Pre-mRNA Missplicing as a Cause of Human Disease

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Abstract. Regulated alternative splice site selection emerges as one of the most important mechanisms to control the expression of genetic information in humans. It is therefore not surprising that a growing number of diseases are either associated with or caused by changes in alternative splicing. These diseases can be caused by mutation in regulatory sequences of the pre-mRNA or by changes in the concentration of trans-acting factors. The pathological expression of mRNA isoforms can be treated by transferring nucleic acids derivatives into cells that interfere with sequence elements on the pre-mRNA, which results in the desired splice site selection. Recently, a growing number of low molecular weight drugs have been discovered that influence splice site selection in vivo. These findings prove the principle that diseases caused by missplicing events could eventually be cured.

1 Importance of Alternative Splicing for Gene Regulation

The sequencing of various eukaryotic genomes has demonstrated that a surprisingly small number of genes generate a complex proteome. For example, the estimated 20,000–25,000 human protein-coding genes give rise to 100,000–150,000 mRNA variants as estimated by EST comparison. Array analysis shows that 74% of all human genes are alternatively spliced (Johnson et al. 2003) and a detailed array-based analysis of chromosome 22 and 21 suggests that every protein-coding gene could undergo alternative splicing (Kampa et al. 2004). Extreme examples illustrate the potential of alternative splicing: the human neurexin 3 gene could form 1,728 transcripts (Missler and Sudhof 1998) and the Drosophila DSCAM gene could give rise to 38,016 isoforms, which is larger than the number of genes in Drosophila (Celotto and Graveley 2001).

Unlike promoter activity that predominantly regulates the abundance of transcripts, alternative splicing influences the structure of the mRNAs and their encoded proteins. As a result, it influences binding properties, intracellular localization, enzymatic activity, protein stability, and post-translational modification of numerous gene products (Stamm et al. 2005). The magnitude of the changes evoked by alternative splicing are diverse and range from a complete loss of function to very subtle, hard to detect effects (Stamm et al., 2005). Alternative splicing can indirectly regulate transcript abundance.

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About 25–35% of alternative exons introduce frameshifts or stop codons into the pre-mRNA (Stamm et al. 2000; Lewis et al. 2003). Since approximately 75% of these exons are predicted to be subject to nonsense-mediated decay, an estimated 18–25% of transcripts will be switched off by stop codons caused by alternative splicing and nonsense mediated decay (Lewis et al. 2003). Finally, several proteins that regulate splice-site usage shuttle between nucleus and cytosol where they regulate translation (Sanford et al. 2004).

1.1 Splice Sites are Selected Through Combinatorial Control

Proper splice site selection is achieved by binding of protein and protein: RNA complexes (trans-factors) to weakly defined sequence elements (cis-factors) on the pre-mRNA (Fig. 1A). Binding of the trans-factors occurs cotranscriptionally and prevents the pre-mRNA from forming RNA:DNA hybrids with the genomic DNA. RNP complexes forming around exons promote binding of U2AF and U1 snRNP at the 3′ and 5′ splice sites respectively, which marks the sequences to be included in the mRNA. Sequences located in exons or the flanking introns can act as splicing silencers or enhancers. All cis-elements can only be described as consensus sequences that are loosely followed (Black 2003) and in general, they bind only weakly to trans-acting factors. The action of the cis-elements depends on other surrounding elements, and due to this sequence context the same sequence can either promote or inhibit exon inclusion (Carstens et al. 1998). In order to achieve the high fidelity of splice site selection, multiple weak interactions are combined (Maniatis and Reed 2002; Maniatis and Tasic 2002) and as a result of this combinatorial control, splice site selection is influenced by multiple factors (Smith and Valcarcel 2000). This combinatorial control is mirrored in the complex composition of splicing regulatory complexes that often combine overlapping enhancing and silencing parts that collaborate to regulate exon usage (Singh et al. 2004b; Pagani et al. 2003b).

The formation of a specific protein:RNA complex from several intrinsically weak interactions has several advantages: (1) it allows a high sequence flexibility of exonic regulatory sequences that puts no constraints on coding requirements; (2) the protein interaction can be influenced by small changes in the concentration of regulatory proteins, which allows the alternative usage of exons depending on a tissue and/or developmental-specific concentration of regulatory factors; (3) phosphorylation of regulatory factors that alter protein:protein-interactions can influence splice site selection; (4) the regulatory proteins can be exchanged with other proteins after the splicing reaction, allowing a dynamic processing of the RNA.

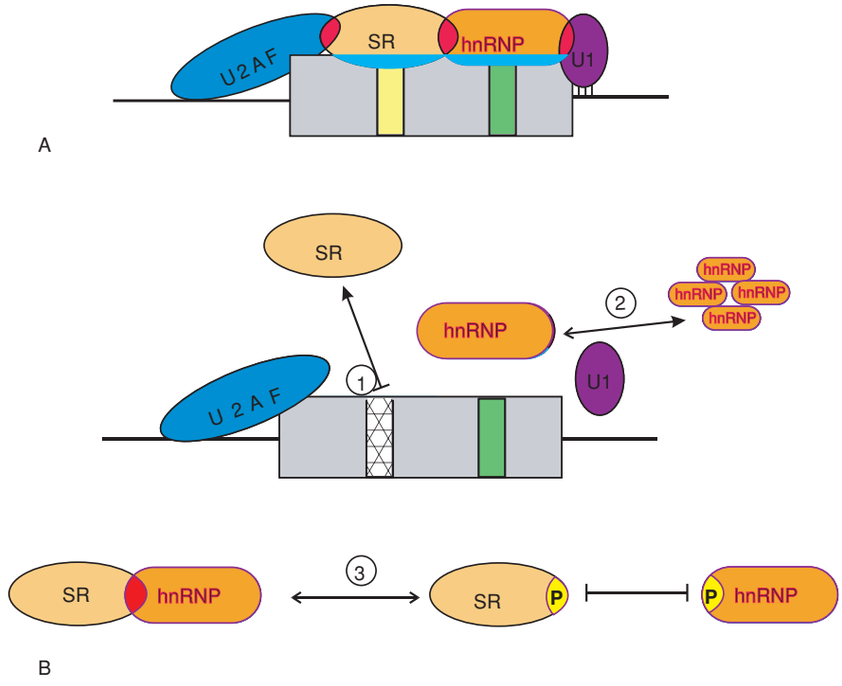

Fig. 1A, B. Change of splice site selection during disease. **A**: Formation of RNP complexes to recognize splice sites. The exon is shown as a *gray square*, the intron as *lines*. The formation of a complex between SR proteins and hnRNPs on two exonic enhancers (*small boxes* in the exons) is shown. This complex stabilizes the binding of U2AF to the 3′ splice site and of U1snRNP to the 5′ splice site of the exon (*dashed lines* show RNA:RNA binding). Multiple intrinsically weak protein:protein (*red*) interactions allow the formation of a specific complex. **B**: Mechanisms to change exon recognition. The formation of RNP complexes around exons can be disturbed by different ways. *1*: Mutations in regulatory sequences can abolish binding of regulatory factors. *2*: The concentration of regulatory factors can be altered, either by sequestration in different compartments or through a change of their expression level. *3*: Phosphorylation events change the interaction between regulatory proteins, which interferes with exon recognition

The usage of alternative exons changes during development or cell differentiation both in vivo and in cell cultures. Furthermore, numerous external stimuli have been identified that change alternative splicing patterns. In most cases, these changes are reversible, indicating that they are part of a normal physiological response (Stamm 2002).

2
Human Diseases that are Caused by Mutation in Splicing Signals

Since alternative splicing plays such an important role in gene expression, it is not surprising that an increasing number of diseases are caused by abnormal splicing patterns (Stoilov et al. 2002; Faustino and Cooper 2003; Garcia-Blanco et al. 2004; Fig. 1B). There is a positive correlation between the number of splice sites and the likelihood of a gene causing a disease, suggesting that many mutations that cause diseases may actually disrupt the splicing pattern of a gene (Lopez-Bigas et al. 2005). The disease-causing mechanism can be subdivided into changes in cis- and trans-factors. Changes in cis-factors are caused by mutations in splice sites, silencer and enhancer sequences, and through generation of novel binding sites in triplet repeat extensions. Alterations in trans-acting factors are frequently observed in tumor development, where the concentration and ratio of individual trans-acting factors change. Mutations can be seen as new sources for alternative splicing regulation. For example, the alternative splicing patterns of different histocompatibility leukocyte antigens (HLA) are regulated by allele-specific mutations in the branchpoint sequences. Since the variability of HLAs are the basis of the adaptive immune response, these mutations strengthen the immunity by enlarging the number of potential HLA molecules (Kralovicova et al. 2004).

2.1
Mutation of Cis-acting Elements

Mutations of cis-acting elements can be classified according to their location and action. Type I mutations occur in the splice sites and destroy exon usage, type II mutations create novel splice sites that cause inclusion of a novel exon, type III and IV mutations occur in exons or introns, respectively, and affect exon usage. Type I and II mutations are the simplest mutation to be recognized. About 10% of the mutations stored in the Human Gene mutation database affect splice sites. They have been compiled in that (Stenson et al. 2003) and in specialized databases (Nakai and Sakamoto 1994).

Although bioinformatics resources such as the ESE finder (Cartegni et al. 2003), or the RNA workbench (Thanaraj et al. 2004) help to predict type III and IV mutations, the theoretical models often do not fit the experimental findings (Pagani et al. 2003a). However, the increase of genotype screening in human diseases has identified numerous exonic and intronic variations. Their association with a disease phenotype is often unclear since apparently benign polymorphism, such as codon third position variations or conservative amino acid replacement, are difficult to assess. A list of

well-studied mutations in splicing regulatory elements is given in Table 1 and is maintained at the alternative splicing database web site (http://www.ebi.ac.uk/asd/).

2.2 Examples of Diseases

As examples, we discuss two well-studied pathologies: cystic fibrosis and spinal muscular atrophy. Cystic fibrosis is a recessive disease caused by loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) gene occurring with an incidence of 1:3,500. The CFTR gene encodes a cAMP-regulated chloride channel that controls the hydration of mucus. Currently, 1,388 mutations of CFTR have been described, 185 of which are splicing mutations. Twenty of these splicing mutations are located in exons, the rest in introns (http://www.genet.sickkids.on.ca/cftr/), which roughly reflects the exon/intron composition of the gene. Mutations changing exons' 9 and 12 usage have been studied in detail. Both exons are alternatively spliced in healthy individuals and the ratio of exon inclusion varies between individuals (Hull et al. 1994), which could be attributed to variable concentrations of trans-acting factors between them. Complete skipping of these exons is caused by several splice-site mutations. These mutations result in the classical clinical picture of cystic fibrosis that shows chronic respiratory and digestive problems, and affects the lower respiratory tracts, pancreas, biliary system, male genitalia, intestine, and sweat glands. In contrast, type III and IV mutations change the ratio of exon inclusion and cause non-classical forms of cystic fibrosis that affect only a subgroup of organs or appear later. A detailed analysis of the mutations showed that they are part of a larger regulatory element, the composite exonic regulatory element of splicing (CERES). CERES contains multiple overlapping silencing and enhancing elements that work only in the particular CERES context and cannot be moved into heterologous sequence contexts. Several neutral polymorphisms in CERES can influence splicing and therefore contribute to the disease. Finally, the isoform ratio evoked by CERES mutation was depending on the cell type, which would explain why the mutations affect only a few organs (Pagani et al., 2003a; Pagani et al., 2003b). Thus, mutations affecting alternative splicing contribute to a very heterogeneous clinical phenotype that makes genotype–phenotype correlation difficult.

Spinal muscular atrophy is a neurodegenerative disorder with progressive paralysis caused by the loss of alpha motor neurons in the spinal cord. The incidence is 1:6,000 for live births and the carrier frequency is 1 in 40, making SMA the second most common autosomal recessive disorder and the most frequent genetic cause of infantile death. SMA is caused by the loss of the SMN1 gene that encodes the SMN protein, which regulates

Table 1. Examples of enhancer mutations involved in human diseases. The table lists examples of mutations in regulatory motifs that cause aberrant splicing. The list is updated at the alternative splicing database website (www.ebi.ac.uk/asd/). *Large letters* indicate exonic mutations, *small letters indicate* intronic mutations. The *top line* of each sequence indicates wild type, the *lower line* the mutant

Disease	Gene	Mutation	Reference
FTDP-17<?2>	tau	T>G at pos. 15 of Exon 10 (N279 K)	Clark et al. (1998)
		ATTAA**T**AAGAAG	
		ATTAA**G**AAGAAG	
FTDP-17<?2>	tau	AAG del at 16 of Exon10 (280 K)	Rizzu et al. (1999)
		ATTAATAAGAAGCTG	
		ATTAAT–AAGCTG	
FTDP-17<?2>	tau	T>C at pos. 30 of Exon 10 (L284L)	D'Souza et al. (1999)
		CTGGATCT**T**AGCAAC	
		CTGGATCT**C**AGCAAC	
FTDP-17<?2>	tau	G>A at pos. 92 of Exon10 (S305 N) improves the splice site	Iijima et al. (1999)
		GGCA**G**TGTGA	
		GGCA**A**TGTGA	
Thrombasthenia of Glanzmann and Naegeli<?1>	Integrin GPIIIA	AC**G**GTGAGgt AC**A**GTGAGgt	Jin et al. (1996)

Menkes disease<?1>	MNK	GATCTTCTGGA GATCT–GGAT	Gu et al. (2001)
Metachromatic leukodystrophy<?1>	Arylsulfatase A	CAGAC**G**AGGTC CAGAC**A**AGGTC	Hasegawa et al. (1994)
Immunodeficiency<?1>	TNFRSF5, tumor-necrosis factor receptor superfamily, member 5 (CD40);	CTAC**A**GGG CTAC**T**GGG	Ferrari et al. (2001)
Cerebrotendinous xanthomatosis<?1>	CYP27A1	CCTATG**G**GCCGTT CCTATG**T**GCCGTT	Chen et al. (1998)
Marfan syndrome<?1>	Fibrillin-1	GGGATCAT**C**GTGGGA GGGATCAT**T**GTGGGA	Liu et al. (1997)
Acute intermittent porphyria<?1>	Porphobilinogen deaminase	GTGATTCG**C**GTGGGT GTGATTCG**G**GTGGGT	Llewellyn et al. (1996)
Hereditary tyrosinemia<?1>	Fumarylacetoacetate hydrolase	CTTATGAA**C**GACTGG CTTATGAA**T**GACTGG	Ploos van Amstel et al. (1996)
Leigh's encephalomyelo-pathy<?1>	Pyruvate dehydrogenase E1 alpha	GGGC**G**CTGG GGGC**A**CTGG	De Meirleir et al. (1994)
Immunodeficiency<?1>	Adenosine deaminase	GGGGAG**C**GAGACTTC GGGGAG**T**GAGACTTC	Santisteban et al. (1995)

(*Continued*)

Table 1. Examples of enhancer mutations involved in human diseases. The table lists examples of mutations in regulatory motifs that cause aberrant splicing. The list is updated at the alternative splicing database website (www.ebi.ac.uk/asd/). *Large letters* indicate exonic mutations, *small letters indicate* intronic mutations. The *top line* of each sequence indicates wild type, the *lower line* the mutant—(*Cont'd*)

Disease	Gene	Mutation	Reference
2-methylbutyryl-coa dehydrogenase deficiency/ short/branched-chain acyl-coa dehydrogenase (SBCAD)<?1>	Short/branched-chain acyl-CoA dehydrogenase	GAGTGG**A**TGGGGG GAGTGG**G**TGGGGG	Matern et al. (2003)
Homocystinuria<?1>	Methionine synthase	TCAGCC**T**GAGAGGA TCAGCC**C**GAGAGGA	Zavadakova et al. (2002); Zavadakova et al. (2005)
Bardet-Biedl Syndrome<?1>	MGC1203	GGCCTT**C**G GGCCTT**T**G	Badano et al. (2006)

snRNP assembly. Humans posses an almost identical gene, SMN2 that was generated through a recent duplication. Although both genes are almost identical in sequence, due to a translationally silent C>T change at position 6 in exon 7, they have different splicing patterns and exon 7 is predominantly excluded in SMN2. This exon-skipping event generates a truncated, less stable and probably nonfunctional protein. Therefore, SMN2 cannot compensate the loss of SMN1. The SMN protein functions in the assembly of snRNPs. The SMN protein is absent from all cells in SMA patients. However, this protein deficiency becomes only apparent in motor neurons that eventually die. The loss of the motor neurons causes SMA. The disease can manifest in four phenotypes (type I to IV) that differ in onset and severity. The phenotypes correlate roughly with the number of SMN2 copies in the genome, most likely because more SMN2 copies produce more SMN protein. Since stimulation of SMN2 exon 7 usage would increase SMN protein levels and potentially cure the disease, work has concentrated on understanding the regulation of exon 7. As for CFTR exon 9 and 12, multiple factors determine the regulation, including a suboptimal polypyrimidine tract (Singh et al. 2004c), a central tra2-beta1-dependent enhancer (Hofmann et al. 2000) and the sequence around the C>T change at position 6 that can either bind to SF2/ASF or hnRNPA1 (Cartegni and Krainer 2002; Kashima and Manley 2003). Recent large scale mutagenesis studies indicate that again a composite regulatory exonic element termed EXINCT (extended inhibitory context) is responsible for the regulation of exon 7 inclusion (Singh et al. 2004a; Singh et al. 2004b).

These two examples illustrate some of the general principles of diseases caused by misregulated splicing: mutations in splicing regulatory sequences can be hard to detect and translationally silent point mutations or intronic mutations can have drastic effects. The effect of the identical mutation on splice site selection can vary between cell types, which can cause specific, sometimes atypical, phenotypes. Identical mutations show also different penetrance when different individuals are analyzed, suggesting that alternative splicing could be a genetic modifier (Nissim-Rafinia and Kerem 2002).

3
Changes of Trans Factors Associated with Diseases

Knock-out experiments indicate that the complete loss of splicing factors NOVA-1, SRp20, SC35, and ASF/SF2 causes early embryonic lethality (Jensen et al. 2000; Jumaa et al. 1999; Wang et al. 2001; Xu et al. 2005). Up to now, knock-outs of splicing regulatory factors are largely absent in libraries of ES cells where one allele was silenced through gene

trapping. This indicates that the proper concentration of regulatory factors is necessary for cell survival. However, the loss of splicing factors in differentiated cells can be tolerated and leads to specific phenotypes (Xu et al. 2005).

Mutations in proteins implicated in splicing have been observed in retinitis pigmentosa, a progressive loss of photoreceptor cells during childhood, where PRP31 is mutated (Vithana et al. 2001) and forms of azospermia, where RBMY has been deleted (Venables et al. 2000).

Changes in the concentration or localization of splicing factors are frequently observed in tumorigenesis. For example, the concentration of SC35, ASF/SF2, and tra2-beta1 are altered in ovarian cancer (Fischer et al. 2004). An array-based study of changes in Hodgkin's lymphoma revealed 2–5 fold changes in seven general splicing factors as well as the ectopic expression of the neuron-specific splicing factor NOVA-1 and NOVA-2 (Relogio et al. 2005). In addition, numerous splicing events were altered, but it is not possible to explain how these changes are related to alterations of trans-acting factors.

4
Human Diseases Associated with Aberrant Splice Site Selection Without Obvious Mutations

A number of diseases have been described that are associated with a change in alternative splicing patterns in the absence of mutations or alterations in trans-acting factors. For example, in schizophrenia, the alternative splicing patterns of the gamma2 subunit of gamma amino butyrate type A receptor (Huntsman et al. 1998), the N-methyl-D-aspartate (NMDA) R1 receptor, and the neuronal cell adhesion molecule (Vawter et al. 2000) were altered. Recent results show that the alternative splicing of tau exon 10 is significantly altered in sporadic Alzheimer's disease (Umeda et al. 2004; Glatz et al. 2006). Changes of alternative splicing patterns have been frequently reported to be associated with cancer development, e.g., Wilms' tumor, breast cancer, melanoma, and prostate cancer (Table 2). Furthermore, EST analysis demonstrates widespread changes of alternative splicing patterns in cancer cells (Xu and Lee 2003) when compared with normal cells. However, these changes have to be interpreted with caution, since they are not always reproducible by RT-PCR analysis (Gupta et al. 2004). Strikingly, in the majority of cancer tissues, mutations in the genes giving rise to altered mRNA isoforms have not been observed. It is therefore likely that these changes are caused by altered concentration of regulatory factors, or through changes in their subcellular localization or phosphorylation state (Rafalska et al. 2004; Fig. 1B).

Table 2. Human diseases associated with aberrant splice-site selection without obvious mutations

Gene	Disease	Reference
Estrogen receptor	Breast-cancer	Pfeffer et al. (1993)
Gris1: Graffi Integration Site 1	leukemia	Denicourt et al. (2003)
BAFF	cancer	Gavin et al. (2003)
MDM 2	cancer	Steinman et al. (2004); Lukas et al. (2001)
ADAR	inflammation	Yang et al. (2003)
HOX2.2	cancer	Shen et al. (1991)
WT1	cancer	Baudry et al. (2000)
Bin1	cancer	Ge et al. (2000)
FGFR-2	cancer	Kwabi-Addo et al. (2001)
EAAT2	Sporadic amyotrophic lateral sclerosis	Lin et al. (1998)
NOS	Sporadic amyotrophic lateral sclerosis	Catania et al. (2001)
Ich-1	ischemia	Daoud et al. (2002)

5
Treatment of Diseases Caused by Missplicing

5.1
Gene Transfer Methods

Type I and II mutations either destroy splice sites or activate cryptic splice sites. Antisense nucleic acids can suppress point mutations and promote the formation of the normal gene products. Special chemistries were devised to prevent RNAseH-mediated cleavage of the RNA and to lower toxicity (Sazani and Kole 2003). Oligonucleotides have been used to target cryptic splice sites that are activated in beta thalassemias (Lacerra et al. 2000), to suppress exon usage in Duchenne muscular dystrophy (Mann et al. 2001) and to block HIV replication (Liu et al. 2004).

The antisense approach was further developed in ESSENCE (exon-specific splicing enhancement by small chimeric effectors). ESSENCE uses bifunctional reagents that contain a peptide effector domain and an anti-sense-targeting domain. The effector domains of these protein–nucleic acids

were arginine–serine (RS) repeats that mimic the effect of SR proteins (Cartegni and Krainer 2003).

Related to ESSENCE is the use of bifunctional oligonucleotides in TOES (targeted oligonucleotide enhancer of splicing), where a part of the oligonucleotide binds to an SR protein, which promotes exon inclusion (Skordis et al. 2003). Several RNA based approaches have been tested in cell culture. They include the use of RNAi to suppress unwanted isoforms (Celotto and Graveley 2002), spliceosome-mediated RNA trans-splicing (SmaRT) to correct factor VIII deficiency in a mouse model (Chao et al. 2003) and ribozymes that use trans-splicing to replace defective p53, beta-globin mRNA and a chloride channel in cell culture (Lan et al. 1998; Watanabe and Sullenger 2000; Rogers et al. 2002). Finally, antisense oligonucleotides have been used to modify U7 snRNA, which results in the nuclear accumulation of the oligonucleotide sequences in stable U7snRNP complexes (Asparuhova et al. 2004) that interact with the mutant target gene.

5.2 Low Molecular Weight Drugs

It is well known that small molecules can interact with RNA, and this principle is used by several RNA-binding antibiotics, such as gentamicin, chloramphenicol, and tetracycline (Xavier et al. 2000). Therefore, several chemical screens were performed to identify small-molecular-weight molecules that interfere with splice site selection. It was found that (–)-epigallocatechin gallate (EGCG), a polyphenol and component of green tea (Anderson et al. 2003), as well as kinetin and the related benzyladenine, a plant hormone (Slaugenhaupt et al. 2004), promotes correct splice-site usage in the IKAP gene, involved in familial dysautonomia. Histone deacetylase inhibitors, such as sodium butyrate and valproic acid, have been used to increase the correct level of SMN2 splicing (Chang et al. 2001; Brichta et al. 2003). SMN2 splicing was also influenced by the phosphatase inhibitor sodium vanadate (Zhang et al. 2001), the cytotoxic anthracycline antibiotic aclarubicin (Andreassi et al. 2001) and the nonsteroidal anti-inflammatory drug indoprofen (Lunn et al. 2004). A major disadvantage of most of the inhibitors is their low specificity. However, surprisingly, indole derivatives were found to act on specific SR proteins that regulate specific ESE sequences (Soret et al. 2005). Since these substances block HIV replication by interfering with early viral splicing events, they open the intriguing possibility of a specific pharmacological treatment for splicing disorders.

5.3 Diagnostics

Up to now, the majority of studies analyzing splice site selection were done by RT-PCR (Stamm et al. 2000). Recently, microarray formats have successfully

been used to detect changes in splice site selection associated with diseases (Fehlbaum et al. 2005; Relogio et al. 2005). These microarrays use several oligonucleotides located within the exon and on the exon–exon junctions to infer the presence and connections of alternative exons. The arrays detect the usage of a single exon, and it is currently not possible to infer the composition of complete mRNAs using microarrays. One important finding of microarray analysis is that diseases can be associated with a large number of small changes in alternative splice site selection, rather than with a few large changes. It will therefore be necessary to analyze data obtained with exon-specific microarrays with different software tools that use gene ontologies to detect coordinated small changes in groups of exons (Ben-Shaul et al. 2005).

6 Conclusions

Misregulated alternative splicing emerges as a new cause for human diseases. Recent progress shows that misregulation of alternative splicing can be reversed. Most of the treatment paradigms are in the experimental stage. However, the growing list of drugs interfering with splice-site selection promises that some treatment options will be moved to the clinic soon.

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