Mechanism of alternative splicing and its regulation (Review)

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Abstract. Alternative splicing of precursor mRNA is an essential mechanism to increase the complexity of gene expression, and it plays an important role in cellular differentiation and organism development. Regulation of alternative splicing is a complicated process in which numerous interacting components are at work, including cis-acting elements and trans-acting factors, and is further guided by the functional coupling between transcription and splicing. Additional molecular features, such as chromatin structure, RNA structure and alternative transcription initiation or alternative transcription termination, collaborate with these basic components to generate the protein diversity due to alternative splicing. All these factors contributing to this one fundamental biological process add up to a mechanism that is critical to the proper functioning of cells. Any corruption of the process may lead to disruption of normal cellular function and the eventuality of disease. Cancer is one of those diseases, where alternative splicing may be the basis for the identification of novel diagnostic and prognostic biomarkers, as well as new strategies for therapy. Thus, an in-depth understanding of alternative splicing regulation has the potential not only to elucidate fundamental biological principles, but to provide solutions for various diseases.

Contents

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1. Introduction

The discovery of the phenomenon that viral sequences are removed from a pre-mRNA and the remaining sequences are joined together led to a fundamental principle governing biology, known as RNA splicing. The identification stimulated theories for protein diversity, such as alternative splicing, which over time have been realized repeatedly through experiments. Gilbert (1) first proposed the concept of alternative splicing in 1978, which is currently the mechanism that accounts for the discrepancy between the number of protein-coding genes (~25,000) in humans and the >90,000 different proteins that are actually generated (2,3). The notion of 'one gene-one RNA-one protein' is no longer relevant. More than 95% of human genes have been found to undergo splicing in a developmental, tissue-specific or signal transduction-dependent manner (4).

Constitutive splicing is the process of intron removal and exon ligation of the majority of the exons in the order in which they appear in a gene. Alternative splicing is a deviation from this preferred sequence where certain exons are skipped resulting in various forms of mature mRNA. Weaker splicing signals at alternative splice sites, shorter exon length or higher sequence conservation surrounding orthologous alternative exons influence the exons that are ultimately included in the mature mRNA (5). This process is mediated by a dynamic and flexible macromolecular machine, the spliceosome, which works in a synergistic and antistatic manner (as explained below) (6,7). Three possible mechanisms, exon shuffling, exonization of transposable elements and constitutively spliced exons, have been proposed for the origin of alternative splicing (8).

Numerous studies have reiterated the critical and fundamental role of alternative splicing across biological systems (9). The species of higher eukaryotes have been discovered to exhibit a higher proportion of alternatively spliced genes, which is an underlying indication of a prominent role for the mechanism in evolution. Alternative splicing mediates diverse

biological processes over the entire life span of organisms, from before birth to death (10,11). Conserved splicing to species-specific splice variants play a significant functional role in species differentiation and genome evolution (12,13), as well as in the development of functionally simple to complex tissues with diverse cell types, such as the brain, testis and the immune system. Alternative splicing even participates in RNA processing itself, from pre- to post-transcriptional events.

Thus, alternative splicing has a role in almost every aspect of protein function, including binding between proteins and ligands, nucleic acids or membranes, localization and enzymatic properties. Taken together, alternative splicing is a central element in gene expression (14).

2. Molecular mechanisms of alternative spicing

Systematic analyses of ESTs and microarray data have so far revealed seven main types of alternative splicing (12) (Fig. 1). The most prevalent pattern (~30%) is the cassette-type alternative exon (exon skipping) in vertebrates and invertebrates (Fig. 1C), while in lower metazoans, it is intron retention (Fig. 1F) (15). Intron retention in human transcripts is positioned primarily in the untranslated regions (UTRs) (16) and has been associated with weaker splice sites, short intron length and the regulation of cis-regulatory elements (17).

Alternative selection of 5' or 3' splice sites within exon sequences (~25%) may lead to subtle changes in the coding sequence (Fig. 1D and E), and an additional layer of complexity arises with mutually exclusive alternative exons (Fig. 1B). One example of a transcript that undergoes alternative splicing, which generates variation in the protein, is *FGFR2*. Differences in the splicing machinery in different cell types and unique cis-acting elements in the *FGF-R2* pre-mRNA lead to altered tissue specific choices that create either *FGF-R2IIIb* or *FGF-R2IIIc* mature transcripts (18).

The protein expression is further regulated by alternative polyadenylation of mRNA, which influences the coding potential or the 3'UTR length by modifying the binding availability of microRNA or RNA (19). Of note, it has been demonstrated that each type of alternative splicing can operate in a stochastic manner, and different splice-site identification and processing mechanisms do not necessarily occur at the same frequencies among all biological kingdoms (20).

The mechanisms outlined above are just one indication of the complexity, as numerous molecules are involved in alternative splicing in a coordinated manner. Even the basic nucleotide components and the essential molecules that recognize them can introduce diversity in the synthesis of mature transcripts.

Two major steps constitute the basic process of splicing: Assembly of the spliceosome followed by the actual splicing of pre-mRNA. The spliceosome is mainly composed of U1, U2 small nuclear ribonucleic proteins (snRNPs) and the U4/U6.U5 tri-snRNP, and configure in identify a core set of splicing signals: The 5' splice site, the branch point sequence and the 3' splice site (Fig. 2). Specific spliceosomal complexes (E, A, B and others) and eight evolutionarily conserved DExD/H-type RNA-dependent ATPases/helicases assemble in a proposed stepwise manner and execute multiple splicing steps that result in exon ligation and intron excision. Numerous steps in the pathway are reversible (21).

The exons that end up in the mature mRNA during the process of alternative splicing is entirely defined by the interaction between cis-acting elements and trans-acting factors. Cis-acting elements include exonic splicing enhancers (ESEs) and intronic splicing enhancers (ISE) that are bound by positive trans-acting factors, such as SR proteins (serine/arginine-rich family of nuclear phosphoproteins), whereas exonic splicing silencers (ESSs) and intronic splicing silencers are bound by negative acting factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs). The collaboration between these elements results in the promotion or inhibition of splicesome assembly of the weak splice sites, respectively (Fig. 2) (22,23). In general, the cis-acting elements function additively. The enhancing elements tend to play dominant roles in constitutive splicing, while the silencers are relatively more important in the control of alternative splicing (22). Enhancer activity has been shown to be abolished by a stable stem-loop structure as short as 7 base pairs in an RNA transcript owing to the mechanisms of physical competition, long-range RNA pairing, a structure splice code and co-transcription splicing (24,25). Furthermore, the specificity of cis-acting enhancer elements for introns or exons has been investigated. In these experiments, an ESE was found to act as an ISE depending on its location in an exon or intron (26).

HnRNPs are highly conserved from nematodes to mammals and have several critical roles in pre-mRNA maturation. Their function is to bind to the ESS to the exclusion of SR proteins. A looping out pre-mRNA leads to exonic sequestration from the rest of pre-mRNA transcript (27). HnRNPs A/B are a family of RNA-binding proteins, its diversification roles in the modulation of alternative splicing have evolved based on differing affinities for their cognate nucleic acids (28). HnRNP H and F serve to alter the proteolipid protein (PLP/DM20) ratio via the variation in the recruitment of U1 snRNP (29). Similarly, the antagonistic role of hnRNP M to the splicing factor Nova-1 generates alternatively spliced dopamine receptor pre-mRNAs, which create isoforms associated with diverse key physical functions, such as control, reward, learning and memory (30). In addition, hnRNP L and phosphorylation of ser513 have been recently shown to be involved in the regulation of alternative splicing through dynamic membrane depolarization and Ca^{2+}/calmodulin-dependent protein kinase IV activation (31,32).

In addition to the coupling of SR proteins to enhancer elements, SR proteins interact with U1 snRNP and the 35 kDa subunit of the heterodimeric factor, U2AF. The second subunit of U2AF, $U2AF^{65}$, binds SF1 and the pyrimidine tract simultaneously, on the basis of the arginine/serine (RS)-rich domain, which results in recognition and stability of the branch point, as well as polypyrimidine tract sequences. Approximately 10-12 serines in the N-terminal region of the RS domain are rapidly phosphorylated by the binding of SR-specific protein kinase to serine/arginine-rich splicing factor 1 with an unusually high affinity. This continuous phosphorylation/dephosphorylation cycle of SR proteins facilitates the shuttling of SR proteins between the nucleus and the cytoplasm, and is critically required for the regulation of alternative splicing by growth signals transduced to the nucleus (33,34). SR proteins have also been proposed to participate in post-splicing activities, such as mRNA nuclear

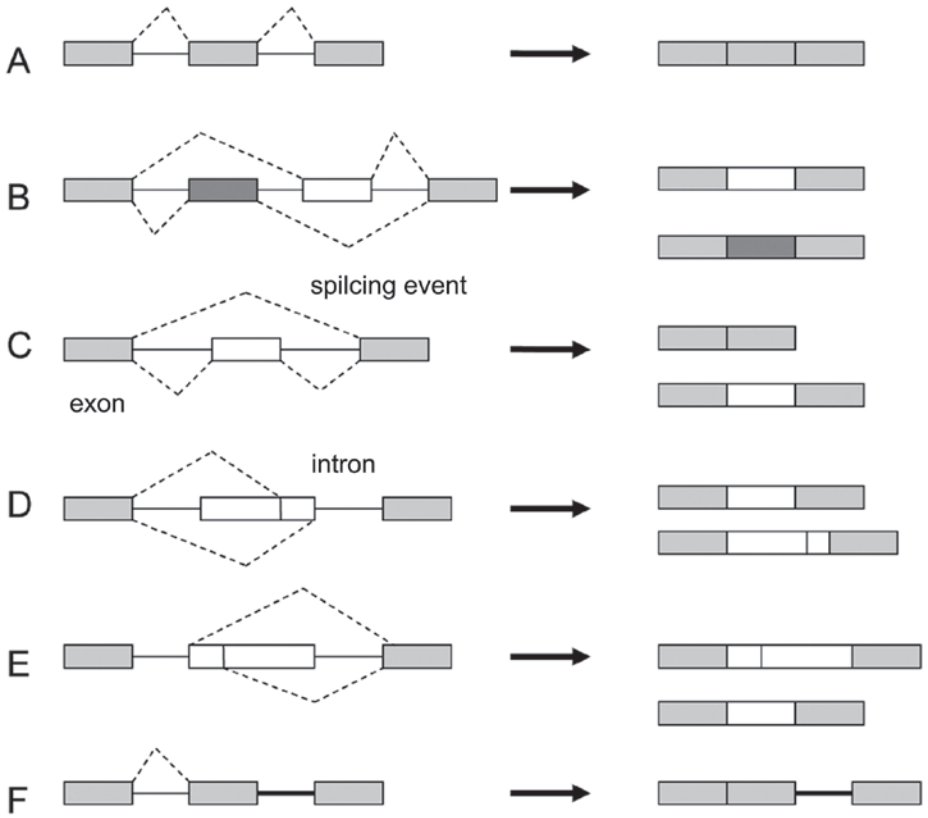

Figure 1. Five main types of alternative splicing events are depicted. (A) Constitutive splicing; (B) mutually exclusive exons; (C) cassette alternative exon; (D) alternative 3' splice site; (E) alternative 5' splice site; and (F) intron retention.

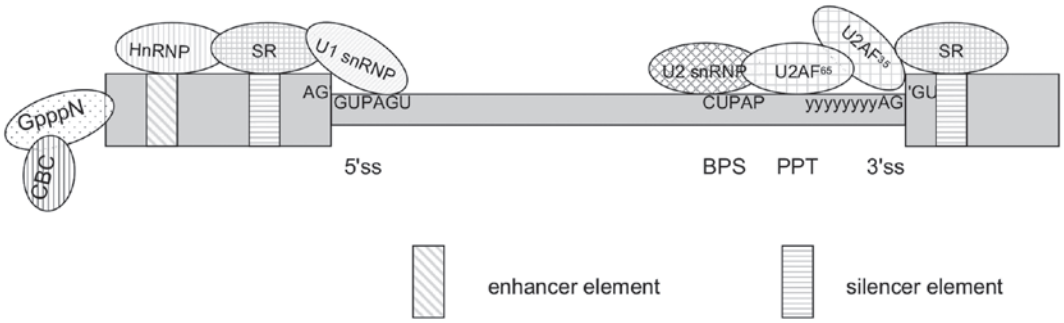

Figure 2. Schematic representation of the sequence elements and proteins at 5' and 3' exon-intron boundaries in an RNA transcript. The diagram illustrates the appropriate relative distributions of the molecules and core splicing signals with its consensus sequence in regulation of the alternative splicing. The enhancer elements [(exonic splicing enhancers (ESEs) and intronic splicing enhancers (ISEs)] are recognized by activator proteins (the SR protein family), and the silencer elements [exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs)] are bound by repressor proteins [the heterogeneous nuclear ribonucleoproteins (hnRNP) protein family]. These two protein families are engaged to promote or inhibit spliceosome assembly at weak splice sites, respectively.

export, nonsense-mediated decay (NMD) and mRNA translation (35).

In general, positive or negative splice-site recognition is regulated through various mechanisms, such as the local concentration or activity of splicing regulatory factors, under diverse physiological or pathological conditions. How these elements function together to precisely select a regulated splice site is, however, only partially explained by these results (36).

3. Coupling of alternative splicing to transcription

Since the first significant observation of co-transcriptional spliceosome assembly from electron micrographs of *Drosophila melanogaster* embryonic transcription units (37), increasing evidence supports the idea that transcription and splicing are physically and functionally coupled, and has also uncovered the intricate association between mRNA splicing, RNA polymerase II (Pol II) and chromatin structure (38,39).

A large number of components associated with the physical interaction between splicing and transcription have been purified, with particular attention on the carboxyl terminal domain (CTD) of the large subunit of RNAPII (40). The CTD consists of 52 tandem repeats of the heptapeptide YSPTSPS in mammals (26 tandem repeats in yeast) (41), which act as a special platform to recruit different factors to the nascent transcripts via dynamic phosphorylation of serine residues.

Kinases that phosphorylate specific CTD serine residues have been identified and are components of the protein apparatus driving the specific function. For example, ser5 phosphorylation is associated with transcription initiation through cyclin-dependent kinase 7 (CDK7) of the general transcription factor IIH (TFIIH), whereas ser2 phosphorylation is preferentially linked with CTD activity at the 3'-end of genes through CDK9 of the positive transcription elongation factor b (42). In addition, phosphorylation of ser7 has been found to facilitate elongation and splicing (43). Thus, phosphorylation is a mechanism that clearly demonstrates that functional coupling exists between transcription and alternative splicing.

CTD participates in gene expression-related functions ranging from 5' capping, splicing, poly-adenylation and chromatin remodeling (44). Of note, mutation and deletion analysis of CTD has revealed multiple defects in mRNA processing (45), therefore, CTD and additional components of the two machineries have emerged as a central element in governing the interactions between transcription and splicing. Taken together, functional coupling appears to maintain an important role in alternative splicing in driving determinative physiological changes, and fine-tune gene expression in mathematical modeling approaches (46).

Two models have been suggested to explain the co-transcription process of how transcription coupled repair influences alternative splicing. The mechanism of the recruitment model may mainly depend on specific features of CTD (as mentioned above), whereas the kinetic model is based on the different elongation rates of Pol II, which in turn determine the timing of the presentation of splices sites (47,48).

Fundamentally, the aforementioned mechanism influences patterns of alternative splicing via the variations in Pol II elongation and recruitment of splicing factors by specific histone marks (49). Thus, alternative splicing is highly influenced not only by transcription, but also by the chromatin structure, which underscores chromatin as another layer in the regulation of alternative splicing. The resultant mature mRNA is thus a reflection of numerous DNA modifications, such as patterns of histone methylation at exons, modulation of histone modifications and increased DNA methylation at exons (50,51). Conversely, a previous study indicated that splicing may mediate chromatin remodeling via deposition of histone marks on DNA or numerous associations between splicing factors and elongation proteins (38).

Adding additional complexity to the regulation network is alternative transcription initiation (ATI) and alternative transcription termination (ATT) sites. ATI and ATT significantly contribute to the diversity of the human and mouse transcriptomes to a degree that may exceed alternative splicing, when considering the number of possibilities available through alternative nucleotides, isoforms and introns (52,53). In contrast to the prevalence of alternative splicing that occurs within coding sequences (CDSs), the dominant class of alternative events, which includes ATI and ATT, occur in UTRs. This discovery reflects the preferential regulation of large distinct groups of genes with different mechanisms, such as strong coupling with alternative splicing in 5' and 3'UTRs (54).

Despite the strong correlation between alternative splicing and transcription, alternative transcription mainly results in variations of the transcript number or the 5'/3' terminal protein variants due to differential transcriptional start or terminal sites. By contrast, alternative splicing associated alterations mostly lie within the protein sequence, potentially affecting almost all areas of protein function (14,55).

4. Alternative splicing and nonsense-mediated decay

NMD is an extensive and complicated mechanism, ranging from yeast to human, exploited to achieve another level of robustness in post-transcriptional gene expression control. Studies have revealed that up to one-third of human alternative splicing events contain premature termination codons (PTC), which are recognized and lead to the degradation of transcripts containing NMD cis-elements in their 3' UTRs (56,57). In vertebrates, it has been proposed that the coupling of the exon junction complex (EJC) to mRNA transcripts, followed by binding of 3'UTRs to EJCs, triggers vertebrate specific NMD (58). The sensitivity of mRNA transcripts to NMD is modulated by alternative splicing events in the 5' or 3'UTRs and aids with the wide range of protein biosynthesis (59). Furthermore, analysis of quantitative alternative splicing microarray profiling has demonstrated that individual knockdown of NMD factors [Up-Frameshift (UPF)] strongly affects PTC-introducing alternative splicing events, indicating a role for different UPF factor requirements in alternative splicing regulation (60). In a second example, regulation of intron retention by alternative splicing-NMD in a specific differentiation event has been recently observed (61).

5. Trans-splicing

Trans-splicing is a common phenomenon in trypanosomes, nematodes, *Drosophila* and even humans, and refers to the novel and unusual splicing of exons from independent pre-mRNAs (62,63). The phenomenon has been explored as a therapeutic option for a variety of genetic diseases, particularly in the treatment of cancer (64). The carcinoembryonic antigen (CEA), for example, is associated with a variety of neoplastic processes and was exploited as a target for trans-splicing. A CEA RNA-targeting trans-splicing ribozyme was designed to perform RNA replacement through a trans-splicing reaction specifically in CEA expressing cells (65). The activity of the ribozyme simultaneously reduced CEA expression and introduced the thymidine kinase gene, which rendered the cells sensitive to ganciclovir treatment. RNA trans-splicing has also been utilized for the potential treatment of neurodegenerative diseases through a novel technology, spliceosome mediated trans-splicing (SMaRT). SMaRT was successfully used *in vivo* to re-engineer tau mRNA transcripts to include E10, and therefore, offers the opportunity potential to correct tau mis-splicing and treat the underlying disease (66).

6. Alternative splicing and non-coding RNA

Non-coding RNAs (ncRNAs), including microRNA and small interfering RNA, have recently emerged as novel regulators in alternative splicing, generally through the modulation of

the expression of key splicing factors during development and differentiation (67).

7. Alternative splicing and disease

Stringent regulation of alternative splicing is necessary for the functional requirements of complex tissues under normal conditions, whereas aberrant splicing appears to an underlying cause for an extremely high fraction of dysfunction and disease (68). Aberrant splicing has been suggested to root in alterations of the cellular concentration, composition, localization and activity of regulatory splicing factors, as well as mutations in components of core splicing machinery (69). A changed efficiency of splice site recognition is the immediate consequence, while irregularities in protein isoforms in different systems ultimately establish the disease state. Any of these alterations affecting alternative splicing can facilitate the appearance of characteristics in cancer cells, including the inappropriate proliferation, migration, methylation changes and resistance to apoptosis and chemotherapy (70). Alternative splicing has been implicated in nearly all aspects of cancer development, and therefore, is a main participant in the disease.

Understanding the basic mechanisms and patterns of splicing in tumor progress will shed light on the biology of cancer and lay the foundation for diagnostic, prognostic and therapeutic tools with minimum treatment toxicity in cancer (71). Extensive research efforts have already committed to developing drugs that target specific cancer protein isoforms. Several examples are genes associated with apoptosis [*BCL2L1* (BCL-X), *FAS*, *BIRC5* (survivin) and *MDM2*], immortality (human telomerase reverse transcriptase), and angiogenesis (vascular endothelial growth factor-A) (72,73).

However, limited success has been achieved by simply activating or inhibiting cancer-associated genes, possibly due to the expression of target genes in normal and cancers cells, such as angiogenic and anti-angiogenic isoforms (74). The lack of specificity of numerous molecular targets for cancer cells favors the development of isoform-specific diagnostic markers as therapeutic targets (75). Therefore, the key task for cancer treatment in the future should be to detect and target the expression of a gene at the gene level.

8. Conclusion

The combination of an alternative splicing database, tandem mass spectrometry, and even the latest synthetic alternative splicing database may aid with the identification, analysis and characterization of potential alternative splicing isoforms. Over two-thirds of human genes and 40% of *Drosophila* genes contain one or more alternative exons, and >90% of the protein-coding genes associated with alternative splicing events according to the >60,000 studies since the discovery of splicing (76). Alternative splicing appears to be prevalent in almost all multi-exon genes. However, what limits our insight into a more complete and accurate usage of alternative splicing are factors such as biased coverage of ESTs toward the 5'- and 3'-ends of transcripts, insufficient widespread analyses, subtle alternative splicing associated changes and advanced alternative splicing networks involved in various mechanisms and numbers of regulatory proteins. All these deficiencies lead to an incomplete understanding of the alternative splicing mechanism and may prevent the correct prediction of splice events in other species, such as the chimpanzee or plant (77,78). Distinguishing alternative splicing from other regulatory mechanisms in the gene regulation is also difficult. Alternative splicing, alternative trans-splicing, NMD, transcriptional efficiency, exon duplication and RNA editing (79) all contribute to an extensive mechanism for generating protein diversity. In addition, the difference between artificial experimental systems and real-life scenarios makes it challenging to transfer functional studies from cells to whole organisms. Numerous questions remain regarding the global impact of alternative splicing on cellular and organismal homeostasis, as well as its underlying molecular mechanisms. Finally, with regards to cancer-associated alternative splicing, whether a particular splice site selection causes the observed effect or is merely the result of the cancerous transformation is hard to distinguish. The data collected regarding alternative splicing is likely to represent only the tip of the iceberg, with further information yet to be revealed in future studies.

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