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Coupling of RNA polymerase II transcription elongation with pre-mRNA splicing

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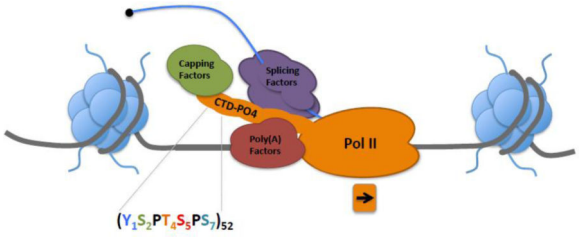
Abstract

Pre-mRNA maturation frequently occurs at the same time and place as transcription by RNA polymerase II (pol II). The co-transcriptionality of mRNA processing has permitted the evolution of mechanisms that functionally couple transcription elongation with diverse events that occur on the nascent RNA. This review summarizes current understanding of the relationship between transcriptional elongation through a chromatin template and co-transcriptional splicing including alternative splicing decisions that affect the expression of most human genes.

Graphical abstract



Keywords

RNA polymerase II; CTD; transcription elongation; alternative splicing; kinetic coupling; histone modification; nucleosome occupancy

Introduction

An important shift occurred in our perception of eukaryotic transcription when it was realized that transcription is not a stand-alone process, but rather is functionally coupled to maturation of the RNA transcript. Thus the major mRNA processing steps of capping, splicing and cleavage/polyadenylation, as well as messenger ribonucleoprotein (mRNP)

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assembly, initiate co-transcriptionally rather than post-transcriptionally [1; 2; 3; 4]. Co-transcriptionality permitted the co-evolution of transcription and processing factors with the result that in some cases they perform their functions in an interdependent or coupled fashion. Transcription and co-transcriptional RNA metabolism are integrated with one another by both spatial and kinetic coupling mechanisms. RNA polymerase II (pol II) is uniquely equipped with an essential appendage, the C-terminal heptad repeat domain (CTD) of the large subunit that is required for all three major mRNA processing reactions [5; 6; 7] and for recruitment of splicing factors to sites of transcription [8]. The co-transcriptional nature of mRNA maturation means that the physiological substrate of the processing factors is not a full-length freely diffusible pre-mRNA, but a transcription elongation complex (TEC) with a growing RNA that is extruded at average rates of 0.5–4 kb/min on human genes[9]. A comprehensive understanding of mRNP maturation must therefore take into account the "co-transcriptionality" of this process. This perspective considers the cycle of transcription initiation, elongation and termination in the context of the processing of nascent transcripts. The "mRNA factory" is a useful model for how transcription and RNA processing occur at the same time and place within a dynamic macromolecular complex [6] that comprises both the RNA synthetic and processing machines (Fig. 1). In this review we focus on the elongation phase of the transcription cycle and its relation to splicing of the nascent transcript.

Transcription elongation is far from a smooth ride for the RNA polymerase. Each journey made by a pol II TEC along a given gene follows a unique narrative punctuated by acceleration, deceleration, backtracking, pausing and release; and premature termination may sometimes end the journey before the 3' end of the gene [9; 10; 11; 12]. Each passage that pol II makes along a gene is influenced by numerous factors that govern elongation and ultimately determine how the nascent RNA grows. These effectors of elongation include sequence elements near the 3' end of the RNA [13], nucleosomal barriers, and factors that bind and modify pol II such as positive transcription elongation factor PTEFb (Cdk9/CycT), negative elongation factors NELF, DRB (5,6-dichloro-1-β-D-ribofuranosyl-1H–benzimidazole) sensitivity inducing factor DSIF (Spt4/5), and TFIIS [12; 14]. Average elongation rate is a function of the rates of catalysis of phosphodiester bonds and associated enzyme translocation as well as the duration of numerous pauses some of which can be several minutes long [15; 16; 17]. Regulated polymerase pausing is used in prokaryotes to coordinate transcription with co-transcriptional translation [18; 19] and recent studies suggest that pausing may operate in eukaryotes to coordinate co-transcriptional pre-mRNA splicing [20; 21; 22; 23]. The rate of nascent RNA growth can also affect the way it folds [24] and RNA structure is an important determinant of how the transcript will be processed by the splicing machinery.

Alternative splicing affects the expression of about 95% of human genes and abnormal proportions of alternatively spliced mRNA isoforms are hallmarks of the transcriptome in many diseases including cancer [25; 2627]. Most alternative splicing decisions are probably made co-transcriptionally [1; 2; 2829] and how elongation rate affects co-transcriptional spliceosome assembly and function is an important challenge that promises to deliver a much deeper understanding of how normal and abnormal alternative splicing decisions are made.

Spatial coupling: recruitment of processing factors to the site of transcription

Coupling in space is achieved by recruitment of factors to the TEC often through interaction with the CTD “landing pad” (Fig. 1). The paradigm for recruitment coupling is that the instructions for processing factor association with the TEC are provided by dynamic phosphorylation of CTD heptad repeats (YS_2PTS_5PS) in a way that is synched with the transcription cycle [30; 31]. Hence phosphorylation of the CTD repeats on Ser5 residues is essential for capping enzyme recruitment at 5’ ends of genes [32; 3334] and phosphorylation on Ser2 facilitates recruitment of cleavage/polyadenylation factors at 3’ ends [35; 36]. How the phospho-CTD code influences co-transcriptional splicing is less well understood, but recent work implicates CTD phosphorylation in recruitment of U2AF65 that binds polypyrimidine tracts and stabilizes U2snRNP binding [37; 38]. Although pol II co-purifies with SR proteins and U1 snRNP [39; 40], and the CTD is required for the SR protein SRp20 to regulate alternative splicing [41], it is currently unclear whether recruitment of these splicing factors requires direct contacts with the polymerase. Expression of a pol II CTD mutant (S2A) that cannot be phosphorylated on Ser2 reduced co-transcriptional splicing in a promoter-specific way as well as U2AF65 and U2 snRNP recruitment [38]. One should note however that mutation of one residue in the CTD heptad repeats could indirectly affect phosphorylation of other residues. Conversely, inhibition of splicing was reported to specifically reduce Ser2 CTD phosphorylation (Ser2-P) suggesting a role of splicing factors in maintenance of this modification [42]. Consistent with this idea, the FUS protein, a regulator of alternative splicing [43] involved in the pathogenesis of ALS, binds the CTD and helps maintain Ser2-P [44]. Furthermore, FUS acts as an adaptor for U1 snRNP binding to pol II [39]. Whether FUS-mediated recruitment of U1 snRNP is affected by Ser2-P and how this recruitment might regulate alternative splicing remain to be resolved.

Recruitment of processing factors to the site of transcription is not exclusively through the pol II CTD landing pad. The mediator complex that binds promoters and enhancers can influence alternative splicing through its Med23 subunit that contacts the splicing factors hnRNPL, SF3B and Eval1 [45]. This effect of mediator might in part account for the seminal observation that promoter sequences can determine how a transcript is spliced [46]. In addition, as discussed below, a growing body of work has identified the chromatin template as a recruitment site for splicing factors.

Kinetic coupling: how elongation rate affects nascent pre-mRNA processing

Kinetic coupling between transcription and co-transcriptional RNA transactions is less well understood than spatial coupling. The “window of opportunity” or “first come first served” model [47; 48; 49; 50] is a helpful way of thinking about this form of coupling. The idea is that when upstream and downstream events on the nascent transcript compete, then the upstream site will have a head start, and therefore a competitive advantage. That advantage is greater when elongation is slow, and smaller when elongation is fast. Potentially competing co-transcriptional events that could be modulated by elongation rate include alternative splice

site and poly(A) site recognition, RNA binding site recognition by regulatory proteins, and base-pairing of competing sequences with a common complementary element as the transcript folds (Fig. 2).

Recent work has underscored the widespread importance of kinetic coupling in determining the outcome of co-transcriptional splicing reactions. The average pol II elongation rate in mammals is 0.5–4.0 kb/minute, however it varies extensively between genes and even between different regions within a gene [9; 11; 17; 51]. The “window of opportunity” model predicts that elongation rate controls alternative splicing by modulating the competition between upstream and downstream 3’ splice sites (Fig. 2A) and it can explain why a slow mutant pol II enhanced inclusion of alternative exons in the fibronectin and NCAM genes [47; 52] and constitutive splicing in yeast [53]. Changing the elongation rate can potentially alter the window of opportunity for binding of both positive and negative splicing factors, which accounts for why slow elongation can also favor exon skipping. For example slow elongation enhances CFTR exon 9 skipping by favoring association of the negative splicing factor, ETR-3 that competes with U2AF65 for binding to the polypyrimidine tract [54].

We used pol II rate mutants to investigate the impact of elongation rate on alternative splicing genome-wide in human cells. Both slow and fast transcription changed the alternative splicing of thousands of exons [55]. Unexpectedly, while there were some cases where slow and fast elongation had opposite effects on splicing outcomes as predicted by the “window of opportunity” model, there were many more examples where slow and fast mutants both increased or both decreased inclusion of a particular alternative exon. This result suggests an optimal rate or “goldilocks” model in which most rate-sensitive splicing events require an elongation rate that is neither too fast nor too slow but “just right” to produce a proper balance of alternatively spliced RNA isoforms. It remains to be determined what differentiates rate-sensitive from rate-insensitive alternatively spliced exons but chromatin environment, RNA structure, or specific sequence motifs could be responsible. In future, it will be of interest to investigate whether elongation rate is regulated under physiological conditions to modulate alternative splicing programs. In addition, kinetic and spatial coupling of transcription with mRNA processing are likely to be interdependent because factors that control elongation rate could be differentially recruited to the TEC and conversely elongation rate could influence recruitment of processing factors.

Detailed understanding of kinetic coupling will require high-resolution analysis of elongation rate, pausing, and nascent RNA structure. Newly developed methods are helping to address this technical challenge (see [9] for a review). Elongation rate can be assayed in a genome-wide fashion by sequencing nascent pol II transcripts at time intervals after pol II is released from a block near the transcription start site induced by the drug, DRB [51; 56]. In this way one can follow the progress of a wave of pol II as it progresses along genes. A low resolution way to detect pausing is by anti-pol II ChIP-seq that measures polymerase density within genes. A pause or slow down can be inferred from a local peak of pol II density, but there are other possible interpretations that are difficult to eliminate such as differential epitope availability (discussed in [57]). Pausing is most directly measured by native elongating transcript sequencing (NET-seq), that precisely maps the 3’ ends of nascent transcripts enriched by high salt urea wash [21 58] or anti-pol II immunoprecipitation [59]. There are

potential caveats to NET-seq experiments however since precipitated pol II complexes may include RNAs that are not directly tethered to the template by pol II including excised introns [59]. Furthermore anti-pol II immunoprecipitation could be biased by epitope availability. These reservations notwithstanding, an elevated frequency of NET-seq reads at a particular location strongly suggests that transcription has paused there. Recently long-range sequencing of nascent transcripts from their 3' ends revealed that in yeasts, co-transcriptional splicing can be completed by the time pol II has extended only 25–30 bases beyond the 3' splice site [58]. This remarkable finding implies that splicing can be completed on the nascent RNA when only about 10 bases beyond the 3' ss have emerged from the pol II RNA exit channel.

Relationship between pausing and splicing

Two important recent studies in human cells applied NET-seq to provide global maps of the 3' ends of nascent transcripts at single nucleotide resolution [21; 22]. Strikingly these studies revealed a high frequency of 3' ends, very close to 3' and 5' splice sites that flank exons. Since splicing intermediates were computationally filtered from the NET-seq datasets, the results suggest that splice sites are potent pol II pausing signals. Pausing at 3' splice sites at the beginning of exons is particularly remarkable because it occurs before exon definition [60] while the splice site is sequestered within the pol II ternary complex. These observations therefore suggest the surprising conclusion that the 3' splice site is recognized by the transcription machinery as a pause site before it is recognized by the splicing machinery as a processing site. NET-seq employing immunoprecipitation with phospho-specific anti-CTD antibodies suggests that pol II paused at 3' and 5' splice sites is Ser5 hyperphosphorylated. In addition CTD Ser2-P appears to increase as pol II is released from the 3' splice site and moves into the exon (Fig. 3). Release from splice site associated pauses might be stimulated by U2AF65 that associates with pol II elongation complexes and inhibits pausing in vitro [61]. In summary, these NET-seq studies show that pausing of specific pol II phosphoisoforms is strongly correlated with splice sites although one potential caveat is that some antibodies against the phosphorylated CTD can cross react with abundant highly phosphorylated SR splicing factors [62]. One attractive idea is that pausing at intron-exon boundaries or elsewhere in exons, provides a window of opportunity for some splicing steps to be completed co-transcriptionally, but whether the duration of transcriptional pauses is long enough to have a meaningful effect on co-transcriptional splicing has not been established [20, 23].

The remarkable coincidence of pause sites with splice sites begs the question of whether pausing at exon intron boundaries is a cause or consequence of splicing. Seminal work in budding yeast using anti-pol II ChIP-seq strongly suggests that at splicing-dependent transcription pause occurs at 3' splice sites [20; 63] however these pause sites have yet to be confirmed by nascent RNA sequencing [58] Pausing near yeast 3' splice sites was reduced by splice site mutations and restored by suppression of a branch point mutation with altered specificity U2 snRNA [20]. Interestingly pol II paused at yeast 3' splice sites has CTD Ser5-P [63] like the pause at mammalian 3' splice sites [22]. When splicing was blocked by *prp5* or U2 snRNA mutations pausing of pol II in a high Ser5-P, low Ser2-P form occurred further upstream within introns suggesting that a splicing-dependent checkpoint must be satisfied before pol II is permitted to resume elongation [63]. This model resembles the switch that

occurs at 5’ ends of metazoan genes from paused Ser5-P pol II to actively elongating Ser2-P pol II [9, 10]; a transition that may involve a capping-dependent checkpoint. The splicing-dependent pause model suggests some interesting questions for future studies: Does the relationship between splicing and pausing differ between organisms like yeast where splicing is specified by intron definition and humans where exon definition predominates? What happens if a splicing checkpoint is not satisfied? One possibility is that transcription could terminate prematurely aided by the Xrn2 5’-3’ exonuclease [64]. How could a splicing-dependent checkpoint signal that is generated far from the pol II active site induce pausing? Intriguingly the yeast U2 snRNP-associated Cus2 protein is required for this pause [63] and its metazoan homologue, TAT-SF1, interacts with PTEFb that functions as a CTD Ser2-Ser5 kinase [65]. Additional interactions between PTEFb and the SR protein, SC35 [66], and the U5snRNP subunit, SKIP, [67] suggest further biochemical links between splicing and transcription elongation. In summary recent insights point to a remarkably precise connection between pol II pausing and recognition of exon-intron junctions and the underlying mechanism is a fascinating problem for future investigation.

Pol II elongation factors and co-transcriptional splicing

The kinetic coupling of co-transcriptional splicing suggests that factors which modulate pol II elongation could affect splicing decisions. As pol II moves through a gene, nucleosomes and other DNA binding proteins can cause it to arrest and backtrack, dislodging the 3’ end of the nascent transcript from the active site [68, 69, 70, 71]. Backtracked pol II can be rescued by the transcription factor TFIIS, which inserts into a side channel in the enzyme and interacts with the active site to stimulate its intrinsic endonuclease activity [72, 73, 74]. RNA cleavage realigns the 3’ end with the active site and allows transcription to continue. TFIIS reduces the duration of pol II pausing and accelerates elongation in vitro [75, 76] and in vivo [59]. Remarkably, depletion of TFIIS in yeast [49] and expression of dominant negative TFIIS in *Arabidopis* [77] both enhanced exon inclusion. These results therefore suggest that pausing of backtracked pol II provides a window of opportunity to complete some splicing reactions co-transcriptionally. Further work will be required to determine whether TFIIS participates in normal control of alternative splicing. It will also be of interest to determine whether additional regulators of pol II pausing including TFIIF, G-Down1 [78], Spt5 [79], elongin [12], and components of the super elongator complex [14] affect co-transcriptional pre-mRNA splicing decisions.

Nucleosome occupancy and co-transcriptional splicing

A corollary of pervasive co-transcriptional splicing, is that this reaction occurs largely in a chromatin environment that can differ between different regions of a gene. The revelation that nucleosomes, and the histone modifications that mark them are unequally distributed between exons and introns [80, 81, 82 83] was an important conceptual breakthrough. Nucleosomes are more densely packed on exons than introns in many organisms because of their higher average G-C content [80, 83, 84] and nucleosome occupancy is higher on constitutive exons than alternative exons [80, 85]. Furthermore exons flanked by weak splice sites have greater enrichment of nucleosomes than those with strong splice sites [83] suggesting a functional connection between chromatin structure and local splicing activity.

Nucleosomes are major barriers to transcription elongation in vitro, but in vivo they are efficiently evicted from transcribed chromatin. When pol II encounters a nucleosome, elongation pauses [59, 72, 86, 87, 88, 89, 90] preferentially at the entry point and 45 bases into the nucleosome at the contact point between DNA and the H3/H4 tetramer [68, 87]. Nucleosome displacement from the template by pol II *in vitro*, is greater when elongation is faster suggesting a two-way relationship between elongation rate and the chromatin speedbumps[91]. Consistent with the speedbump effect of nucleosomes, ChIP-seq and NET-seq showed that pol II occupancy is higher [23, 82, 92, 93], and elongation rate is slower, in exons than introns [17, 94] with the possible exception of fission yeast [95]. Kinetic coupling suggests that pausing at nucleosomes positioned near exons could tip the balance between competing splice sites thereby influencing alternative splicing decisions (Fig. 2). Two recent studies assessed the relationship between nucleosome occupancy and alternative splicing. One study found that near alternative exons that are preferentially included in progesterone treated breast cancer cells, the chromatin structure switched from poorly positioned to well-positioned nucleosomes and this rearrangement correlated with increased pol II occupancy consistent with pausing [96]. A second study addressed whether changes in nucleosome occupancy can cause changes in splice site selection by restricting the histone supply in colon carcinoma cells through inhibition of histone mRNA 3' end formation. In histone-depleted cells, chromatin accessibility and pol II elongation rate increased on several genes and many transcripts had elevated intron retention and altered alternative splicing [97]. Together these studies suggest that positioned nucleosomes enhance splice site recognition and a likely mechanism is through kinetic coupling by nucleosome-induced pol II pausing.

Chromatin remodelers and co-transcriptional splicing

Histone chaperones and remodelers affect the movement of pol II by promoting the assembly or disassembly of nucleosomal roadblocks within transcribed genes. Kinetic coupling implies that histone dynamics could influence RNA processing decisions by affecting pol II pausing. The most important regulators of co-transcriptional histone dynamics within genes are the H2A/H2B chaperone FACT (facilitates chromatin transcription) and the H3 chaperone Spt6, that both increase the rate of pol II elongation through chromatin [98, 99, 100].

Chaperones may also influence pre-mRNA processing indirectly by controlling the deposition of histone variants that can affect the nucleosomal barrier to pol II elongation [101, 102]. One such variant, H2A.Z, which is deposited by SWR-C, stimulates elongation rate in yeast [103] possibly due to enhanced nucleosome exchange and reduced pausing [68]. Mammalian, H2A.Z may also influence splicing through recruitment of processing factors since it can can bind SF3B1, a component of the U2 snRNP [104].

The ATP-dependent chromatin remodelers SWI/SNF and CHD1 promote pol II progress through nucleosomes [105, 106]. They also co-immunoprecipitate with splicing factors and their depletion changes alternative splicing patterns [105, 107, 108, 109, 110]. The effects of the Brm SWI/SNF subunit on splicing correlate with altered pol II pausing, but unexpectedly they are independent of its ATPase activity [105, 108]. It is therefore possible that Brm affects splicing in a chromatin-independent way through its incorporation into hnRNP particles on

nascent transcripts [111]. In summary histone chaperones and remodelers can exert influences over co-transcriptional splicing in at least two ways: 1) by modulating elongation through effects on nucleosome density and deposition of histone variants, and 2) by affecting recruitment of splicing factors to the chromatin template.

Covalent histone marks and splicing

Exons and introns differ in nucleosome occupancy and in the density covalent histone marks. After normalizing for total histone content, H3K27 me1, me2, and me3, H3K36me3, H3K79me1, H4K20me1, and H2BK5me1 are detectably enriched within exons [80, 81, 82, 92, 93, 112, 113] while introns are relatively enriched for H3K79me2, H2BK5me1, H2Bub, H3K4me1, H3K4me2, H3K9me1, H3K23ac, H3K79me1, H3K79me2, H3K79me3 and H4K20me1 [93, 113]. Which of these modifications actually affect splicing and by what mechanisms is an exciting area of investigation (reviewed in [114 115]). Several histone marks associated with actively transcribed genes including H3K36me3, H2BK120 monoubiquitylation (H2Bub1) and H3, H4 N-terminal tail acetylation,are implicated in control of splicing.

The H3K36me3 mark is added co-transcriptionally by the SETD2 methyltransferase, that is recruited to the TEC, either directly by the Ser2-P CTD [116] or indirectly by Spt6 [117]. The importance of H3K36me3 for splicing is highlighted by the fact that SETD2 mutant kidney tumors have splicing defects in about 25% of expressed genes including extensive intron retention [118]. The level of H3K36me3 within alternative exons correlates with their inclusion in spliced transcripts [81, 82] and inhibition of splicing re-positions this mark within genes suggesting that its deposition is sensitive to local splicing activity [119] probably through differential SETD2 recruitment [120]. Not only is H3K36 methylation responsive to splicing, but it can also control splicing by recruiting polypyrimidine tract-binding protein (PTB) and Srsf1 to the chromatin template through contacts with the “chromatin readers” MRG15 and Psip1 [121, 122]. Other covalent histone marks may also recruit splicing factors including SR proteins and U2 snRNP to chromatin, from whence they are presumed to hop onto the nascent transcript [123, 124]. An independent way that H3K36me3 could modulate co-transcriptional splicing is through kinetic coupling since this mark helps maintain nucleosome occupancy within genes [125, 126] and could thereby affect splicing by limiting elongation rate.

Another histone modification implicated in control of co-transcriptional splicing is H2Bub1, which is enriched on genes with fast elongation rates [127]. H2Bub1 cooperates with FACT [128] and SWI/SNF [129] to facilitate elongation by mobilizing nucleosomes within genes. Depletion of the H2B deubiquitinase, USP49 caused extensive changes in alternative splicing marked by preferential skipping of exons with elevated H2Bub1 and reduced association of the U1 and U2 snRNPs with chromatin [130].

Histone H3, H4 hyperacetylation is associated with enhanced exon skipping and acceleration of transcription following depolarization of excitable cells [52, 131, 132]. The proposed kinetic coupling mechanism is that nucleosomes with hyperacetylated N-terminal tails pose a lower barrier to transcription elongation, and indeed single molecule studies show slightly reduced

pausing at a mock-acetylated nucleosome [89]. Interestingly there may be a two-way interaction between the nascent transcript and histone acetylation since RNA-binding proteins, including splicing factors, that associate with the nascent transcript can recruit regulators of histone acetylation including a histone acetyl transferase [133], a deacetylases (HDAC) [134], and an HDAC inhibitor [132].

Heterochromatin, Argonauts, and alternative splicing

Repressive histone modifications in heterochromatin can also influence specific alternative splicing decisions. Surprisingly Argonaut (AGO) proteins, best known as effectors of RNA-guided heterochromatin formation, can also function as regulators of alternative splicing (reviewed in [29; 114]). This connection was discovered by synthetic siRNA targeting of AGO1 to an alternative exon in the fibronectin gene which caused an increase in its inclusion in spliced transcripts [135]. Furthermore, knock down of AGOs changes alternative splicing of many transcripts in human and Drosophila cells [136; 137; 138]. In some cases AGO-regulated alternative splicing of specific exons correlates with sites of H3K9 and H3K27 methylation [135 133]. The latter heterochromatin mark is also implicated in control of FGFR2 alternative splicing by a long non-coding antisense RNA that directs polycomb-mediated H3K27 methylation and promotes a specific alternative splicing pathway for the FGFR2 mRNA [139]. Heterochromatin marks could modulate splicing by kinetic coupling through a localized slow down in transcription elongation [135]. Confirmation of this idea will require accurate determination of elongation rates at defined positions within genes, which remains an important technical challenge. Another potential mechanism of alternative splicing regulation by AGOs is suggested by the finding in *C. elegans* that an AGO complex appears to inhibit transcription elongation directly [140]. Yet another regulatory mechanism is suggested by the observation that splicing factors can associate with AGO1/2 and the H3K9me binding protein HP1 [136; 141; 142 143]. While co-purification of RNA binding proteins with chromatin-associated factors should be interpreted with care because of the potential for indirect interaction through contaminating RNA, these studies suggest that splicing can be modulated by recruitment of splicing factors to the chromatin template through AGO and heterochromatin marks. How AGOs are targeted to alternatively spliced transcripts under normal conditions is poorly understood, but in Drosophila, at least, this mechanism appears to be independent of siRNAs [137].

In summary while much remains to be learned about the connections between co-transcriptional splicing and chromatin structure, a substantial body of evidence points to two mechanisms by which chromatin acts as an effector of splicing: 1) by inducing localized transcriptional pausing that could shift the balance between competing splicing reactions through kinetic coupling (Fig. 2) and 2) by serving as a scaffold for recruitment of splicing factors that might subsequently be handed off onto the nascent transcript.

DNA methylation and splicing

Another feature of the chromatin template that can exert a major influence on co-transcriptional splicing is DNA 5-methyl-CpG methylation (5meCpG), a modification that is depleted in alternative exons that are skipped relative to those that are included [144]. Remarkably inclusion of about 20% of alternative exons is affected by DNA methylation

based on experiments using methylation deficient ES cells [141]. This mechanism of splicing control is mediated by methyl sensitive DNA binding proteins including MeCP2 [144] and CTCF whose binding is inhibited by 5meCpG. CTCF binding to an oxidized hydroxymethyl C-modified sequence element in CD45 exon 5 induces pol II pausing and inclusion of this exon in the mRNA [145, 146]. An alternative potential mechanism by which DNA methylation could affect co-transcriptional splicing is via interaction of RNA binding proteins with methyl sensitive DNA binding proteins. This idea is suggested by binding of the splicing regulator YB-1 to MeCP2, which might account for the splicing defects in Rett syndrome patients with MeCP2 mutations [147].

RNA structure and alternative splicing

A relatively little studied aspect of co-transcriptional pre-mRNA processing to date is the effect of nascent RNA folding. While nascent RNA is often depicted as a passive, linear structure it actually contains multiple layers of information in the form of secondary and tertiary structures [148]. These structures can dictate important events in the life of an RNA molecule, including alternate splice site selection [149]. For example, pre-mRNA structure could mask or unmask sites for RNA binding proteins (RBPs), sequester non-productive, cryptic binding sites and bring sequences such as 5' and 3' splice sites closer together to favor specific splicing events [150, 151, 152, 153 154]. Folding can even impact RNA sequences by recruiting ADARs (Adenosine to Inosine Deaminases Acting on RNA) that catalyze conversion of adenosine to the non-canonical base inosine exclusively in double-stranded RNA. A–I RNA editing frequently affects the sequences of splice sites or splicing control elements [155]. Further, inosine containing RNA is bound by Vigilin proteins [156] that are involved in heterochromatin formation [157], suggesting the possibility that RNA editing could influence transcription elongation rate by affecting chromatin structure.

Recent technological advances have provided the first glimpses into global *in vivo* mRNA structure by structure-seq a strategy that combines chemical probing of secondary structure with deep-sequencing [158, 159, 160]. One observation made so far is that stable structures at 5' splice sites in *Arabidopsis* correlate with unspliced introns [159]. Future insights into the relationship between transcriptional elongation, RNA structure and co-transcriptional pre-mRNA processing should emerge from structure-seq studies of nascent RNA populations. Not only is pol II elongation rate likely to affect how nascent transcripts fold as previously observed in prokaryotes and in vitro [24, 161, 162, 163], but RNA structure may also feed back to control elongation rate. I*n vitro* pol II synthesis of highly structured transcripts is faster than less structured transcripts, probably due to inhibition of backtracking by RNA hairpins [164]. Future investigations of the relationship between pol II elongation and the structure of the nascent transcript seems likely to reveal novel mechanisms of co-transcriptional splicing regulation.

Conclusions and Future Directions

The functional relationship between transcriptional elongation and co-transcriptional RNA metabolism is a fascinating one that is beginning to yield up its secrets thanks to powerful new methods that permit genome-wide analysis of growing RNA ends at single nucleotide resolution. A major remaining challenge is to relate elongation at defined positions within

genes to the splicing and folding of the nascent RNA. Important outstanding questions about how functional coupling of transcription and splicing works include:

1. Is the variation in pol II elongation rate between human genes a result of regulation, and what is its functional significance for mRNA processing?
2. What is the source of the signal(s) that pauses pol II at exon-intron boundaries and how does a splicing dependent checkpoint work? How does the CTD phosphorylation code influence co-transcriptional splicing?
3. How is nascent RNA structure affected by elongation rate and how important is RNA folding for co-transcriptional RNA processing decisions?
4. How does transcription elongation affect RNA binding protein association and spliceosome assembly on nascent transcripts?
5. What is the relative importance of chromatin-mediated regulation of alternative splicing by kinetic coupling versus splicing factor recruitment? Is there a mechanism that transfers splicing factors from chromatin to the nascent RNA?

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Research Highlights

- Kinetic coupling of transcription elongation with pre-mRNA splicing is widespread.
- Transcriptional pausing and mRNA splicing appear to be functionally interdependent
- Chromatin influences splicing through effects on pausing and splicing factor recruitment

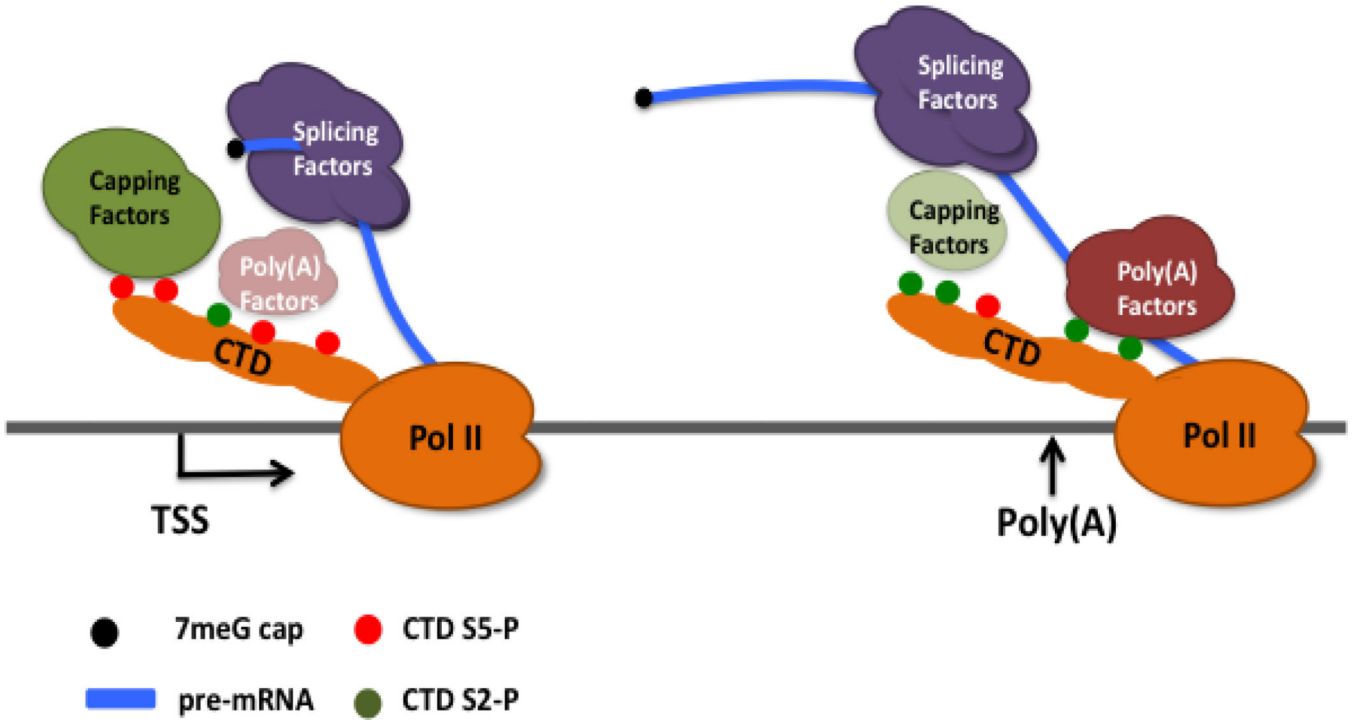

Figure 1.

The mRNA factory model. Coupling of transcription with pre-mRNA processing within a complex that contains both the synthetic and processing machines. Recruitment of RNA processing factors to the transcription elongation complex (TEC) occurs through dynamic interactions with the CTD "landing pad". According to the CTD code hypothesis [30], interactions with the CTD are instructed by dynamic phosphorylation of the CTD heptad repeats including Ser2 and Ser5 residues (S2-P and S5-P, red and green dots) in ways that are synched with the transcription cycle. Capping enzyme and the cleavage/polyadenylation (poly(A)) complexes interact directly with S5-P and S2-P CTD isoforms that are enriched at 5' and 3' ends of genes respectively. Note that capping factors are detected also at 3' ends and polyA factors at 5' ends by ChIP. Whether splicing factors interact directly with the CTD has yet to be confirmed at the structural level. Whether different processing factors can simultaneously localize on the CTD is also not known but it is unlikely to be prohibited on steric grounds. The 7-methyl guanosine cap (7meG) is shown at the 5' end of the nascent RNA (blue line).

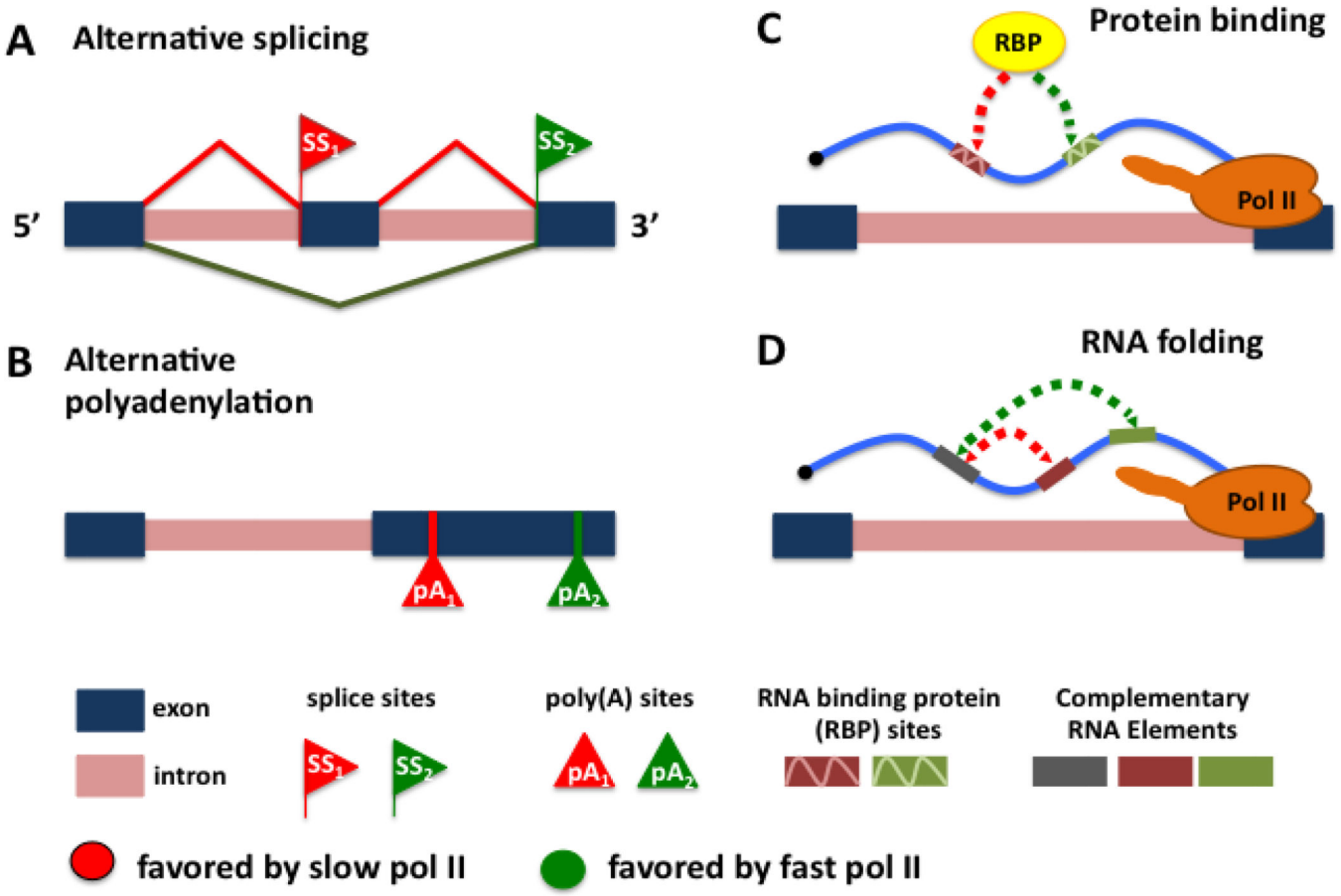

Figure 2.

"Window of opportunity" model for kinetic coupling of nascent RNA metabolism with transcription elongation. When upstream and downstream events on the nascent transcript compete, then the upstream site will have a head start, and therefore a competitive advantage. Slow elongation lengthens the window of opportunity for upstream events to occur before they face competition from downstream sites. Competing upstream and downstream sites on the nascent RNA include: A. 3' splice sites of alternative exons; B. polyadenylation sites; C. RNA binding protein (RBP) recognition sites; D. complementary RNA sequence elements that base pair as the RNA folds. Sites favored by slow and fast elongation are colored in red and green hues respectively.

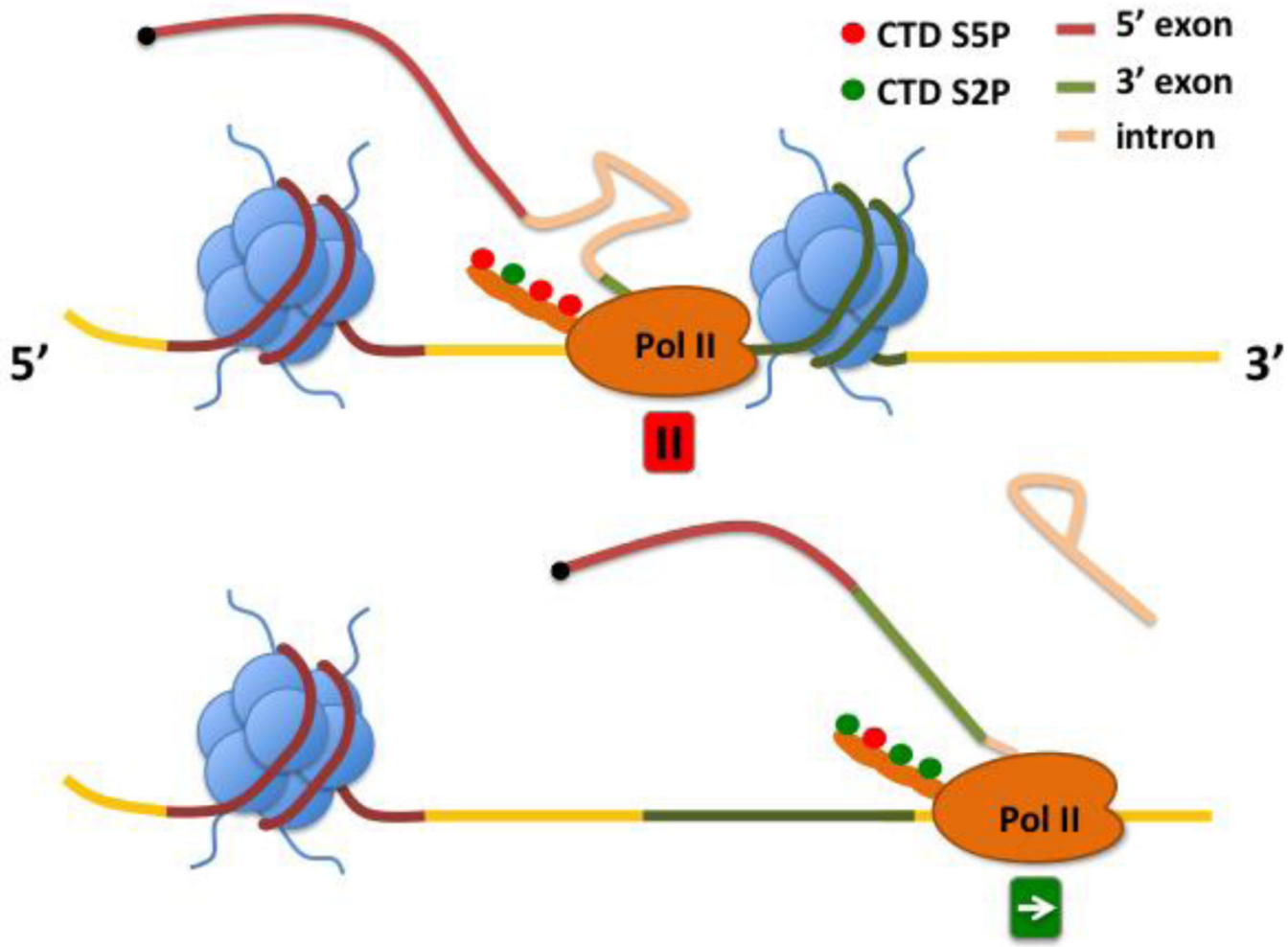

Figure 3.

Splicing-dependent pol II pausing. Commitment to splicing induces pol II pausing close to the 3' splice site at the start of exons, which have higher nucleosome densities than introns. This pause is accompanied by CTD Ser5 phosphorylation. Pol II pauses with high CTD Ser5 phosphorylation (S2-P). Release from this pause is proposed to be contingent on a splicing-dependent checkpoint being satisfied (grey arrow, lower panel) that may be accompanied by increased CTD Ser2 phosphorylation (S2-P) reminiscent of the switch in CTD phosphorylation that occurs following release from the promoter-proximal pause at transcription start sites. This is a speculative model based on references [20_23, 63].