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Splicing fidelity, enhancers, and disease

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1. ABSTRACT

Eukaryotic pre-mRNA splicing allows for a large, diverse proteome to be coded by a relatively small genome. Alternative splicing events are well regulated, but when mutations disrupt the splice sites or regulatory elements, disease can occur. Similarly, mutations can cause disease through aberrant transcript production. Enhancers, one of the splicing regulatory elements, are frequent targets of disease causing mutations. This review provides an overview of the splicing reaction and mechanisms of alternative splicing and provides examples of enhancer defects that cause disease.

2. INTRODUCTION

Eukaryotic gene transcripts contain alternating protein coding (exons) and non-coding (introns) regions. For transcripts to encode functional protein, introns must be efficiently removed and exons accurately spliced together. Fidelity in the process of pre-mRNA splicing is essential for the proper flow of genetic information. Disruption of splicing, incomplete splicing, and splicing errors can all lead to aberrant protein products that cause disease. Here, we review the mechanisms and regulation of splicing and show how disruption of splicing results in a set of specific diseases.

3. SPLICING MECHANISM

3.1. Canonical splice sites

In higher eukaryotes, the splice site sequences that are recognized by the splicing machinery in the process of exon and intron definition are not highly conserved. Splice site sequences for the major class of introns are the 5′ splice site (AGguaagu), 3′ splice site (yagG), branch point (ynyur**a**y), and polypyrimidine tract (exons - upper case, introns - lower case, branch point - bold) (1, 2). In contrast, splice sites in lower eukaryotes such as the yeast *Saccharomyces cerevisiae*, are defined by strong consensus sequences (5′ splice site, GUAUGU; branch point, UACUAAC; 3′ splice site, YAG) (3). The prevalence of alternative splicing in higher eukaryotes compared to a lack of such activity in *S. cerevisiae* is related to the conservation of splices sites. The variability observed in higher eukaryotes, especially humans, opens the door to two related but opposite outcomes. The first is that variation in the strength of splicing signals enables regulated splicing so specific splice sites can be joined to generate wide diversity in protein function while maintaining a much smaller genome compared to proteome. The flip side is that misregulation of splicing, splicing errors, incorrectly recognized splice sites, and incomplete splicing can all result in aberrant protein production, of which some isoforms may be deleterious and cause disease.

3.2. Spliceosome assembly

Canonical splice sites are recognized by components of the spliceosome, which is composed of 5 RNA-protein complexes known as small nuclear ribonucleoproteins (snRNPs). Each snRNP contains 1-2 snRNAs and several proteins, both unique and shared (4, 5). snRNPs recognize *cis*-acting elements that identify exon/intron boundaries, and they assemble on these elements in a step-wise fashion.

During spliceosome assembly discrete and detectable complexes have been identified (Figure 1). In the first complex (E), U1 snRNP binds to the 5′ splice site through base pairing of the 5′ end of U1 snRNA with the 5′ splice site, U2AF binds to the polypyrimidine tract and 3′ splice site through its 65 and 35 kDa subunits, respectively, and SF1 interacts with the branch point sequence (6-11). The second complex (A) is ATP dependent and is formed by U2AF65 recruiting U2 which base pairs with the branch point sequence (12-18). Subsequently, the U4/U6-U5 tri-snRNP joins, forming the B complex (19). The final catalytic complex (C) involves a rearrangement of assembled proteins, RNA-protein interactions, and RNA-RNA interactions (20). U4/U6 base pairing is destabilized allowing U6 to pair with the 5′ splice site and with U2 (21). As a result, U1 and U4 are displaced. U5, the most highly conserved snRNA interacts with both the 5′ and 3′ splice sites and is thought to help align the exons for ligation at the catalytic core of the spliceosome (22, 23). Most evidence suggests that overall catalysis is RNA based but that active site formation is facilitated by protein function (24).

3.3. Splicing reaction

The pre-mRNA splicing reaction occurs as two sequential *trans*-esterification reactions. Based on conservation of phosphoester bonds, ATP is not formally required for splicing, but several spliceosome assembly steps require ATP. In the first step, the 5′ splice site phosphate undergoes nucleophilic attack by the 2’ hydroxyl of the branch point adenosine. This results in cleavage of the upstream exon with a free 5′ hydroxyl and coincident ligation of the 5′ end of the intron to the branch point forming an exon-intron lariat structure. In the second reaction, the free hydroxyl at the 5′ splice site attacks the 3′ splice site phosphate resulting in exon ligation. The free intron lariat is subsequently debranched and degraded, and the spliceosomal components are recycled. During splicing, protein complexes (Exon Junction Complexes) are deposited nearby ligated splice sites that link export out of the nucleus to accurate, complete splicing (25, 26).

4. ALTERNATIVE SPLICING

The spliceosome is faced with the challenge of efficiently and precisely identifying splice sites. This is an especially daunting task as transcripts can contain 10 times more potential splice sites than correct ones and many transcripts contain sequences that are better matches to consensus splice sites than the correct sites (27, 28). In addition, introns are typically long (averaging around 3000 nucleotides but extending to as long as 300 kb and constituting roughly 25% of the genome) while exons are short (averaging 140 nucleotides and accounting for only 1.1% of the genome) (29-31). Thus, it is not surprising that within the “sea” of RNA encountered by the splicing machinery, there exists sequences that better match the short consensus elements that typically flank introns. Canonical splice site sequences are necessary for the identification of correct exon/intron boundaries but they are not sufficient to rule out potential but incorrect sites (32). One result of the abundance of degenerate and weak splice sites is that the same splice site may not be used every time, sometimes in a regulated fashion, others as simple mistakes. For regulated splice site choice, multiple protein products can be generated from a single gene (33, 34).

4.1. Prevalence of Alternative Splicing

Several mechanisms exist to generate naturally occurring alternative transcripts. These events can occur by usage of alternative 3′ or 5′ splice sites, intron retention, exon skipping or inclusion, and selection between mutually exclusive exons (Figure 2). Analysis of alternative splicing events occurring in the genes on chromosome 22 estimated that 60% of genes generate at least 2 mature transcripts (35-37). Of all alternative splicing events, about 80% affect the open reading frame and thus the encoded protein (38). As more and more cDNAs are isolated, especially with advances in modern sequencing technologies, it is possible that nearly all protein coding genes will be found to utilize alternative splicing to some extent.

4.2. Aberrant splicing and NMD

Aberrant splicing can result from either the disruption of constitutive splicing or incorrect use of

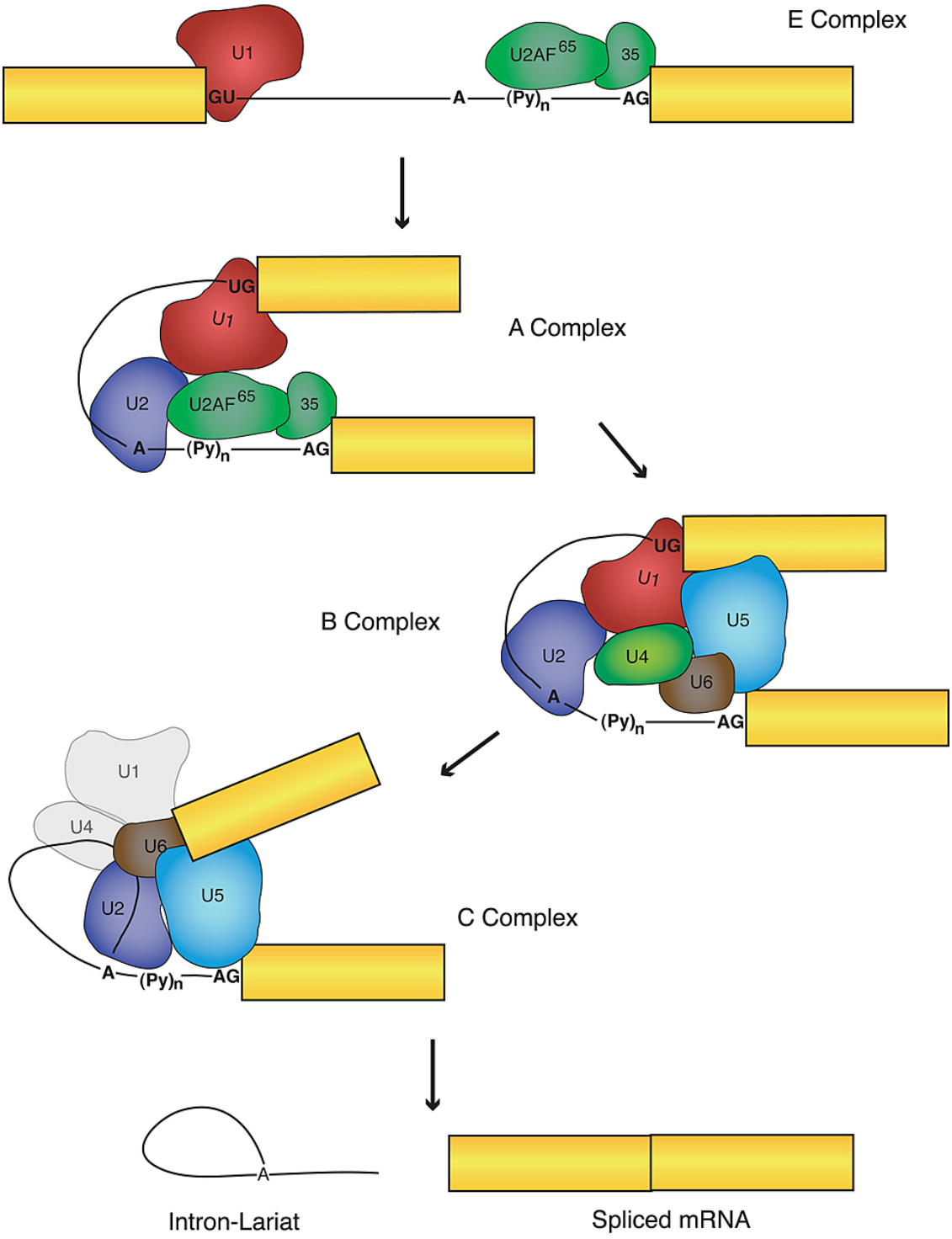

Figure 1. Step-wise spliceosome assembly. Spliceosome assembly occurs in a step-wise manner with the formation of four discrete complexes. In the E complex, U1 binds the 5′ splice site, U2AF65 binds the polypyrimidine tract, and U2AF35 binds the 3′ splice site. U2AF65 recruits U2 to the branch point, forming the A complex. The B complex forms when the U4/U6-U5 tri-snRNP joins, and then rearrangement of the components forms the C complex. U4/U6 base pairing is destabilized, allowing U6 to displace U1 at the 5′ splice site and base pair with U2. U1 and U4 are displaced from the complex. U5 interacts with the 5′ and 3′ splice sites, bridging the exons. It is in the final complex where the two sequential transesterification reactions occur. The end result is the fully spliced mRNA and the intron lariat, which is debranched and degraded.

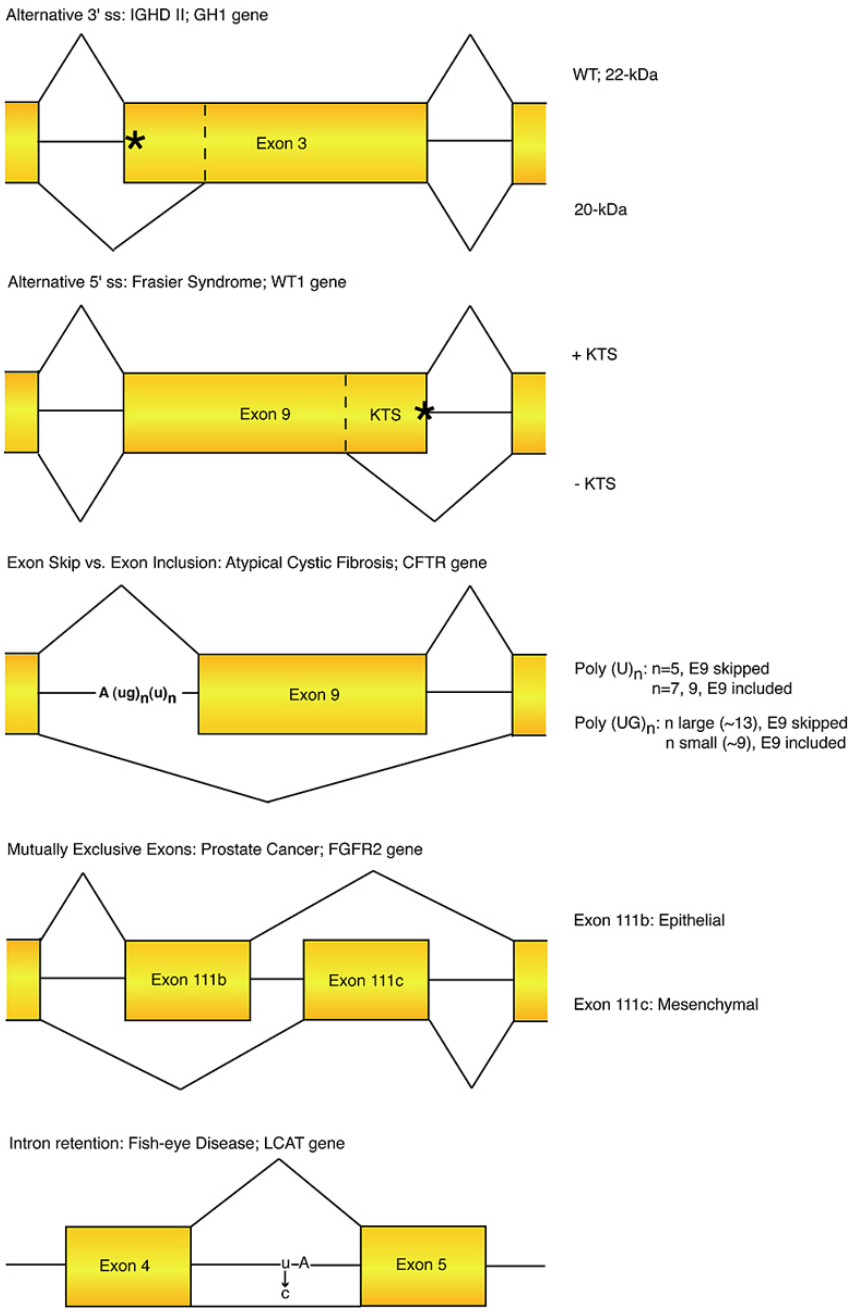

Figure 2. Types of alternative splicing with disease-related examples. A. Isolated growth hormone deficiency type II is caused by exon 3 skipping in the growth hormone1 (GH1) gene. There is also a cryptic splice site in exon 3 that produces a product lacking the first 45 nucleotides of exon 3. Mutations at the 5′ end of exon 3 result in loss of this isoform. B. Exon 9 of the Wilms' tumor suppressor (WT1) gene has two alternative 5′ splice sites. These two sites are nine nucleotides apart and those nucleotides code for the amino acid sequence KTS. Normally, the proximal 5′ splice site is favored resulting in production of the +KTS isoform. In Fraiser syndrome, mutations inactivate the proximal 5′ splice site and result in –KTS isoform production (199). C. Intron 8 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene contains variable length poly UG and poly U tracts downstream of the branch point. The lengths of these tracts effects exon 9 inclusion, and lengths that result in exon 9 skipping cause atypical cystic fibrosis (200). D. The fibroblast growth factor receptor 2 (FGFR2) gene has mutually exclusive exons IIIb and IIIc, which are included in epithelia and mesenchyme, respectively. There are many regulatory elements in the exons and the introns on either side of them. In prostate cancer, loss of the exon IIIb containing isoform in the epithelia promotes an epithelial to mesenchymal transition, which corresponds to a transition from a well-differentiated tumor to an aggressive tumor (201). E. Fish-eye disease is caused by a mutation two base pairs upstream of the branch point in intron 4 of the lecithin:cholesterol acetyltransferase (LCAT) gene, which results in intron 4 retention (202).

alternative splice sites. These errors can result in an upset in the ratio of alternative transcripts. Another possible result is exon skipping or cryptic splice site activation resulting in a frame shift of the downstream coding region. Aberrant splicing events that result in frame shifts are likely to produce transcripts that contain premature stop codons (PTCs). If the PTC lies more than 50 nucleotides upstream of the last exon-exon junction (marked by Exon Junction Complexes), then the transcript will be subject to nonsense mediated decay (NMD) and no protein will be produced from the erroneous transcript (26, 39-41). The ability to couple alternative splicing and NMD may actually serve as a rheostat allowing control over precise levels of a given protein isoform (42-44).

5. ENHANCERS AND SR PROTEINS

The presence of short and mostly degenerate splice sites highlights the need for additional *cis*-acting signals to guide the spliceosome away from potential but incorrect splice sites and towards proper splice sites. The best characterized of these additional *cis*-acting signals are enhancer and silencer elements (45, 46).

5.1. Enhancer structure and function

Splicing enhancers are purine-rich sequences found in both exons and introns. Exonic enhancers direct U2AF and U2 binding to the 3′ splice site and U1 binding to the 5′ splice site, respectively. For bona fide enhancers it appears that the specific sequence is more important than the overall purine content (47). If the purine content was a more important variable than the specific sequence, then changes to the sequence that do not affect purine content would be expected to have no consequence. However, such mutations disrupt enhancer activity (48). Similarly, if purine content is the major factor, replacement of one purine-rich enhancer sequence of a certain length with another equally purine-rich sequence of the same length should result in similar splicing outcomes. But again, this is not the case (47). These examples highlight the importance of specific sequences, in addition to purine-content, in defining enhancer elements. It is apparent that both purine content and sequence context—position within introns and exons, and distance from splice sites—can affect enhancer function

5.2. SR protein structure and function

Exonic and intronic splicing enhancers (ESEs and ISEs) are purine rich sequences that are usually recognized by essential, non-snRNP splicing factors known as SR proteins. Members of the SR protein family have one or two N-terminal RNA recognition motifs (RRMs) and a C-terminal domain rich in serine and arginine dipeptides (RS domain) (49). The RRM imparts substrate specificity on each SR protein through its sequence-specific RNA binding ability. The RS domain participates in protein-protein interactions that guide spliceosomal components to splice sites and define exon boundaries in a phosphorylation-dependent manner.

The core SR protein family has ten members: SRp20 (X16, RBP1), SRp30c, 9G8, SRp40, SRp55, SRp54, SRp46, SRp75, ASF/SF2 (SRp30a), and SC35 (SRp30b) (50). *In vitro* splicing in HeLa cytoplasmic S100 extracts, which contain all of the required splicing components except SR proteins, requires the presence of at least threshold levels of one SR protein. Based on S100 complementation assays, it was first thought that SR proteins are functionally redundant (51-55). However, even though they can function interchangeably *in vitro*, *in vivo* they are functionally distinct (56-61).

SR proteins are required for identification of constitutive splice sites and also for regulating alternative splicing decisions (62). In general, SR proteins enhance the usage of intron-proximal splice sites whenever there is a choice between competing 5′ or 3′ splice sites (63-69). SR proteins define exon boundaries by two mechanisms that are not mutually exclusive. First, SR proteins enhance splicing in an RS domain-independent manner by antagonizing adjacent silencer elements (70-72). Second, they recruit the splicing machinery to splice sites in an RS domain-dependent manner. These RS domain–splicing factor interactions result in the formation of bridge complexes across introns and exons. In higher eukaryotes, exon definition through exon bridging is more common due to the large and prohibitive size of introns (73-77). Intron recognition occurs more readily with small introns that have a length less than 250 nucleotides (78, 79). In addition, enhancer bound SR proteins are able to promote U2 binding to the branch point sequence and help recruit the U4/U6-U5 tri-snRNP (80). In the case of a weak polypyrimidine tract or 3′ splice site, SR proteins recruit U2AF35 to the 3′ splice site which in turn recruits U2AF65 to the polypyrimidine tract (68, 77, 81-83). ASF/SF2 and SC35 can interact with the U1 snRNP protein component, U1-70K, and with U2AF35 thus physically bridging the 3′ and 5′ splice sites (56, 68, 76, 77, 82, 84-88). This illustrates how SR proteins play a role throughout the entire spliceosome cycle (89).

5.3. Silencers and hnRNPs

In opposition to enhancer elements, exons and introns also contain silencers (90). Unlike enhancers, which are more commonly exonic sequences, silencers are typically intronic sequences (91). Silencers repress splice site usage by interfering with spliceosome assembly, exon bridging, and adjacent enhancer-bound SR protein function. Silencers are usually bound by heterogeneous nuclear ribonucleoproteins (hnRNPs) and alter the selection between alternative splice sites (92-95). hnRNPs have 1-2 RRMs and an auxiliary domain involved in protein-protein interactions. Two of the better characterized hnRNPs are hnRNP A1 and Polypyrimidine Tract Binding protein (PTB) (96, 97). Each of these proteins illustrates one way of repressing splicing. When hnRNP A1 binds to a silencer, it stimulates the cooperative assembly and nucleation of hnRNPs on the pre-mRNA (72). This results in the silencer and the area around the silencer being bound by hnRNPs and in SR proteins being blocked from binding to adjacent enhancers. Repression of splicing by PTB can occur in multiple ways. First, PTB binding can outcompete

binding of U2AF to the polypyrimidine tract thereby blocking splicing (98, 99). Also, PTB can block exon definition by binding to silencer elements on either side of an exon resulting in the looping out of the exon, making it inaccessible or unrecognizable to the splicing machinery (97, 100, 101).

5.4. Combinatorial control and tissue specificity

Alternative splicing events are regulated by many signals, both positive and negative. In and around alternative exons and splice sites there are multiple elements that define the proper splice sites by forming a network of interactions (102). The sum of these interactions determines the outcome. Since these regulatory elements depend on the actions of multiple activators and repressors, changes in the regulation or levels of splicing factors can combinatorially alter the outcome (103). For example, loss of a single SR protein can result in exon skipping of exons that require the function of that SR protein to counteract the action of an hnRNP. Likewise, mutation of enhancer or silencer elements can alter splicing regulation. The presence of RNA secondary structures can influence access to splice site and *cis*-elements (104-107). Splicing decisions that vary between cell types and developmental stages are regulated by natural variations in SR protein levels and also by the ratio between SR proteins and repressors. For example, the molar ratio of ASF/SF2 and hnRNP A1 varies between different tissues in rat (92, 95, 108-110). Also, tissue-specific splicing can be regulated by tissue-specific splicing factors. Neurons contain two splicing factors, NOVA-1 and nPTB, that regulate neuron-specific splicing decisions (111-115).

6. ENHANCERS AND DISEASE

Mutations at canonical splice sites have been proposed to account for at least 15% of disease-causing point mutations (116). However, this number mostly likely dramatically underestimates the impact of splicing aberrations on disease (117). Exonic mutations, distinct from splice sites, are typically thought of as causing changes in amino acid sequence (missense mutations) or truncated proteins (deletions or nonsense mutations). However, these mutations can affect the function of splicing regulatory elements such as ESEs and ESSs. Also, silent mutations, which are often thought to have no consequence, can affect these same elements. Intronic sequences are often not analyzed for disease causing mutations because they are non-coding, even though mutations within ISEs and ISSs can cause disease. Mutations that alter the strength of an enhancer or silencer can have various effects on splicing decisions. For example, if they disrupt an ESE they can result in exon skipping and large internal deletions in the protein product. If the exon skipping event does not maintain the reading frame, then the transcript will likely be degraded by NMD because of the presence of a PTC. In addition to exon inclusion and exclusion, mutations can affect splicing by activating cryptic splice sites or disrupting the usage of alternative splice sites. Altering the ratio of alternative splice site usage can lead to inappropriate expression in a cell-type or developmental stage-specific manner.

6.1. Spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disease involving progressive degeneration of motor neurons in the spinal cord resulting in wasting of voluntary muscles and weakness (118). Genetically, the disease is characterized by homozygous loss of the survival of motor neuron 1 (SMN1) alleles (119, 120). The specific functions of SMN1 are unknown, but it is thought to be involved in the cytoplasmic assembly and maturation of core snRNPs (121, 122).

The SMA locus on chromosome 5 contains 2 SMN genes, SMN1 and SMN2. SMN2 contains only 5 nucleotide differences compared to SMN1 and the two genes theoretically encode identical proteins. However, SMN2 is unable to compensate for the loss of SMN1. Analysis of mature SMN2 transcripts revealed that only about 20% contain exon 7. SMN protein lacking the region encoded by exon 7 is unstable and inactive (123). There are several *cis*-elements that contribute to the splicing difference between SMN1 and SMN2 (124, 125). A single, silent nucleotide difference in the sixth position of exon 7 (E7 + 6 C→T) (126, 127) disrupts an enhancer sequence that binds ASF/SF2 and creates a silencer sequence that is bound by hnRNP A1 (Figure 3A) (128-130). Another nucleotide difference between the two genes is in intron 7. This difference also creates an hnRNP A1 binding site in SMN2, which causes exon 7 skipping (131). Also, there is a *cis*-element downstream of the 5′ splice site of intron 7 that negatively affects the strength of that splice site (132). And there is a RNA stem-loop structure at the 3′ end of exon 7 that prevents inclusion (106). The overall result of this change is skipping of exon 7 (133).

Another cause of SMA involves mutations in the SMN1 gene that produce an exon 7 skipped transcript. A single G to T transversion at the sixth position in intron 7 leads to exon skipping by disruption of U1 binding site at the 5′ splice site (126, 134). Mutated SMN1 essentially acts as a second SMN2 allele with each allele producing small amounts of full-length SMN protein. Since the two SMN genes together produce more full-length product than SMN2 alone, these mutations result in a less severe phenotype. However, loss of SMN1 and production of only 20% full-length transcript from SMN2 causes a more severe disease.

6.2. Frontotemporal dementia with parkinsonism linked to chromosome 17

Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) is an autosomal dominant disease characterized by progressive dementia. FTDP-17 causing mutations have been linked to the microtubule-associated protein tau (MAPT) (135). The MAPT protein product, tau, functions in microtubule assembly and microtubule-dependent transport in axons. Exon 10 of MAPT encodes the last of four repeated microtubule binding motifs and is alternatively included or excluded. The normal ratio of exon 10+ and 10- transcripts is 1:1. Mutations that alter this ratio, specifically mutations that result in more exon 10 inclusion, result in disease (Figure 3B) (136). An excess of exon 10+ protein products

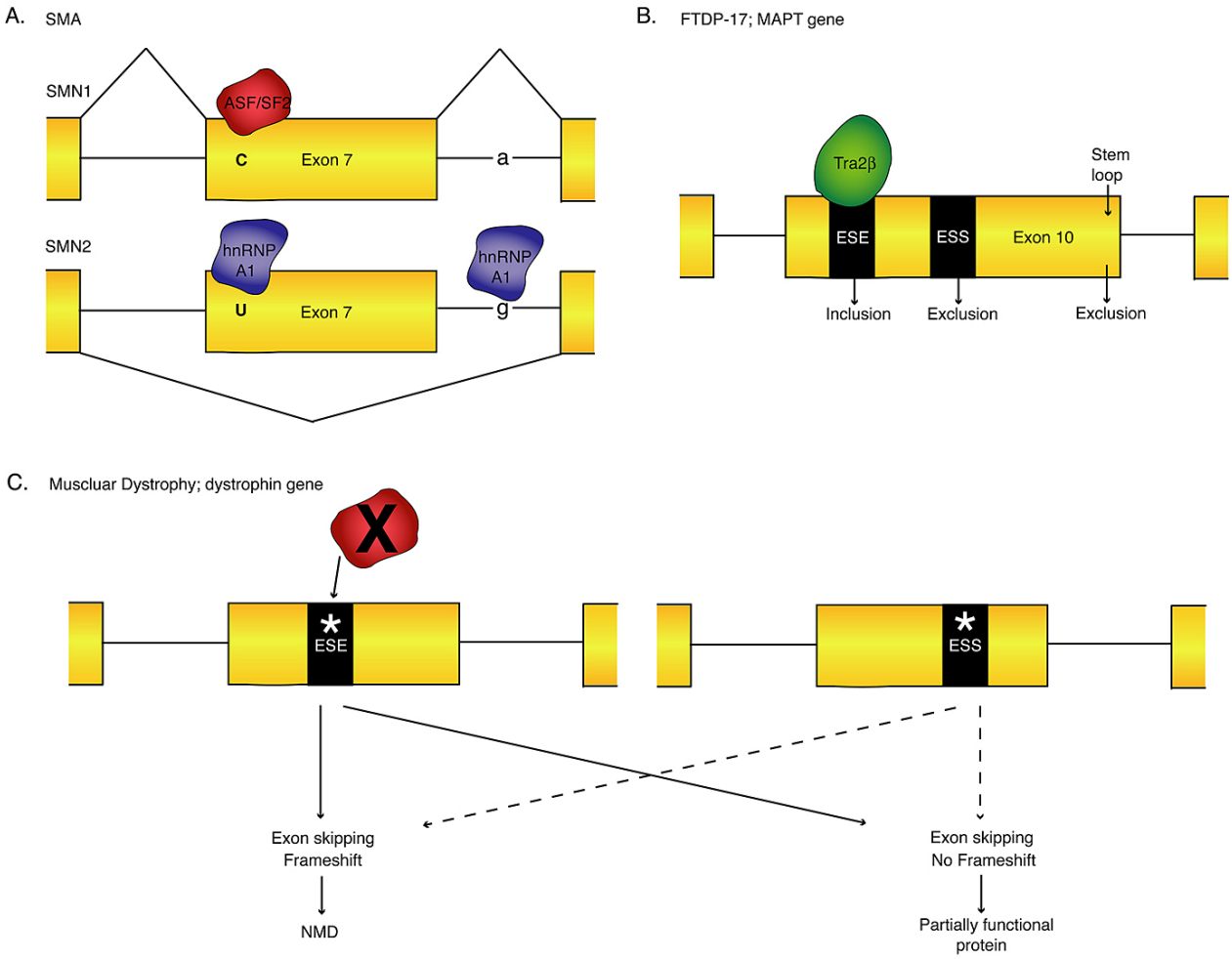

Figure 3. Enhancers and disease. A. Spinal muscular atrophy (SMA) is caused by homozygous loss of the survival of motor neuron 1 (SMN1) alleles, for which the survival of motor neuron 2 (SMN2) gene is unable to compensate (115, 116). SMN2 contains a single nucleotide (E7 + 6 C → T) different from SMN1 (120, 121). This nucleotide is part of an enhancer sequence in SMN1 that is bound by ASF/SF2 and activates exon 7 inclusion (122-124). The change in SMN2 results in disruption of the enhancer and creation of a silencer that is bound by hnRNP A1 resulting in exon 7 skipping. B. Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) is caused by alteration of the normal 1:1 ratio of exon 10 skipping to exon 10 inclusion in the microtubule associated protein tau (MAPT) gene (127). There are three main regulatory elements in exon 10, and disruption of any of them can result in disease. C. Muscular dystrophy is caused by mutations that affect exon inclusion in the dystrophin gene. These mutations can either disrupt an enhancer or create a silencer and result in exon skipping (138, 139). If the exon skipping event results in a frame shift then the transcript will be degraded by nonsense mediated decay, and if the event does not result in a frame shift then a partially functional protein will be produced.

causes aggregation of insoluble, filamentous tau, neurofibrillary tangles, and neurodegeneration (137, 138).

Exon 10 of the MAPT gene contains three regulatory elements that combinatorially control exon 10 splicing. An RNA stem loop at the 3′ end of exon 10 prevents exon 10 inclusion by blocking U1 snRNP from accessing the 5′ splice site of intron 10 (107). Four FTDP-17 mutations at the 5′ end of intron 10 (+3 G->A, +13 A->G, +14 C->T, +16 C->T) all disrupt formation of the regulatory stem loop resulting in increased exon 10 inclusion (105). The mutation I 10+3 G->A also increases binding of U1 snRNP to the 5′ splice site. Exon 10 also contains both an ESE and an ESS (139-142). A missense mutation (N279K) strengthens the ESE, and a silent mutation (L284L) weakens the ESS. Since each of these mutations causes FTDP-17 and each affects only one of the regulatory elements, it seems that all three elements are required to maintain the proper ratio of exon 10+ and 10- isoforms (139, 143).

6.3. Muscular dystrophy

Muscular dystrophy is an X-linked recessive disease caused by mutations in the dystrophin gene. It is characterized by progressive wasting and degeneration of skeletal muscle resulting in weakness and impaired

movement. In the more severe form of the disease, Duchenne muscular dystrophy (DMD), there is an absence of dystrophin, and in the less severe form, Becker's muscular dystrophy (BMD), there are insufficient amounts of fully functional dystrophin. The dystrophin gene is about 2500 kb long, contains 79 exons, and produces a 14 kb muscle specific mRNA (144). Dystrophin, a structural cytoskeletal protein, maintains membrane stability and is involved in communication between the extracellular matrix and the cytoskeleton. The large number of exons, the size differences between exons and introns, and the overall length of nascent transcripts encoding dystrophin all underscore the engineering challenge that both the transcription and splicing machinery face to synthesize and process functional mRNA templates.

Both DMD and BMD are caused by mutations that affect exon inclusion (Figure 3C). Some mutations, both point mutations and deletions, cause aberrant exon skipping by disrupting natural splice sites. Other mutations do so by disrupting enhancer elements or creating strong silencer elements (145, 146). In some cases, disruption of a natural splice site results in activation of a cryptic splice site. The factor that determines the severity of disease, and thus DMD versus BMD, is whether the aberrant splicing event caused by the mutation results in a downstream frame shift and creation of a PTC (147). If a frame shift occurs, then the PTC will trigger NMD of the transcript resulting in complete loss of the protein product and the more severe DMD (146). However, if the aberrant splicing event maintains the reading frame, then a protein product with an internal deletion will be produced. If this partially functional protein can fulfill some of dystrophin's role, then the less severe BMD is seen.

Several of the mutations that cause BMD result in nonsense codons at the site of the mutation. However, these mutations are not present in the mature transcript since the mutated exon is aberrantly skipped. While one possible explanation for how these mutations influence splicing decisions could involve active screening and recognition of the nonsence codon by the cell, this does not seem to be the case (148). Analysis of these mutations on exonic splicing regulatory elements has shown that they either disrupt enhancer elements or create silencer elements, which are then responsible for the aberrant splicing events (146, 149, 150). It would be interesting to see if knock down of hnRNP A1, in a case where a nonsense mutation creates a strong silencer element, would allow for inclusion of the mutated exon and destruction of the transcript by NMD rather than production of an internally deleted protein.

6.4. Growth Hormone Deficiency

Human growth hormone (GH), which is made by somatotrophs in the anterior pituitary, regulates postnatal growth and is a major determinant of overall body size (151). Growth hormone is secreted in pulsatile bursts that peak at night (152). The amplitude of the bursts, rather than the average growth hormone concentration, determines growth (153-155). Growth hormone deficiency occurs in 1/4000–1/10,000 live births (156-159). While most cases are sporadic, 5-30% of cases have an affected first-degree relative and are genetic (160, 161).

The growth hormone gene, GH1, lies on chromosome 17 (17q23) in a cluster of related genes: growth hormone 1 (GH1), chorionic somatomammotropin genes 1 & 2 (CSH1&2), chorionic somatomammotropin pseudogene (CSHP1), and growth hormone 2 (GH2 or GH-V) (162-166). All five genes have a five exon, four intron structure (162, 167). CHS1&2 are expressed in the placenta and code for identical proteins, which are partially responsible for fetal growth (168). GH2 is also expressed in the placenta and differs from GH1 by 13 amino acids (169).

6.4.1. GH1 gene structure

The GH1 gene produces five protein isoforms, four of which are the result of aberrant splicing (170). The 22 kDa isoform is encoded by transcripts containing all five exons and is the major, biologically active form of the protein. Activation of an in-frame cryptic splice site in exon 3 results in loss of the first 45 nucleotides of exon 3 and produces a biologically active 20 kDa protein (170-172). Transcripts encoding the 20 kDa isoform account for 5-10% of GH1 transcripts. Transcripts that skip exon 3 produce a 17.5 kDa protein and account for 1-3% of GH1 transcripts (173). This isoform acts in a dominant negative fashion to block the activity of the full-length 22 kDa protein. Trace amounts of GH1 transcripts skip exons 3-4 or exons 2-4 and produce 11.3 kDa or 7.4 kDa protein, respectively (174).

6.4.2. IGHD II

Isolated growth hormone deficiency type II (IGHD II) is an autosomal dominant disorder characterized by short stature. Additional signs and symptoms are fasting hypoglycemia, micro penis, delayed skeletal development, truncal obesity, delayed dentition, and youthful facial expression (169).

IGHD II is caused by increased exon 3 skipping and production of the 17.5 kDa isoform. This isoform acts dominant negative to the full-length 22 kDa isoform by suppressing GH secretion in both cell culture and transgenic mice (175). In cell culture, the 17.5 kDa isoform is retained in the ER, disrupts the Golgi apparatus, and impairs GH trafficking (176-179). Additionally, it slightly reduces the stability of the 22 kDa isoform (176-179). Mice overexpressing the 17.5 kDa isoform have a defect in the maturation of GH secretory vesicles and lose the majority of their somatotrophs resulting in anterior pituitary hypoplasia (180, 181). Consistent with a secretory defect, treatment with growth hormone releasing hormone does not increase serum GH levels (182). It is not entirely clear why loss of exon 3 results in a dominant negative isoform but current models are most consistent with the inability to properly fold the 17.5 kD isoform and/or create GH dimers, both of which ultimately lead to an unfolded protein response (183, 184). Exon 3 of GH1 codes for the linker domain between two helices in the protein and this

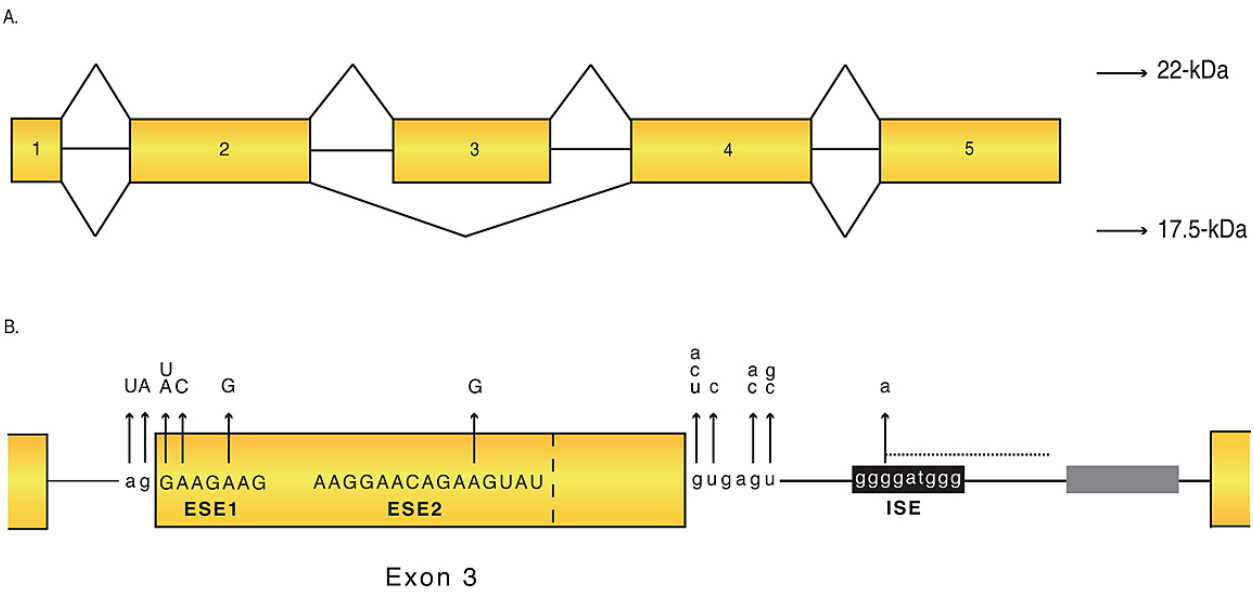

Figure 4. GH1 gene structure with IGHD II-causing point mutations. Mutations that result in exon 3 skipping in the growth hormone 1 (GH1) gene cause isolated growth hormone deficiency type II. These mutations commonly occur at the exon/intron boundaries on either side of exon 3, but they can also occur in enhancer elements within exon 3 and intron 3. The cryptic splice site in exon 3 is illustrated by a vertical dashed line. A deletion mutation in intron 3 (IVS3 del 28-45) is illustrated by a horizontal dotted line, and another deletion mutation in intron 3 (IVS3 del 56-77) is illustrated by a gray box.

linker contains a cysteine that forms an internal disulfide bridge (185). Disruption of the disulfide bridge would result in the free sulfhydryl of the unpaired cysteine forming aberrant intermolecular disulfide bonds that would disrupt the storage and secretion of the 22 kDa isoform. However, mutation of the unpaired cysteine to alanine did not correct the secretory defect (179, 182).

6.4.3. Enhancer mutations and exon 3 skipping

As noted earlier, wild-type GH1 produces small amounts of aberrant, non-full-length transcripts (173). This is because the splice sites across introns 2 and 3 are fairly weak. The 5′ and 3′ splice sites of intron 2 and the 5′ splice site of intron 3 are all weaker than the consensus splice sites (169, 181). Also, the cryptic splice site in exon 3 that is used to produce the 20 kDa isoform is a stronger splice site than the 3′ splice site of intron 2. To overcome these weak splicing signals, a variety of enhancer elements have been identified. Two purine-rich enhancer elements in exon 3 (ESE1 and ESE2) and one in intron 3 (ISE) function to maintain accurate splicing, patients with mutations in these regions display IGHD II (Figure 4) (186, 187). ESE1 consists of the first seven nucleotides of exon 3, ESE2 is a 15 nucleotide sequence upstream of the cryptic splice site (E3 + 19-33), and ISE is a nine nucleotide sequence within intron 3 (IVS3 + 26-34) (181, 188, 189). Mutations of the splice sites flanking introns 2 and 3 (182, 190-196) and also mutations that disrupt enhancer function all result in increased exon 3 skipping (181, 182, 186-188, 197). However, these mutations are not all equal. Patients with IGHD II present with variable ages of onset, clinical severity, and rates of progression. This clinical variation correlates with variation in the levels of the 17.5 kDa isoform (178, 181, 182, 186-189, 197). Splice site mutations result in production of only the 17.5 kDa isoform and result in an earlier average age of onset and greater clinical severity (187, 198). Enhancer mutations result in an increase in the percentage of 17.5 kDa isoform produced and cause less severe height reductions than splice site mutations (180, 181, 187, 189, 198).

6.4.4. ESE2 – a repressor

Most of the mutations that cause IGHD II do not directly affect the splice sites around exon 3 and instead are found within ESE1 or ISE. A recently identified mutation lies within ESE2 (E3+29 A->G) (197). ESE2 is recognized by ASF/SF2 and SC35. At first glance, it would seem likely that the mutation disrupts the binding of both SR proteins resulting in loss of enhancer function. However, recent experiments demonstrate that the mutation actually creates a stronger SC35 binding site and that SC35 actually functions to repress splicing of exon 3 in this case. In contrast, when ASF/SF2 binds, exon 3 inclusion is activated. It appears these two proteins compete for binding to ESE2 and play antagonistic roles. This work adds to the complexity of splicing regulation by SR proteins since not all SR proteins apparently activate splicing and can instead actually repress exon inclusion. The disease-causing point mutation within ESE2 creates an SR protein binding site that leads to repression of exon inclusion.

7. CONCLUSIONS

Degeneracy of the canonical splice sites in higher eukaryotes provides a means of regulating splice site selection. Current estimates are that 60% of genes are

alternatively spliced. Alternative splicing events are highly regulated by enhancer and silencer elements in exons and introns. These elements bind splicing factors that either promote or block spliceosome assembly and affect splice site usage decisions. Mutations that disrupt this regulation cause disease. These disease causing mutations can easily be overlooked because they can be silent point mutations or occur in introns, which are not expected to cause disease. However, both silent mutations and mutations that would be expected to affect amino acid sequence can cause disease by affecting splicing regulatory elements.

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Abbreviations: snRNA: small nuclear ribonucleicacid; snRNP: small nuclear ribonucleoprotein; kDa: kilodalton; PTC: premature stop codon; NMD: nonsense mediated decay; RRM: RNA recognition motif; PTB: polypyrimidine tract binding protein; hnRNP: heterogeneous nuclear ribonucleoprotein; ESE: exonic splicing enhancer; ESS: exonic splicing silencer; ISE: intronic splicing enhancer; ISS: intronic splicing silencer; SMA: spinal muscular atrophy; SMN1/2: survival of motor neuron 1/2 gene; FTDP-17: frontotemporal dementia with parkinsonism linked to chromosome 17; MAPT: microtubule-associated protein tau gene; DMD: Duchenne muscular dystrophy; BMD: Becker's muscular dystrophy; GH: growth hormone; IGHD II: isolated growth hormone deficiency type II; WT1: Wilms' tumor suppressor gene; FGFR2: fibroblast growth factor receptor 2 gene; CFTR: cystic fibrosis transmembrane conductance regulator gene; LCAT: lecithin:cholesterol acetyltransferase gene

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