Review

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Pause locally, splice globally

Fernando Carrillo Oesterreich, Nicole Bieberstein and Karla M. Neugebauer

Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany

Most eukaryotic protein-coding transcripts contain introns, which vary in number and position along the transcript body. Intron removal through pre-mRNA splicing is tightly linked to transcription by RNA polymerase II as it translocates along each gene. Here, we review recent evidence that transcription and splicing are functionally coupled. We focus on how RNA polymerase II elongation rates impact splicing through local regulation and transcriptional pausing within genes. Emerging concepts of how splicing-related changes in elongation might be achieved are highlighted. We place the interplay between transcription and splicing in the context of chromatin where nucleosome positioning influences elongation, and histone modifications participate directly in the recruitment of splicing regulators to nascent transcripts.

Co-transcriptional splicing

Transcription of protein-coding genes by RNA polymerase II (Pol II) produces a complementary RNA copy of the DNA template strand, including exons and introns. Pre-mRNA splicing, the removal of introns and ligation of exons, is carried out by the spliceosome, a megadalton complex comprised of five small nuclear RNAs (snRNAs) stably assembled with specific proteins into small nuclear ribonucleoproteins (snRNPs) as well as numerous non-snRNP proteins [1]. The seminal observation in *Drosophila* that chorion gene transcripts appear shortened while still attached to chromatin by Pol II has given rise to the concept that splicing can take place, at least in part, co-transcriptionally; that is, during the process of transcription [2,3]. The interpretation that electron-dense particles mapping to intron positions might be spliceosomes later achieved support from imaging and chromatin immunoprecipitation (ChIP) experiments in other systems, which localized spliceosomal snRNPs to transcribing genes [4–7].

The demonstration that a good deal of spliceosome assembly takes place co-transcriptionally still left the general question of whether introns are partially or completely removed from nascent RNA unanswered [8]. Because nascent RNA is such a vanishingly small proportion of the total mRNA in cells, further evidence for co-transcriptional splicing accumulated over the years, based on the study of selected endogenous or reporter genes in a variety of species [7,9–11]. Very recently, the number of examples of genes that undergo co-transcriptional splicing has expanded, due to the development of highly sensitive RT-PCR and high-density tiling microarray assays [12–15]. In budding yeast, global analysis has established that the majority of introns are co-transcriptionally excised [16]. Nevertheless, the question of how widespread co-transcriptional splicing is among genes or among specific introns within genes remains an open question in most species.

Why is it important whether introns are excised co-transcriptionally? The crucial experiment would be to block co-transcriptional splicing and thereby force splicing to occur post-transcriptionally. Would post-transcriptional splicing be less efficient, as suggested by *in vitro* experiments that couple transcription and splicing [17,18]? Would alternative splicing outcomes differ? Would mRNP assembly, subsequent nuclear export and/or mRNP stability suffer [19]? It is currently not possible to separate transcription and splicing *in vivo*, so we cannot yet answer these important biological questions. However, the notion that spliceosome assembly and splicing catalysis are co-transcriptional has stimulated researchers to address possibilities for coupling among the transcription and splicing machineries [20]. Recent progress through *in vivo* approaches has started to pay off. In this review, we will focus on exciting recent developments in two areas: evidence for direct coupling between transcription elongation and splicing and the demonstrated involvement of histone modifications in splicing factor recruitment to nascent transcripts.

Functional coupling of splicing and transcription elongation

Assembly of the catalytically active spliceosome depends on the stepwise recruitment of splicing factors as well as regulated, energy-dependent structural rearrangements, leading to the sequential formation of two catalytic centers on the substrate pre-mRNA [1]. This cascade of assembly events requires a substantial amount of time. Estimates of the time required for splicing *in vivo* lie between 30s and 10 min [3,6,10,13,21]; the 30s duration from intron recognition to catalysis measured in fly, yeast and human cells is likely to be a good estimate, due to the high resolution of the assays used (Box 1). Splicing occurs co-transcriptionally only if catalysis finishes before transcriptional termination. Thus, co-transcriptional splicing is in 'kinetic competition' with transcription elongation and transcript cleavage at the polyadenylation site. This is nicely illustrated by the observation that promoter-proximal introns are co-transcriptionally removed to a greater extent than downstream introns, suggesting that many introns might be post-transcriptionally excised in metazoans [4,12].

Traditionally, transcription elongation was seen as a continuous process with a uniform rate (nucleotides/min), potentially showing stochastic pausing along the gene body

Corresponding author: Neugebauer, K.M. (neugebau@mpi-cbg.de).

Box 1. *In vivo* determination of splicing rates

In principle, there are two rates that are relevant to co-transcriptional splicing: the rate of spliceosome assembly and the rate of catalysis. The recent measurement of splicing rates in human cells was achieved by fluorescence correlation spectroscopy of spliceosomal snRNPs as they diffuse throughout the nucleoplasm and interact with endogenous pre-mRNAs; snRNPs bound to pre-mRNAs within seconds and splicing took place within 15-30 seconds [21]. This rate includes both spliceosome assembly as well as catalysis and reflects a rate averaged over all expressed introns in the cell.

Because the spliceosome assembles in a step-wise manner [1], it follows that the occurrence of specific steps in spliceosome assembly depends strongly on gene architecture and will vary from gene to gene. For example, early factors such as the U1 snRNP can associate with the 5′ splice site as soon as it is transcribed [5,6]; however, further spliceosome assembly and catalysis cannot occur until the entire intron and 3′ splice site are present. Therefore, a useful parameter for determining splicing rates is the distance in nucleotides between the 3′ splice site and the downstream location of exon ligation; this has been done in two ways: 1) direct observation of intron lariat formation and nascent RNA shortening in electron microscopic images of chromatin preparations [2,3]. In the fly, intron loop removal and/or nascent RNA shortening was observed 1-4.5 kb downstream of the 3′ splice site. 2) An alternative method in yeast is to use ChIP of the MS2 binding protein to measure the position of MS2 stem-loop formation by splicing or, conversely, removal of an intronic MS2 stem loop [8]. In this case, both measurements indicated that splicing occurred ~1 kb downstream of the 3′ splice sites. Both methods yield distances, not rates. A time frame can be calculated, assuming an average elongation rate; in yeast, splicing of the MS2 reporter therefore occurs ~30 seconds after 3′ splice site synthesis [8]. In a recent study of a distinct, inducible reporter gene, splicing was found to occur within a time window of 60 seconds, when assayed independently of transcription elongation rate by RT-qPCR [10].

How splicing rates differ among genes or introns is poorly understood; yet it is clear that introns vary in their rate of excision, and intron removal does not always proceed according to the order of synthesis [12,15]. These differences could be due to the combined influence of gene architecture and splicing regulation on spliceosome assembly. It is currently assumed that catalysis by active spliceosomes follows uniform kinetics, but future work might challenge that assumption.

(Figure 1a). Accordingly, elongation rates have been determined by measuring the average time needed to transcribe a defined sequence (Box 2). Elongation rates *in vivo* determined by independent methods in different species reside in the same order of magnitude, ranging from ~1 to ~4.5 kb/min [14,22–25]. The elongation rate is a crucial determinant of the co-transcriptional availability of introns and exons for splicing [16]. In agreement, decreased elongation rates caused by a mutant Pol II or external stimuli alter splicing patterns [26–28]. Here, an apparently uniform decrease in elongation rate delays the synthesis of downstream competing splice site sequences

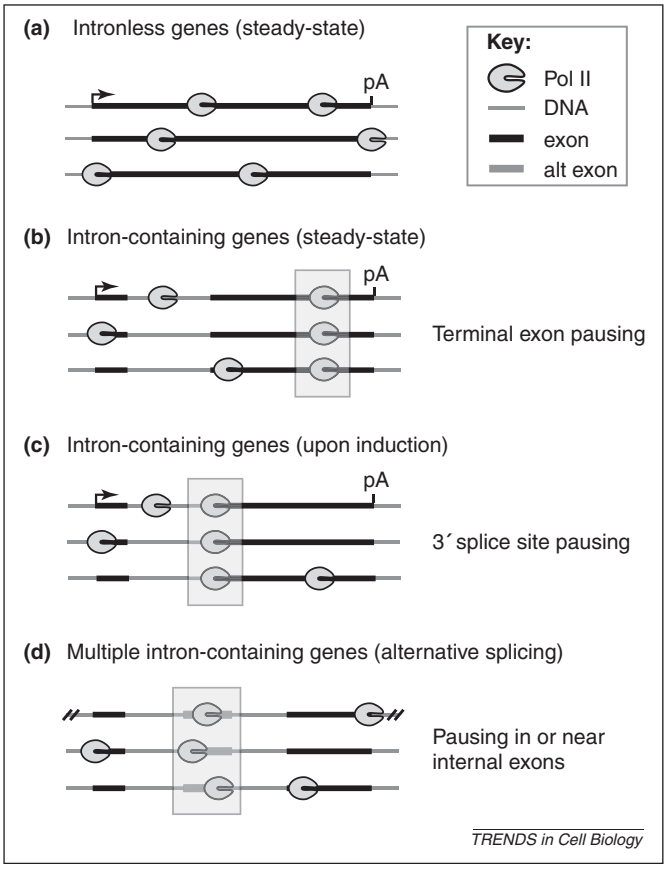

Figure 1. Three transcriptional pausing events associated with splicing. (**a**) Pol II does not reproducibly pause at particular sites along intronless genes in budding yeast. By contrast, intron-containing genes display Pol II pausing at discrete sites with respect to gene architectural landmarks: (**b**) In terminal exon pausing, detected in exponentially growing, unsynchronized cells, Pol II pauses ~250 nt before the end of the terminal exon. (**c**) In 3′ SS pausing, detected in transcriptionally induced genes with introns, Pol II pauses transiently at or near 3′ SS. (**d**) In metazoans, Pol II enrichment in or around internal exons is consistent with a role for pausing in alternative cassette exon inclusion.

Box 2. *In vivo* determination of transcription elongation rates

Regulation of transcriptional elongation to control co-transcriptional splicing is an emerging concept in the field. One major challenge towards a better understanding of this regulation is to accurately quantify elongation rates *in vivo*. To this end, it is necessary to quantify the movement of Pol II along the DNA template at high resolution. At present, absolute, numerical values for elongation rates are gained by determining the average time needed to transcribe a defined length of DNA. Two different approaches have been pursued: 1) Quantification of Pol II progression along a gene at different time points after transcription induction or shut-off is measured either by Pol II ChIP [22] or by analysis of RNA synthesized by Pol II [14]. The movement of the ''Pol II wave-front'' is used to deduce average elongation rates. 2) Quantification of RNA synthesis during steady-state transcription. Here, the reporter RNA is detected *in vivo* by association of a fluorescently labeled protein, and fluorescence recovery after photobleaching (FRAP) allows determination of synthesis rates [23,25,92]. A related approach uses *in situ* hybridization to count the nascent RNA molecules associated with endogenous genes; normalization to gene length provides the average elongation rate [24].

In vitro experiments nicely illustrate the downside of averaging elongation rates over genes: Local fluctuation of elongation rates, including Pol II backtracking, renders average elongation rate over a stretch of several thousand bases inaccurate and potentially misleading [38]. Changes in elongation rates within genes appear to be the rule rather than the exception *in vivo* [51], yet these can currently only be estimated as relative changes by Pol II ChIP or nascent RNA preparation analyzed on high density tiling arrays [16,95–97,101]. In agreement with non-uniform elongation rates, Pol II density varies enormously along genes. Although, these studies show changes in elongation behavior at high resolution, it is impossible to deduce absolute rates. To fulfill the need for high-resolution quantification of *in vivo* elongation rates, both existing methods might in future be combined through modeling.

and thus promotes the inclusion of weak upstream exons. A similar effect is observed when pause sites occur locally [29–31]. By contrast, local increases in elongation rates lead to skipping of an alternative exon [32,33]. Thus, accumulating evidence that elongation rates can vary *in vivo*, suggests that regulation of transcription elongation, either globally/locally or induced/constitutive, might control co-transcriptional splicing (Figure 1).

Combination of known splicing rates and constant elongation rates along intron-containing genes in *Saccharomyces cerevisiae* predicts that most splicing in yeast is post-transcriptional due to the shortness of terminal exons [8,16]. However, two recent reports show that the assumption of uniform elongation is incorrect [16,34]. First, Pol II elongation slows at specific sites before termination of endogenous genes, allowing sufficient time for splicing to occur co-transcriptionally [16]. This phenomenon, termed terminal exon pausing (Figure 1b), was discovered by high-density tiling array analysis of nascent RNA purified from a chromatin fraction. This approach allowed determination of local changes in Pol II density at high resolution as well as quantification of co-transcriptional splicing. Consistent with terminal exon pausing, the majority of yeast intron-containing transcripts were spliced co-transcriptionally. Taken together, these data, based on the steady-state situation of exponentially growing budding yeast, show that terminal exon pausing delays 3' end cleavage and provides time for co-transcriptional splicing. Therefore, terminal exon pausing is an example of functional coupling between splicing and transcription.

Another splicing-dependent Pol II pausing event was described in a parallel study [34]. Here, a series of inducible reporter constructs based on the previously characterized Ribo1 gene [10] were integrated into the *S. cerevisiae* genome. Pol II distribution as well as the phosphorylation state of its carboxy-terminal domain (CTD) was measured by ChIP and quantified by qPCR. This innovative experimental set-up quantifies the changes in Pol II profiles with high temporal resolution. Upon induction of transcription, a transient accumulation of Pol II was detected around the 3′ splice site (3′ SS) of the reporter (Figure 1c). Accumulated Pol II was phosphorylated at serine residues 2 and 5 of its CTD. Pol II enrichment and CTD phosphorylation reoccurred at later time-points, at least twice within a time window of 14 min. Strikingly, the transient Pol II accumulation was shown to be splicing dependent: Neither intronless nor reporter constructs defective in either step of splicing showed accumulation of Pol II. Likewise, base-pairing between U2 snRNA and the pre-mRNA was essential for pausing. These data suggest a model whereby co-transcriptional splicing leads to pausing at 3' splice sites, likely through the activity of a second-step splicing factor or by completion of the splicing reaction itself.

While both studies demonstrate a functional link between splicing and transcription, the nature and position of the observed pausing differ (Figure 1): The pause described by the Beggs laboratory is transiently formed shortly after induction of transcription. Interestingly, Pol II accumulation fades out and reappears in a periodic fashion. To our knowledge, this study is the first kinetic description of Pol II distribution and pre-mRNA splicing before mRNA levels reach steady state [34]. By contrast, terminal exon pausing is detected in exponentially growing, unsynchronized cells on endogenous genes. On average, terminal exon pausing begins ~200 nt downstream of the 3′ SS and ~250 nt upstream of the poly(A) site. Stalling or slowing transcription at this position extends the time the nascent RNA, including the complete intron, is accessible to the spliceosome, thereby increasing the chance of co-transcriptional splicing [16]. The Pol II accumulation described by the Beggs laboratory is situated at the end of the intron near the 3′ SS, resulting in longer exposure of at least part of the intron to splicing factors. A release from pausing might optimize later steps in splicing. The discovery of widespread co-transcriptional splicing of endogenous genes in concert with identification of 3′ SS and terminal exon pausing [16,34] indicates that co-transcriptional splicing might indeed confer important advantages for gene expression.

The strong evidence for splicing-specific alteration of Pol II elongation begs further mechanistic insights into the cause and nature of the respective pauses. The best characterized pausing mechanism so far is backtracking. A backtracked polymerase 'slides' in reverse on the template DNA resulting in loss of the free RNA 3' end in the catalytic center [35]. The biological significance of backtracking was established by the demonstration that promoter proximal Pol II stalling in *Drosophila* correlates with backtracking [36]. Apart from recruitment of TFIIS, an elongation factor that releases backtracked Pol II [37,38], RNA secondary structure could positively or negatively alter backtracking behavior [39]. Interestingly, both the chance of entering backtracking as well as the duration of a backtrack-induced pause could lie in the thermal stability of the DNA–RNA duplex in the catalytic core of the polymerase as well as in the DNA–DNA duplexes either melted upstream or annealed downstream of the transcribing polymerase. Likewise, the influence of RNA secondary structure on backtracking, as well as the formation of R-loops (base-pairing between nascent RNA and single-stranded DNA behind transcribing Pol II), another potential regulator of elongation rates [40], depends on the thermodynamic stability of the underlying sequence. Strikingly, exons are characterized by a high GC content compared to introns [41–43], raising the possibility of exon-specific elongation rates, dictated solely by the thermodynamic stability of the DNA duplex.

The increased GC content in exons relative to introns favors another potential influence on local elongation rates, nucleosome positioning [42–44]. Nucleosome disfavoring sequence elements are located at exon–intron boundaries resulting in a depletion of nucleosomes over the 5' and 3' splice sites [42,45]. In agreement, nucleosomes are reported by several independent studies to be positioned on internal exons in various species and cell types [42–44,46–48] but one study challenges the generality of this phenomenon [49]. An appealing hypothesis is that nucleosome positioning can regulate elongation kinetics. Indeed, *in vitro* as well as *in vivo* data show that nucleosomes impose a natural barrier to transcribing Pol II [50,51]; *in vivo* data show a significantly higher density of Pol II over exons compared to introns, suggesting

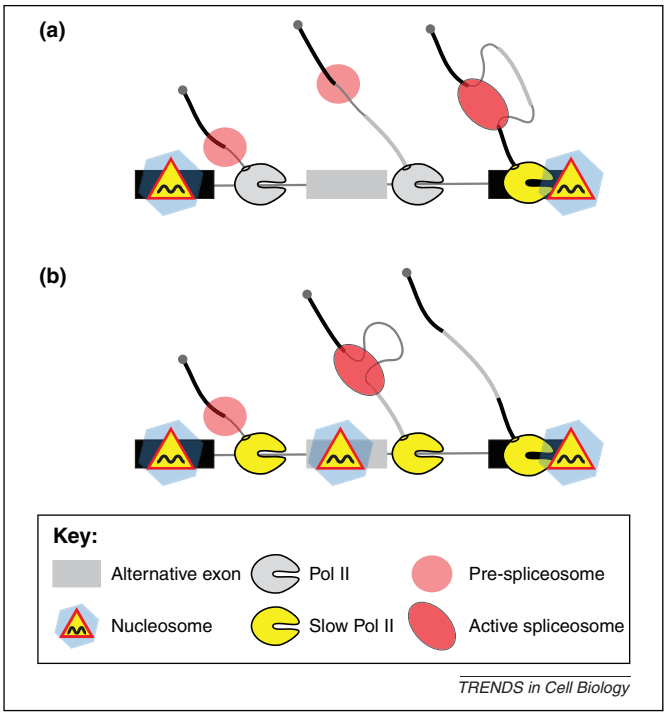

Figure 2. Positioned nucleosomes obstruct elongation across internal exons. In metazoans, nucleosomes (blue hexagons) are positioned over internal exons (filled boxes) and might act as 'speed bumps' to slow Pol II. Local decreases in transcription around alternative exons (gray boxes) prolongs the time splicing sequences in the nascent transcript are exposed to the splicing factors (red shapes). (**a**) Depletion of nucleosomes from alternative exons results in fast Pol II (gray shape) and quick synthesis of the 3′ SS of the constitutive downstream exon, competing with weak splice sites of the alternative exon. Thus, strong 5′ and 3′ SSs of constitutive exons are chosen, resulting in alternative exon skipping. (**b**) By contrast, nucleosomes positioned on an alternative exon act as speed bumps, slowing Pol II (yellow shape); resulting in delayed synthesis of competing splice sites and thus recognition and inclusion of the alternative exon.

pausing [42,44,48,49,52]. Slowing transcription within metazoan exons might regulate co-transcriptional splicing by increasing the time available for splicing of upstream introns, similar to terminal exon pausing seen in yeast (Figure 2). If pausing occurs near the 3′ SS, as observed in yeast [34], synthesis of alternative 3′ splice sites might be delayed, allowing inclusion of upstream exons. In agreement with splicing regulation, exons flanked by weak splice sites show a stronger enrichment of nucleosomes than exons with strong splice sites, and nucleosome occupancy is correlated with inclusion levels [42,43]. Moreover, pseudo-exons are depleted of nucleosomes [43,46], suggesting that impaired nucleosome positioning inhibits exon recognition. Interestingly, nucleosome positioning on exons is independent of transcription, suggesting that this epigenetic mark is not determined by splicing.

To pinpoint the immediate cause of altered Pol II elongation, future research has to meet the challenge of quantifying the influence of these different mechanisms on transcription elongation *in vivo* (Box 2). Given the recent discoveries of pausing at the 3′ SS and in terminal exons, it is now crucially important to determine if either or both of these phenomena occur in other species. If, as discussed above, nucleosome positioning at exons leads to pausing in higher organisms, these local pausing events could contribute significantly to alternative splicing on a global level (Figures 1d and 2).

Transcription and splicing in the context of chromatin

Co-transcriptional splicing takes place in the context of chromatin and recent studies have indicated that chromatin organization, including nucleosome positioning (see above) and histone modifications, are coupled to splicing [53,54]. One emerging view is that nucleosomes and specific histone modifications help mark exons in a sea of introns to facilitate splicing factor recruitment. In addition to the observed nucleosome positioning over exons, ChIP-chip and ChIP-Seq data show specific enrichment of certain histone modifications over exons. Several groups reported that H3K36me3 is positioned over exonic sequences [42,46,48,49,55]. In contrast to nucleosome positioning, the enrichment of this histone modification is transcription-dependent, because the H3K36 methyltransferase Set2 associates and travels with Pol II along the gene, thereby co-transcriptionally methylating H3K36 [56–58]. The enrichment of H3K36me3 over exons does not appear to simply reflect the underlying nucleosome distribution, an issue that has been addressed by a number of data-normalization schemes. Although the field has yet to reach a consensus on which histone modifications are specifically enriched over exons, H3K36me3, H3K27me1, H3K27me2, H3K27me3, H4K20me1, H3K79me1 and H2BK5me1 levels are reported to be elevated over exons even after normalization to either total H3 or MNase digestion [42,46,48,49,59]. Moreover, peaks of 10 additional histone marks were localized to promoter-proximal introns extending downstream of the transcription start site until internal exons are reached: H2BK5me1, H2Bub, H3K4me1, H3K4me2, H3K9me1, H3K23ac, H3K79me1, H3K79me2, H3K79me3 and H4K20me1 [49,59]. These histone marks appear to have a reciprocal pattern along genes compared to H3K36me3, which increases in downstream gene regions [59].

Due to the complex relationship between histone tail modifications and gene architecture, it is not clear whether particular histone marks show specific enrichment over all exons. Disagreements might arise from the use of different data sets for analysis; (i.e. ChIP-Seq or ChIP-chip and variation in experimental protocols [42,49]). Moreover, differences in methods of data analysis among groups could lead to further differences in results and their interpretation, even when working with the same raw dataset. Additionally, commercially available antibodies directed toward histone modifications vary in specificity and quality. Egelhofer *et al*. tested 246 antibodies against 57 histone modifications [60]; notably, 25% failed specificity tests in dot blot or western blot, and 20% of the specific antibodies failed in ChIP[*]. Histone modification antibodies must therefore be tested thoroughly. Unspecific binding can be blocked pre-incubation of the antibody with the undesired modified peptides [61]. These findings highlight the need to resolve the issue of chromatin modifications positioned over exons, using improved or complementary methods.

Can histone modifications influence alternative splicing? A recent study used membrane depolarization of neuronal cells to study NCAM alternative splicing under physiological conditions [32]. Upon depolarization, NCAM

[*] The database used is available at: http://compbio.med.harvard.edu/antibodies/.

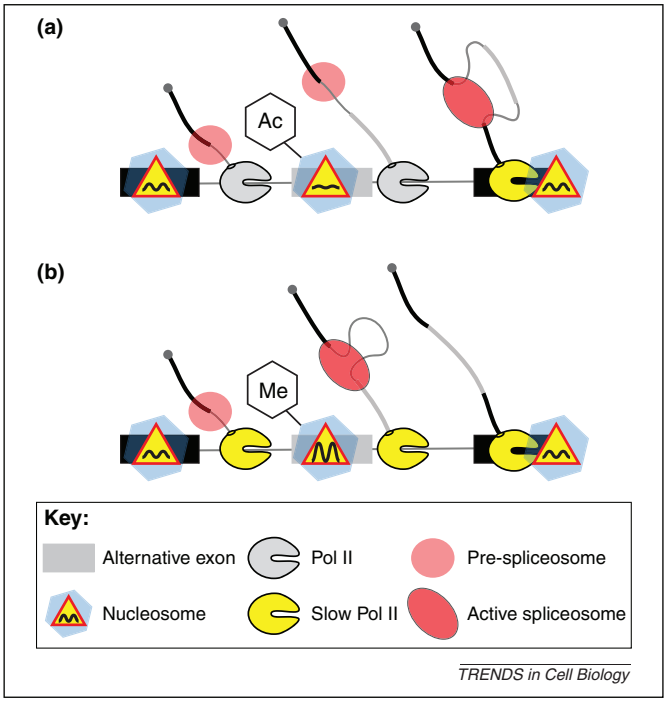

Figure 3. Chromatin environment regulates transcription and splicing. Histone modifications lead to local changes in elongation and thereby affect co-transcriptional splicing. (**a**) Acetylation of histones leads to a more open chromatin structure around an alternative exon (gray box). Here, nucleosomes (blue hexagon) show decreased 'speed bump' potential, allowing high Pol II elongation rates (gray Pol II) and quick synthesis of competing downstream splice sequences. Consequently, spliceosome formation (red shapes) occurs on strong splice sequences surrounding the alternative exon, which is skipped. (**b**) Methylation of histones leads to a compact chromatin structure around an alternative exon. Local slowing of Pol II (yellow Pol II) results in longer exposure of alternative exon splice sites to the spliceosome and exon inclusion.

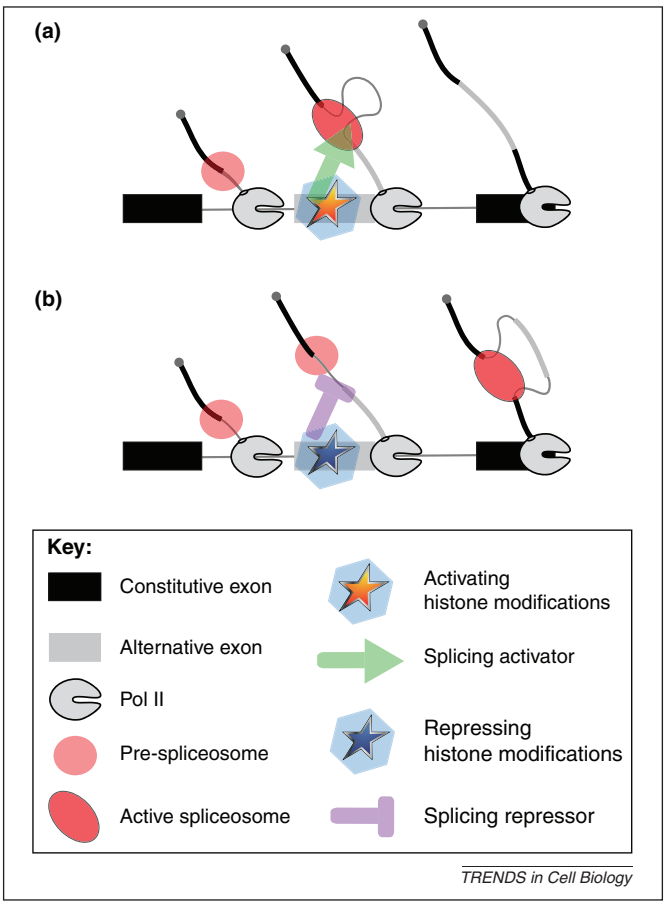

Figure 4. Nucleosomes (blue hexagons) marked by histone modifications directly recruit factors that influence co-transcriptional splicing. (**a**) Specific histone modifications (orange star) around an alternative exon (gray box) might positively influence splicing by directly recruiting splicing activators (green arrow) or spliceosomal components (red shapes). (**b**) By contrast, repressive histone modifications (blue star) recruit splicing repressors (purple T) impairing inclusion of the alternative exon. See the text for specific examples.

exon 18 was skipped and this shift in splicing outcome was associated with chromatin relaxation, hyperacetylation of H3K9 and increased levels of H3K36me3 surrounding the region of the alternative exon. In addition, inhibition of histone deacetylases in HeLa cells led to skipping of the fibronectin EDII exon, which was correlated with increased H4 acetylation, higher elongation rates up- and downstream of EDII and reduced association of positive splicing regulators with the nascent RNA [62]. Thus, open, acetylated chromatin would promote fast Pol II elongation and thus skipping of alternative exons (Figure 3). Conversely, triggering local transcriptional gene silencing with an siRNA directed against an intron downstream of the alternative fibronectin EDI exon led to an increase in the heterochromatin marks H3K9me2 and H3K27me3 and increased inclusion of the EDI exon [63]. These observations invoke a scenario (see above) in which repressive chromatin slows Pol II elongation, allowing for inclusion of weak exons (Figure 3). Taken together, these examples illustrate how chromatin modifications over alternative exons are correlated with changes in Pol II elongation behavior and splicing outcome. A challenge for the future is to understand whether histone modifications cause pausing or whether Pol II elongation rates determine the extent of histone modifications.

In addition to splicing regulation via changes in elongation rates, histone modifications can interact directly with splicing factors (Figure 4). For instance, the splicing factors SRp20 and ASF/SF2 bind to the unmodified H3 tail and to H3K9Ac, H3K14Ac and H3K9me peptides *in vitro* [64]. Components of the U2 snRNP interact with the human STAGA complex [65] that links the human SAGA complex to H3K4me3 [66], and CHD1 that in turn binds H3K4me3 [67]. In yeast, strong genetic interactions were found between U2 snRNP proteins and Gcn5, the histone acetyltransferase component of the SAGA complex [68,69]. Deletion of Gcn5 reduced the co-transcriptional recruitment of U1 and U5 snRNP, demonstrating that histone acetylation is required for co-transcriptional spliceosome assembly. Moreover, direct interactions between splicing factors and chromatin modifications clearly impact alternative splicing [54]. For example, the splicing repressor PTB is recruited to the histone modification H3K36me3 via an adaptor protein, MGR15, leading to exon skipping [70]. Knockdown of MGR15 as well as a reduction in H3K36me3 resulted in increased exon inclusion. Thus, histone modifications (H3K36me3) can modulate alternative splicing through chromatin readers (MGR15) that recruit splicing regulators (PTB) to an alternative exon.

A major challenge in the field is to perturb distinct histone modifications in ways that can distinguish global effects on overall chromatin state from specific effects on splicing. Are observed changes in alternative splicing due

to direct recruitment of splicing factors or to changes in Pol II elongation rates? Are secondary effects on gene expression levels responsible for the observed phenomena? How specific are histone methyltransferases or acetyltransferases? Are there additional unidentified substrates? It is important to note that many RNA processing factors are also post-translationally modified by phosphorylation, acetylation, methylation and ubiquitinylation [1,71]. Ubiquitinylation of Prp8 is required for U4/U6 duplex unwinding during spliceosome assembly, and defects in the ubiquitin pathway compromise splicing due to reduced levels of functional tri-snRNPs [72]. Intriguingly, arginine methylation of histones and snRNP proteins can also affect splicing outcome [73–75]. Thus, global inhibition of post-translational modifications cannot necessarily be interpreted as an effect on chromatin alone; understanding the roles of post-translational modifications on all components of the gene expression machinery is an area that deserves further attention.

The emerging view that recruitment of splicing factors to nascent RNA can be modulated by chromatin modifications contrasts with a previous model focusing on direct recruitment of splicing factors by Pol II itself. This proposal was stimulated by the finding that mRNA 5' end capping factors bind directly to the CTD, which is composed of numerous repeats of a heptapeptide containing serine residues at positions 2, 5 and 7 that undergo dynamic cycles of phosphorylation during transcription [76,77]. The notion that highly charged splicing factors, such as SR proteins, could bind to the phospho-CTD was particularly appealing [78]. However, early evidence that Pol II co-immunoprecipitates with splicing factors has since been clouded by the demonstration that antibodies specific for Pol II bind charged domains on splicing factors and vice versa [79]. Two types of data have argued against the strict recruitment of snRNPs and splicing factors through direct binding to Pol II: (i) Mass spectrometry studies of purified spliceosomes failed to identify any subunit of Pol II, and similar analysis of Pol II does not identify splicing factors [80–86], and (ii) In yeast and mammalian cells, splicing factors are not recruited to paused genes or actively transcribed intronless genes at which Pol II is abundantly concentrated [6,7,87,88]. Indeed, the CTD appears not to be specifically required to enhance splicing in yeast or mammalian cells [89,90]. Nevertheless, under some conditions U1 snRNP does co-purify with Pol II and can concentrate on at least some intronless genes [17,91,92] but it is not clear whether this interaction is dependent on the CTD. Interestingly, regulation of alternative splicing by the SR protein SRp20 (SRSF3) has been shown to be CTD-dependent, suggesting that the CTD might influence splicing factor recruitment or activity by low-affinity or indirect interactions [93]. Interestingly, a recent study showed that changes in splicing pattern result from dissociation of the spliceosome-associated factor YB-1 from the Pol II subunit Rpb7 [94]; this exciting finding could signify a new direction in the field towards a role for the Pol II holoenzyme in co-transcriptional spliceosome assembly

Recent genome-wide ChIP-chip studies in budding yeast provide insights into the dynamics of CTD phosphorylation during elongation [95–97]. The switch from Ser5 to Ser2 phosphorylation occurs at a constant distance from the transcriptional start site [95,96] and might be especially important for the regulation of co-transcriptional splicing. Binding of the SR protein SF2/ASF (SRSF1) correlates with Ser2 phosphorylation of CTD [98] and another SR protein, SC35 (SRSF2), regulates transcription by recruitment of pTEFb, an elongation factor responsible for Ser2 phosphorylation [99]. In budding yeast, induced transcription of a reporter gene results in splicing-dependent, transient phosphorylation of Ser2 and Ser5 around the 3′ SS [34]. Furthermore, changes in splicing patters upon UV irradiation of tissue culture cells are preceded by hyperphosphorylation of the CTD residues Ser2 and Ser5 [27]. Nagging questions persist regarding antibody specificity and the correspondence between ChIP signals and the number of phosphorylated serine residues of the 26–52 CTD repeats [77]. Although splicing regulation and CTD phosphorylation might be correlated, it is difficult to make simple equations between binding and observed recruitment *in vivo*. For example, the yeast termination factor Pcf11 binds Ser2 phosphorylated CTD *in vitro* [100], but its *in vivo* recruitment pattern is not correlated directly with the profile of CTD-Ser2 phosphorylation [95,96]. Therefore, it will be important to determine the exact nature of CTD phosphorylation, direct and indirect effects of CTD phosphorylation and the consequences for splicing in future.

Conclusions and outlook

Functional coupling between splicing and transcription is an emerging property of gene expression, suggesting that co-transcriptional splicing has broad biological consequences. One clear mechanism of coupling is local regulation of transcription elongation rates, which influences co-transcriptional splicing by determining the amount of time the nascent RNA substrate is available to splicing factors before 3' end cleavage and release. First, local changes in elongation can be caused by sequence-specific thermodynamic differences in the transcription bubble. Second, nucleosome positioning can influence elongation and co-transcriptional splicing by (i) locally stalling Pol II and/or (ii) providing a local scaffold for recruitment of positive or negative splicing regulators via modified histone tails. Third, specific recruitment of transcription and RNA processing factors to the Pol II holoenzyme and/or CTD plays additional roles. In the future, it will be important to understand how these three features of transcriptionally active chromatin combine to influence co-transcriptional splicing.

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