated at the promoter region, pol II molecules display a different distribution inside the gene with a clear accumulation within the variable region, peaking on exon v4. This peak disappears when endogenous Brm is knocked down by RNAi, but is higher when cells are treated with phorbol esthers, that activate ERKs. Furthermore, in this region the phosphorylation status of the CTD shows an enrichment of the non-elongating phospho-ser5 form of RNA pol II (typical of promoters). These findings strongly suggest that activation of Sam68 by ERK triggers the formation of macromolecular complexes together with pol II and Brm at the central block of variable exons where the nascent transcript is exposing binding sites for Sam68, resulting in the stalling of pol II molecules and the subsequent inclusion of the variable exons into mature mRNA, in agreement with the kinetic coupling model. This work suggests the existence of internal road-blocks regulated in vivo by external signals, that can affect alternative splicing according to the kinetic model (Fig. 5) [89].

It is clear now, that modulation of histone post-translational modification patterns can affect the outcome of alternative splicing, bringing into our attention a new field of research with the premise that chromatin structure is likely to be a key player in the effects of transcription over alternative splicing regulation. In a neuronal depolarization model, for instance, it was found that depolarization triggers the skipping of

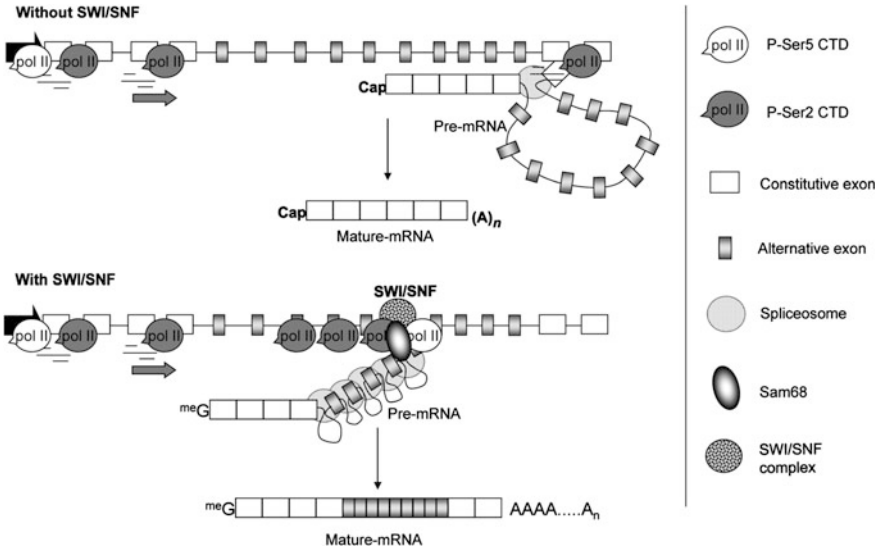

Fig. 5 SWI/SNF stimulates inclusion of alternative exons in the CD44 gene by creating a "road block" to pol II elongation at the variable region. The pause is the consequence of multiple protein interactions involving SWI/SNF, pol II, the splicing regulator Sam68 and spliceosomal components. The phosphorylation pattern of pol II CTD associated to Brm is changed from phospho-Ser2 to phospho-Ser5. This might cause the stalling of pol II molecules coming behind, even if they are phosphorylated at the elongation-competent Ser2

exon 18 from the neuronal cell adhesion molecule (NCAM) mRNA and that this correlates with increased acetylation of lysine 9 of Histone H3 (H3K9ac) between exons 17 and 19, with no parallel increase at the promoter region [90]. Another histone mark associated with transcription elongation, [54, 55] H3 lysine 36 trimethylation (H3K36me3) was also found to increase in distal regions of the *ncam* gene upon depolarization. This is consistent with an increase in RNA pol II pocessivity observed at the area affected by the acetylation changes which explains the decreased recognition of the alternative exon [90].

In a different work [91], targeting transfected small interfering RNAs (siRNAs) to intronic regions downstream of the fibronectin alternative exon E33, increased its inclusion into the mRNA due to local facultative heterochromatin formation, in a mechanism resembling transcriptional gene silencing [92–94]. Moreover, this effect was abolished by treating cells with drugs that favors chromatin relaxation: TSA and the DNA methyltransferase inhibitor 5-azadeoxycytidine, implying a causal relationship between the local repressive chromatin structure and the splicing outcome. In light of these observations, then, it would not be surprising to find out that endogenous noncoding RNAs have a relevant role in alternative splicing regulation through epigenetic modulation.

Recently, genome-wide analysis of nucleosome positioning and histone marks distribution, has revealed striking patterns that can be understand, at least partially,

from the perspective of pre-mRNA processing. In several metazoan organisms, nucleosomes were found to be preferentially positioned on exons, strongly suggesting a role of nucleosome occupancy in exon definition [95, 96]. Accordingly, pseudoexons (nonincluded intronic sequences flanked by strong splice sites) were depleted of nucleosomes, whereas exons flanked by weaker splice sites showed more nucleosome enrichment than those with strong splice sites [96] and included alternative exons were more highly enriched in nucleosomes than excluded ones [95], suggesting a role in alternative splicing regulation as well. Consistently with the kinetic model of coupling, pol II levels were shown to be increased along exons, suggesting that positioned nucleosomes might slow its elongation rate, favoring exon definition [95]. Regarding histone marks, genome-wide analysis revealed a nonrandom distribution as several modifications are enriched in exons but not in introns [95, 96]. The H3K36me3 mark, for instance, was enriched along exons of active genes, [95–97] although this pattern partially reflects underlying nucleosome occupancy [96]. Interestingly, H3K36me3 signal was reduced on alternative exons in comparison with constitutive ones [97]. Altogether, this compelling evidence suggests a role of nucleosome positioning, chromatin structure, and histone modifications in alternative splicing regulation. This influence can be achieved through pol II elongation rate modulation or, alternatively, by recruitment of splicing factors through direct or indirect recognition of the different chromatin marks [52, 53].

8 Concluding Remarks

The combinatorial model once proposed to explain alternative splicing regulation was initially thought to deal with post-transcriptional processing. During this chapter, we wanted to introduce new insights into the complexity of this model when considering transcription-coupled alternative splicing. Although the resulting model is far from being simple, there is little doubt in that it represents a much more realistic picture of the process.

The first issue that must be taken into account is that different splicing events will differ in their degree of co-transcriptionality, and that a specific splicing event can be processed at different relative times depending on the transcriptional status. This degree of co-transcriptionality would affect splicing choices *per se* (according to the elongation model) and would also modulate the effect of *trans*-acting factors (especially those that interact with the transcription machinery) and secondary structures on alternative splicing.

This complex picture can be seen as an epi-phenomenon of the necessity to couple transcription and splicing (an advantageous situation itself as it would facilitate a more efficient processing of the pre-mRNA). However, it is also an opportunity for a multi-step subtle regulation of the alternative splicing process, a physiologically relevant issue when considering different splicing patterns in different tissues or in response to external signals. For example, it is known that the epigenetic status of particular sequences is altered during tissue differentiation, resulting in specific template and transcription patterns for each cell type. It is expected that these

transcriptional differences would impact on alternative splicing choices. In addition, cell signaling can induce changes in transcriptional status of specific genes and localized remodeling of chromatin and epigenetic marks, further affecting alternative splicing.

We also should reconsider previous reports of altered genes or gene expression in some cancer types in light of the generalization of transcription/splicing coupling. One example could be the fusion protein originated in human myeloid leukemia between TLS and ERG proteins. Whereas both normal and fused TLS can interact with RNA pol II, only TLS recruits splicing regulators such as TASR1, also known as SRSF10 (formerly SRp38). This makes TLS/ERG fusion protein act as an inhibitor of TASR-mediated splicing [98].

At this point, it should be noticed that there are plenty of levels where the coupling between transcription and alternative splicing is probably altered in cancer cells, resulting in abnormal expression patterns. The ongoing effort of scientists to put all the regulatory processes together will test and clarify this possibility and define its role in the disease.

Acknowledgments We thank the members of our lab and Anabella Srebrow for criticisms and helpful discussions. This work was supported by grants from the Fundación Antorchas, the Agencia Nacional de Promoción de Ciencia y Tecnología of Argentina, the European Union Network of Excellence on Alternative Splicing (EURASNET), and the University of Buenos Aires. I.E.S. and L.G.A. are recipients of fellowships from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina (CONICET). A.R.K. is a Howard Hughes Medical Institute international research scholar and a career investigator of the CONICET.

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