Misregulation of pre-mRNA splicing that causes human diseases. Concepts and therapeutic strategies

Review Article

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Summary

About one third of all human genes are subject to alternative splicing. The molecular mechanisms that regulate alternative splice site usage are beginning to emerge and show that transcription and pre-mRNA processing are integrated processes that can be modified by cellular signals. Several diseases are caused by mutations in sequences that regulate pre-mRNA processing. Their molecular characterization indicates that contributions of pre-mRNA splicing defects to human diseases have been underestimated and could account for pleiotropic phenotypes. The understanding of the molecular mechanisms allows the development of therapeutic strategies.

Abbreviations: **ACTH**: adrenocorticotrophic hormone; **CFTR**: cystic fibrosis transmembrane conductance regulator; **CKI**: casein kinase I; **Clk**: Cdc2-like kinase; **ConA**: Concanavalin A; **EGF**: epidermal growth factor; **ESE**: exonic splicing enhancer; **ESS**: exonic splicing silencer; **FGF**: fibroblast growth factor; **hnRNP**: heterogeneous ribonucleoprotein; **IFN**: interferon; **IL**: interleukin; **ISE**: intronic splicing enhancer; **ISS**: intronic splicing silencer; **MKK**: mitogen-activated protein kinase kinase; **NGF**: nerve growth factor; **PDGF**: platelet-derived growth factor; **PI3-K**: Phosphatidylinositide-3-kinase; **PKC**: protein kinase C; **PKG-I**: protein kinase G-I; **polII**: polymerase II; **PTP-1B**: protein tyrosine phosphatase 1B; **SAM68**: src associated in mitosis; **SELEX**: systematic evolution of ligands by exponential enrichment; **snRNP**: small nuclear riboprotein; **SR**: protein: protein with serine-arginine-rich domain; **SRPK**: SR protein kinase; **STAR**: signal transduction and activation of RNA; **TCR**: T cell receptor; **TNF**: tumor necrosis factor; **U2AF**: U2 auxiliary factor; **UTR**: untranslated region.

I. Introduction

A. Splicing and basal splicing machinery

In eukaryotes, the primary transcript generated by polymerase II undergoes an extensive maturation process that involves capping, polyadenylation, editing, and pre-mRNA splicing. Pre-mRNA splicing removes intervening sequences (introns) and joins the remaining sequences (exons) to form mature mRNA that is finally exported into the cytosol. With a few exceptions, all human polII transcripts are spliced, and it is estimated that about 30% of all pre-mRNA transcripts are subject to alternative splicing (Brett et al., 2000; Mironov et al., 1999). Alternative splicing is a mechanism where parts of the pre-mRNA are either excluded from, or included in the mature mRNA. This process can be regulated in a cell-type or developmental-specific way (Stamm et al., 1994, 2000), that can be used to regulate gene expression at the level of pre-mRNA processing. Furthermore, it allows different protein isoforms to be created from a single gene. In many cases, stop codons are introduced by alternative splicing (Stamm et al., 1994, 2000), which usually changes the carboxy terminus of proteins. This can affect the

physiological function of a protein, as shown by several examples: **(i)** creation of soluble instead of membrane-bound receptors (Baumbach et al., 1989; Eipper et al., 1992; Hughes and Crispe, 1995; Tabiti et al., 1996; Toksoz et al., 1992; Zhang et al., 1994); **(ii)** altered ligand affinity (Sugimoto et al., 1993; Suzuki et al., 1995; Xing et al., 1994); **(iii)** protein truncations producing inactive variants (Duncan et al., 1995; Eissa et al., 1996; Sharma et al., 1995; Swaroop et al., 1992; van der Logt et al., 1992); and **(iv)** changes of endocytotic pathways (Wang and Ross, 1995). In addition, inclusion or skipping of alternative exons can **(v)** add or delete protein modules that change the affinity towards ligands (Danoff et al., 1991; Giros et al., 1991; Guiramand et al., 1995; Strohmaier et al., 1996); **(vi)** modulate enzymatic activity (O'Malley et al., 1995); **(vii)** create different hormones (Amara et al., 1982; Courty et al., 1995); and **(viii)** change properties of ion channels (Kuhse et al., 1991; Sommer et al., 1990; Xie and McCobb, 1998). Finally, **(ix)** numerous transcription factors are subject to alternative splicing, which contributes to control of gene expression (reviewed in Lopez, 1995). A recent compilation and statistical analysis of alternative exons (Stamm et al., 2000) is available on the world wide web under www.stamms-lab.net.

Proper splicing regulation is important for an organism, since it has been estimated that up to 15% of genetic defects caused by point mutations in humans manifest themselves as pre-mRNA splicing defects caused by changing splice site sequences (Krawczak et al., 1992; Nakai and Sakamoto, 1994). These mutations can be viewed as new sources of variation in human evolution that was probably accelerated by alternative splicing mechanisms, allowing the combination of different RNA processing events to generate appropriate mRNAs as a result of changing cellular needs (Herbert and Rich, 1999).

Significant progress has been made in understanding the mechanism of constitutive splicing. Three major cis-elements on the RNA define an exon, the 5' and 3' splice sites and the branch point (Berget, 1995). These elements are recognized by the spliceosome, a 60S complex containing small nuclear RNAs (U1, U2, U4, U5, U6) and over 50 different proteins (Neubauer et al., 1998). In the spliceosome, U1 snRNP, U2AF, SF1, U6 snRNP, and U2 snRNP are the trans-acting factors that ultimately recognize the 5', 3' splice sites and the branch point (reviewed in Elliot, 2000; Green, 1991; Krämer, 1996; Moore, 2000). Sequence compilations of the 5', 3' splice sites and the branch point revealed that they follow only a loose consensus sequence (Breitbart et al., 1987). Only the GT and AG nucleotides directly flanking the exon, together with the branch point adenosine (**Figure 1A**), are always conserved, whereas in all other positions nucleotides can deviate from the consensus. However, some positions in splice sites adhere better to a consensus than others (Breitbart et al., 1987; Stamm et al., 1994; Stamm et al., 2000). Interestingly, a new class of exons has been discovered that uses AT and AC instead of the GT/AG flanking nucleotides (Tarn and Steitz, 1997). Due to the degenerate nature of splice sites, it is difficult to predict exons in genomic sequences, and current computer programs cannot accurately predict exons from genomic DNA (Thanaraj, 2000). This contrasts the high accuracy and fidelity characteristic for splice-site selection *in vivo*.

One reason for the specificity observed *in vivo* are additional regulatory elements known as exonic or intronic enhancers or silencers (**Figure 1, Table 1**). These elements are again characterized by loose consensus sequences. The enhancers can be subdivided into purine-rich (GAR-type), pyrimidine-rich and AC-rich (ACE) enhancers (Cooper and Mattox, 1997). Enhancers bind to proteins that are able to recruit components of the basal splicing machinery, which results in recognition of splice-sites located near an enhancer (Hertel et al., 1997). The degeneracy of splicing enhancers is most likely necessary to allow for the amino acid usage needed. The importance of splice-site enhancers becomes apparent when they are changed by mutation, which can alter their interaction with trans-acting factors.

Interestingly, some of these mutations are silent, e.g. they do not change the amino acid usage, but generate an aberrant gene product by causing an abnormal splicing product. It remains to be determined whether all missense mutations cause a pathological state by an amino acid exchange or are actually unrecognized splicing mutations. Such an analysis could be made by analyzing the pre-mRNA processing of the mutation by RT-PCR or RNAse protection. An overview of diseases caused by splicing enhancers/silencers is shown in **Table 2**.

Proteins binding to enhancer or silencer sequences can be subdivided into two major groups: members of the SR family of proteins (Manley and Tacke, 1996) and hnRNPs (Weighardt et al., 1996). Binding of individual proteins to enhancer sequences is intrinsically weak and not highly specific. However, multiple proteins bind to all known exon enhancers forming a complicated RNA:protein complex. This binding involves protein:protein as well as protein:RNA interactions, and results in the specific recognition of an exon (Figure 1). As a result, proper splice-site recognition is governed by the ratio of various proteins involved, as well as the enhancer and silencer sequences.
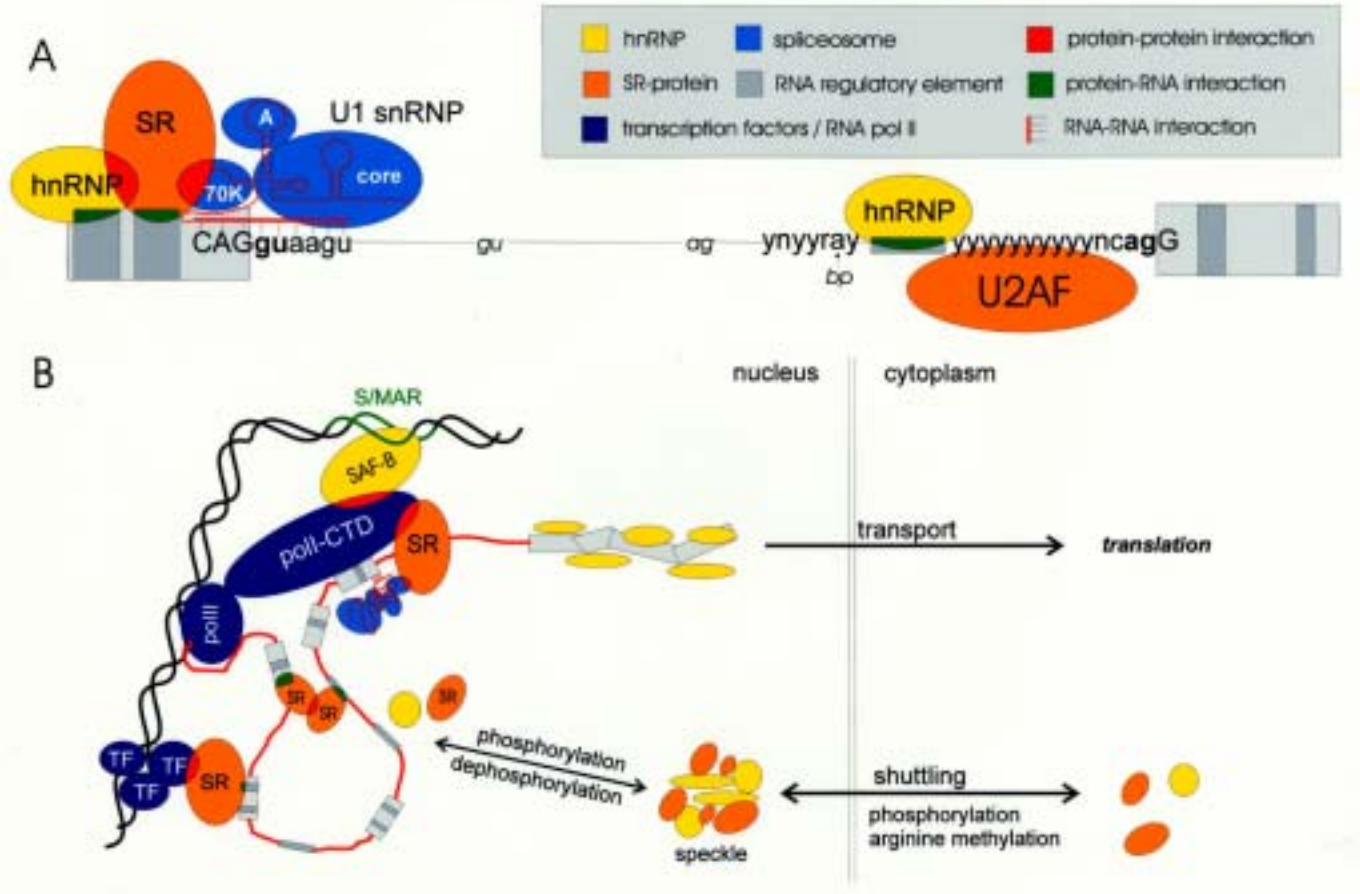
Figure 1: cis and trans factors involved in pre-mRNA splicing.

A) **Elements involved in alternative splicing of pre-mRNA**. Exons are indicated as boxes, introns as thin lines. Splicing regulator elements (enhancers or silencers) are shown as gray boxes in exons or as thin boxes in introns. The 5' splice-site (CAGguaagu) and 3' splice-site $(y)_{10}$ncagG, as well as the branch point (ynyyray), are indicated (y=c or u, n=a, g, c or u). Upper-case letters refer to nucleotides that remain in the mature mRNA. Two major groups of proteins, hnRNPs (yellow) and SR or SR related proteins (orange), bind to splicing regulator elements; the protein:RNA interaction is shown in green. This protein complex assembling around an exon enhancer stabilizes binding of the U1 snRNP close to the 5' splice-site, due to protein:protein interaction between an SR protein and the RS domain of U170K (shown in red). This allows hybridization (thick red line with stripes) of the U1 snRNA (red) with the 5' splice-site. The formation of the multi-protein:RNA complex allows discrimination between proper splice-site (bold letters) and cryptic splice-sites (small *gu ag*) that are frequent in pre-mRNA sequences. Factors at the 3' splice-site include U2AF which recognizes pyrimidine rich regions of the 3' splice-sites, and is antagonized by binding of several hnRNPs (e.g hnRNP I) to elements of the 3' splice-site. orange: SR and SR related proteins; yellow: hnRNPs; green: protein:RNA interaction; red: protein:protein interaction; thick red line with stripes: RNA:RNA interaction

B) The RNA factory. RNA is generated after genes are recognized by transcription factors (TF) by RNA polymerase II (polII) (dark blue). Exons present on the RNA are recognized by SR proteins and hnRNPs that interact with exonic or intronic sequence elements. SR proteins interact with factors assembled around the promoter and can form protein networks across exons. SR proteins directly interact with the carboxy terminal domain of RNA polII (polII-CTD), which assembles proteins near active sites of transcription. Among polII interacting proteins is scaffold attachment factor B (SAF-B) that can couple SR proteins and RNA polII to chromatin organizing elements (S/MAR, thick green line). The processed RNA is coated with hnRNPs and transported into the cytoplasm, where it is translated into protein. SR proteins and hnRNPs are recruited from storage sites (speckles) through phosphorylation. Some SR proteins and hnRNPs shuttle between nucleus and cytoplasm. Protein shuttling can be regulated by phosphorylation or arginine methylation.

Gene/Exon	Element			Reference
	Type	Sequence	Meth.	
Human CFTR Exon 9	ESE	GAUGAC	1	(Pagani et al., 2000)
Human CFTR Exon 9	ESS	UUAUUUCAAGA **SF2/ASF, SRp55, SRp75**	1, 2	(Pagani et al., 2000)
Human Fibronectin EDA exon	ESS	CAAGG	1, 2	(Staffa et al., 1997)
Human fibronectin EDA exon	ESE	GAAGAAGA	2	(Caputi et al., 1994)
Human fibronectin EDA exon	ESE	AGGGUGACC	1, 2	(Staffa et al., 1997)
rat fibronectin intron downstream of exon EIIIB	ISE	UGCAUG repeat		(Huh and Hynes, 1994)
mouse IgM exon M2	ESE	GGAAGGACAGCAGAGACCAAGAG	3	(Tanaka et al., 1994)
cardiac troponin T exon 17	ISE	GUAAGUCAGGCUGCAUGCCUCCCACCACA CCUGUGCUGCAUGACACCUGGGGGCUGACC UGCAACAGAAGUGGGGCUGAGGGAAGGA CUGUCCUGGGGACUGGUGUCAGAGCGGG GUUGGUGACUCUCAGGAUGCCCAAAAUGC CCA	2	(Carlo et al., 1996)
Chicken cTNT	ESE	AAGAGGAAGAAUGGCUUGAGGAAGACGA CG **SRp55**	2, 3, 4, 5	(Nagel et al., 1998)
Chicken cTNT Intron 4	ISE	GCGCUUCUUCCCUUCCCUCCUCCCUGGCUC AG	5, 2	(Cooper, 1998)
Chicken cTNT Intron5	ISE	CUCCACCUUUCUU	5, 2	(Cooper, 1998)
Chicken cTNT Intron5	ISE	UGUGUCCUGUGCCUUUCCCUGCU	5, 2	(Cooper, 1998)
Chicken cTNT Intron5	ISE	AUUCUUUCACUUCUCUGCU	5, 2	(Cooper, 1998)
Human GH-1 Intron 3	ISE	GGXXXXGGG	5	(McCarthy and Phillips, 1998).
Human Dystrophin Exon27		UAAAGAAGAGGcccaacaaAAAGAAGCGAAA G	5, 3	(Shiga et al., 1997)
Human Calcitonin (CT) Exon 4	ESE	GGAAAGAAAAGGGA	5, 2	(Zandberg et al., 1995)
Human Calcitonin (CT) Exon 4	ESE	ACUUCAACAAGUU	5, 2	(Zandberg et al., 1995)
Drosophila dsx exon 4	ESE	CUUCAAUCAACAU or CAACAAUCAACAU **SR proteins, Tra, Tra2**	3	(Tian and Maniatis, 1993)
Drosophila dsx exon 4	ESE	UCWWCRAUCAACA **Tra/Tra2**	3	(Bruzik and Maniatis, 1995).
Drosophila dsx exon 4	ESE	AAAGGACAAAGGACAAAA **tra/Tra2**	6	(Lynch and Maniatis, 1995)
mouse c-src intron B downstream of exon N1	ISE	AGGCUGGGGGCUGCUCUCUGCAUGUGCUU CCU **KSRP, p53/hnRNP F**	4, 6, 7, 3	(Min et al., 1997);
mouse c-src intron B downstream of exon N1	ESE	TGCATG	2, 5 , 1	(Modafferi and Black, 1997).
human a-tropomyosin gene exon 5/SK	ESS	UAAGUGUUCUGAGCUGGAGGAGGAGCUG AAGAAUGUCACCAACAACCUCAAGUCUCU UGAGGCUCAGGCGGAGAAG	2, 12	(Graham et al., 1992)

Gene/exon	Type	Sequence	Meth.	Reference
HIV-1 tat-rev exon 3	ESS	AGAUCCAUUCGAUUAGUGAA	3, 11	(Amendt et al., 1995).
HIV-1 exon 6D	ESE	GAGAAAGGAGAGA	5, 2	(Wentz et al., 1997)
HIV-1 tat exon 3	ESS	AGAUCC and UUAG	3 , 8	(Si et al., 1998)
HIV-1 tat-rev exon 3	ESS	GAAGAAGAAGGUGGAGAGAGAGAGACAG AG	3, 11	(Amendt et al., 1995).
mouse CGRP intron 3	ISE	CAGGUAAGAC **PTB, U1 snRNA**	8 , 7	(Lou et al., 1996)
rat GABA(A) receptor gamma2 intron	ISE	UUCUCU (within pyrimidine context) **PTB**	7, 8	(Ashiya and Grabowski, 1997)
rat beta-TM exon 5, 6	ESE	GARGARGAR	3	(Tsukahara et al., 1994)
human caldesmon exon 5	ESE	(GAGGAAGAGAAAAGGGCAGCAGAGGAG AGGCA)x4	2	(Elrick et al., 1998; Humphrey et al., 1995)
chicken β tropomyosin intron IVS AB	ISS	CCCUCUCUCUAUCGCUGUCUCUUGAGCCA CGCC	5, 2	(Libri et al., 1990; Libri et al., 1991)
Chicken β-Tropomyosin intron 6	ISE	(A/U)GGG repeat	3, 7	(Sirand-Pugnet et al., 1995)
B3, artificial	ESE	UCGACAUUGGGAGCAGUCGGCUCGUGC **SRp40**	9	(Tacke et al., 1997)
human FGFR-2 K-SAM(IIIb)	ESS	UAGGGCAGGC	2	(Del Gatto and Breathnach, 1995)
Rat Clathrin Light Chain B exon EN	ESE	ACAAAGCGUUCUACCAGCAGCCAGAUGCU G	4	(Stamm et al., 1999)
MHC-B exon N30 upstream intron	ISE	UGCAUGUCGUACUGCAUGU	1, 2	(Kawamoto, 1996)
human insulin receptor exon B upstream intron	ISE	GUAGUGGGACCCAGAGACGGCAGAAGGG UGGGUGGAGUCUGAAUGGAG	1, 2	(Kosaki et al., 1998)
human insulin receptor exon B upstream intron	ISS	UUACUCGGACACAUGUGGCCUCCAAGUGU CAGAGCCCAGUGGU	1, 2	(Kosaki et al., 1998)
human CD44 exon v5	ESS	AGACAGAAUCAGCACCAGUGCUCAUGGAG AAAAUUGGACCCCGGAACCACAGCCUCCU UUCAAUAACCAU	1, 2	(Meijer et al., 1997)
human CD44 exon v5	ESE	GAGUAUCAGGAUGAAGAGGAGACCCCAC AUGCUACAAGCA	1, 2	(Konig et al., 1998)
CT/CGRP intron downstream from exon 4	ISE	CUCCGCUCCUCUUC **PTB**	5, 2, 4, 7, 10, 12	(Lou et al., 1999)
CT/CGRP intron downstream from exon 4	ESE	UUAUUUUUCCC **PTB**	5, 7, 4, 8	(Lou et al., 1999)
BPV-1	ESS	CUGUCUCUUCUUUGCUCGGCUCCCCCGCCU GCGGUCCCAUCAGAGCAG **U2AF(65), PTB, and SR Proteins**	3, 11, 7, 10	(Zheng et al., 1999)
BPV-1	ESE	GAAGGACCUGAAGGACCCUGCAGGAAAA GCCGAGCCAGCCCAGCC	1, 3	(Zheng et al., 1999)
Rous Sarcoma Virus gag gene	ESS	CGAAUCGACAAAGGGGAGGAAGUGGGAG AAA **HnRNP H**	7,10, 4, 6	(Fogel and McNally, 2000)
Rous Sarcoma Virus gag gene	ESS	AUCGAGAAACCAGCAACGGAGCGGCGAAU CGAC **SF2/ASF**	3, 2, 5, 7, 4,	(McNally and McNally, 1996; McNally and McNally, 1998)

Table 1. Compilation of RNA elements that influence splice-site selection (previous two pages)

The first column shows the gene and exon that contains the RNA element, which is characterized in the next columns according to its type (ESE: exonic sequence element; ISE: intronic sequence element) and sequence. Trans-acting factors are indicated in bold under the sequence if they were identified experimentally. Most RNA elements will work in combination with additional RNA regulatory sequences that are not shown. Meth. indicates the experimental method used: 1: deletion analysis; 2: *in vivo* splicing assay; 3: *in vitro* splicing assay; 4: gel mobility shifts; 5: mutagenisis; 6: *in vitro* binding; 7: UV-crosslink; 8: competition experiments; 9: SELEX; 10: immunoprecipitation; 11: spliceosomal complex formation; 12: nuclease protection. R= G or A; W=A or U.

B. Splice-site recognition is influenced by the relative concentration of proteins that form a complex to recognize exon-intron borders

In contrast to constitutive splicing, the mechanisms regulating alternative exon usage are less well understood. It is clear that the relative concentration of splicing-associated proteins is responsible for alternative splice-site selection (Black, 1995; Grabowski, 1998; Varani and Nagai, 1998) . It has been shown experimentally, that the relative concentration of SR proteins and hnRNPs can dictate splice-site selection, both *in vivo* and *in vitro* (Cáceres et al., 1994; Mayeda and Krainer, 1992; Screaton et al., 1995; Wang and Manley, 1995). The expression levels of various SR proteins (Ayane et al., 1991; Mayeda and Krainer, 1992; Screaton et al., 1995; Zahler et al., 1993) and hnRNPs (Kamma et al., 1995) vary amongst tissues and could therefore account for differences in splice-site selection. Several examples of antagonistic splicing factors have been described (Cáceres et al., 1994; Gallego et al., 1997; Jumaa and Nielsen, 1997; Mayeda et al., 1993; Polydorides et al., 2000). Here, one factor promotes inclusion of an exon and the other factor promotes its skipping. In most of these cases, it remains to be determined whether this antagonistic effect is achieved by (**i**) an actual competition of the factors for an RNA binding site, (**ii**) through sequestration of the factors by protein:protein interaction and, (**iii**) by changes in the composition of protein complexes recognizing the splicing enhancer. In addition, cell-type specific splicing factors have been detected. In *Drosophila,* for example, the expression of the SR protein transformer is female-specific (Boggs et al., 1987) and determines the sex by directing alternative splicing decisions. Other tissue-specific factors include the male germline specific transformer-2 variant in *D. melanogaster* (Mattox et al., 1990) and *D. virilis* (Chandler et al., 1997), an isoform of its mammalian homologue htra2-beta3 that is expressed only in some tissues (Nayler et al., 1998a), the muscle specific protein Nop30 (Stoss et al., 1999a) , the neuron-specific factor NOVA-1 (Jensen et al., 2000) as well as testis and brain enriched factor rSLM-2 (Stoss et al., 2001) and NSSR (Komatsu et al., 1999). For most of these factors, the tissue-specific target genes remain to be determined. However, a combination of knockout experiments and biochemical analysis allowed the identification of doublesex, fruitless, and transformer-2 as a target of the transformer-2/transformer complex in *Drosophila* (Heinrichs et al., 1998; Hoshijima et al., 1991; Mattox and Baker, 1991) and glycine receptor alpha2 and GABA(A) pre-mRNA as a target for NOVA-1 (Jensen et al., 2000). Although this analysis is currently limited, it is likely that a given splicing factor will influence several pre-mRNAs.

C. Coupling of splicing and transcription

Transcription and splicing can be separated by biochemical means. Especially when RNA splicing is studied i*n vitro*, the two processes are uncoupled, since the RNA is made synthetically. However, when transcription and splicing factors were analyzed by microscopy methods (Misteli, 2000) and in yeast two-hybrid screens, an intimate association became apparent. A number of studies used the carboxyl terminal domain of the RNA polII and found several interacting proteins, some of which, e.g. snRNPs and SR like proteins, were most likely involved in pre-mRNA processing (Bourquin et al., 1997; Corden and Patturajan, 1997; Du and Warren, 1996; Kim et al., 1996; Yuryev et al., 1996). Furthermore, these complexes are most likely associated with S/MAR elements (Bode et al., 2000) via SAF-B, an hnRNP-like protein that can link factors involved in pre-mRNA processing, and the CTD to chromatin-organizing elements (Nayler et al., 1998c). Finally, it was shown directly that RNA polymerase II can stimulate the splicing reaction *in vivo* (Hirose et al., 1999) and targets splicing factors to sites of active transcription (Misteli and Spector, 1999). These close interactions most likely influence splice-site usage and it has been shown that the particular promoter usage influences splice-site selection (Cramer et al., 1999). Together, these data indicate that pre-mRNA is generated and processed by a large complex that was termed 'RNA factory' (McCracken et al., 1997). It is most likely that 5' capping (Cho et al., 1997), polyadenylation (McCracken et al., 1997), and editing (Higuchi et al., 2000) activities are also part of this complex.

When cells are stained with antibodies against splicing factors, the proteins are concentrated in 20-40 large nuclear structures that are called speckles. Using imaging techniques, it has been shown that splicing and transcription take place concomitantly in the vicinity of speckles (Huang and Spector, 1996; Jiménez-Garcia and Spector, 1993). Within speckles, no transcriptional activity could be detected (Fay et al., 1997), indicating that these structures serve as storage particles. Speckles are dynamic (Misteli et al., 1997) structures that can release splicing components when these are phosphorylated (Nayler et al., 1998b). A new subnuclear structure, the YT bodies, was discovered that forms around speckles and often partially overlaps with them (Nayler et al., 2000). YT bodies contain YT521-B, a protein that binds to factors implicated in pre-mRNA processing and is subject to tyrosine phosphorylation through src kinases (Hartmann et al., 1999). YT bodies change in response to the tyrosine phosphorylation status of the cell (Nayler et al., 2000 and our unpublished results) and harbor sites of transcription (Nayler et al., 2000), suggesting that the RNA factory can be modulated by tyrosine phosphorylation in YT bodies.

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Disorder/Gene	Mutation	Effect	Reference
Spinal Muscle Atrophy (SMA) SMN2	Silent C>T coversion in exon 7	disrupts ESE	(Coovert et al., 1997; Jablonka et al., 2000; Lefebvre et al., 1995; Lefebvre et al., 1997; Lorson et al., 1999; Monani et al., 2000; Vitali et al., 1999)
Sandhof disease (β-hexsamindase β-subunit	exon 11 P417L C>T conversion at nucleotide 8 intron 10 A>G conversion at position −17.	disruts an ESE. Causes use of a cryptic splice site at nucleotide +112 generates cryptic splice site at position −37, dirupts an ESI and putative branch point	(Fujimaru et al., 1998)
Frontotemporal Dementia with Parcinsonism tau	Intron 10: +13 A>G, +14C>T, +16C>T, IVS10+3 G>A Exon10: L284L T>C, S305S T>C, S305N G>A Exon10: N297K T>G, del280K (AAG deletion)	disrupt ISI IVS10+3 G>A improves splice site consensus disrupt ESI S305N G>A improves splice site consensus dirupts ESE	(D'Souza et al., 1999; Hutton et al., 1998; Iijima et al., 1999; Rizzu et al., 1999; Spillantini et al., 1998; Stanford et al., 2000)
Acute intermittent porphyria porphobilinogen deaminase	R28R, C>G	skipping of exon 3	(Llewellyn et al., 1996)
Glanzmann thrombasthenia integrin GPIIIa	C>A at position +16 and silent G>A at +134 of exon 9	skipping of exon 9	(Jin et al., 1996)
Hereditary tyrosinemia type 1 fumarylaceto acetate hydrolase	N232N C>T	skipping of exon 8	(Ploos van Amstel et al., 1996)
Leigh's encephalomyelopathy pyruvate dehydr-ogenase(E1α)	silent A>G	aberrant splicing of exon 6	(De Meirleir et al., 1994)
Menkes disease MNK	Gly>Arg G>A	skipping of exon 8	(Das et al., 1994)
severe combined immunodeficiencies adenosine deaminase	R142X G>A and C>T in the same codon	skipping of exon 5	(Santisteban et al., 1995)
metachromatic leukodystrophy arylsulfatase A	Thr409Ile C>T	activates a cryptic splice site	(Hasegawa et al., 1994)
Marfan syndrome fibrilin-1	silent C>T in exon 51	skipping of exon 51	(Liu et al., 1997)
cerebrotendinous xanthomatosis CYP 27	silent G112G G>T in exon 2	creates a cryptic splice site and causes skipping of exon 2	(Chen et al., 1998)
lethal when mutation is homozygous CD45	silent C>G at position 77 of the alternative exon 4	constitutive skipping of exon 4	(Zilch et al., 1998)
β-thalasemia β-globin	T->G tansversion in posstition 705 of intron 2	activates cryptic 3' splice site in intron 2	(Dobkin et al., 1983)
Myotonic dystrophy DMPK	Expanded (CUG)>40 in the 3' UTR of	either sequestration or change in alternative exon usage	(Philips et al., 1998; Tiscornia and Mahadevan, 2000)

Table 2: Mutation in RNA regulatory elements that cause disease
Mutations that disrupt RNA regulatory elements and cause a disease are listed. The name of the gene is under the disease and is underlined. Point mutations that change splice-sites (Krawczak et al., 1992; Nakai and Sakamoto, 1994) are not added to the table, if they are not part of exonic elements. ESE: exonic sequence element; ISE: intronic sequence element, IVS: intron.

Finally, proteins implicated in splicing shuttle between the nucleus and the cytosol. After a stress-induced change of the cellular phosphorylation status they accumulate in the cytosol (van Oordt et al., 2000), which affects pre-mRNA processing patterns in the nucleus, because the nuclear concentration of the splicing factors is changed.

Together, these data suggest that the concentration of factors involved in splice-site selection, which dictates exon usage, can be controlled by several ways: (i) a specific amount of the factor expressed in a tissue, (ii) release from storage sites by phosphorylation, (iii) export from the nucleus, (iv) sequestration by protein-binding partners that, e.g., assemble at an active promoter, and (v) local concentration at different sites of the nucleus. Together, these mechanisms allow the cell to process a given pre-mRNA with a specific set of splicing regulatory proteins, such as SR proteins and hnRNPs

II. Change of splice-site selection in response to an external stimulus
A. Overview

Alternative splicing pathways are not static, since the use of alternative exons can change during development (for a summary see: Stamm et al., 1994, 2000), or in response to outside stimuli. For example, insulin administration influences the incorporation of the alternative exon 11 of the insulin receptor (Sell et al., 1994) and activates exon ßII inclusion in the PKC gene (Chalfant et al., 1998); serum deprivation alters usage of the serine/arginine-rich protein 20 (SRp20) exon 4 (Jumaa and Nielsen, 1997); and neuronal activity changes the alternative splicing pattern of clathrin light chain B, the NMDAR1 receptor, and c-fos (Daoud et al., 1999). In the brain, stress changes splicing patterns of potassium channels (Xie and McCobb, 1998) and of acetylcholin esterase (Kaufer et al., 1998). ConA has been shown to change splicing patterns of the class 1b major histocompatibility complex molecule Qa-2 (Tabaczewski et al., 1994) and the splicing patterns of tumor necrosis factor ß are regulated by src kinases (Gondran and Dautry, 1999; Neel et al., 1995). Finally, programmed cell death is concomitant with a change in the alternative splicing patterns of several cell death regulatory proteins (reviewed in Jiang and Wu, 1999).

As can be seen in **Table 3**, numerous extracellular stimuli, such as growth factors, cytokines, calcium concentration, and extracellular pH can change alternative exon usage. Since these alternative exons are in mRNAs of diverse biological functions, it is likely that the change of alternative splicing in response to an extracellular signal is a general regulatory mechanism in higher eukaryotes. Although for some cases the signal transduction pathways have been established, the molecular mechanism that transduces the signal to the spliceosome remains largely obscure.

For several systems, it was demonstrated that changes in alternative splicing do not require *de novo* protein biosynthesis. Examples include the splicing of exon v5 of the CD44 gene in response to TPA (König et al., 1998) or the differential splicing of the Ca-ATPase transcript upon a rise in intracellular calcium (Zacharias and Strehler, 1996). It is likely that these changes in splicing patterns are the result of posttranscriptional modifications of regulatory proteins, e.g. phosphorylation, methylation, and glycosylation. However, it is largely unknown which factors are affected. In the following, we summarize several protein groups that are likely endpoints of signal transduction pathways in the spliceosome.

B. SF1

Some signal transduction pathways to spliceosomal components have been investigated in detail. One paradigm is SF1 (Berglund et al., 1998; Rain et al., 1998), a factor that recognizes the branch point and is therefore important for the formation of the spliceosomal A complex. SF1 has recently been identified as a target of PKG-I (Wang et al., 1999). This kinase is activated by cGMP. The cGMP level itself can be regulated by a membrane bound guanylyl cyclase receptor that is activated by natriuretic peptides or by a cytoplasmic guanylyl cyclase which is activated by nitric oxide (NO). Phosphorylation of SF1 on Ser20 inhibits the SF1-U2AF65 interaction, leading to a block of pre-spliceosome assembly.

C. hnRNPA1

hnRNP A1 has also been implicated as a mediator of signal transduction. This protein antagonizes the action of SR proteins that promote distal 5' splice-site usage in E1A and ß-globin pre-mRNAs bearing thalassemia mutations (Cáceres et al., 1994; Mayeda and Krainer, 1992). In addition, hnRNPA1 controls inclusion of exon 7b of its own transcript (Blanchette and Chabot, 1999) and of exon 2 of the HIV Tat-pre-mRNA (Caputi et al., 1999). Two signal transduction pathways have been described to change the RNA binding properties and the intracellular localization of hnRNPA1. Stimulation of the PDGF receptor causes phosphorylation of hnRNP A1 by PKCzeta (Municio et al., 1995). This phosphorylation impairs the RNA binding and strand annealing activity of hnRNPA1.

Furthermore, hnRNP A1 is phosphorylated by the MKK3/6-p38 signaling cascade after cellular stress induced by osmotic stress or UV irradiation, but the direct kinase remains to be determined (van Oordt et al., 2000). Stress induced phosphorylation leads to the cytoplasmic accumulation of hnRNP A1 and results in a change of the

Stimulus	Signal transduction	Alternatively spliced transcript	*de novo* protein	Reference
Phorbolester Concanavalin A	PKC Ras	CD44 exon v5	no	König et al., 1998
Concanavalin A	?	Qa-2 exon 5	?	Tubaczewski et al., 1994
Phorbolester	PKC Ras	Cd45 exon 4	yes: SF2, SRp55	Lynch et al., 2000 Lemaire et al., 1999
Phorbolester PDGF, EGF	?	PTP-1B	yes	Shifrin and Neel, 1993
Phorbolester, Insulin	?	PKCβ exon βII	?	Chalfant et al., 1995, 1998 Ishizuka et al., 1996
Insulin	?	Insulin receptor exon 11	?	Sell et al., 1994
FGF-1, FGF-2	?	FGF-receptor-2 and –3, exon III	?	Scotet and Houssaint, 1998
TNFα, IFNγ	?	CD44, exons v6, v9	?	Mackay et al., 1994
IFN-γ, IL-1β TNF-α, LPS	?	Nitric-oxide synthase (iNOS)	?	Eissa et al., 1996
IL-1β, PDGF TGF-β	?	Fibronectin, exon IIICS	?	McKay et al., 1994
TGF β	?	Fibronectin EIIIA	?	Wang et al., 1991 Balza et al., 1988
Dexametasone	?	Insulin receptor	?	Kosaki and Webster, 1993
Hypophysectomy, ACTH	?	Slo	?	Xie and McCobb, 1998
NGF, Dexametasone	?	NF1	?	Metheny and Skuse, 1996
NGF	Ras	Agrin	?	Smith, 1997
pH	?	ATP synthase gamma-subunit, exon 9	?	Hayakawa et al., 1998
pH	?	Tenascin-C	?	Borsi et al., 1995
Osmotic shock UVC irradiation	MKK3/6-p38	Adenovirus E1A	no	Van der Houven van Oordt et al., 2000
Change in neuronal activity	?	Htra2-beta, exon 3; Clathrin light chain B, exon EN	?	Daoud et al., 1999
Induction of Long-Term potentiation	?	Syntaxin 1, 3	?	Helme-Guizon et al., 1998 Rodger et al., 1998
Hippocampal kindling	?	Glutamate receptor-A and –B exon FLIP, FLOP	?	Kamphuis et al., 1992
Rise in intracellular Ca	?	Ca^{2+}-ATPase	No	Zacharias et al., 1996

Table 3: Stimuli that change alternative splicing patterns
Signals known to influence alternative splicing patterns are shown in the first column. Known proteins involved in signal propagation to the splicing machinery are indicated in the second column and the gene which changes its alternative splicing pattern is shown in the third column. In some cases, it has also been determined whether *de novo* protein biosynthesis is necessary for the observed change of a given splicing pattern, which is shown in the fourth column.

alternative splicing pattern of the adenovirus E1A pre-mRNA splicing reporter.

D. STAR proteins

Other likely candidates for proteins that can transduce a signal to the spliceosome are STAR proteins. STAR is an abbreviation for signal transduction and activation of RNA (Jones and Schedl, 1995; Vernet and Artzt, 1997). This protein family is also called GSG for GRP33, Sam68, GLD-1 (Jones and Schedl, 1995). Its members belong to an expanding group of proteins that share an amino-terminal maxi-KH-RNA binding domain as well as proline and tyrosine-rich regions present in many adapter proteins involved in signal transduction (Richard et al., 1995). The binding properties of STAR proteins suggest their involvement in splice-site selection. For example, Sam68 has been found to crosslink to a splicing regulatory region on the rat tropomyosin pre-mRNA (Grossman et al., 1998), and also binds to FBP21, a protein implicated in splicing (Bredford et al., 2000). A protein related to SAM68 is SLM-2, for Sam68 like molecule (Di Fruscio et al., 1999). In humans, the protein is called T-STAR and was shown to interact with RBM, an hnRNP G like protein previously implicated in splice-site regulation (Venables et al., 1999, 2000). We identified the rat homolog and demonstrated that it regulates alternative splicing of CD44, htra2-beta, and tau pre-mRNAs by binding to purine-rich enhancer sequences (Stoss et al., 2001). The physiological importance of STAR proteins becomes apparent in mutations of the quaking locus. The molecular defect is a mutation in the STAR family member QKI, which results in severe defects in myelination in the nervous system (Ebersole et al., 1996).

SAM68 is phosphorylated by the tyrosine kinases Src or Fyn during mitosis (Fumagalli et al., 1994). SAM68 tyrosine phosphorylation is inducible by insulin in fibroblasts, or after TCR stimulation (Fusaki et al., 1997; Lang et al., 1997; Sanchez-Margalet and Najib, 1999). The tyrosine phosphorylation results in a decrease of the RNA binding affinity and leads to the dissociation of Sam68 multimers which could have a direct influence on the regulation of alternative splicing (Chen et al., 1997; Wang et al., 1995). We were able to show that rSam68, rSLM-1, and rSLM-2 bind to the scaffold attachment factor B (SAF-B), a component that binds to DNA-nuclear matrix attachment regions as well as to RNA polymerase II and various SR proteins (Stoss et al., 2001). This association again emphasizes the intimate connection between pre-mRNA processing, transcription, and chromatin structure. The exact change of protein:protein interaction caused by tyrosine phosphorylation of complex components and its influence on alternative splicing remain to be determined, but will most likely influence splice-site selection.

E. SR proteins and their kinases

Finally, in humans, SR proteins are phosphorylated by four different Cdc2-like kinases of the LAMMER family (Clks) and two structurally related kinases called SRPK1 and SRPK2 (Gui et al., 1993; Koizumi et al., 1999; Nayler et al., 1997; Prasad et al., 1999; Stojdl and Bell, 1999; Wang et al., 1998). These kinases all contain an RS domain; they interact with SR proteins and their overexpression leads to the disassembly of speckles (Nayler et al., 1998b). A direct involvement of Cdc2-like kinases in the regulation of alternative splicing has recently been shown for the Clk1-, E1A- and SRp20-, and tau pre-mRNAs (Duncan et al., 1997; Hartmann et al., 2000; Stoss et al., 1999b). However, there are important differences among these kinases. First, the Cdc2-like kinases are ubiquitously expressed, whereas the SRPKs show a more differentiated expression pattern with SRPK1 predominantly expressed in testis and SRPK2 in brain (Papoutsopoulou et al., 1999a; Wang et al., 1998). In addition, SRPK1 shows higher specificity towards ASF/SF2 in comparison with Clk/STY, since Clk/STY phosphorylates Ser-Arg, Ser-Lys, or Ser-Pro sites, wheras SRPK1 shows a strong preference for Ser-Arg sites (Colwill et al., 1996a). There is also evidence that these kinases phosphorylate SR proteins at different sites. Finally, there are differences in the intracellular localization, since the Clks are predominantly nuclear and colocalize with speckles, whereas SRPK1 was primarily found in the cytoplasm and is believed to phosphorylate the cytosolic SR protein fraction.

In addition, there are three other kinases that are able to phosphorylate SR proteins: CKIa, the Lamin B receptor and topoisomerase I (Gross et al., 1999; Nikolakaki et al., 1996; Rossi et al., 1996). In contrast to the Cdc2-like kinases, the substrates of CKIa must already be phosphorylated, suggesting a phosphorylation hierarchy in the regulation of SR proteins. The lamin B receptor is part of a nuclear envelope complex that also phosphorylates lamin A and B. This kinase phosphorylates RS domains and seems to have substrate specificities identical to SRPK1 (Papoutsopoulou et al., 1999b).

Phosphorylation of SR proteins has numerous effects on protein:RNA and protein:protein interactions. For example, phosphorylation of SRp40 enhances its RNA binding affinity (Tacke et al., 1997) and phosphorylation of ASF/SF2 stimulates its binding to U170K, a component of the U1snRNP (Xiao and Manley, 1998). This type of covalent modification of SR proteins has a direct effect on their activity in the splicing reaction (Prasad et al., 1999; Tazi et al., 1993).

An important consequence of SR protein phosphorylation is the release of these proteins from their storage compartments, the speckles (Colwill et al., 1996b; Gui et al., 1993; Koizumi et al., 1999), and their recruitment to sites of transcription (Misteli, 2000; Misteli et al., 1998).

In addition, phosphorylation influences the ability of some SR proteins to shuttle between the nucleus and the cytoplasm (Caceres et al., 1998; Yeakley et al., 1999). In a manner that is similar to hnRNP A1 (van Oordt et al., 2000), this could result in a rapid change of the nuclear concentration of SR proteins, which changes alternative splice-site selection.

Together, these data show that pre-mRNA splicing can be regulated by external stimuli. Some of the signal transduction pathways to the spliceosome are beginning to emerge and result in regulated serine and tyrosine phosphorylation of splicing proteins.

III. Diseases caused by splicing defects
A. Overview

Most of the diseases associated with defects in pre-mRNA processing result from a loss of function due to mutations in regulatory elements of a single gene. These mutations have previously been compiled (Krawczak et al., 1992; Nakai and Sakamoto, 1994) and are available on the web (cookie.imcb.osaka-u.ac.jp/nakai/asdb.html). A few diseases have been attributed to a change in trans-acting factors. Knockout experiments of essential splicing factors have proven lethal (Hirsch et al., 2000; Wang et al., 1996). However, the knockout of an enzyme involved in pre-mRNA editing (Higuchi et al., 2000) and overexpression of mutated SR proteins in *Drosophila* (Kraus and Lis, 1994) causes phenotypes that cannot be linked to a single mRNA, which shows that defects in pre-mRNA processing factors can result in a complex pathological state. The combinatorial nature of trans-acting factors raises the interesting possibility that pleiotropic diseases with a variable phenotype might be caused by alterations of trans-acting factors. Using minigenes of CFTR mutations, it has been shown that the splicing patterns of mutated alleles strongly depend on the cell type (Nissim-Rafinia et al., 2000), indicating that variations in trans-acting factors could be the reason for a variable penetrance of mutations among individuals with different ethnic backgrounds (McInnes et al., 1992; Rave-Harel et al., 1997). Similarly, a contribution of pre-mRNA processing could explain why natural mutations in human genes frequently have tissue- or cell-type specific effects. In these cases, the mutated gene is similarly expressed in all cells, but is processed in a tissue specific manner, since the relative concentrations of splicing factors vary among tissues (Hanamura et al., 1998).

Since diseases caused by splicing defects have been recently reviewed (Philips and Cooper, 2000), we will concentrate on three diseases, FTDP-17, spinal muscular atrophy, and ß-thalassemia to illustrate the complex relationships between trans-acting factors and their corresponding cis-elements, and to outline possible therapeutic approaches.

B. FTDP-17: frontotemporal dementia with parkinsonism linked to chromosome 17

Frontotemporal dementias (**Figure 2A**) represent a rare form of presenile dementias that are clinically defined by behavioral and personality changes, psychomotor stereotypes, as well as loss of judgment and insight. The neuropathological findings include an asymmetric frontotemporal atrophy and the presence of filamentous tau deposits. The disease was mapped to the tau locus on chromosome 17. Tau is a microtubule-associated protein. Knockout experiments revealed that tau is not essential for brain formation (Harada et al., 1994), although it is involved in the pathology of several neurodegenerative diseases (Spillantini and Goedert, 1998). Tau transcripts undergo complex regulated splicing in the mammalian nervous system. The alternative splicing of one of its exons, exon 10, is species-specific. This exon is alternatively spliced in adult humans, but is constitutively used in the adult rodent brain. In addition, the usage of exon 10 is regulated during development and increases when neuronal development proceeds. In the protein, it encodes one of the four microtubuli binding sites of tau. Some of these tau mutations that activate exon 10 usage were shown to cause an accelerated aggregation of tau into filaments (Nacharaju et al., 1999), which is a hallmark of several neurodegenerative diseases, e.g. Alzheimer's disease.

A disruption of the proper balance of tau isoforms with three and four microtuble binding sites is observed in the pathology of several tauopathies, including FTDP, Picks disease, corticobasal degeneration, Guam amytrophic lateral sclerosis/parkinsonism dementia complex. Secondary structure predictions suggest a stem loop structure at the 5' splice-site of exon 10 that contributes to its regulation (Grover et al., 1999; Jiang et al., 2000); however the *in vivo* relevance of this structure remains to be proven. In addition, mapping of elements in tau exon 10 revealed a complicated set of cis-acting elements that is disrupted by natural mutations (D'Souza et al., 1999). Some of these mutations, such as L284L, are silent, but lead to disease by interrupting an exonic element that causes missplicing.

Since alternative splice-site usage can be regulated by the relative concentration of SR proteins or hnRNPs, several such trans-acting factors were tested *in vivo*, and it was found that some SR or SR related proteins (SF2/ASF, SRp75 and U2AF65) stimulate exon 10 skipping (Gao et al., 2000). It was shown that SR proteins are released from their storage compartments, the speckles, by Cdc2-like kinases (clk1-4). Those kinases were found to strongly inhibit missplicing of exon 10, even in several mutations
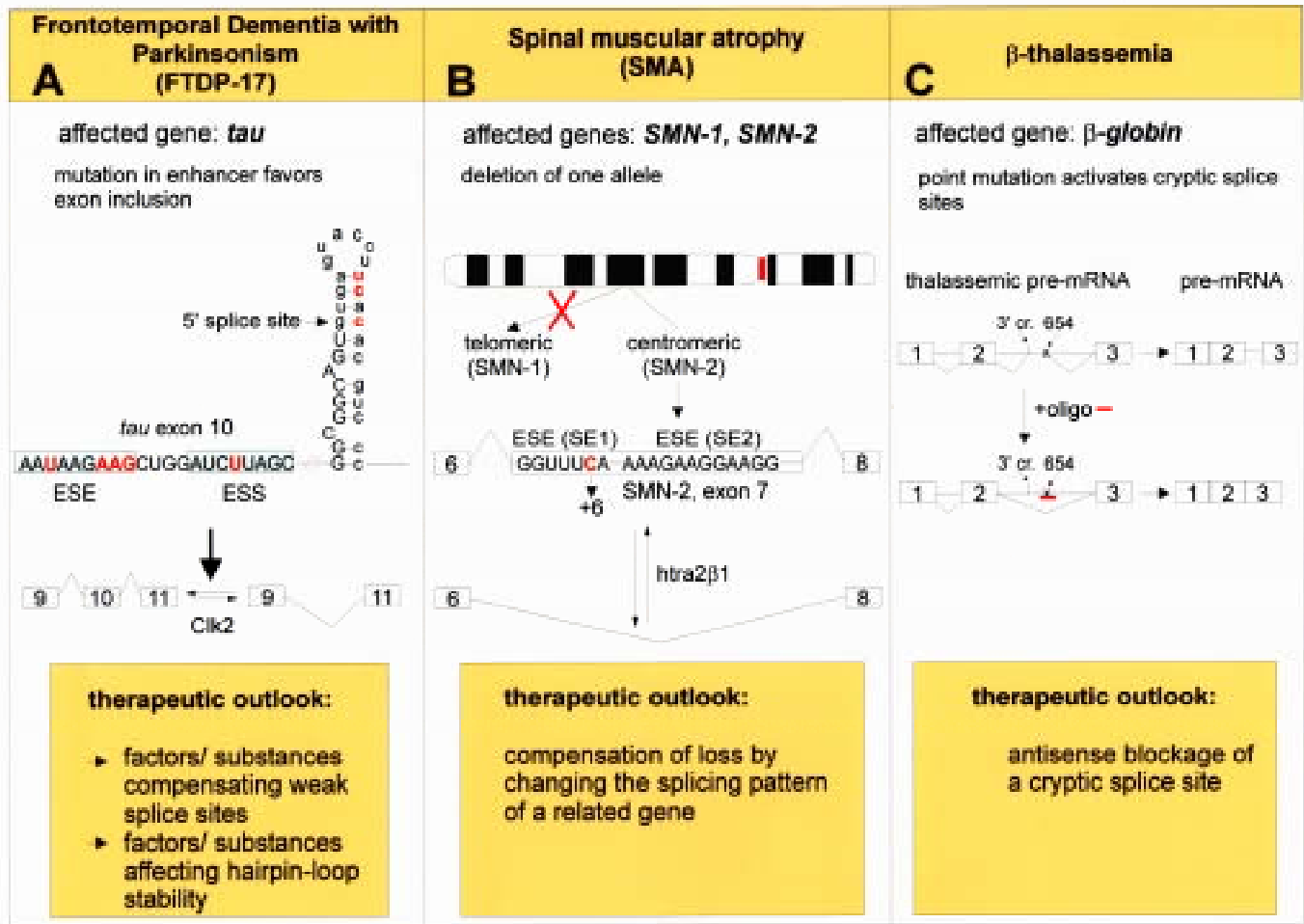
Figure 2: Examples of diseases that are caused by errors in pre-mRNA splicing

A) Frontotemporal dementia with Parkinsonism (FTDP-17) as an example of mutation in splicing enhancers and silencers. FTDP-17 is caused by a misregulation of tau exon 10 usage. Exon 10 splicing is tightly regulated by exonic enhancer and silencer sequences in exon 10. It is unclear whether a secondary structure that masks the 5' splice-site is important *in vivo*. Mutations close to exon 10, which are observed in FTDP-17 patients (shown in red), favor exon 10 inclusion. The Cdc2-like kinases clk1-4 are able to revert the increase in exon 10 inclusion. This opens new therapeutic possibilities to treat FTDP-17 and related diseases by screening for factors or substances that selectively affect the regulation of alternative splicing.

B) Spinal muscular atrophy (SMA): compensation of a non-functional gene. Positional cloning strategies led to the identification of the survival of motorneuron (SMN) genes as one of the genes affected in SMA. Two non-equal copies of SMN exist on chromosome 5q13. Full-length SMN can only be generated from the telomeric copy (SMN-1). A silent C/T transition of the sixth nucleotide of the centromeric copy (SMN-2) (in red) disturbs splicing of exon 7, leading to the generation of a truncated SMN protein. SMA is caused by failure to express the telomeric SMN-1 gene. One possibility to compensate for the loss of SMN-1 expression is overexpression of htra2-beta1. This SR like protein can switch the splicing pattern of the SMN-2 pre-mRNA towards the inclusion of exon 7, leading to a functional SMN-2 copy.

C) β-thalassemia: A point mutation activates a cryptic splice-site. The second intron of the thalassemic β-globin gene harbors a C to T mutation at nucleotide 654. This creates an additional 5' splice-site that activates a cryptic splice-site at nucleotide 579 of the β-globin pre-mRNA, leading to the retention of an intronic region. Antisense oligonucleotides can be used to mask the aberrant splice-sites, resulting in the formation of the desired gene product.

that activate exon 10 usage (Hartmann et al., submitted).

These examples show that missplicing can be reversed *in vivo* by activating regulatory proteins through their kinases. It is possible that lower molecular weight substances can be isolated that cause the release of specific regulatory factors, by either activating the appropriate kinase or blocking the corresponding phosphatase. In addition, recent results show that drugs such as aminoglycoside antibiotics which can directly interact with regulatory RNA structures, may also have a therapeutic potential (Varani et al., 2000).

C. Spinal muscular atrophy (SMA)

Proximal spinal muscular atrophy (SMA, **Figure 2B**) is a neurodegenerative disorder with progressive paralysis caused by the loss of alpha-motor neurons in the spinal cord. With an incidence of 1 in 10,000 live births and a carrier frequency of 1 in 50, SMA is the second-most common autosomal recessive disorder and the most frequent genetic cause of infantile death (Pearn, 1980). The gene responsible for the disease was identified as SMN1 (survival of motor neurons, Lefebver, 1995) and the disease is caused by loss of (96.4%) or mutations in (3.6%) the SMN1 gene (Wirth, 2000). A nearly identical copy of the SMN1 gene exists but cannot compensate for the absence of SMN1, because it is processed differently. Due to a single nucleotide difference in exon 7, this exon is skipped in SMN2. Therefore the proteins generated by both genes differ in their carboxy terminus, which is most likely crucial for the function. The protein generated by SMN2 encodes a truncated, less stable protein with reduced self-oligomerization activity (Coovert et al., 1997; Lefebvre et al., 1995, 1997). The exon enhancer containing the singlenucleotide difference has been characterized (Lorson et al., 1999) and was found to be of the GAR type. A systematic search for trans-acting factors identified human transformer2-beta, a member of the SR related family of proteins (Hofmann et al., 2000). An increase of the concentration of htra2-beta1 results in stimulation of exon 7 increase. A mRNA generated by this pathway would encoded for a protein that can complement for the loss of SMN1.

This example demonstrates that pre-mRNA processing can be maniplulated *in vivo* to complement the loss of a gene product.

D. β-thalassemia

β-thalassemias are autosomal recessive diseases (**Figure 2C**), in which the amount of β-globin is reduced. Individuals carrying a single mutated gene are less prone to malaria infection. About 3% of the world population, mostly people living in regions endemic with malaria, or their descendants, are carriers. β-thalassemia causes a hypochromic, microcytic and hemolytic anemia. The

imbalance of globin synthesis causes the α-chains to precipitate and damages the red blood cells. More than 100 mutations of the β-globin gene leading to thalassemia have been described (Wetherall and Clegg, 1981), among them are at least 51 point mutations, which mostly affect pre-mRNA processing (Kazzazin and Boehm, 1988; Krawczak et al., 1992). These mutations either destroy the 5' or 3' splice-sites or generate cryptic splice-sites that are usually located in the introns (**Figure 2C**). As a result, no functional β-globin protein is produced. Since point mutations often manifest themselves as defects in pre-mRNA splicing, missplicing of β-thalassemias is a model for a large number of mutations (Krawczak et al., 1992; Nakai and Sakamoto, 1994). Cryptic splice-sites of thalassemic β-globin can be changed *in vivo* by overexpression of the SR protein SF2/ASF (Cáceres et al., 1994). Furthermore, the mutated cryptic sites can be blocked by a complementary oligonucleotide (Schmajuk et al., 1999; Sierakowska et al., 2000), that enhances the formation of the desired β-globin product in cell-culture systems.

This example illustrates that cryptic splice-sites can be masked *in vivo*, which promotes the formation of the desired gene product.

IV. Detection and treatment of splicing defects

A. Alternative splicing as an indicator of disease

Since pre-RNA pathways can adapt according to environmental signals, the splicing pattern of pre-mRNAs is most likely a reflection of the cellular state. There are numerous examples in which a change of exon usage is associated with a pathological state. The most prominent example is cancer. Some of the changes found in splice-site selection that are associated with cancer are shown in **Table 4**. One of the best described genes that shows the importance of pre-RNA processing for tumor progression and metastasis is CD44. In this gene, at least 12 exons are alternatively spliced (Screaton et al., 1992) and their usage relates to metastatic potential.

With the completion of the human gene project and the progress in array techniques, it will be possible to detect differences in splicing patterns between a normal and pathological state. Arrays with exon-specific oligonucleotides will make it possible to discover changes in alternative splicing patterns. Given the numerous examples in which changes in exon usage are associated with disease, the development of such a DNA exon chip might help in the diagnosis of cancer and the elucidation of the underlying molecular pathology. Furthermore, such a tool might be

Gene	Cancer type	Reference
AML1	leukemia	(Ogawa et al., 1995)
calretinin	colon cancer	(Schwaller et al., 1995).
deoxycytidine kinase	myeloid leukemia	(Veuger et al., 2000)
E-cadherin	gastric carcinomas	(Becker et al., 1994)
fas	Acute leukemia	(Inaba et al., 1999)
fibronectin	various cancers	(Mandel et al., 1992)
	liver tumor	
interleukin-6R alpha	Multiple myeloma	(Thabard et al., 1999)
K-SAM	cancer	(Itoh et al., 1994)
MCL1	Myeloid cell	(Bae et al., 2000)
	leukemia	
n-cam	lung cancer	(Carbone et al., 1991)
P2XM	varios tumors	(Nawa et al., 1999)
parathyroid hormone-like peptide	tumors	(Yasuda et al., 1989)
prostatic secretory protein of 94 amino acids	prostate cancer	(Xuan et al., 1995)

Table 4: Examples of genes that change their splicing pattern during cancer formation and progression. Only a few examples are given

used to detect the effect of trans-acting factors *in vivo* and could ultimately be used to unveil misregulation of trans-acting factors in complex diseases.

B. Suppression of point mutations by oligonucleotides

The majority of known pathological states associated with splicing are generated by point mutations that either destroy splice-sites or generate new, cryptic sites in the vicinity of normally used exons (Krawczak et al., 1992). It has been demonstrated that antisense nucleic acids binding to the aberrant splice-sites can inhibit the usage of the wrong sites and promote the formation of the normal gene product. Among nucleic acids, modified RNA oligonucleotides have been used (Sierakowska et al., 2000). Blocking the aberrant splice-sites forces the splicing machinery to reselect the original splice-site and can restore the correct gene product. Currently, 2'-O-methyl oligoribonucleoside phosphoro-thioates are the most widely used nucleic acids, since they do not induce RNase H mediated cleavage of targeted RNA and seem to have only minor effects on cell viability, morphology, and growth rates. Diseases targeted include ß-thalassemias (Schmajuk et al., 1999), cystic fibrosis (Friedman et al., 1999), muscular dystrophy mRNA (Wilton et al., 1999), and eosinophilic diseases (Karras et al., 2000). Furthermore, apoptosis can be influenced by oligonucleotides directed against Bcl-x splice variants (Taylor et al., 1999). The oligonucleotide approach offers a high specificity to target a mutated gene. Most studies have been performed in cell culture systems, where the oligonucleotide approach works in multiple cellular contexts, which argues for its broad applicability to suppress an aberrant splice-site selection. It remains to be seen whether this approach can also be used to modify exon enhancer usage.

C. Modification of trans-acting factor action

Since the selection of splice-sites is dependent on the relative concentration of regulatory proteins, a change in the concentration of a protein could possibly correct a pathological ratio of exon inclusion to exon skipping. For example, overexpression of SR proteins and their kinases clk1-4 can revert missplicing of tau exon 10 (Gao et al., 2000; Hartmann et al., submitted); overexpression of htra2-beta1 can change the splicing pattern of SMN2 to complement loss of SMN1 in spinal muscular atrophy (Hofmann et al., 2000); and the levels of hnRNPA1 and SF2/ASF regulate alternative splicing of mutated alleles of the cystic fibrosis transmembrane conductance regulator (Nissim-Rafinia et al., 2000) and mutated ß-globin genes (Cáceres et al., 1994). Since most splicing factors are released from nuclear storage compartments, a promising strategy might be the identification of specific antagonist/agonists for splicing factor kinases from a chemical library. Furthermore, it is likely that release of splicing factors can occur in response to a stimulation of a receptor and specific agonists could be found to arrest this process. Such molecules will be easier to deliver pharmacologically because of their small size; however such agonists remain to be identified, and their specificity proven.

V. Conclusions

The basic mechanisms regulating alternative splice-site selection have been deciphered in recent years. The abundant usage of alternative pre-mRNA processing has most likely generated an evolutionary advantage for vertebrates, since the formation of protein isoforms required for specialized functions was greatly accelerated. A disadvantage of this mechanism is the misregulation of pre-mRNA processing that is apparent in several human diseases. We hypothesize that the role of pre-mRNA defects in human disease is just beginning to emerge and is currently largely underestimated. Since splice-site selection is regulated by extracellular signals, the analysis of these signal transduction pathways might provide new insights and novel chances for therapy. The connection of the molecular mechanisms governing splice-site selection with the detailed analysis of human diseases will provide opportunities for diagnosis and treatment.

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