Regulation of Alternative Splicing Through Coupling with Transcription and Chromatin Structure

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Abstract

Alternative precursor messenger RNA (pre-mRNA) splicing plays a pivotal role in the flow of genetic information from DNA to proteins by expanding the coding capacity of genomes. Regulation of alternative splicing is as important as regulation of transcription to determine cell- and tissue-specific features, normal cell functioning, and responses of eukaryotic cells to external cues. Its importance is confirmed by the evolutionary conservation and diversification of alternative splicing and the fact that its deregulation causes hereditary disease and cancer. This review discusses the multiple layers of cotranscriptional regulation of alternative splicing in which chromatin structure, DNA methylation, histone marks, and nucleosome positioning play a fundamental role in providing a dynamic scaffold for interactions between the splicing and transcription machineries. We focus on evidence for how the kinetics of RNA polymerase II (RNAPII) elongation and the recruitment of splicing factors and adaptor proteins to chromatin components act in coordination to regulate alternative splicing.

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INTRODUCTION

The split nature of eukaryotic genes, organized in exons and introns, and the concomitant appearance of precursor messenger RNA (pre-mRNA) splicing seem to have had at least two evolutionary advantages. First, at the phylogenetic level, new genes could be generated from ancestor ones through nondisruptive recombination at introns by means of a process known as exon shuffling (1). The second evolutionary advantage is that alternative splicing, a mechanism that operates at the ontogenetic level by allowing a single gene to make two or more mature mRNA variants, expands the coding capacity of eukaryotic genomes. The number of protein-coding genes in vertebrates is not radically different from the number in invertebrates: ~20,000 genes both in vertebrates and in the worm *Caenorhabditis elegans*. However, the number of genes whose transcripts undergo alternative splicing and the average number of spliced isoforms per gene are higher in vertebrates (2), strongly suggesting that the prevalence of alternative splicing in these organisms is important for their higher complexity. As a compelling example, alternative splicing occurs in nearly 95% of mammalian genes (3, 4). Nevertheless, the difference in alternative splicing prevalence between vertebrates and invertebrates is not as great as the difference between multicellular and unicellular eukaryotes. The number and length of introns, and therefore the possibility of alternative splicing, are much higher in multicellular than in unicellular eukaryotes. The latter, which include, for example, trypanosomes, have mostly genes without introns (5). In the budding yeast *Saccharomyces cerevisiae*, introns are rather short and are present in a subset of genes, so although splicing exists, alternative splicing is very rare (6).

Alternative splicing has been recently shown to parallel transcriptional regulation in determining tissue- and species-specific differentiation patterns and the etiology of hereditary disease and cancer. The emerging evidence places alternative splicing in a central position in the eukaryotic genetic information flow, where this RNA processing mechanism is the target of various signaling pathways involving not only the splicing machinery but also transcription factors and chromatin structure (7). Transcription of genes encoding mRNAs is performed by RNA polymerase II (RNAPII) and starts at a nucleotide conventionally numbered +1, which marks the 5′ end of the first exon. RNAPII proceeds downstream of the cleavage/polyadenylation site of the pre-mRNA to terminate at positions that vary from gene to gene. In this way, the 5′ end of the transcript is determined by the +1, but its 3′ end is not marked by the transcriptional termination site but by the cleavage/polyadenylation site. The unspliced pre-mRNA that is produced is usually known as a primary transcript. The splicing process is performed by the spliceosome, a ribonucleoprotein megaparticle that assembles around splice sites in each intron. Each splice site consists of a consensus sequence that mediates its recognition by spliceosomal components (**Figure 1*a***). Strong splice sites (i.e., those that are more similar to the consensus sequence) are

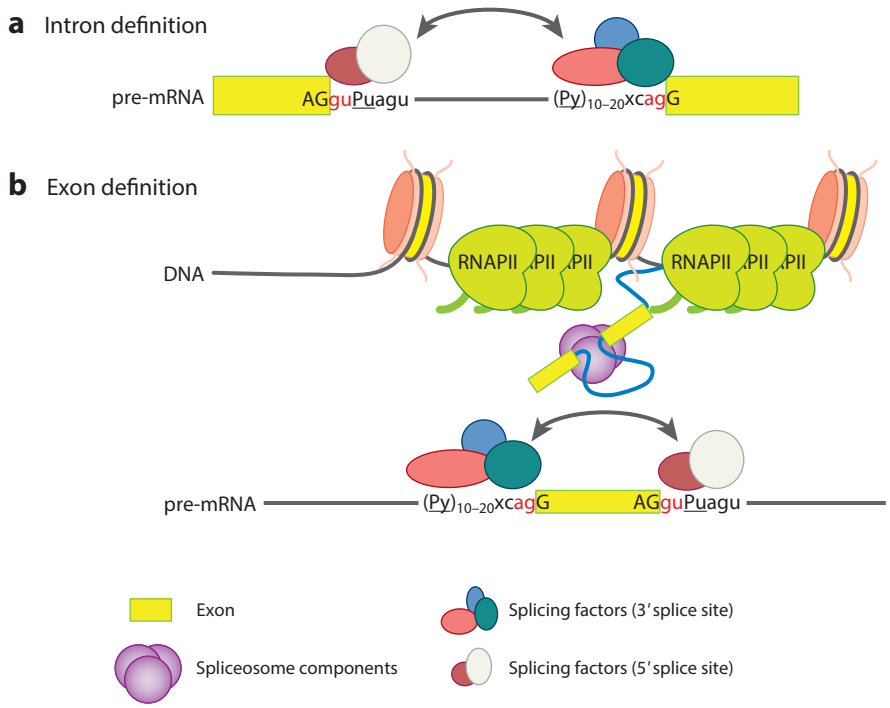

Figure 1

Two mechanisms proposed for intron and exon definition. (*a*) Intron definition takes place at the precursor messenger RNA (pre-mRNA) level. Spliceosome components are recruited to the splice sites flanking an exon on the mRNA precursor. Interactions between the 3′ splice-site and 5′ splice-site complexes favor exon recognition. Panel based on Reference 16. (*b*) Exon definition occur at the DNA level. Nucleosomes are preferentially positioned in exons, which may act as speed bumps for RNA polymerase II (RNAPII), helping in the cotranscriptional recruitment of splicing factors to the nascent pre-mRNA and improving exon definition. This architecture is preferred by exons with higher guanine–cytosine content than in the flanking introns (18, 26, 119, 120). Panel based on References 36 and 119–123.

more efficiently recognized and used than weak or suboptimal splice sites. It is the proximity of competing strong and weak splice sites along a nascent pre-mRNA that leads to alternative splicing (8). However, because of the cotranscriptional nature of splicing (discussed below in the section titled Connections Between Transcription and Splicing), full-length primary transcripts may not exist as such, particularly in the case of very long genes, because the excision of introns occurs cotranscriptionally, following the synthesis of relevant splicing sites and splicing regulatory sequences.

The relative positions of weak and strong splice sites give rise to the different modes of alternative splicing: cassette exons, mutually exclusive cassette exons, alternative 5′ splice sites (5′ ss), alternative 3′ splice sites (3′ ss), and intron retention. Alternative mRNA cleavage/polyadenylation and alternative initiation of transcription at different promoters are not considered alternative splicing.

Alternative splicing is regulated by both *cis*-regulatory sequences and *trans*-acting factors. The former include exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers, and intronic splicing silencers, depending on their locations and on how they affect the usage of a splice site. *Trans*-acting factors work through binding to splicing enhancers and silencers and include members of well-characterized serine–arginine (SR)-rich and heterogeneous nuclear ribonucleoprotein (hnRNP) families of proteins, as well as tissue-specific factors, such as polypyrimidine tract–binding protein (PTB) (9), NOVA (10), and FOX (11). Some of these factors activate, whereas others inhibit, the use of splice sites. In some cases, the direction of the effect depends on the position of the binding site on the pre-mRNA (12).

The multiple roles and mechanisms of action of these *cis*-regulatory sequences and *trans*-acting factors have been extensively studied (for comprehensive reviews, see References 13–15). However, the situation is in fact more complex because splicing is coupled to transcription, and factors that regulate transcription also affect alternative splicing.

The model reviewed here takes into account the coordinated influences of chromatin organization, histone marks, and RNAPII association with splicing factors, as well as the effect of the RNAPII elongation rate on splicing. This model suggests that splicing, transcription, and chromatin organization machineries interact and ensure that splicing is controlled temporally and spatially. Evolutionary studies and genome-wide analyses suggest that there are two different mechanisms of recognition of the splicing unit: intron definition and exon definition (**Figure 1**) (16–18). Gene structure, epigenetic markers, and RNAPII likely play different roles in the recognition mechanisms.

COUPLING ALTERNATIVE SPLICING TO TRANSCRIPTION

Alternative splicing is regulated by the abundance and posttranslational modifications of splicing factors, as well as by functional and physical interactions between the transcription and splicing machineries. More than 25 years ago, Eperon and colleagues (19) showed that alternative splicing patterns for a single gene differed depending on whether splicing took place in vivo (during ongoing RNA synthesis) or in vitro (on a premade pre-mRNA template), which suggested coupling. Later, the Kornblihtt laboratory (20, 21) found that different RNAPII promoters placed in front of the same transcriptional unit elicited different proportions of two splicing isoforms, which introduced the idea that alternative splicing can be coupled with transcription. As we discuss in this and the following sections, not only promoters but also transcription factors, coactivators, transcriptional enhancers, proteins with dual activities as transcription and splicing factors, chromatin remodelers, and factors affecting chromatin structure were subsequently shown to influence alternative splicing decisions.

Connections Between Transcription and Splicing

The evidence for the co-occurrence of splicing and transcription has been accumulating without pause during the past decades. But we normally start telling the story with one of the oldest and yet maybe most striking evidence for cotranscriptional splicing: the electron microscopy observation of transcriptional units prepared from *Drosophila* embryos, which revealed the presence of RNA loops in the nascent transcript (22). More than 20 years later, RNA sequencing technology produced a similar image in a genome-wide manner. For example, total RNA sequencing from human liver and brain shows an accumulation of reads forming a 5′-to-3′ decreasing slope along long introns, but these increase abruptly on exon–intron junctions, a pattern explained by the combination of ongoing transcription and cotranscriptional splicing (23).

Cotranscriptional processes can be obtained by purification of nuclear and/or chromatin-associated RNA fractions. Genome-wide studies of chromatin-associated transcripts have been performed in several biological systems, including yeast (24), fly heads (25), and human cell lines (26). All of these confirm that most introns initiate their splicing cotranscriptionally. A detailed quantitative polymerase chain reaction–based analysis of two multiexonic human genes showed cotranscriptional excision of most introns, normally following the order of transcription, although introns flanking alternative exons behave differently (27). In yeast, the evidence is somewhat contradictory. Earlier reports suggested that a majority of splicing events occurred posttranscriptionally and that this effect was more pronounced for genes with short terminal exons (28). However, the general agreement for both yeast and mammalian cells is that the recruitment of splicing factors always takes place while the transcript is contacting the DNA via RNAPII (29–31). This conservation between distant organisms indicates a strong selective pressure for the cotranscriptional recruitment of splicing factors, a matter we discuss in more detail in the following section.

In order to obtain a precise picture of how splicing and transcription interact in vivo, the kinetics of both processes need to be quantitatively determined in living cells. This is not an easy task for endogenous genes, because steady-state levels of RNA present in the cell normally mask the changes occurring in real time. Two general approaches have been used.

The first approach involves using normal RNA-detection techniques on inducible genes, analyzing the RNA population at different time points after induction. This approach allows the study of transcriptional elongation on endogenous genes and the simultaneous detection of intron removal. A good example of this strategy is the study of tumor necrosis factor α–induced genes in cultured human umbilical vein cells (32). By using tiling arrays to detect the accumulation of the newly synthetized RNA, an average speed of 2.9 to 3.3 kb/min was calculated. Also, the position of pausing events was determined, and intragenic insulator elements characterized by the binding of RAD21 and CTCF proteins were almost always associated with paused RNAPII. Although the cotranscriptional removal of at least the first introns was corroborated, as described in a paper by Wada et al. (32), a more recent report using high-throughput sequencing of chromatin-associated RNA during a lipopolysaccharide-induced response in macrophages showed abundant accumulation of fully transcribed and partially unspliced transcripts still bound to chromatin, with splicing readily proceeding before release to nucleoplasm (33). This finding suggests a lag between complete synthesis of the genes and release from chromatin, which decreases the level of pure posttranscriptional splicing. A more general method, based on the principle of inducible gene expression but usable for noninducible genes as well, is the use of a reversible transcriptional blocker, such as 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB). DRB blocks transcript elongation shortly after initiation but allows most engaged RNAPIIs to complete transcript synthesis and terminate transcription. After removal of the drug, synthesis of new transcripts resumes synchronously, allowing researchers to measure rates of transcript elongation.

Using this method for several human noninducible genes, the average transcription rate was estimated to be 3.8 kb/min, and intron removal occurred within 5 to 10 min of intron synthesis with little variation caused by intron size, even for minor spliceosome (U12) events (34). Recently, extensions of this technique were developed and, in theory, were able to measure genome-wide elongation rates. Investigators also used DRB inhibition but coupled it with BrU metabolic labeling of nascent RNA, followed by immunoprecipitation and high-throughput sequencing (35, 36). This approach allowed measurement of elongation rates on an unprecedented scale, confirming the same order of magnitude for elongation rates (∼1.5–2 kb/min) and starting the association of gene-to-gene variation in elongation rate with topological features, such as gene length and exon content, as well as with specific chromatin marks. A remarkable result of these studies is that the exon density of a gene is negatively associated with elongation rate, which is probably the result of RNAPII pausing events at most exons. This finding suggests a direct or indirect influence of splicing on elongation and/or a role for nucleosomes, preferentially positioned in exons, as speed bumps for elongation (see the section titled Chromatin Organization and Its Impact on Constitutive and Alternative Splicing, below).

The second strategy for measuring transcription and splicing kinetics is to devise reporter genes amenable to imaging-based interrogation. Here, the main advantage is the possibility of analysis in single cells, which allows for the discovery of specific features and variation normally hidden in the average population. Typically, these genes contain multiple insertions of a sequence that, once transcribed, forms a stem loop that can be recognized by a specific protein. The most widely used system is the one obtained from the MS2 phage. The MS2-binding protein is introduced into the cell fused to a fluorescent protein that binds very tightly to its cognate RNA loop. Because several copies of the MS2 RNA sequence are included in the reporter, and normally several copies of the genes are introduced in tandem, the result is a transcription-dependent fluorescent spot in the locus of the reporter gene. Then, the FRAP (fluorescence recovery after photobleaching) technique can be used, whereby the replacement of the bleached protein is mostly the result of a new transcription of the sequence and association with a free MS2-binding protein from the nucleoplasm. By use of this technique, together with mathematical modeling, the transcriptional elongation rate was estimated in single cells to be between 1.8 and 4.3 kb/min (37, 38), which is in very good agreement with the biochemical measurements discussed above. By use of the same approach with versions of the reporter with the MS2 located in an intron, cotranscriptional splicing was supported by showing faster turnover of the intron with respect to the distal exon (39). A more recent study used a similar reporter with two different RNA stem loops and two different labels on a *b-globin*-inducible reporter, but instead of using fluorescence recovery after photobleaching, the fluorescence fluctuations were measured in a tight time frame using spinning-disk confocal microscopy after induction (40). This measurement confirmed a similar transcription rate but estimated a very rapid intron excision rate (within 20 to 30 s) and determined that a weakening of the splicing signals leads to slower splicing kinetics.

A single-cell imaging approach in an inducible system was also undertaken using single-molecule imaging combined with live imaging (41). In agreement with the findings discussed above regarding splice-site strength and slower kinetics, this research showed that almost all post-transcriptional splicing events were caused by inefficient recognition of the polypyrimidine tract. Also, when the investigators analyzed endogenous genes, they found that the alternative exons showed the most posttranscriptional splicing. This result is in agreement with findings from both single-gene and genome-wide biochemical studies, which show that alternative exons tend to be spliced late when compared with constitutive exons (26, 27). This observation can be explained either by the reduced efficiency of the splice sites for intron flanking alternative exons or by a delay in splicing finalization, which allows regulatory events to take place.

Although these detailed kinetic analyses have provided strong support for the idea of coupled splicing–transcription processes in the whole genome, the first evidence of mechanistic coupling came from a functional analysis of alternative splicing regulation in individual genes, with the above-mentioned comparison between in vitro and in vivo splicing patterns (19), and promoter swapping experiments (20, 21, 42). These reports showed that this effect is not a consequence of differences in promoter strength, suggesting a qualitative difference in transcription driven by the different promoters. A further mechanistic analysis using a Gal4-based recruitment system to assess the impact of different transcription factors suggested that the ability of a transcription factor to stimulate RNAPII elongation rates can be crucial in the splicing outcome (43).

In an early example of the physiological relevance of this promoter effect, transcriptional hormone response elements located in the promoter were also shown to change alternative splicing decisions (44). Follow-up studies of this phenomenon uncovered the role of different transcriptional coactivators in this hormone-responsive coupling (45). One of these coactivators, the protein CoAA, is an RNA-binding protein itself, although its activity on alternative splicing regulators depends on the promoter used (46), demonstrating the existence of dual proteins involved in both transcription and RNA processing. Some other examples of dual proteins include the coactivator PGC-1 (47), the transcription factor Spi-1/PU.1 (48), the protein TCERG1/CA150 (49, 50), and the Y-box-binding protein 1 (YB-1) (51).

A key player in the coupling of transcription and splicing seems to be the RNAPII complex itself. In a proteome study of RNAPII-interacting proteins, many known splicing regulators were recovered (52). Also, RNAPII and, in particular, the C-terminal domain (CTD) of its largest subunit seem to be necessary for the accumulation of splicing factors to sites of transcription in vivo (53).

Functional Implications of Coupling

Why are transcription and splicing coupled? As mentioned above, a major driving force for the existence of this mechanism seems to be the need for efficient recruitment of processing factors to the nascent RNA and, therefore, efficient splicing. Because coupling is an RNAPII-dedicated mechanism, this can be assessed by transcribing intron-containing genes with promoters for other RNA polymerases. In all cases, this process causes splicing defects (54–57). Removal of RNAPII's CTD consistently leads to defects in all steps of processing on the *b-globin* transcript (58). An alternative approach to assess coupling consisted in the use of an in vitro system for coupled transcription and splicing (52, 59, 60), which allows testing of the effects of cotranscriptionality on splicing, but probably does not have the refinement of in vivo coupling (61). With this system, transcription by RNAPII leads to transcript stabilization, more efficient splicing, and more efficient recruitment of U1 small nuclear ribonucleoproteins (snRNPs) and SR proteins, whereas transcription by the RNA polymerase of phage T7 causes the RNA to accumulate in nonspecific complexes with hnRNP proteins, where RNA remains unspliced and is degraded.

Even for budding yeast, where splicing is limited to a minor proportion of genes with a single intron, the influence of transcription on splicing can be observed. The small group of efficiently spliced genes in yeast shows pausing of RNAPII downstream of one of the two exons. Mathematical modeling supports the notion that pausing is necessary to allow cotranscriptional splicing (24), suggesting that cotranscriptional splicing is an evolutionarily conserved mechanism. The coupling of splicing with transcription has an effect on the coordination of multiple alternative splicing events. In genes in which multiple alternative exons exist, coordination of splicing events favors some specific combinations over others. Although in many cases alternative splicing events are supposed to occur independently, some specific cases of coordination have been reported

(62–64), and a statistical analysis of ESTs (expressed sequence tags) suggests that it might be more common than generally thought (63). Coordination could also arise in posttranscriptional splicing if, for example, different events are regulated by a shared set of splicing factors. However, in at least one case, the influence of one event on the other seems to be preferentially occurring in the direction of transcription (63). This polarity of the effect implies that the effect is at least partially cotranscriptional and that changes in transcription can affect the outcome of coordination.

Just as transcription influences splicing, there is also evidence for the influence of splicing on transcription. Experts in genetic engineering and expression of genes in heterologous systems probably know that including an intronic sequence in a mammalian expression vector very often leads to higher expression levels of the desired mRNA. In addition, intron-mediated enhancement of gene expression is now thought to be important for the expression of endogenous genes; budding yeasts are living examples, with most of their few introns located in the most highly expressed genes (65).

The influence of splicing on gene expression is found in several steps along the transcriptional process and even beyond in the subsequent events of the mRNA life cycle, including export and translation (reviewed in References 66 and 67). Several reports have noted the importance of the presence of a promoter-proximal 5′ ss and the actions of splicing factors, such as the U1 snRNP, for early steps of transcription and the efficient transition to the elongating RNAPII complex (68–70). Although U1 components, similar to other splicing regulators, are not recruited to the gene body in absence of a functional intron (31), these components can be found associated with the RNAPII complex and at active transcription foci independent of splicing, probably through interactions with the CTD (52, 71, 72). The U1 snRNP seems to be important in guaranteeing preferential productive transcription in the sense direction of bidirectional promoters. Most promoters in human cells seem to initiate transcription in both directions (73), but the enrichment of promoter-proximal U1 recognition sites in sense transcripts and their relative depletion in antisense transcripts seem to be responsible for their ultimate directionality, given that knocking down U1 components increases bidirectional transcription (74).

Although an effect of functional splicing on the transcriptional elongation rate was suggested from the aforementioned early studies, a more recent report, using an imaging approach to measure pre-mRNA and RNAPII kinetics in an inducible reporter in mammalian cells, showed no difference in elongation kinetics across the gene when comparing intron-containing versus intronless versions of the construct or when there was pharmacological manipulation of the splicing process (72). It is still possible that an effect of splicing on elongation rates depends on the specific splicing unit as well as on the specific class of promoter driving transcription. For example, the auxiliary splicing factor SRSF2 (also known as SC35) stimulates RNAPII elongation and/or processivity in a subset of genes (75). A more recent report from the same group has shown that this SR protein is present in the positive transcription elongation factor b (P-TEFb) sequestering the 7SK complex and that its recruitment to binding sites in the nascent RNA helps the recruitment of P-TEFb, assisting the release of RNAPII from promoter-proximal pausing (76).

In addition to its role in the early steps of transcription, the splicing process may regulate RNAPII pausing on promoter distal locations. Study of an integrated inducible reporter and endogenous intron-containing genes in budding yeast showed a transient pause of RNAPII and rephosphorylation of the Rpb1 CTD on Ser5 when reaching the end of the first (and, in this case, only) intron (77). Ser5 phosphorylation strictly depended on functional splice sites, suggesting the existence of a splicing-mediated checkpoint in transcription. The existence of this checkpoint in yeast was further supported by later research showing that pausing relies on the U2-associated protein Cus2 and that release from pausing depends on the activity of the spliceosomal ATPase Prp5 (78).

MODELS FOR THE COUPLING OF ALTERNATIVE SPLICING TO TRANSCRIPTION

Two mechanisms that are not mutually exclusive have been proposed to explain how coupling works: recruitment coupling and kinetic coupling.

Recruitment Coupling

This mode of coupling involves splicing factor recruitment to transcription sites by the transcription machinery. The RNAPII CTD has a key role in functionally coupling transcription with factors affecting capping and 3′ processing, and it has also been implicated in alternative splicing (79). The presence of a repetitive CTD in its largest subunit is a unique feature of RNAPII. In mammals, the CTD comprises 52 tandemly repeated heptapeptides with the consensus sequence Tyr–Ser–Pro–Thr–Ser–Pro–Ser. Corden and colleagues (80) discovered that the CTD was necessary for transcriptional activation by almost all transcriptional enhancers tested, and in 1997, Bentley's group (58, 81) found that the roles of the CTD were not limited to transcriptional activation but also involved all three pre-mRNA processing reactions, namely capping, splicing, and 3′ end processing; these authors also introduced the concept of an mRNA factory governed by the CTD (81). The idea of an mRNA factory containing RNAPII and processing factors gave strong support to the concept of coupling of transcription and pre-mRNA processing in which both processes can influence each other and occur in a highly coordinated manner within the cell nucleus. Most of the multiple functions of the CTD (79, 81–85) depend on phosphorylation of specific CTD residues: Ser5 and Ser2 phosphorylations (Ser5-P and Ser2-P) are important for the recruitment of capping enzymes (86) and for 3′ mRNA processing (87), respectively. Ser7-P and Thr4-P also function in 3′ processing of small nuclear RNAs (88) and histone mRNAs (89). The functions of Tyr1-P in mammalian CTD are less clear, although the tyrosine residue itself seems to be necessary for CTD stability (85).

In vitro experiments revealed that 22 repeats from either the conserved or divergent half of the CTD are sufficient to stimulate splicing and 3′ end cleavage (90). By contrast, several alternative splicing factors associate with the CTD (52), but it is not yet clear whether these interactions are direct or mediated by the nascent mRNA. The role of the SR protein SRSF3 (formerly named SRp20) in promoting inclusion of cassette exon 33 (E33) in mature fibronectin mRNA depends on the CTD, as a mutant polymerase with a truncated CTD abolishes E33 upregulation upon SRSF3 knockdown (91). An example of a CTD-independent recruitment mechanism was reported for the thermogenic activator PGC-1, which modulates inclusion of fibronectin exon 25 into the mature mRNA only if it can bind the promoter of the gene (47). More recently, a multicomponent transcription complex known as Mediator has been implicated in alternative splicing (92). One of its subunits, MED23, physically links transcription factors bound to regulatory DNA sequences with general transcription factors at core promoters. Many RNA processing factors bind MED23, including the alternative splicing regulator hnRNPL. Moreover, most alternative splicing events regulated by hnRNPL are also regulated by MED23.

Kinetic Coupling

In this mechanism, the rate of RNAPII elongation influences alternative splicing by affecting the pace at which splice sites and regulatory sequences emerge in the nascent pre-mRNA during transcription. Not all alternative splicing events are affected by transcriptional elongation. In most, but not all, of the affected cassette exons, DNA sequences that induce RNAPII pausing (93) or

drugs that inhibit RNAPII elongation or processivity (94, 95) promote higher exon inclusion into the mature mRNA, whereas drugs that cause a more open chromatin state (96, 97) or factors (43) that promote elongation increase exon skipping. For example, the DNA-binding protein CTCF, implicated in targeting gene insulators (i.e., sequences that isolate sets of genes coregulated by the same DNA *cis*-acting sequences), promotes inclusion of an alternative exon 5 in *CD45* by binding to a target site in its downstream intron, thus creating a roadblock to RNAPII elongation. Interestingly, DNA methylation of this intronic site prevents CTCF binding, releases RNAPII, and reverses the effects on exon 5 splicing (98, 99). In other studies, DNA methylation was found to be enriched with alternative exons and to regulate alternative splicing through the binding of the methyl-CpG-binding protein MeCP2 (100, 101). These reports stress the importance of considering the effects of intragenic DNA methylation patterns on chromatin structure so as to understand the regulation of gene expression, instead of focusing entirely on how methylation affects promoter activity.

The effects of factors such as CTCF in inducing intragenic transient pausing during elongation may be counterbalanced by factors that enable RNAPII to overcome these blocks. For example, the DBIRD protein complex binds to RNAPII and promotes exclusion of a set of exons embedded in adenine–thymine (AT)-rich DNA sequences. These sequences are intrinsically difficult for RNAPII to transcribe, and DBIRD acts by facilitating RNAPII elongation through them (102).

The most direct evidence for kinetic coupling involved the use of slow RNAPII mutants that harbor an amino acid substitution in the catalytic domain of its large subunit and that show a reduced elongation rate both in vitro (103) and in vivo (38). Transcription by these mutants increases inclusion of several cassette exons, including fibronectin E33 (104) and *NCAM* E18 (97), but it also affects the choice of alternative 5′ ss for *Bcl-x* (105), regulates alternative splicing of the *ultrabithorax* (*Ubx*) gene in *Drosophila* (104), and modulates the inclusion of an artificially created alternative exon in yeast (6). A global analysis showed that dozens of alternative splicing events are affected by treatment of human cells with pharmacological inhibitors of elongation (95). Most drug-sensitive genes also displayed increased levels of alternative exon inclusion when transcribed by a slow RNAPII mutant compared with wild-type RNAPII. By contrast, none of the drug-insensitive genes were affected by the slow RNAPII.

One interpretation of the kinetic model is that slow elongation favors the removal of the intron that lies upstream of an alternative exon before the downstream intron emerges. However, such a mechanism would not be compatible with the exon definition (**Figure 1*a***). Alternatively, slow elongation might favor the recruitment of splicing factors to the upstream exon before the downstream exon is synthesized. Once commitment to include the exon is achieved, the order of intron removal in fact becomes irrelevant. This is supported by evidence that slower elongation induces higher inclusion of fibronectin E33 without affecting the order of intron removal, suggesting that slow elongation favors the commitment to exon inclusion during spliceosome assembly (106). Single-molecule imaging analyses indicate that, although catalysis of constitutive splicing is cotranscriptional, catalysis of alternative splicing occurs posttranscriptionally (41). These experiments assessed only the timing of splicing catalysis but not the timing of splicing factor recruitment needed for commitment.

If slow elongation favors recruitment of constitutive splicing factors, it can also lead to greater skipping of alternative exons by creating a window of opportunity for negative regulatory splicing factors to bind to their target sequences in the upstream intron of the pre-mRNA. Several studies have shown that cell treatments known to reduce RNAPII elongation can also promote alternative exon skipping (95, 107, 108). A detailed mechanism was recently provided for alternative splicing at the *CFTR* locus. Inclusion of the *CFTR* cassette exon 9 is regulated by a UG-repeat polymorphism, which is located just upstream of the polypyrimidine tract in the 3′ ss and is the target site for the

negative factor ETR-3 (109). Treatments that slow down RNAPII elongation, including the use of a slow RNAPII mutant, promote ETR-3 binding to this UG-repeat sequence and displace the constitutive auxiliary factor U2AF65 from the polypyrimidine tract, which hinders the recognition of the *CFTR* exon 9 as a proper exon and results in the skipping of this exon (110). So, the positive or negative effects that kinetic coupling may have on exon inclusion depend on the identity and architecture of the particular regulatory sequences that surround each alternative splicing event.

Interestingly, Bentley and colleagues (111) recently used both slow and fast RNAPII mutants and confirmed that slow elongation promotes skipping or inclusion, depending on the particular alternative exon. They found many alternative cassette exons that, as expected, respond in opposite ways to the fast and slow mutants, but they also found a subset of exons that respond in the same direction whether elongation is faster or lower. These findings led the authors to conclude that, within a narrow range, proper elongation speed is critical for exon inclusion.

Are the Kinetics of Transcription and Splicing Compatible with the Kinetic Coupling Model?

Genome-wide studies (95) clearly indicate that not all alternative splicing events are subjected to elongation control. Also, we ignore the specific sequence and/or structural features that would make a particular splicing event sensitive to elongation. In fact, real-time imaging of cotranscriptional splicing has demonstrated that the observed kinetics of splicing fit better with a mathematical model in which splicing has not one rate-limiting step (assembly or catalysis) but three, one of which occurs at the level of transcriptional elongation (39). This model predicts that changes in elongation rates similar to those observed with the slow RNAPII mutant (which slows elongation by about twofold) would have a high impact on alternative splicing decisions only if there are three rate-limiting steps that affect kinetic coupling (as opposed to only one).

The generation of an in vitro splicing system in 1983 enabled mechanistic analyses of the splicing reaction. As in vitro reactions are complete after ~30 min of incubation (112–114), a diffusion-based model for the interactions of splicing factors with pre-mRNA was assumed. However, recent in vivo analyses revealed that splicing is completed within ~20 s (115) and that the mRNA precursor remains associated with chromatin until all introns have been removed (33). This strongly suggests that the diffusion model is inaccurate and that at the level of either factor recruitment or subsequent catalysis, the chromatin serves as a scaffold on cotranscriptional splicing.

CHROMATIN ORGANIZATION AND ITS IMPACT ON CONSTITUTIVE AND ALTERNATIVE SPLICING

Chromatin Density and Nucleosome Occupancy

Recent advances in high-throughput sequencing have provided high-resolution insights into the multilayered regulation of gene expression. Innovative approaches have evaluated three-dimensional DNA interactions and have revealed close ties between chromatin structure and alternative splicing. The structural units of chromatin are formed by nucleosomes. Each nucleosome consists of a 147-bp stretch of double-stranded DNA wrapped around a single histone octamer unit. It has now become clear that changes in nucleosome organization not only compact DNA and neutralize its negative charges but also affect transcription. Variations in chromatin organization are linked to widespread alterations in RNA processing, including splicing

anomalies in ~25% of all expressed genes (116, 117, 118). Mapping of nucleosome locations at the genome-wide level revealed that a substantial fraction of nucleosomes are not randomly located but instead occupy exons more frequently than introns, although only when the exon has a higher guanine–cytosine (GC) content than its flanking introns (**Figure 1*b***) (18, 26, 119, 120). Nucleosomes are preferentially positioned within constitutively spliced rather than alternatively spliced exons (120, 121). Interestingly, without distinction between constitutive or alternative behavior, stable nucleosome occupancy has been reported to be stronger in exons with weak splice sites (122). Nucleosomes are rarely located over intronic sequences that flank splice sites owing to the presence of polypyrimidine sequences that are part of the 3′ splice signals and that also serve as barriers for nucleosome occupancy (120, 122, 123). This phenomenon appears to be evolutionarily conserved (120, 124). Together, these studies suggest that the chromatin structure acts to direct exon selection.

The speed bump model (125) suggests that nucleosomes affect transcriptional elongation by providing barriers to RNAPII, thus favoring exon definition and promoting exon inclusion into the mature mRNA (**Figure 1**) (125–127). A nucleosome role in exon recognition was suggested by analysis of pseudoexons, namely intronic sequences that are not included in mRNA but are flanked by strong splice sites. These regions are bound less frequently by nucleosomes than other intronic regions (122). Additional support for the significance of nucleosome occupancy in splicing was provided by the demonstration that perturbation of chromatin structure alters localization of splicing factors in speckle compartments (128) and changes splicing patterns (129). These revelations provided the foundation for the theory that the chromatin structure may reflect the underlying intron–exon recognition of the gene (130, 131).

Nucleosome positioning and chromatin organization can be directly altered by multisubunit remodeler complexes. Remodelers are conserved throughout evolution, contain an ATPase domain, and are classified into four subfamilies: SWI/SNF, ISWI, CHD, and INO80 (132). The SWI/SNF remodeler modulates transcription of many genes by altering nucleosome organization at promoter regions (133, 134) and in gene bodies (135). SWI/SNF also regulates both splicing and the RNAPII elongation rate by interacting with either splicesome components or the RNAPII-phosphorylated CTD domain (136–140). The SWI/SNF ATPase catalytic subunit Brahma (Brm) can interact with U5 snRNP or with Sam68, an hnRNP-like protein that influences exon recognition (136). This interaction facilitates the recruitment of the splicing machinery to variant exons in the *CD44* gene with weak splice sites (136). Interestingly, the regulation of gene splicing by Brm is independent of its ATPase activity (140). SAP155, a U2 snRNP component, interacts with WSTF, an ISWI-type chromatin remodeler (139). Another aspect of regulation is carried out by the recruitment of SWI/SNF to nascent pre-messenger ribonucleoprotein (mRNP) alternative exons, where it enhances inclusion levels (136, 137).

Members of the CHD family of chromatin remodelers also regulate both transcription and splicing. CHD1 and Brm colocalize with elongating RNAPII (141). CHD1 overexpression and depletion affect alternative splicing in vitro and in vivo and lead to impaired association of U2 snRNP components with pre-mRNA (142, 143). Human CHD1 copurifies with RNAPII elongation factors such as FACT and PAFc and with the SF3A subunit of the spliceosome (143). CHD1 also copurifies with spliceosome components SRSF3 and U2 snRNP (144) and associates with the transcriptional corepressor NCoR and with mRNA splicing proteins such as mKIAA0164, SRSF3, and SAF-B (142). The histone mark H3K4me3 facilitates pre-mRNA maturation by bridging the CHD1 and spliceosome components (143). These observations suggest that chromatin remodelers and RNAPII mediate interactions with splicing.

In conclusion, nucleosomes seem to demarcate exons at the DNA level and may help the splicing machinery recognize transcribed exons (RNA) and promote their splicing by pausing RNAPII.

Another possible role of nucleosome association with exons is to protect coding sequences and splice sites from mutational hazards (145).

Histone Modifications and DNA Methylation

Tissue- and feature-specific eukaryotic chromatin structure is determined by a code involving DNA methylation and histone modifications. Although this code is usually referred to as the epigenetic code, the word epigenetics should in theory be reserved for those modifications that are truly inheritable, which include DNA methylation but not all histone modifications. In any case, we use the term epigenetic *sensu lato*, that is, to refer to the above-mentioned modifications even if there is no formal proof of inheritance.

Regions of chromatin have unique profiles of DNA methylation, and there are more than 50 possible histone tail modifications. The protein modifications affect binding affinity among histones and between histones and DNA; thus, their presence can dictate the higher-order chromatin state. Deregulation of histone marking results in a variety of human diseases (146–150). Different histone modifications are associated with different functions, such as transcription activation, silencing, or DNA repair. Histone modification over promoters is predictive of gene expression levels (151). Several histone modifications are enriched at RNAPII transcription start sites, most notably H3K4me3 (151). Certain histone modifications, such as H3K36me3, H3K79me1, H2BK5me1, H3K27me1, H3K27me2, and H3K27me3, are more abundant over internal exons versus introns and are clearly correlated to exon expression (119, 120, 152–155). Of these modifications, H3K36me3 is the most strongly associated with exonic sequences and correlates with the level of gene expression and exon inclusion (119, 154).

Several histone modifications can affect chromatin–histone attraction through their negative charge (e.g., modification by histone acetylation or phosphorylation) or by introducing bulk and/or recruitment of other effectors (e.g., modification by histone ubiquitylation). The negatively charged histone tails can repel the negatively charged DNA, leading to the formation of a more open structure, whereas the positively charged histones further condensed chromatin. H2B monoubiquitylation correlates with cotranscriptional splicing and the RNAPII elongation rate. Exonic depletion of H2Bub1 is highly correlated with RNAPII pausing at exons (156). Conversely, histone hyperacetylation leads to increased local RNAPII elongation rates and decreased inclusion of exons (97, 157). Additional accumulated evidence has associated histone acetylation with cotranscriptional splicing. Inhibition of HDAC1, HDAC2, or SRSF1 (the SR protein formerly known as SF2/ASF) expression results in alternative splicing of *MCL1* (158). Further evidence was provided by a genome-wide study in which HDAC inhibition altered the splicing pattern of more than 700 genes and reduced cotranscriptional association of the splicing regulator SRSF5 (formerly known as SRp40) with its target exons (144). Depolarization of neuronal cells triggers hyperacetylation surrounding exon 18 of the gene encoding the neural cell adhesion molecule (NCAM), increasing transcriptional elongation and resulting in the skipping of this exon (127), together with a decrease of U2AF65 recruitment to 3′ ss (128). These findings illustrate how epigenetic marks may affect splicing as they impact nucleosome organization, the RNAPII elongation rate, and accessibility to spliceosome components.

Recent research suggests that histone marks may also have a more direct impact on splicing, as certain histone modifications recruit splicing factors through chromatin-binding proteins (143, 159). This recruitment can affect alternative splicing regulation. For example, H3K36me3, a significantly enriched modification in actively transcribed gene bodies and around the intron–exon boundaries of included exons, recruits MRG15, a chromodomain-containing protein that is part of several histone-modifying complexes. MRG15, in turn, drafts the splicing factor PTB.

PTB binds to specific sequences on pre-mRNA and directs the outcome of alternative splicing, depending on the location of the binding site relative to the affected exon (159, 160). The complex H3K36me3–MRG15–PTB forms a splicing–chromatin cotranscriptional architecture that can maintain scaffolding and retains the splicing machinery in the vicinity of chromatin.

Additional examples of histone modification–splicing factor associations include the H3K36me3-binding protein Psip1 with the splicing factor SRSF1 (118, 161); the chromatin remodeler CHD1 with components of the U2 snRNP (143); and binding of the splicing factor SRSF3 with unmodified histone H3 and with H3K9Ac, H3K14Ac, and H3K9Me (162). The BS69 protein mediates recognition of the U5 snRNP to H3K36me3 to antagonist U5 activity and promotes intron retention (163).

Another aspect of recognition occurs through the interaction of splicing factors with histone-modifying enzymes: hnRNP-L and Aly/Ref1 associate with Kmt3a methyltransferase (164), and hnRNP-M and hnRNP-U are found in a complex with Kmt4 methyltransferase (165). Similarly, Gcn5 acetyltransferase recruits the U2 snRNP to H3ac (166). The Hu splicing regulators interact with HDAC2 and inhibit its deacetylase activity (157). Inhibition of USP49, a histone H2Bu deubiquitylase, results in association of the splicing factors U1A and U2B with chromatin and nascent pre-mRNA (167). In *S. cerevisiae*, Bre1 monoubiquitylation of H2B mediates its interaction with the SR-like protein Npl3 and is required for efficient cotranscriptional recruitment of the splicing machinery (168). This complex not only promotes monoubiquitylation of H2B (169) but also regulates the establishment of H3K4me and H3K79me marks, which result in gene silencing (170). Furthermore, monoubiquitylation of H2B stimulates recruitment of splicing machinery components onto nascent pre-mRNA. SART3, a recycling factor that promotes formation of the U4/U6 snRNP, binds histones, and enhances deubiquitylation of H2B (171). This variety of mechanisms by which splicing factors are associated with different chromatin components via specific histone modifications may assist in the recruitment of splicing factors onto the pre-mRNA in a way that can be dependent or independent of the RNAPII complex, or could also generate splicing-dependent chromatin patterns (as discussed further in the section titled Influence of Splicing on Chromatin Structure, below).

DNA methylation also seems to affect splice-site recognition and occurs with higher frequency in exons than in introns (172). Exons that are generally excluded by the splicing machinery have lower levels of DNA methylation than do constitutively included exons (172, 173). DNA methylation is observed at higher levels in the 5′ ss regions than at 3′ ss regions (174). DNA methylation may mediate cotranscriptional splicing through the actions of methyl-binding domain proteins (MBDs). MBDs recruit histone-modifying enzymes to alter the surrounding chromatin, which could interfere with the RNAPII elongation rate (175, 176). MBD2 (previously known as MeCP1) and MeCP2, which is an MBD, are capable of binding to Sin3A HDAC (177, 178). The MECP2–Sin3a–HDAC and MBD2–HDAC (NuRD) complexes provide a mechanistic link between DNA hypermethylation and histone deacetylation in transcriptional repression. MeCP2 binds to alternatively spliced exons (101), and overexpression or inhibition of expression of MeCP2 alters alternative splicing patterns (179). The MeCP2 complex contains the splicing factor Prpf3, and it can associate with the splicing factor YB1 in an RNA-dependent manner (179). MBD2 binds to both the splicing factor hnRNP C1/C2 and SWI/SNF (180, 181). This finding is very interesting in terms of cotranscriptional splicing regulation and because hnRNP C competes with U2AF65 at many splice sites and affects the exonization of *Alu* elements (182).

The HP1 family of heterochromatin-associated proteins may also facilitate cross talk between DNA methylation and splicing. HP1 interacts with the histone chaperone complex FACT (183) and binds specifically to H3K9me2 and H3K9me3 in heterochromatin, which contains methylated DNA (184). In *Drosophila*, HP1a binds not only to heterochromatin but also to active chromatin

regions in an RNA-dependent way. In addition, it interacts with different RNA-binding proteins of the hnRNP family and mediates the association between hnRNPs and heterochromatin (185). Thus, HP1 may serve as an adaptor among methylated H3K9, DNA methylation patterns (in vertebrates), and pre-mRNA processing (186). Recently, HP1 was shown to bind the SR proteins SRSF1 and SRSF3 in mammalian cells (187, 188), and the U2 snRNP complex was shown to be recruited by HP1 to DNA methylated regions (187).

Small interfering RNAs (siRNAs) can also influence alternative splicing through effects on chromatin. When targeted to promoter sequences, siRNAs promote the deposition of silencing marks (H3K9me2, H3K27me3) that inhibit transcription, a mechanism known as transcriptional gene silencing, which differs from the well-established cytoplasmic roles of siRNAs in posttranscriptional gene silencing. Through a transcriptional gene silencing–like mechanism, siRNAs, designed to target the intron downstream of an alternative exon, generate intragenic roadblocks to elongation and effectively upregulate exon inclusion via kinetic coupling (189). This requires the presence of the small RNA–associated protein Argonaute 1 (AGO1) and the heterochromatin-binding protein HP1α (**Figure 2**). Another member of the HP1 family, HP1γ, also facilitates exon inclusion in *CD44* and other genes by interacting with intragenic H3K9me3 marks and inhibiting RNAPII elongation (190). A general role for Argonaute proteins in the nucleus has been proposed (191) in light of the finding that immunopurified human AGO1 and AGO2 from chromatin-embedded proteins associate with chromatin modifiers and splicing factors. Both AGO1 and AGO2 facilitate spliceosome recruitment and increase histone H3K9 methylation on variant exons to modulate the RNAPII elongation rate, thereby affecting alternative splicing (189).

In summary, several chromatin architectures associated with the regulation of cotranscriptional splicing have been suggested. The formation of a cotranscriptional splicing scaffold would improve cross talk between these processes and might use chromatin cues to mark exons for the splicing machinery.

Influence of Splicing on Chromatin Structure

There may be a reciprocal relationship between chromatin structure and splicing. Recent results reveal not only that epigenetic modifications modulate splicing patterns, but also that splicing actively modulates the epigenetic pattern of chromatin. For example, U1 snRNP binding to the 5′ ss of a pre-mRNA alters chromatin organization, and overexpression of the splicing factors SRSF2 (previously named SC35) and SRSF1 promotes nucleosome depletion, whereas overexpression of hnRNP A1 increases it (129). Additional support for splicing influence on chromatin organization includes the findings that Hu splicing regulator proteins can induce local histone acetylation in regions surrounding alternative exons. Disruption of Hu-binding sites leads to increased inclusion of alternative cassette exons as well as significantly lower levels of H3 and H4 acetylation on the nucleosomes formed on surrounding exons (157).

Other studies propose that splicing establishes and maintains epigenetic marks, such as trimethylation of H3K36 (192, 193), which is considered a signature mark for exons and transcriptional activation (194, 195). Genome-wide analyses indicate that splicing mechanistically couples the recruitment of HYPB/Setd2 methyltransferase to the elongating RNAPII itself and to H3K36me3-associated proteins (192). Inhibition of expression of the splicing factor SAP130 leads to reduced levels of H3K36me3 and to the subsequent reduced recruitment of HYPB/Setd2 (192). H3K36 trimethylation by histone methyltransferase Kmt3a depends on pre-mRNA splicing. Inhibition of splicing by disruptive mutation of the splice sites of an integrated *b-globin* reporter gene caused a shift in the relative distribution of H3K36 trimethylation away from the 5′ end and toward the 3′ end of the gene (193). The use of pharmacological splicing inhibitors, such as sudemycin E,

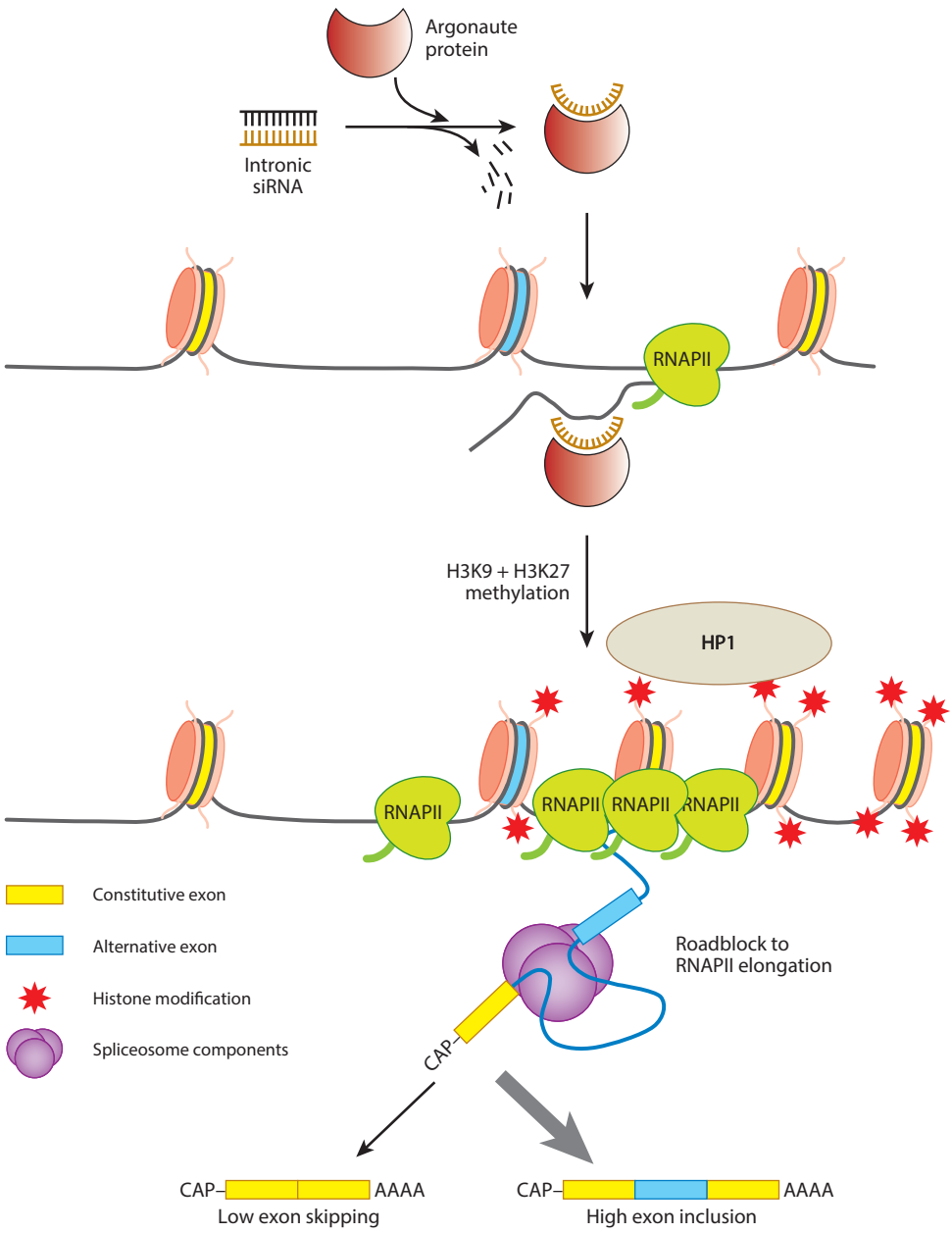

Figure 2

Small interfering RNAs (siRNAs) regulate alternative splicing through transcriptional gene silencing. Transfection with siRNAs targeting the intron downstream of an alternative exon promotes dimethylation and trimethylation of H3K9 and H3K27, processes that require Argonaute proteins acting in the nucleus. These silencing marks recruit the heterochromatin protein HP1 and create roadblocks for RNAPII elongation, which in turn upregulates or downregulates alternative exon inclusion, depending on the nature of the alternative splicing event, through kinetic coupling. Experiments supporting this model are described in References 189 and 220.

that inhibit the activity of the U2 snRNP component SF3B1 results in loss of the H3K36me3 mark (196). Depletion of SF3B3, a core spliceosome component, decreases Kmt3a recruitment to chromatin and reduces H3K36me3 levels (197). Kmt3a recruitment could be related to splicing efficiency because alternative exons have lower levels of H3K36me3 than do constitutive exons (154). These examples indicate that pre-mRNA splicing affects the writing and/or maintenance of epigenetic marks with resulting changes in the chromatin state.

THE FUNCTIONAL IMPACT OF TRANSCRIPTIONAL COUPLING ON SPLICING REGULATION

Participation of Transcriptional Coupling in Tissue-Specific Alternative Splicing

The physiological importance of cell- and tissue-specific alternative splicing is evidenced by the many roles of this mechanism in development and disease. Embryonic development is accompanied by a number of tissue-specific splicing events that are crucial to cell identity (198–202), such as embryonic segmentation (203), postnatal heart remodeling (204), neuronal development (205), blood cell formation, and epithelium/mesenchyme differentiation (206). Approximately 14% of the exons of humans and chimpanzees are expressed differently between sexes (207, 208). In *Drosophila*, the mechanism of sex determination, mediated by the protein encoded by *Sex lethal* (*Sxl*), serves as a model for alternative splicing during development (209). Upon human aging, dramatic changes in alternative splicing of more than 5,000 exons are observed, primarily in genes encoding proteins involved in metabolic processes (199). Splice-site selection aberrations cause 15% of all human diseases (210, 211). Thus, alternative splicing plays a critical role in gaining intrinsic properties and functions for the cell.

How prevalent is tissue-specific alternative splicing regulation? An analysis of the human cell line transcriptome revealed differences in more than 22,000 tissue-specific isoforms (212). Although tissue- or lineage-specific gene expression is often highly conserved among different species, alternative splicing is conserved in only a subset of cases (208, 213). This finding has made researchers question the functionality of the multiple isoforms found, some of which could result from transcriptional noise. Recent research analyzing tissue-specific transcriptomes in different primate species has reinforced the idea that only a subset of the existing alternative splicing events may participate in tissue-specific functions, as evidenced by the conservation of splicing patterns across species (214).

Differences in splicing patterns among tissues and during an organism's life span often result from the expression of splicing factors (215, 216). However, in many reports, there are suggestions that transcription and chromatin properties may also contribute to the establishment of robust tissue-specific splicing patterns.

As described above, the properties of the promoter of a gene can affect the outcome of alternative splicing in the gene body, so the use of alternative promoters in different tissues can lead to different splicing patterns. RNAPII occupancy maps have revealed that 6,000 promoters are tissue specific, with 37% of protein-coding genes and 31% of noncoding genes having alternative promoters active in different tissues (217). The impact of alternative promoter usage on alternative splicing patterns has not been assessed in a systematic manner, but there are some specific examples suggesting that indeed it can occur, such as the different splicing outcomes from the alternative promoters of the *Bcl-x* gene (218).

Another way in which chromatin and alternative splicing cross talk may be involved in tissue differentiation is the establishment of tissue-specific epigenetic marks and chromatin structures,

which can affect the elongation rate of RNAPII (219) or the recruitment of splicing factors. The latter option is exemplified by the case of H3K36me3 influence on alternative splicing events regulated by the splicing factor PTB, in particular for the *FGFR2* gene, between epithelial cells and mesenchymal stem cells (159). It is noteworthy that, in a genome-wide experiment, the set of splicing events with weak PTB-binding sites is preferentially affected by H3K36 trimethylation and MRG15 occupancy, suggesting that the chromatin mark acts as a reinforcement of the primary regulation in *trans*. This can be better appreciated in a differentiation system, in which the different states and the transition can be studied. For example, as mentioned above, in differentiating neurons the deployment of general intragenic H3K9me2 and H3K27me3 on the *NCAM* gene is associated with the changes in exon 18 alternative splicing, but the chromatin effect explains only a part of the total variation (220). A very interesting case is the recruitment of the splicing regulator HuR to the *Nf1* gene. It has a negative effect on the inclusion of the alternative exon 23a (which is reinforced by RNA, HuR-mediated inhibition of HDAC2, and a concomitant increase in the local histone acetylation levels) (157). This hyperacetylation favors higher elongation rates, which in turn decreases exon 23a inclusion, generating a chromatin-mediated reinforcement of the primary splicing decision.

Differences in DNA methylation can also be causative, as shown above by the example of CTCF-mediated regulation of *CD45* exon 5 splicing (98). In this case, the degree of methylation on the CTCF-binding site, which prevents binding, was lower in circulating naïve lymphocytes than in mature ones. Studies in honey bees have revealed the importance of DNA methylation during development as well as its impact on lineage-specific appearance and behaviors (221). More than 80% of differentially methylated honey bee genes are upmethylated in worker larvae compared with queens. Interestingly, differentially methylated regions are correlated with the level of alternatively spliced exons and the enrichment of the spliceosomal component and factors in methylated genes (222). One case of a gene not only differentially methylated but also alternatively spliced between workers and queens, and between tissues with different metabolic influxes, is anaplastic lymphoma kinase (ALK), an important regulatory enzyme that activates downstream metabolism signaling according to a nutritional context (222).

The latter case exemplifies other less well-characterized mechanisms linking tissue-specific transcriptional activity and chromatin states with alternative splicing: the presence of tissue-specific overlapping noncoding RNAs. An interesting feature of the *ALK* gene is the presence of an antisense noncoding transcript, anti-ALK, in the 3′ region of the gene, which also seems to be regulated between tissues and individuals; *ALK* can be involved in the establishment of methylation patterns and alternative splicing regulation. Another interesting but not well-explored mechanism is the activation of distal transcriptional regulatory elements located close to alternative exons (223). These regulatory elements can create open chromatin regions in a tissue-specific manner, can contact promoter and other regulator elements, and can harbor specific histone marks, which would in turn affect transcription elongation and alternative splicing.

An observation that is gaining increasing support is that alternative splicing regulation is implicated in the transcriptional network that regulates the pluripotency of embryonic stem cells. Alternative splicing events influence transcriptional control of pluripotency genes (201, 224–226), and induced pluripotent stem cells undergo changes in splicing patterns to closely resemble embryonic stem cells (227), indicating that splicing patterns play a role in reversion to a differentiated cell by reprogramming. Downregulation of U2AF35 and SRSF3 suppresses the efficiency of somatic cell reprogramming, suggesting that these splicing factors participate in the molecular network of pluripotency maintenance (227). Also, the splicing factor SON participates in the expression of several pluripotency factors (228). The existence of these dual splicing–transcription

regulatory networks suggests that the coupling between transcription and splicing can have profound implications in the circuitry controlling complex gene expression patterns.

Participation of Transcriptional Coupling in Dynamic Splicing Regulation

Cross talk between the splicing and transcription machineries is most likely a dynamic process because cells need to adjust both their splicing and transcriptional programs as the result of the reception of environmental cues, as well as react to normal changes of the cell state during the cell cycle. Several cases of transcription-coupled alternative splicing alterations caused by extracellular stimuli have been documented. In cases of an acute stimulus triggering transcriptional activation of genes, very efficient cotranscriptional splicing may ensure that the final mRNA is produced in due time, as reported for different genes in estradiol-induced responses (229). These estradiol-responsive genes are also regulated at the alternative splicing level, as mentioned above (44–46, 230).

In the fruit fly, different components of SWI/SNF regulate RNAPII elongation and completion of pre-mRNA splicing in transcripts that depend on ecdysone hormone signaling at critical points during larval development (117). This is not the first report of chromatin-remodeling complexes affecting RNAPII elongation and splicing in response to a stimulus. In human cells, the ability of mammalian SWI/SNF to induce stalling of the polymerase on a series of alternative exons in the *CD44* gene is activated by treatment with phorbol esters (136). This effect is also mediated by the heterochromatin-binding protein HP1γ [which is recruited in response to the stimulus, at least partially due to the preexisting high levels of H3K9 methylation in the intragenic region of the *CD44* gene (190)], as well as by HP1γ-dependent recruitment of the AGO1 and AGO2 proteins to the nascent RNA. This in turn keeps the level of H3K9 methylation high, providing another example of chromatin-mediated reinforcement of a splicing choice (191).

The regulation of alternative splicing in the *NCAM* gene in response to neuronal activation via depolarization of the membrane potential also requires chromatin-mediated regulation of RNAPII elongation rate (97). In this case, increases in chromatin accessibility and histone acetylation levels in a restricted part of the gene surrounding the alternatively spliced exon 18 cause an increase of polymerase and a concomitant decrease in exon inclusion. The chromatin structure at the gene promoter, located more than 200 kb upstream, is not affected by the stimulus, which coincides with no overall changes in total expression levels. This example shows that transcription-coupled alternative splicing regulation does not always need to be accompanied by changes in transcript levels.

The cell not only senses the outside environment but also responds to the intracellular state. An obvious change is progression through the cell cycle. Here, phosphorylation has a major role in histone dynamics and can cotranscriptionally alter the affinity of chromatin-binding proteins. When entering into mitosis, histones undergo massive phosphorylation that affects localization of splicing factors. SRSF1 and SRSF3 are released from hyperphosphorylated mitotic histone, and the inhibition of H3 phosphorylation increases retention of both SR proteins in chromatin (162). At the same time, during the cell cycle, HP1 proteins fluctuate by binding to and detaching from chromatin, despite stationary histone H3K9 trimethylation (231, 232). Inhibition of SRSF1 expression increases the lifetime of HP1 on chromatin and causes a delay in G_0/G_1 entry (162). These findings illustrate a dynamic cross talk between chromatin and splicing factors, which likely affects alternative splicing patterns based on the cell's state.

Also, transcriptional coupling to alternative splicing is essential for the DNA damage response. RNAPII becomes radically hyperphosphorylated after DNA damage caused by UV irradiation

of human cells, causing a slower elongation rate and affecting the alternative splicing of several genes. Some of the spliced genes, such as the apoptotic regulators *Bcl-x* and *caspase-9*, are crucial for the downstream decisions of the damaged cells (233). In addition to genes involved in DNA damage responses and apoptosis, UV treatment also changes the alternative splicing patterns of the regulators of RNA processing, revealing the core of RNA-based responses to genotoxic stress (95). This type of response can be a more general feature of adaptation to stress because starvation in *C. elegans* also produces changes in RNAPII elongation and alternative splicing on RNA processing factors (95).

EVOLUTIONARY PERSPECTIVE

By understanding the origin of alternative splicing and the evolutionary forces that have shaped (and are still shaping) this process, we can gain a better understanding of why nucleosomes are preferentially positioned in exons, linking the chromatin and splicing codes. For the past 15 years, the homologous alternative splicing events that maintain the same pattern of alternative splicing between two distant organisms (such as human and mouse) have been considered as functional events. The logic behind this assumption is that, if the event was present in the last common ancestor of two organisms and if millions of years have passed since their speciation and the event has been maintained, it is likely to be functional. Sequencing of the genomes and transcriptomes of many vertebrates and invertebrates has identified homologous events among multiple species, and these data provide the foundation for research on how these events are controlled.

A comparative analysis of conserved alternative splicing events revealed that exon skipping is the predominant form of alternative splicing in invertebrates and vertebrates, accounting for ~40% of all alternative splicing events. Intron retention is the rarest form of alternative splicing in vertebrates, accounting for less than 3% of all conserved events (234). Interestingly, intron retention is common in plants. It seems that plants and vertebrates use two different approaches to generate genomic diversity. Plants took what appears to be the more logical pathway: Whole-genome duplication is a means to freeing a set of genes for rapid mutational accumulation while leaving functional genes intact. Most mutations abolish gene function; however, a small fraction results in new functions and can lead to speciation. In vertebrates, and especially in mammals, the strategy is totally different: Most mammalian genes are highly conserved, including exon–intron structures and coding sequences (8). Alternative splicing (along with gene transcription regulation) is a major mechanism for generating transcriptome diversification, with a higher amount of alternative splicing in vertebrates compared with invertebrates and a higher number of genes that are alternatively spliced in humans compared with all other examined mammals (3, 212, 234). So what distinguishes alternative exons from constitutive exons?

As mentioned above, alternative exons are flanked by weak splice sites compared with those that flank constitutively spliced exons (235–237). Also, introns flanking alternatively spliced exons have been more elongated during evolution compared with introns flanking constitutively spliced exons (124). These features led to the suggestion that alternative exons are suboptimal: The weak splice sites and long flanking introns generate suboptimal exons that are not recognized as effectively by the splicing machinery as are constitutive exons. Thus, alternative exons are selected by the splicing machinery on one transcript and thus are spliced into the final mature mRNA, whereas on other transcripts, the splicing machinery misses these exons and ligates the flanking exons to form a mature mRNA without the cassette exon (8).

Alternative exons are actually more conserved in their sequence compared with constitutive exons, especially in their beginning and ending regions (237–239). In these two regions of an exon, the binding sites for splicing proteins that regulate the exon inclusion level are found

(237, 240). Conservation also extends into the flanking intron sequences. Approximately 100 nt flanking conserved alternative exons are also highly conserved (238). These intronic sequences flanking alternative exons that are spliced in a tissue-specific manner are more conserved than flanking exons that are alternatively spliced throughout an organism (241). How sequences in the flanking introns modulate splicing is largely unknown, as there do not appear to be extensive splicing regulatory binding sites or conserved secondary structures that might mediate exon selection (238).

Human–mouse comparative genomics revealed that a large fraction of alternative exons is not conserved between the two species (239). Thus, certain alternative exons have been gained or lost during evolution. We currently know of three different mechanisms by which new alternative exons emerge: The first is exonization, an evolutionary process in which mutations in intron sequences generate functional splice sites that are selected by the splicing machinery (242, 243). By this process, intron sequences become exons. The majority of these new exons are alternatively spliced. Very often, mutations generate strong splice sites; thus, constitutively selected exons lead to genetic disorders (243, 244). When the generation of new exons from introns advances via alternative splicing, skipping over these exons, ligation of the flanking exons maintains the original coding sequence and the synthesis of the functional protein (245). Inclusion of new exons via exonization generates a new isoform. Some of these new isoforms are functional, whereas others are mildly or very deleterious. The functional exons that evolve via this exonization process show higher levels of exon inclusion compared with the nonfunctional exons, their length in nucleotides is usually divisible by three, and they lack a stop codon so that exon inclusion inserts a peptide domain without disturbing the original coding sequence (246). In humans, the exonization process is threefold more prevalent than it is in mice. The reason is that in primates there are ~700,000 retroelements, called *Alu*, that have been inserted into intron sequences. A large fraction of these intronic *Alu* elements is one or two mutations away from being exonized (243, 244, 246). So, the exonization process, especially of *Alu* elements in primates, enhances transcriptome and proteome capacity by generating new alternatively spliced exons without destroying the expression of functional genes.

Constitutively spliced exons may also become alternatively spliced; a large fraction of alternatively spliced exons in human originated from constitutively spliced exons (247). During evolution, mutations that weaken splice-site signals cause exons to shift their mode of splicing from constitutive to alternative. Such exons tend to have a number of nucleotides divisible by three, and skipping over these exons does not change the coding sequence of downstream exons. Mutations that lead to the generation of new 5′ ss or 3′ ss are other versions of this mechanism (248). A mutation either within one of the flanking introns of a constitutively spliced exon or within the exon itself may generate a functional splice site that competes with the original splice site for selection by the splicing machinery. Evolutionary forces applied to the distance between the two alternative splice sites again tend to be separated by stretches of DNA whose length in nucleotides is a multiple of three, without insertion of a stop codon, maintaining the original coding sequence downstream from the alternative region.

Finally, exon shuffling may result in the creation of new, alternatively spliced exons. Reverse transcriptase and integrase convert RNA transcripts into DNA. These DNA fragments may then be inserted into new locations within the genome (2). When exons are inserted via this process into introns of other genes, they are usually alternatively spliced in the new location.

Reconstruction of intron length revealed that introns were short in the last common ancestor of all multicellular organisms and contained lower GC content than exons (18). During evolution, segments of homeothermic genomes (mammal and avian species) underwent a GC content increase (172). Genomes of mammal and avian species contain isochores, regions whose GC content

differs from that of adjacent regions. Genes located in high– and low–GC content regions show different exon–intron GC content architectures. In genes that are located in low–GC content regions, introns have lengthened during evolution, and splicing fidelity depends on the lower GC content in introns than in exons. In this group of genes, mutations in splice sites result in exon skipping. In contrast, when genes are located in high–GC content regions, their introns are short, and both exons and introns have high GC content. The likely evolutionary scenario is that, during the transition from cold-blooded to warm-blooded organisms, introns of genes that remained in low–GC content regions were lengthened (for example, by insertion of retroelements), and as long as the exon–intron differential GC content was maintained, the splicing machinery continued to recognize short exons within the long flanking intron sequences. Genes located in regions that accumulated mutations that elevated their GC content were under selection to keep their introns short.

These two exon–intron GC content architectures are likely to relate to the different ways in which the splicing machinery recognizes the primary splicing unit. Two models for the mechanism of exon and intron selection were previously suggested: intron definition and exon definition (**Figure 1**) (16, 249). In intron definition, the splicing machinery recognizes an intronic unit and places the basal splicing machinery across this intron. As this type of recognition is presumably impaired when introns are long, the introns recognized through the intron definition mechanism are probably under evolutionary pressure to remain short. Indeed, in lower eukaryotes, where introns are generally short, intron definition is probably the dominant mode of splicing. By contrast, in higher eukaryotes, such as vertebrates, in which a large fraction of introns is long, the splicing machinery had to adapt to identify short exons among the long introns via the exon definition mechanism. In this mechanism, it is the exon unit that is recognized by the splicing machinery (2, 8, 16, 249–251).

The two different exon–intron GC content architectures also show different chromatin organization and different patterns of DNA methylation (18, 172). In low–GC content regions, the differences in GC content between introns and exons result in higher levels of nucleosome occupancy on exons compared with the flanking introns. By contrast, genes located in the high–GC content regions have higher nucleosome occupancy throughout the gene compared with that found in low–GC content regions, and nucleosomes do not preferentially bind to exons compared with flanking introns. Thus, there must be other markers that distinguish exons from introns, especially for genes located in high–GC content regions. Indeed, high–GC content genes have ∼15% higher levels of DNA methylation in exons compared with the proximal regions of the flanking introns (172). This observation is likely biologically significant because in this group there are no more CG dinucleotides (the site of DNA methylation) in exons than in introns. In genes that are located in low–GC content regions, there are also higher levels of DNA methylation in exons compared with the flanking introns, but the difference is only ∼5%. Therefore, nucleosome positioning and DNA methylation are likely to be conserved evolutionary mechanisms that assist the splicing machinery in splice-site selection.

CONCLUDING REMARKS

Alternative splicing not only expands the coding capacity of the genomes of higher eukaryotes but also explains species- and tissue-specific patterns of gene expression. Multiple mechanisms of differing biochemical nature regulate alternative splicing. These include the interactions of splicing factors with their pre-mRNA target sequences and complex interactions with the transcription and chromatin machineries. Promoter occupation, transcriptional activators and coactivators, mediator and adaptor proteins, histone marks, chromatin compaction, nucleosome positioning, and RNAPII elongation rates have emerged as key features that contribute to alternative splicing

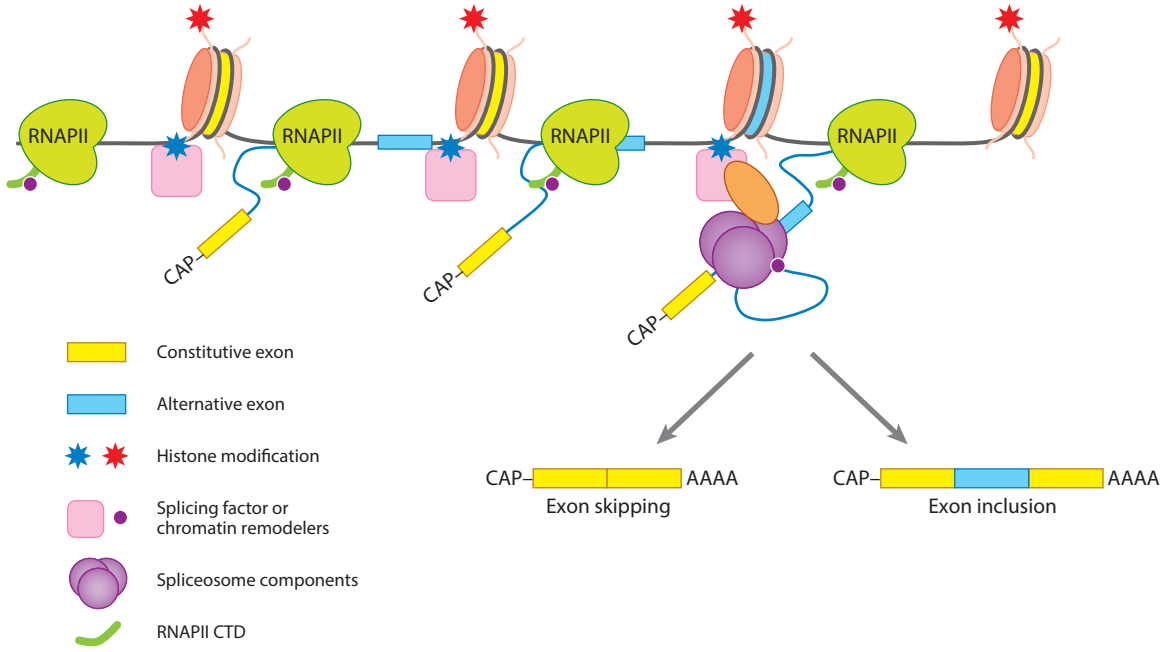

Figure 3

The importance of chromatin location for cotranscriptional splicing regulation. Splicing factors can associate with the RNA polymerase II (RNAPII) C-terminal domain (CTD), chromatin remodelers, histone marks, or methylated DNA. Elongating RNAPII and nucleosomes maintain the scaffolding for nascent precursor messenger RNA.

regulation (**Figure 3**). Remarkable progress made in the field has increased our understanding of the importance pf local chromatin structure's effect on the splicing reaction. Evidence of splicing factors' association with histone modifications, chromatin remodelers, or RNAPII indicates that chromatin acts as a scaffold for cotranscriptional splicing. However, many fundamental questions remain to be answered. How much does alternative splicing contribute to cell fate and tissue or organ differentiation compared with differential transcriptional regulation? What is the physiological relevance of the coupling of alternative splicing with transcription and chromatin? If this coupling is important, how much does it contribute to cell-type-specific alternative splicing patterns? Is there an epigenetic transgenerational memory inheritance of alternative splicing patterns? Do the kinetic and recruitment models, suggested over the years to explain the impact of chromatin on splicing, actually reflect different concepts? It might be that these two models actually represent the same underlying mechanisms, as histone modifications recruit chromatin-modifier enzymes that alter RNAPII elongation and some histone modifiers associate with RNAPII to affect elongation of transcription. Further studies are necessary to clarify the role of asymmetric histone tail modifications and epigenetic influences on splicing. Undoubtedly, answers to these questions will require multidisciplinary approaches and combined efforts through genome-wide and individual gene studies.

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