DNA AND CELL BIOLOGY
Volume 21, Number 11, 2002

Pp. 803–818

Defects in Pre-mRNA Processing as Causes of and Predisposition to Diseases

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ABSTRACT

Humans possess a surprisingly low number of genes and intensively use pre-mRNA splicing to achieve the high molecular complexity needed to sustain normal body functions and facilitate responses to altered conditions. Because hundreds of thousands of proteins are generated by 25,000 to 40,000 genes, pre-mRNA processing events are highly important for the regulation of human gene expression. Both inherited and acquired defects in pre-mRNA processing are increasingly recognized as causes of human diseases, and almost all pre-mRNA processing events are controlled by a combination of protein factors. This makes defects in these processes likely candidates for causes of diseases with complicated inheritance patterns that affect seemingly unrelated functions. The elucidation of genetic mechanisms regulating pre-mRNA processing, combined with the development of drugs targeted at consensus RNA sequences and/or corresponding proteins, can lead to novel diagnostic and therapeutic approaches.

OVERVIEW

RECENT PROGRESS IN THE HUMAN GENOME PROJECT has demonstrated that humans possess a surprisingly low number of genes, estimated to range around 25,000 to 40,000 (Lander *et al.,* 2001). A considerable fraction of the genes identified by different approaches now appears to be nonoverlapping, which implies a somewhat larger number of genes than previously assumed; however, the total number is not drastically modified (Hogenesch *et al.,* 2001). To create the proteome estimated to range between 90,000 and one million proteins (O'Donovan *et al.,* 2001; Harrison *et al.,* 2002; Hodges *et al.,* 2002), humans abundantly process their pre-mRNAs (Modrek *et al.,* 2001; Rubin, 2001) before protein translation occurs. This allows the production of multiple protein isoforms from a single gene. Transcription and pre-mRNA processing are both physically and functionally linked. This occurs by interaction of processing factors with the carboxy terminal domain of RNA polymerase II (Steinmetz, 1997) and by interaction of processing factors with transcription factors (Ge *et al.,* 1998). As a result, the choice of a particular promoter influences splice site selection (Cramer *et al.,* 1999, 2001). Finally, RNA processing factors are linked to chromatin organizing elements by scaffold attachment factor B (Nayler *et al.,* 1998). These associations imply that molecular defects in promoter structure and chromosomal localization of a gene can result in aberrant pre-mRNA processing.

While being transcribed, pre-mRNAs undergo a sequence of structural changes, such as capping, editing, splicing, and polyadenylation. In this process, the fidelity of each maturation step is controlled. Although the individual maturation steps can be biochemically separated, recent reports show that most of them are functionally coupled (Bentley, 1999; Minvielle-Sebastia and Keller, 1999; Maniatis and Reed, 2002). Defects in this fine-tuned RNA assembly line are increasingly recognized as causes of inherited human diseases (Philips and Cooper, 2000; Stoss *et al.,* 2000; Daoud *et al.*, 2000; Dredge *et al.,* 2001; Mendell and Dietz, 2001; Caceres and Kornblihtt, 2002; Nissim-Rafinia and Kerem, 2002). Changes in proteins functioning at different steps during the processing or changes in the efficiency of the processes can result in variation of genetic expression (Herbert and Rich, 1999). This may affect adaptation

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events, when the expression of the genetic information is altered according to changes in physiologic needs (Stamm, 2002). It should be pointed out that apart from pre-mRNA processing, which is a mechanism to enlarge the number of proteins made from the genome, there are a multitude of post-translational modifications, such as phosphorylation, acetylation, glycosylation, cleavage, etc., which create functional diversity among existing protein isoforms (O'Donovan *et al.,* 2001; Hodges *et al.,* 2002).

We propose that pre-mRNA processing has the potential to adapt the information stored in the genome to the physiologic requirements of circumstance, place, and time. The failure of such adaptation is frequently traced to defects in processing. These defects can manifest themselves directly in a disease or may remain silent until some internal or environmental stimulus (e.g., stress) or time (i.e., old age) allows the mutation to become apparent. Furthermore, the sequential nature of pre-mRNA processing raises the interesting possibility that pleiotropic diseases with a variable phenotype may be caused by specific compositions of *trans*-acting factors. For example, sporadic amyotrophic lateral sclerosis (ALS) was shown to be associated with a change in splicing patterns of the glutamate transporter gene EAAT2 (C. Lin *et al.,* 1998), as well as changes in nitric oxide synthase (NOS) mRNA splicing (Catania *et al.,* 2001). This indicates that the basic defect is in the pre-mRNA processing machinery, which gives rise to altered isoforms associated with the disease. While the culprit gene(s) may not yet be known, key proteins or RNAs controlling the affected processes can be subject to therapeutic efforts.

In this review, we will illustrate the variety of pathogenic defects that occur at each step of pre-mRNA processing in eukaryotic nuclei, and present examples for the mechanisms through which several mutations affect the pre-mRNA or the processing apparatus. To unravel the tangled interrelationships between these processes, we present examples of disease-causing defects in each of the relevant stages of mammalian pre-mRNA processing, describe stress-induced changes in pre-mRNA processing as an example of a disease predisposition, and finally discuss potential prospects for the development of novel therapeutic approaches.

DEFECTS IN PRE-mRNA PROCESSING AS A DIRECT CAUSE OF HUMAN DISEASE

pre-mRNA editing

Nucleotides in the pre-mRNA can be chemically modified in a process called RNA editing (Gott and Emeson, 2000; Gerber and Keller, 2001; Bass, 2002). During editing, adenosine or cytosine is deaminated, changing it into inosine (translated as guanine) or uridine, respectively. The editing of adenosines is catalyzed by adenosine deaminases acting on RNA (ADARs) (Reenan, 2001), whereas the editing of cytosines is catalyzed by Apobec-1 (Chester *et al.,* 2000). All of these enzymes are subsequently associated with heterogeneous nuclear ribonucleoprotein (hnRNP) complexes (Raitskin *et al.,* 2001), and can affect the removal of introns in the subsequent splicing reaction. For example, editing changes the splicing process in ADAR2 by changing an AA into an AG at the 3′ consensus splice site of the ADAR2 pre-mRNA, suggesting a close interaction between editing and splicing (Rueter *et al.,* 1999). There are three *ADAR* genes in humans and genomic disruption experiments show that *ADAR1* is essential in mice. The frequency of editing a particular nucleotide is species, tissue, and development stage specific. In obese Zucker rats, editing of hepatic apolipoprotein B mRNA was found to be 42% higher than in lean controls, corresponding to 1.8-fold increase in Apobec-1 catalytic activity in these rats (Phung *et al.,* 1996). ADAR1 expression was found to be upregulated in a mouse model of microvascular lung injury (MLI) as well as in cultured alveolar macrophages (MH-S cells) stimulated with endotoxin, or interferon-gamma, suggesting a plausible role for *ADAR1* in the pathogenesis of MLI through induction by interferon (Rabinovici *et al.,* 2001). B cells with an unusual heavy- and light-chain antibody repertoire due to abnormal RNA editing were observed in patients suffering from rheumatoid arthritis, implying a role for RNA editing in autoimmune diseases (Meffre *et al.,* 2000). Despite the ubiquitous expression of *ADAR1* and *ADAR2*, editing seems to be most prevalent in the brain, where 1 out of 17,000 nt is edited (Paul and Bass, 1998). Assuming an average length of 1 kb for an mRNA, this implies that up to 1 out of 20 brain transcripts is edited.

Perfectly matched RNA:RNA duplexes in the pre-mRNA appear to be the major determinant for *ADAR* editing. Such duplexes are created either by a natural regulatory "antisense" inversely oriented region in the preedited transcripts or by transcriptional readthrough of adjacent genes in antisense orientation. An example for the first mechanism involves Apobec-1, the editing of which requires a conserved 26 nt sequence that contains an 11 nt mooring sequence located 4–5 nt downstream of the edited cytosine (Chester *et al.,* 2000). Another example refers to malignant tumors, which edit the NF1 neurofibromatosis transcripts more efficiently than benign tumors. Overexpressed Apobec-1, which catalyzes deamination of cytidines, induces murine tumorigenesis (Yamanaka *et al.,* 1995), suggesting that the corresponding gene may operate as a proto-oncogene (Chester *et al.,* 2000). Alternative splicing and RNA hyperediting was observed in the hematopoietic tyrosine phosphatase (*PTPN6*) gene in $CD34^+/CD117^+$ progenitor cells from acute myeloid leukemia patients. There, editing of adenosine (7866) to guanine in a putative branch site causes retention of intron 3, leading to abnormal accumulation of the aberrant splice variants (Beghini *et al.,* 2000). ADAR genomic disruption in *Drosophila* causes no obvious developmental phenotype but results in severe adult defects in motor control, flight, and mating. The defects increase in severity with age, concurrent with neurodegeneration (Palladino *et al.,* 2000; Reenan, 2001). Because editing is a prominent mechanism in the brain, impairments in this process were pursued in neurologic diseases. For example, Ca^{++} conductance of AMPA receptors is regulated by the GluR2 subunit that is edited to exchange a glutamine with an arginine residue. GluR2 editing efficiency and mRNA levels were significantly lower in the ventral gray area of patients with ALS than in controls. These changes may account for the enhanced Ca^{++} influx through AMPA receptors, which is a plausible cause for selective neuronal death in ALS (Takuma *et al.,* 1999). Reduced editing efficiency of GluR2 RNA was also observed in the prefrontal cortex of Alzheimer's disease patients and schizophrenics, as well as in the striatum of Huntington's disease patients (Akbarian *et al.,* 1995). This

points to neuronal activity as a contributing element of editing efficacy.

pre-mRNA splicing

Constitutive splicing. Almost all human genes contain introns that account for about 95% of the pre-mRNA (Lander *et al.,* 2001). Higher eukaryotes possess several types of introns, which have slightly different mechanisms of excision splicing: a major one, in which the introns are flanked by GU-AG dinucleotides and which accounts for more than 98% of all introns (Lander *et al.,* 2001), and a minor one in which introns are flanked by AU-AC dinucleotides (Tarn and Steitz, 1997). The major type has a subtype (about 1% of introns), for which the flanking nucleotides are GC-AG (Thanaraj and Clark, 2001).

During mRNA maturation those introns are excised and the remaining sequences, the exons, are joined. This process is performed by the spliceosome, a macromolecular complex comprising five small ribonucleoprotein particles (snRNPs) and at least 45 additional proteins (Neubauer *et al.,* 1998).

The recognition of an exon is guided by three sequence elements of the pre-mRNA: the 5′ and 3′ splice sites and the branchpoint. In addition, short sequences within or near the exon that can act as enhancers or silencers participate in exon recognition. Both the 5′ and 3′ splice site sequences, the enhancer/silencer elements, and the branch point associated with the splicing process are short (8–12 nt) and degenerate. Nevertheless, these sequences appear to be vulnerable to disease-causing mutations that destroy splice sites or create novel ones. Familial hypercholesterolemia is an autosomal dominant genetic lipoprotein disorder caused by defects within the low-density lipoprotein receptor gene. Some of its many mutations were shown to disrupt acceptor splice sites: an A to G substitution in the penultimate 3′-nucleotide of intron 16 (Lombardi *et al.,* 1993) and a G to C transposition at the last nucleotide of intron 7 (Yu *et al.,* 1999). In both cases a cryptic splice site was activated leading to the formation of a mutated receptor protein. Another example of a splice site mutation that leads directly to a disease phenotype is molybdenum cofactor deficiency (MoCoD), an inherited autosoml recessive disease that leads to early childhood death. Two bi-cistronic genes, *MOCS1* and *MOCS2*, are responsible for the generation of the molybdoenzymes, sulfite oxidase, xanthine dehydrogenase, and aldehyde oxidase. A *MOCS1* splice site mutation leads to the deficiency of all these molybdenum cofactor related enzymes (Reiss *et al.,* 1999). Other splice site mutations, including those in the CFTR and beta-globin genes, resulting in cystic fibrosis and thalassemias, were previously compiled (Krawczak *et al.,* 1992; Nakai and Sakamoto, 1994). Mutations that alter the splicing process can occur outside both splice sites and enhancer/silencer elements. An example is the mutation described in a patient with ataxia-telangiectasia (ATM) which represents a deletion in an intron-splicing processing element crucial for accurate intron removal (Pagani *et al.,* 2002). The deletion of four nucleotides in this element abolishes a binding site for U1 snRNP and leads to activation of a cryptic exon, thus producing an abnormal mRNA transcript.

Changes in splicing factors can also be phenotypic. The appearance of splicing-associated diseases is often correlated with the plasticity and longevity of the affected cells. For example, mutations in the human homologs of the splicing factor PRP31 or the splicing factor PRPC8 may cause retinitis pigmentosa, a progressive loss of rods and cones, which causes loss of over 90% of vision during childhood (McKie *et al.,* 2001; Vithana *et al.,* 2001).

Splice sites are highly degenerate, and additional regulatory sequences within the introns (intronic splicing enhancers) and the exons (exonic splicing enhancers) have to be present for proper exon recognition (Hertel and Maniatis, 1998). These sequences are also short and usually degenerate. They are bound by heterogeneous nuclear ribonucleoproteins, serine–arginine-rich (SR) proteins, and SR-like proteins. SR proteins are characterized by an RNA binding motif and an arginine–serine (i.e., RS)-rich domain located at the carboxyl terminus (Tacke and Manley, 1999; Graveley, 2000). This two-domain structure allows binding of SR proteins both to sequence elements on the pre-mRNA and to other components of the spliceosome. As a result, multimolecular complexes that interact with pre-mRNA at multiple sites are formed, allowing the recognition and bringing together of distant sequences in the excision-splicing event (Dreyfuss *et al.,* 2002).

SR protein binding sequences are frequently located in coding exons; therefore, their degeneration and the degeneration of the genetic code allows the flexibility needed for compatibility with the coding requirements of the gene product. The binding of SR proteins to their degenerate recognition sequences is intrinsically weak, resulting in a concentration-dependent regulation, for example, certain sequence elements are recognized only at higher SR protein concentration (Manley and Tacke, 1996). In addition to SR proteins, elements on the pre-mRNA bind to a diverse group of about 30 proteins, which are operationally defined as components of hnRNP complexes.

hnRNPs contain RNA binding motifs as well as several auxiliary domains, allowing those proteins to simultaneously bind to pre-mRNA and other proteins (Weighardt *et al.,* 1996; Krecic and Swanson, 1999). Both SR proteins and hnRNPs are present in tissue characteristic concentrations (Kamma *et al.,* 1995; Hanamura *et al.,* 1998). SR proteins can be stored and released from cellular storage compartments as speckles, through phosphorylation (Misteli *et al.,* 1998). As a result, the proper splice sites are recognized with a high degree of fidelity in an often tissue-specific manner.

An increasing number of alterations, both in normal and in alternative splicing, are being linked to defects in enhancer/silencer sequences (Cooper and Mattox, 1997; Philips and Cooper, 2000; Stoss *et al.,* 2000; Cartegni *et al.,* 2002). Several disease-relevant mutations are compiled in Table 1. As can be seen there, a number of these mutations are present in an exon, but do not change the protein sequence. Because they cause aberrant splice site selection, they can cause a disease, although they do not modify mRNA translation. Examples of these mutations include a T to C (L284L) mutation in tau exon 10 that disrupts an exonic splicing enhancer, causing frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (D'Souza *et al.,* 1999), a C to G (R28R) mutation in porphobilinogen deaminase that results in exon 3 skipping, and which causes porphyria (Llewellyn *et al.,* 1996) and a A to G mutation in pyruvate dehydrogenase resulting in Leigh's encephalomyelopathy (De Meirleir *et al.,* 1994).

Table 1. Mutations in Exonic Regulatory Sequences That Cause Disease

Gene/disorder	*Mutation*	*Effect*	*Reference*
SMN2 Spinal muscle atrophy (SMA)	Silent C>T conversion in exon 7	Disrupts ESE, skipping of exon 7	(Coovert *et al.*, 1997; Jablonka *et al.*, 2000; Lefebvre *et al.*, 1995, 1997; Lorson and Androphy, 2000; Lorson *et al.*, 1999; Monani *et al.*, 2000; Vitali *et al.*, 1999)
SMN1 Spinal muscle atrophy (SMA)	425del5 W102X	Skipping of exon 3	(Sossi *et al.*, 2001)
Beta-hexaminidase Beta-subunit Sandhof disease	Exon 11 P417L C>T conversion at nucleotide 8 Intron 10 A>G conversion at position −17	Disrupts an ESE. Causes use of a cryptic splice site at nucleotide +112 Causes usage of a cryptic splice site at position −37. Disrupts ISE. The conversion also disrupts a putative branch point	(Fujimaru *et al.*, 1998)
Tau Frontotemporal Dementia with Parkinsonism linked to chromosome 17	Intron 10: +13 A>G, +14 C>T, +16 C>T, IVS10+3 G>A Exon 10: L284L T>C, S305S T>C, S305N G>A Exon 10: N297K T>G, del 280K (AAG deletion)	Disrupt ISE IVS10+3 G>A improves slightly the splice site score (from 6.8 to 7.0) Disrupt ESE S305N G>A improves the splice site Disrupts ESE	(D'Souza *et al.*, 1999; D'Souza and Schellenberg, 2000; Hasegawa *et al.*, 1999; Hutton *et al.*, 1998; Iijima *et al.*, 1999; Spillantini *et al.*, 1998; Stanford *et al.*, 2000)
Porphobilinogen deaminase Acute intermittent porphyria	R28R, C>G	Skipping of exon 3	(Llewellyn *et al.*, 1996)
Integrin GPIIIa Glanzmann thrombasthenia	C>A at position +16 and silent G>A at +134 of exon 9	Skipping of exon 9	(Jin *et al.*, 1996)
Fumarylacetoacetate hydrolase Hereditary tyrosinemia type 1	N232N C>T	Skipping of exon 8	(Ploos van Amstel *et al.*, 1996)
Pyruvate dehydrogenase E1 alpha Leigh's encephalomyelopathy	Silent A>G	Aberrant splicing of exon 6	(De Meirleir *et al.*, 1994)
MNK Menkes disease	Gly>Arg G>A	Skipping of exon 8	(Das *et al.*, 1994)
Adenosine deaminase Severe combined immunodeficiency disease	R142X G>A and C>T in the same codon	Skipping of exon 5	(Santisteban *et al.*, 1995)

TABLE 1. MUTATIONS IN EXONIC REGULATORY SEQUENCES THAT CAUSE DISEASE (CONT'D)

Gene/disorder	*Mutation*	*Effect*	*Reference*
Arylsulfatase A Metachromatic leukodystrophy	Thr409Ile C>T	Activates a cryptic splice site	(Hasegawa *et al.*, 1994)
Fibrillin-1 Marfan syndrome	Silent C>T in exon 51	Skipping of exon 51	(Liu *et al.*, 1997)
CYP 27 Cerebrotenidinous xanthomatosis	Silent G112G G>T in exon 2	Creates a cryptic splice site. In addition it causes skipping of the entire exon 2	(Chen *et al.*, 1998)
CD45 Individuals do not suffer from obvious immunodeficiency, but it is notable that no homozygotes have been described. Associated with Multiple sclerosis in the American population.	Silent C>G at position 77 of the alternative exon 4	Disrupts an ESE. Constitutive inclusion of exon 4	(Jacobsen *et al.*, 2000; Lynch and Weiss, 2001; Zilch *et al.*, 1998)
Beta-globin Beta-Thalassemia	T->G transversion in position 705 of intron 2	Activates cryptic 3′ splice site in intron 2	(Dobkin and Bank, 1983; Dobkin *et al.*, 1983)
BRCA1 Breast and ovarian cancer	E1694X G>T	Disrupts ESE and causes skipping of exon 18	(Liu *et al.*, 2001)
NF-1 Neurofibromatosis type 1	Y2264X C>A, C>G R304X C>T Q756X C>T	Skipping of exon 37 Skipping of exon 7 Skipping of exon 14	(Ars *et al.*, 2000a, 2000b; Hoffmeyer *et al.*, 1998; Messiaen *et al.*, 1997)
DMPK Myotonic dystrophy	Expanded (CUG)>40 in the 3′ UTR of DMPK	Two possibilities: CUG repeats sequestrate CUG-BP and therefore prevent it from its normal function; or expanded CUG repeats alter the alternative splicing of the DMPK-mRNA by developing a new 3′ splice site.	(Phillips *et al.*, 1998; Tiscornia and Mahadevan, 2000)

The affected gene (bold) and the disease is listed in the first column, the mutation in the second column. The effect on pre-mRNA splicing and the assumed mechanism are listed in the third column.

Alternative splicing. In mammals, including humans most genes generate several mRNAs from a single gene by involvement of more than one pattern of excision-splicing choices, a process called alternative splicing (Graveley, 2001). For example, it has been estimated that 59% of all human genes on chromosome 22 are alternatively spliced. Because this number is based on comparison of expressed sequence tags (ESTs) with genomic data, the real number is likely to be higher, as low abundant mRNAs are not taken into account, and ESTs are biased towards the mRNA 3′ ends (Lander *et al.,* 2001). It is likely that the fine-tuned balance between SR proteins, hnRNPs, splice sites, and enhancer/silencer elements can be modulated to achieve a change in pre-mRNA exon usage (Grabowski, 1998; Hastings and Krainer, 2001). The levels of SR proteins and hnRNPs vary among tissues (Hanamura *et al.,* 1998), and can be further modulated by releasing SR proteins from intranuclear storage compartments, such as speckles, through protein phosphorylation (Misteli *et al.,* 1998). Alternative splicing is often tightly regulated in a cell type- and/or development-specific manner; cells from the immune and the nervous systems use this mechanism most abundantly (Grabowski and Black, 2001; Lander *et al.,* 2001).

The phenotype of most neurodegenerative diseases manifests itself later in life, and some missplicing events are causally related to such diseases. Mutations in tau exon 10 that are implicated in FTDP-17, are good examples of such a complex phe-

notype. Frontotemporal dementias represent a rare form of presenile dementia, and are clinically defined by behavioral and personality changes, psychomotor stereotypes, as well as loss of judgment and insight. The neuropathologic findings include asymmetric frontotemporal atrophy and the presence of filamentous tau deposits. FTDP-17 was mapped to the tau locus on chromosome 17, and at least 50 kindreds are affected world wide (Hutton *et al.,* 1998). Tau transcripts undergo complex regulated splicing in the mammalian nervous system. The alternative splicing of tau's exon 10 is species specific: this exon is excised in adult humans, but in the adult rodent brain, it is used constitutively. Exon 10 encodes one of the four microtubule binding sites of tau. In the normal brain, tau isoforms containing and lacking exon 10 sequences are balanced. A disruption of the proper distribution of tau isoforms is observed in the pathology of several tauopathies. In FTDP-17, sporadic corticobasal degeneration and sporadic progressive supranuclear palsy, tau proteins containing exon 10 sequences are overproduced, whereas in sporadic Pick's disease tau proteins lacking exon 10 sequences are predominant (Goedert *et al.,* 1999; Spillantini and Goedert, 2001).

Mapping of elements in tau exon 10 has revealed a potential stem-loop structure at the 5′ splice site, which may be involved in regulation of exon 10 splicing (Hutton *et al.,* 1998). In addition, a complicated set of exonic enhancer elements was identified (D'Souza and Schellenberg, 2000; Gao *et al.,* 2000). The disruption of these elements by mutation apparently disturbs the balance between the different tau isoforms and causes the aggregation of improperly spliced gene products associated with neurodegeneration.

Misdirected regulation of alternative splicing is observed in several additional neurologic disorders. One example includes schizophrenia. The alternative splicing of the long (L) and short (S) gamma2 subunit mRNAs of the gamma-amino butyrate type A (GABA-A) receptor was shown to be modified in the prefrontal cortex of schizophrenic patients. The reduction in gamma2S and the increase in gamma2L mRNAs were suggested to result in less active GABA-A receptors with severe consequences for cortical integrative function (Huntsman *et al.,* 1998). Postmortem brain investigations revealed alternative splicing of *N*-methyl-D-aspartate (NMDA) R1 (NRI) carboxy-terminus isoforms in the superior temporal gyrus of schizophrenic patients (Le Corre *et al.,* 2000). Schizophrenia was further shown to be associated with alternative splicing of the neural cell adhesion molecule (N-CAM) mRNAs. Elevated levels of the variable alternative spliced exon (VASE) were found in CSF of schizophrenic patients (Vawter *et al.,* 2000).

3′End processing and nuclear export

Once RNA polymerase II encounters the highly conserved AAUAAA polyadenylation signal, 3′ end processing takes place (Dreyfuss *et al.,* 2002). A minimum of six factors are required for 3′ end formation: cleavage and polyadenylation factors (CPSF), cleavage stimulation factor (CSF), cleavage factors I and II, poly(A) polymerase (PAP), and poly(A) binding protein. These factors recognize the AAUAAA signal and a downstream element and then perform cleavage of the RNA at its 3′ end as well as its polyadenylation.

Thalassemia is one of the world's most common hereditary diseases. Common forms of both alpha- and beta-thalassemia are both associated with point mutations within the polyadenylation signals of alpha-globin and beta-globin genes, respectively (Higgs *et al.,* 1983; Orkin *et al.,* 1985), leading to the generation of abnormal hemoglobin. Patients suffer from moderate anemia with microcytosis and hypochromia.

A guanosine to adenosine mutation in the 3′ end of the prothrombin gene is a common cause for thromboembolic events. This mutation is positioned at the site where the pre-mRNA is cleaved during processing and then polyadenylated. The mutation leads to more efficient cleavage of the pre-mRNA, and consequently, to elevated amounts of prothrombin mRNA and an increase of the blood prothrombin concentration (Gehring *et al.,* 2001). The mutation is common, with an allele frequency of 1.2%, and may represent an advantage in young age by providing better blood clotting, for example, during childbirth (Poort *et al.,* 1996). Another example for misregulation of 3′ end formation is autosomal dominant oculopharyngeal muscular dystrophy (OPMD), an adult-onset disease caused by a (GCG)8–13 repeat expansion in the polyadenylation binding protein 2 (PABP2) gene. Patients suffer from progressive dysphagia, eyelid ptosis, and proximal limb weakness, and its pathologic hallmark is the accumulation of intranuclear inclusions in muscle fibers due to abnormal mRNA processing (Brais *et al.,* 1998). The export of mRNAs into the cytosol is mediated by nuclear export factors (NXFs) believed to be the molecular link between the hnRNP complexes and the nuclear pore complex (Herold *et al.,* 2000). Loss of the NXF5 protein, which is most likely a novel nuclear RNA export factor, results in X-linked mental retardation, but the molecular details remain to be worked out (J. Lin *et al.,* 2001).

Nonsense mutations, exon skipping, and mRNA degradation

Premature stop codons may lead to an alternatively spliced variant that skips the mutated exon (Dietz *et al.,* 1993), a phenomenon referred to as nonsense-mediated altered splicing. Nonsense-mediated altered splicing was thought to require nuclear recognition by the spliceosome of "cytosolic" translational signals but recent research on the *BRCA1* gene suggests it can result from disruption of a splicing enhancer located in the coding sequence (H.X. Liu *et al.,* 2001). A related mechanism is nonsense mediated decay (NMD), also known as RNA surveillance. In both cases, the nascent mRNA is screened for nonsense mutations. NMD takes place if the distance between a stop codon and the downstream exon–exon junction is larger than 50–55 nt (Hentze and Kulozik, 1999; Maquat and Carmichael, 2001). Such stop codons are considered premature, and NMD leads to decapping and degradation of the mRNA. Because about 25% of all alternatively spliced exons introduce alternative stop codons (Stamm *et al.,* 2000), NMD together with alternative splicing is a common mechanism to downregulate gene expression. The location of exon–exon junctions is memorized in the cytosol by binding of the Y14 protein to the junction (Kim *et al.,* 2001; Kim and Dreyfus, 2001). Messenger RNA stability is also decreased by adenylate, uridine-rich instability elements (ARE) that accelerate mRNA deadenylation. AREs are usually located in the 3′UTR and bind to hnRNPs of the HuR/HuA family (Brennan and Steitz, 2001).

Chain termination mutations are frequent causes for disease in humans. For example, 89% of mutations in the ATM gene that causes ataxia-telangiectasia and 77% of mutations in BRCA1 give rise to premature stop codons (Couch and Weber, 1996; Gilad *et al.,* 1996). The mRNAs with premature stop codons rarely produce truncated proteins but are rather subject to nonsense-mediated decay. Typically, these mutations result in a loss-of-function phenotype. The involvement of NMD in beta-thalassemia, gyrate dystrophy, and Marfan syndrome has been recently reviewed (Frischmeyer and Dietz, 1999). A change in RNA stability is often associated with cancer, due to stabilization of mRNAs that promote tumor progression, such as immunosuppressive cytokines and angionogenic growth factors. Interestingly, HuR, a factor that stabilizes mRNAs by binding to AREs is constitutively expressed in brain tumors, and its expression pattern correlates with the grade of malignancy (Nabors *et al.,* 2001).

DEFECTS IN PRE-mRNA PROCESSING THAT PREDISPOSE TO A DISEASE

Stress-induced alterations

A number of mutations do not show a phenotype directly, but rather remain silent, until some external stimulus allows the mutation to become apparent in certain cell types or developmental and aging phases. This implies that diagnostic and therapeutic approaches might be developed to anticipate, and prevent the damage caused by such defects. The consequences of stress present an example of such a circumstance.

In recent years, a growing body of evidence links alternative splicing adaptation to stressful internal and external changes in cells and tissues. The term stress, adapted from physics, spans physical, chemical, and psychologic pressures that are potentially detrimental and sharing common features (McEwen, 1999). The cellular signaling pathways affecting alternative splicing under modified stimuli are poorly understood, although recent efforts by several investigators yielded interesting findings. Activation of the $MKK_{3/6}$-p38 cascade was shown to modify the subcellular distribution of hnRNP A1, resulting in modified alternative splicing (van der Houven van Oordt *et al.,* 2000). The ability of hnRNP K to silence mRNA translation also requires relocalization to the cytoplasm, which is dependent on the stress-activated ERK MAP–kinase pathway (Habelhah *et al.,* 2001). The ERK/MAP–kinase pathway was shown to be involved, upon activation of T cell lymphocytes, in the alternative splicing of CD44 by retaining exon v5 sequence in the mature mRNA (Weg-Remers *et al.,* 2001). Intriguingly, some of the evoked changes in alternative splicing produce transcripts that have antagonistic cellular actions. For example, heat-shock factor 4 generates by alternative splicing both an activator and a repressor of downstream heat-shock genes (Tanabe *et al.,* 1999). Also, alternative splicing of the apoptotic gene, *bcl-x,* substitutes a large protein product, Bcl-xL, which inhibits cell death, for a smaller one, Bcl-xS, which antagonizes this ability under certain conditions (Boise *et al.,* 1993). Neuronal activity-dependent alternative splicing was shown for the rat homolog of the human splicing regulator transformer 2 gene, *hTra2-beta,* in the rat brain (Daoud *et al.,* 1999).

Stress-induced changes in alternative splicing have been demonstrated for the transcripts of a variety of genes, including those for heat-shock factors 1 (Goodson *et al.,* 1995) and 4 (Tanabe *et al.,* 1999), the apoptotic gene *bcl-x* (Boise *et al.,* 1993), genes for several Jun N-terminal kinases (JNKs) involved in regulating transcription factors (Holland *et al.,* 1997; Y. Zhang *et al.,* 1998) and the human genes for the ATF family of transcription factors (Goetz *et al.,* 1996). A change in alternative splicing following stress was demonstrated, for example, for the glucocorticoid receptor (GR). Perinatal manipulations and postnatal handling were shown to selectively elevate GR mRNA containing a hippocampus-specific exon, which facilitates adaptation to the conditions of stress. Prenatal glucocorticoid exposure, in turn, increased hepatic GR expression by producing a minor exon 1 variant (McCormick *et al.,* 2000).

Glucocorticoids also regulate the splicing pattern of the murine *Slo* gene encoding a brain K^+-channel, which depends on neuronal depolarization (Xie and McCobb, 1998). Hypoxia was shown to induce the alternative splicing of the presenilin-2 gene, generating an isoform also found extensively in the brain of Alzheimer's disease patients (Sato *et al.,* 1999). A detailed study of SR proteins under ischemic conditions in the brain revealed that some of them translocate from the nucleus to the cytosol after an ischemic event, which could explain the observed changes in splice site selection (Daoud *et al.,* 2002). Although splice site selection can serve as a physiologic adaptation to a change in the external conditions, the above examples show that these changes may also contribute to pathophysiologic events. One extensively studied example is a change in the acetylcholinesterase (AChE) pre-mRNA processing (Soreq and Seidman, 2001). Here, various external stimuli were shown to induce rapid, long-lasting changes of neuronal AChE pre-mRNA splicing. These include psychologic stress (Kaufer *et al.,* 1998), environmental stimuli (anti-AChE intoxication) (Shapira *et al.,* 2000), head injury (Shohami *et al.,* 2000), and inherited AChE overexpression (Meshorer *et al.,* 2002). Although most of the currently available evidence on this splicing shift refers to animal studies, a recent screen demonstrated accumulation of the stress-induced AChE-R variant in the cerebrospinal fluid (CSF) of patients with stress indices, indicating that these pathologic changes also occur in humans (Tomkins *et al.,* 2001).

Tumorigenesis

Many cases of different cancers are also associated with changes in the splicing pattern of various genes. For example, 43% of neurofibromatosis type 1 (NF1) defects are caused by aberrantly spliced pre-mRNA (Ars *et al.,* 2000b). Furthermore, the splicing pattern of the adhesion molecule CD44 changes during tumorigenesis (Stickeler *et al.,* 1999) and melanomas were found to be associated with elevated levels of the delta1b and deta1d alternatively spliced transcripts of the tyrosinase gene compared to normal skin melanocytes (LeFur *et al.,* 1997). Different splice variants of the estrogen receptor (ER) (e.g., skipping of exons 2, 3, 4, 5, or 7 (Q.X. Zhang *et al.,* 1996)) were found to be associated with human breast cancer. Resistant acute myeloid leukemia was shown to be associated with high incidence of alternatively spliced forms of deoxycytidine

kinase, which could contribute to the process of cytarabine resistance in these patients (Veuger *et al.,* 2000). It was shown that SR proteins display tumor stage-specific changes in mammary tumorigenesis (Stickeler *et al.,* 1999), which suggests that concentration alterations of these regulatory proteins could orchestrate the use of different splice sites in tumors.

Pathologies associated with changes in alternative splicing patterns without specific mutations in splice sites may reflect defects in an upstream splicing modifier. Differential expression of hnRNPs, snRNPs, SR, and SR-like proteins could, in principle, be the cause for such pathologies. For example, positional cloning revealed segregation with prostate cancer of *ELAC2,* which displays sequence similarity to the 73-kD a subunit of mRNA 3′ end cleavage and polyadenylation specificity factor (CPSF73) (Tavtigian *et al.,* 2001), suggesting mRNA processing abnormalities in prostate cancer.

OUTLOOK FOR THERAPY

A general theme in pre-mRNA processing is a high fidelity and specificity achieved by combinatorial control. This is exemplified in the redundancy of the relevant sequences, the modular composition of the corresponding proteins, and the cascade pattern of the involved processes. The examples compiled above show that numerous defects in pre-mRNA processing contribute to human diseases. This, in turn, calls for the development of diagnostic and therapeutic means targeted at pre-mRNA processing. Different approaches have been tested in various models of diseases that are summarised in Table 2.

Antisense oligonucleotides

The danger of nonspecificity in targeting pre-mRNA processing can be avoided by design of selective agents, such as oligonucleotides (Mercatante and Kole, 2000) (Fig. 1A). Beta-thalassemia in human erythroid cells may be treated with modified oligonucleotides binding to mutated splice sites, showing that this approach is feasible (Lacerra *et al.,* 2000). Antisense oligoribonucleotides targeted against an aberrant 5′ splice site were also used to partially reverse the aberrant splicing of beta-globin mRNA in beta-thalassemia/HbE disease cells in culture (Shirohzu *et al.,* 2000). In cultured cells, targeting of splice sites by antisense oligonucleotides was shown to reverse the inclusion of tau exon 10 in a model of FTDP-17 (Kalbfuss *et al.,* 2001) and to increase the inclusion of SMN exon 7, associated with spinal muscular atrophy (Lim and Hertel, 2001). 2′-*O*-Methylated antisense oligoribonucleotides were used to modify the splicing pattern of the dystrophin pre-mRNA in the mdx mouse model of Duchenne muscular dystrophy (DMD). DMD is a severe muscle disease caused by defects within the dystrophin gene. Its milder version, Becker muscular dystrophy, is caused by in-frame deletions that generate a shorter but minimally functional dystrophin protein. This suggested removal of the deficient part in the mutated dystrophin as a therapeutic approach. The oligoribonucleotides blocked binding sites involved in normal dystrophin pre-mRNA splicing, inducing excision of exon 23 with the mdx nonsense mutation, without disrupting the reading frame (Mann *et al.,* 2001).

Finally, the detrimental accumulation of the stress-induced AChE-R mRNA can be prevented by antisense oligonucleotides,

Table 2. Overview of the Therapeutic Strategies

Drug/substance	*Gene/disease tested*	*Reference*
Antisense oligonucleotides		
Modified oligonucleotides	β-thalassemia	(Shirohzu *et al.*, 2000)
	FTDP-17	(Kalbfuss *et al.*, 2001)
	SMN2/Spinal muscular athrophy	(Lim and Hertel, 2001)
	Dystrophin/Duchenne muscular dystrophy	(Mann *et al.*, 2001)
	AChE-R	(Shohami *et al.*, 2000; Lev-Lehman *et al.*, 2000)
RNAi		
RNA oligonucleotide	Multiple	(Cellotto and Graveley, 2002)
Ribozymes		
Ribozyme	Mutant β-globin	(Lan *et al.*, 1998)
	p53 mRNA *trans*-splicing	(Watanabe and Sullenger, 2000)
SMaRT		
Targeting RNA	CFTR exon 10	(Mansfield *et al.*, 2000)
Low molecular weight drugs		
Neomycin	FTDP-17	(Varani *et al.*, 2000)
Aclarubicin	SMN2/spinal muscular athrophy	(Andreassi *et al.*, 2001)
Sodium butyrate	SMN2/spinal muscular athrophy	(Chang *et al.*, 2001)
Expression of trans-acting factors		
tra2-β1	SMN2/spinal muscular athrophy	(Hofmann *et al.*, 2000)
clk-1	tau/FTDP-17	(Hartmann *et al.*, 2001)

The principal action is shown in a gray box. Drugs and substances used are in the first column, genes and diseases tested are listed in the second column.

with potentially promising prospects for the treatment of head injury (Shohami *et al.,* 2000) and exposure to organophosphate acetylcholinesterase inhibitors (Lev-Lehman *et al.,* 2000).

RNAi

The most recent and yet mechanistically illusive phenomenon that may lead to future therapeutic technologies is RNA interference (RNAi). Found by serendipity, RNAi acts to cause target-specific gene silencing by destabilizing cellular RNA (Carthew, 2001; Zamore, 2001). Intriguingly, RNAi was demonstrated to be effective *in vivo* in *Caenorhadditis elegans* when the nematodes digested engineered bacteria that expressed double-stranded RNA of the *C. elegans* unc-22 gene. These nematodes developed similar phenotypes of unc-22 mutants (Timmons and Fire, 1998). RNAi was shown to suppress gene-expression levels in mammalian cells using either short (e.g., 22-mer) double-stranded RNAs (Elbashir *et al.,* 2001), or long (e.g., 500-mer) dsRNAs (Paddison *et al.,* 2002), as well as stable "knock-down" of genes using long hairpin dsRNA (Paddison *et al.,* 2002). Recently, RNAi was used to selectively degrade alternatively spliced mRNA isoforms in *Drosophila* by treating cultured cells with dsRNA corresponding to an alternatively spliced exon (Celotto and Graveley, 2002). This powerful tool may come to occupy an important role in future gene therapy (Fig. 1B).

Ribozymes

Ribozymes are RNAs that catalyze a limited number of reactions in cells, especially cleavage of other nucleic acids (Lewin and Hauswirth, 2001). The ribozymes under development for therapy are based on small naturally occurring RNAs, such as the hammerhead and the hairpin, derived from plant virus satellite RNA, the tRNA processing ribonuclease P (RNase P), and group I and group II ribozymes, which occur as introns in organelles and bacteria but can be engineered to act in *trans* on RNA or DNA. Group I introns have been used to repair defective mRNAs by *trans*-splicing, for example, to replace defective p53 mRNA with the wild type, resulting in close to full restoration of the wt p53 activity (Watanabe and Sullenger, 2000). A *trans*-splicing group I ribozyme was also shown to be a useful candidate for altering the mutant beta-globin transcripts in erythrocyte precursors derived from peripheral blood of sickle cell disease subjects. Sickling beta-globin, the beta-chain of HbS, transcripts were converted into messenger RNAs that encode the nonsickling beta-globin (Lan *et al.,* 1998).

SMaRT

Another novel approach for gene therapy is Spliceosome-Mediated RNA *Trans*-splicing (SMaRT) (Puttaraju *et al.,* 1999). SMaRT is an emerging technology in which RNA molecules are designed to code a specific pre-mRNA by utilizing *trans*-splicing reaction between the introduced RNA and its pre-mRNA target (Fig. 1C). During the splicing reaction, a part of the pre-mRNA is first excised in a concerted reaction, followed by the remaining exons being ligated. This reaction occurs normally in *cis*, for example, on a single pre-mRNA molecule that is transiently attached to the spliceosome. However, splicing can also take place between two independently transcribed sequences, a process called *trans*-splicing that is found in trypanosomes, nematodes, flatworms, and plant mitochondria. Because the mechanisms of *trans*- and *cis*-splicing are similar, it is possible to generate RNA molecules that would be processed by the spliceosome in a *trans*-splicing reaction, which can be used to repair a defective pre-mRNA molecule by exchanging parts of it (Puttaraju *et al.,* 1999).

SMaRT was demonstrated successfully using plasmids expressing mutant CFTR minigenes. When 293T cells were co-transfected with both the mutated and the normal constructs, they produced a *trans*-spliced mRNA in which the mutant exon 10 was replaced by a normal one. This *trans*-splicing reaction was further shown to produce mature CFTR protein (Mansfield *et al.,* 2000).

Low molecular weight drugs

The use of RNA-biding antibiotics such as gentamicin, chloramphenicol, and tetracycline clearly shows that RNA and/or RNA interacting proteins can be targeted by drugs (Varani *et al.,* 2000; Xavier *et al.,* 2000). Targeting pre-mRNA processing pathways could therefore be a new therapeutic approach. Aclarubicin, an anticancer drug, was shown to change the alternative splicing pattern of SMN2 in cells derived from spinal muscular atrophy patients. In this disease, the SMN1 gene is deleted, and an almost identical human gene, SMN2, cannot compensate for the loss, because it is differently spliced. Aclarubicin treatment can reverse this wrong splicing pattern, which results in formation of full length SMN protein. Although it is yet unknown how aclarubicin changes alternative splicing (Andreassi *et al.,* 2001), this is a promising example of pre-mRNA therapeutics (Fig. 1D).

Indirect effects

The combinatorial regulation of pre-mRNA processing may also result in indirect effects that have to be considered for any therapeutic approach. This is illustrated by the change in alternative splicing patterns of human cardiac troponin T in patients with myotonic distrophy. Myotonic dystrophy is caused by a CUG repeat extension in the 3′ UTR of the cAMP-dependent protein kinase gene (Korade-Mirnics *et al.,* 1998). This repeat extension causes a sequestration of a CUG-binding protein, which results in a change of cardiac troponin T pre-mRNA splicing patterns (Philips *et al.,* 1998; Lu *et al.,* 1999). Increased expression of the CUG-binding protein in skeletal muscle tissue of myotonic dystrophy patients further results in aberrant alternative splicing of the insulin receptor pre-mRNA, with predominant expression of the nonmuscle isoform (Savkur *et al.,* 2001). Finally, the splicing pattern of mutated alleles, for example in the cystic fibrosis CFTR gene, strongly depends on the cell type (Rave-Harel *et al.,* 1997), explaining why natural mutations frequently have a tissue- or cell type-specific effect [e.g., male sterility due to impaired testicular splicing of the CFTR transcripts (Kerem and Kerem, 1996]). A possible explanation would be a cell type-specific set of regulatory factors. Skipping of CFTR exon 9 was shown to depend on expression of a factor binding a polymorphic repeated sequence in its 3′ splice site, TDP-43 (Buratti *et al.,* 2001). However, this

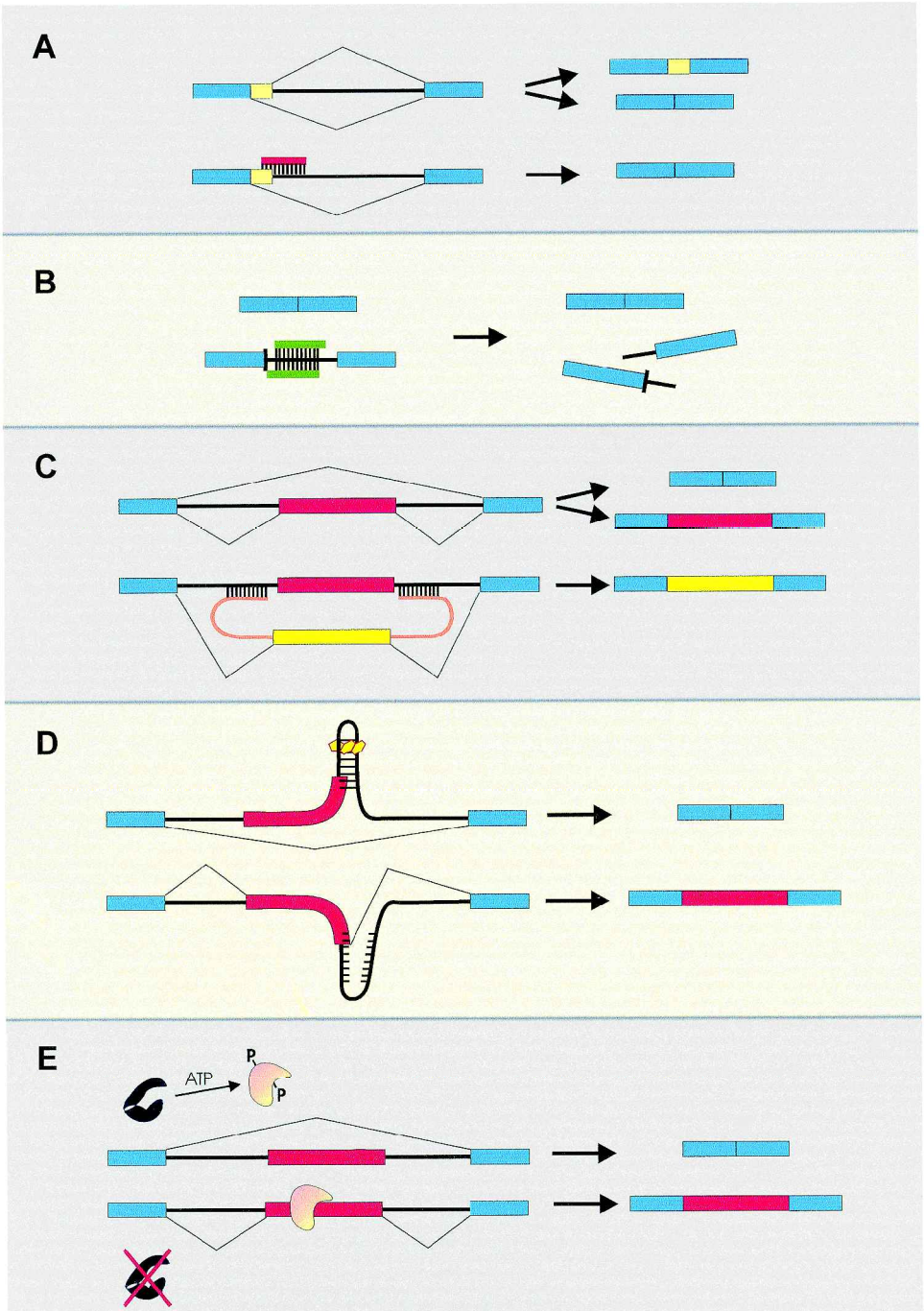

FIG. 1. Principles of different therapeutic strategies. Exons are indicated as boxes, introns as thick lines. Different coloring indicates alternative exon usage. Splicing patterns are shown as thin lines. Small molecular weight substances are shown as small yellow polygons. Base pairing is indicated by thin lines. (**A**) Antisense oligonucleotides (thick red line) can bind to a splice site and prevent its usage (left), which favors exon skipping (right). (**B**) Misspliced mRNAs can be eliminated by RNAi using specific siRNAs (green lines). As a result, the undesired splice product is eliminated (right). (**C**) Undesired exons (red) can be replaced using SMaRT (yellow) constructs, which results in the exchange of an exon in a *trans*-splicing reaction (right). (**D**) Small molecular weight molecules (yellow) can change splicing patterns, for example, by stabilization of secondary pre-mRNA structures. (**E**) Phosphorylation of splicing regulatory proteins changes their interaction with RNA or other proteins and can result in an altered splicing product in the presence (top) or absence (bottom) of a kinase activity (black enzyme).

effect also depends on the simultaneous expression of SR proteins. It is clear that this combinatorial control will impact on every therapeutic intervention; therefore, the effects of existing drugs on pre-mRNA processing of yet unknown gene products, in addition to their target molecules, should be taken into consideration. Nevertheless, the importance of such processes points to the RNA transcripts themselves, as well as their cognate proteins, as novel targets for therapeutic intervention.

Trans-Acting factors

Spinal muscular atrophy illustrates how the knowledge of the molecular mechanisms regulating pre-mRNA processing allows the development of rational therapies. The disease is caused by the loss of the Survival of Motor Neuron gene 1 (*SMN1*) and subsequent loss of the SMN protein. As noted above, a nearly identical gene, *SMN2*, gives rise predominantly to a shorter mRNA form, producing a modified SMN protein. Therefore, SMN2 cannot compensate for the absence of SMN protein. The loss of the *SMN1* gene can, however, be compensated in cultured cells by manipulating the splicing pattern of *SMN2*, through increasing the amount of a regulatory factor, tra2-beta1 that binds to a purine-rich enhancer of the alternative exon (Hofmann *et al.,* 2000). Several SR proteins bind to this enhancer, and their ratio can also be changed by sodium butyrate. Administration of this drug to lymphocytes of spinal muscular atrophy patients results in an altered splicing pattern of *SMN2*, which could also compensate for the loss of *SMN1* (Chang *et al.,* 2001).

In FTDP-17 mutations, splicing of tau exon 10 is mis-regulated through complex changes in the composition of several splicing enhancers (D'Souza and Schellenberg, 2000). These mutations can be overcome by expressing cdc2 like kinases (clk1-4) (Nayler *et al.,* 1997). These kinases phosphorylate SR proteins and alter tau exon 10 splicing, most likely by changing the phosphorylation-dependent composition of the enhancer complex (Hartmann *et al.,* 2001; Fig. 1E). Low molecular weight drugs activators of clk1-4 kinases, when identified, may be therapeutically beneficial for this devastating disease.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB473, Sta399/5-1, Sto456/1-1 and Sta399/7-1, to S.S.) and the U.S. Army Medical Research and Materiel Command (DAMD 17-99-1-9547, to H.S.).

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Received for publication April 9, 2002; received in revised form July 20, 2002; accepted July 31, 2002.