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Alternative Splicing and Disease

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Preface

Splicing of the primary RNA transcript, i.e., removal of introns and joining of exons to produce mature mRNAs competent for translation into proteins, is a quasi-systematic step of gene expression in higher eucaryotes. The full biological significance of splicing has not been appreciated until it was discovered that alternative splicing, that process which allows a primary transcript to yield several distinct mRNAs and consequently several different proteins, is a widespread mechanism. Although we had already recently published in this same series (2003, Volume 31 Ph. Jeanteur, ed.) a book dedicated to the regulation of alternative splicing, we thought it timely to follow up in this direction by focusing on pathological and therapeutic aspects.

Interestingly, estimates of the proportion of human genes that can undergo at least one alternative splicing event increased from about 50%, as mentioned in the preface to this previous book, to 75% in most recent ones, suggesting that alternative splicing is the rule rather than the exception for mammalian genes. An obvious benefit is the creation of protein diversity but there is another side to the coin. The flexibility required for the large repertoire of potential alternative splice sites that goes with a high level of sequence degeneracy at splice junctions has two important consequences.

The first one is that point mutations, or even "neutral" polymorphisms, can be sufficient to abrogate weak alternative sites or, conversely, to create new ones with pathological consequences. It should be noted that most current state-of-the-art transcriptomics is limited to a global quantitative assessment of mRNA sequences originating from a given transcription unit and overlooks the qualitative diversity generated by alternative splicing. Many ongoing studies now address this issue to provide the basic knowledge of alternative splicing events essential for complex multigenic diseases like cancer. The paper by Bracco et al. describes methods and core facilities for quantitation of splice variants expression while that by Novoyatleva et al. examines the generality of missplicing as a cause of human disease.

The second one is that additional information is required to define splice sites to be used among a wealth of cryptic ones, especially in very large introns. This information is provided by regulatory sequences recognized by protein factors. Both these types of elements define potential therapeutic targets: anti-sense strategies are an obvious approach to target RNA sequences in the pre-messenger that has been examined for quite some time already. A detailed account of this type of strategy is given by Garcia-Blanco. In contrast, targeting protein factors by small chemicals is much more novel and is specifically addressed in the chapter by Soret, Gabut, and Tazi.

Targeting human monogenic hereditary diseases offers a wealth of distinct situations where specific therapeutic strategies can be envisioned

mostly at the proof-of-concept stage. The following chapters address, albeit not exhaustively, various pathological situations where splicing aberrations have been well characterized: neurological conditions involving missplicing of genes coding tau proteins (Andreadis), spinal muscular atrophy (Wirth et al.) or myotonic dystrophy (Kuyumcu-Martinez and Cooper), muscular dystrophy (Wilton and Fletcher), premature aging phenotypes (De Sandre-Giovannoli and Levy) of cystic fibrosis (Nissim-Rafinia and Kerem).

Although no chapter is exclusively focused on cancer, the number of gene alterations involved in the development of any single solid tumor is so high and diverse that there are many opportunities for missplicing events. In the face of such a complexity, therapeutic approaches based on redirecting alternative splicing will probably come in the longer term after proof-of-concepts have been obtained in the better-defined and simpler situations offered by the monogenic diseases mentioned above.

Philippe Jeanteur

Contents

Methods and Platforms for the Quantification of Splice Variants' Expression

Laurent Bracco, Emeline Throo, Olivier Cochet, Richard Einstein, Florence Maurier

Abstract. The relatively limited number of human protein encoding genes highlights the importance of the diversity generated at the level of the mRNA transcripts. As alternative RNA splicing plays a key role in mediating this diversity, it becomes critical to develop the tools and platforms that will deliver quantitative information on the specific expression levels associated with splice isoforms. This chapter describes the constraints generated by this global transcriptome analysis and the state-of-the-art techniques and products available to the scientific community.

1 Introduction

Gene profiling assays have become a standard approach to study physiological or pathophysiological processes as genes that are identified as being up- or down-regulated will point to distinct and relevant signaling pathways. Such analyses usually rely on technologies, such as RT-PCR Differential Display, Substractive Hybridization Libraries, SAGE, and pan-genome microarrays that are often described as being able to scan the whole gene expression space. In fact, these technologies are not well-suited to detect and/or quantify isoforms generated by alternative RNA splicing, as they are all based on the 3′ regions of mRNAs. It is now well admitted that alternative splicing is one of the main factors responsible for generating expression diversity at the mRNA level. Large-scale bioinformatics analyses and in-depth transcriptome analysis from selected chromosomes have reported or suggested high rates of alternative splicing, with over 60% of all human genes expressing multiple mRNAs (Lander 2001; Modrek and Lee 2002; Kampa et al. 2004). Alterations of the splicing patterns, by mutation or defects at the level of the spliceosome machinery, can lead to profound cellular deregulations and be the cause of human diseases (Faustino and Cooper 2003; Garcia-Blanco et al. 2004; see also Cooper et al., Stamm et al., Wirth et al., Graveley et al. in this review). *Cis*- or *trans*-acting mutations at the pre-mRNA level can affect RNA splicing and can also alter the ratio between the expression levels of two or more isoforms. Such ratios are thus critical parameters that need to be quantified as some isoforms can exert dominant negative or positive effects. For example, the

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ratio of Bcl-xS/Bcl-xL can determine whether cells undergo apoptosis (Rohrbach et al. 2005), and alteration of this ratio can change the ultimate fate of the cells (Taylor et al. 1999; Akgul et al. 2004). The importance of tau isoforms' ratios in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17; de Silva et al. 2003; Umeda et al. 2004) and of SMN isoforms' ratios in SMA (Feldkotter et al. 2002; Harada et al. 2002) constitute two other well documented examples.

Alternative splicing is also likely to provide the basis for two important medically related applications that will heavily rely on quantitative data. First, as alternative splicing can be deregulated in human diseases, it has the potential to generate novel biomarkers for disease detection and/or screening, prognosis, or treatment outcome. In addition, splice variants' expression ratios could also provide better biomarkers than the mere absolute levels of either variant as it has been demonstrated in the association between acetylcholine esterase splice variants and treatment outcome for Alzheimer's disease patients (Darreh-Shori et al. 2004). It can be anticipated that, as most diseases are complex and heterogeneous, a panel of individual markers will be required to optimize sensitivity and specificity, enhancing the critical issue to properly monitor their expression.

Second, splicing deregulations in human pathologies can point to altered or defective signaling pathways. Traditional pharmacology targeting selected gene products within these highlighted cascades could next be developed to generate, for instance, specific receptor agonists or enzyme inhibitors to somehow "mend" the defective pathway(s).

Novel strategies based on the correction of the deregulated splicing event itself at the RNA level are now being more and more documented. Whether such approaches rely on small molecular weight compounds (Slaugenhaupt et al. 2004; Solier et al. 2004; Soret et al. 2005; Tazi et al. 2005; see also Wirth et al. and Soret et al. in this book), antisense oligonucleotides targeting splice sites or regulatory regions (Sierakowska et al. 1999; Kalbfuss et al. 2001; Bruno et al. 2004; Scaffidi and Misteli 2005; see also Wilton et al in this review), more complex chimeric entities (Suter et al. 1999; Cartegni and Krainer 2003), or through gene therapy vectors (Goyenvalle et al. 2004), their performances will heavily depend upon the availability of appropriate platforms to quantify their efficacies and to adequately monitor their specificities vis-à-vis other splice events.

How relevant is a splice isoform? Has it been generated simply by leakage of the spliceosome machinery? Is it likely to bear a functional impact? The availability of robust and high-throughput tools to scan and quantify the whole transcriptome, including splice variant will clearly empower the scientists to resolve some of these questions. These tools and technologies will be described in this chapter. This review will mainly focus on the technologies available to detect and quantify the expression of known or predicted splice variants at the RNA level. Known or predicted variants

indicate that nucleotide sequence information is available to constitute the basis of the measurement of the expression level. Obviously, if an isoform mRNA gets properly translated, its product can be detected with traditional immunological assays and this will be briefly discussed at the end of this review.

2 General Principles: Specificity is the Key Issue

Alternative splicing produces an extra layer of complexity when dealing with specificity. Not only the selected assay needs to be able to discriminate from other genes that may be homologous, but it needs to discriminate the variant from other isoforms. Indeed, the co-expression of several splice variants in any given biological sample will be the rule more than the exception. This represents a technical challenge as isoforms will share a high degree of homology. The different types of splicing events will dictate the strategy that needs to be developed (Fig. 1). Insertional events such as intron retention (IR), novel alternative exons (NE), or certain uses of 5′ or 3′ cryptic sites (3′ or 5′ ASD/ASA) will generate additional sequence

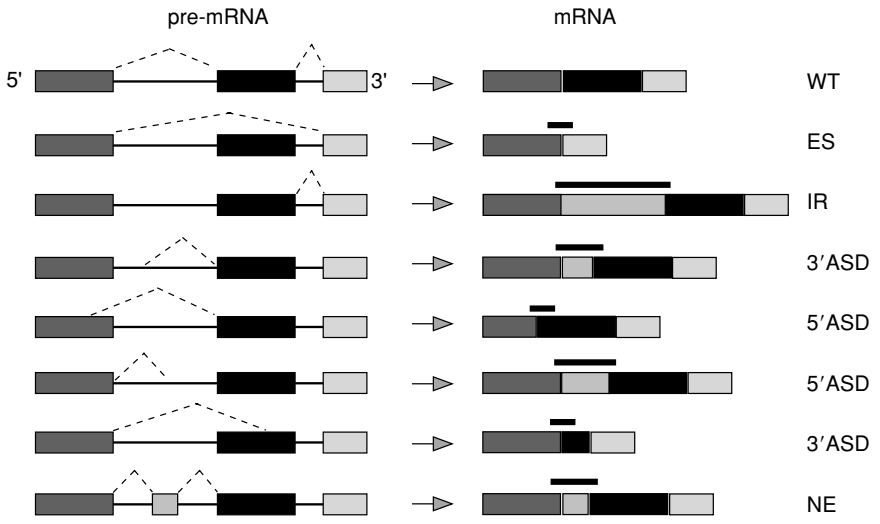

Fig. 1. Types of alternative splicing and effects at the mRNA level. *WT*: wild-type, *ES*: exon skipping, *IR*: intron retention, *ASD*: alternative splice donor (*3′* and *5′*), *ASA*: alternative splice acceptor (*3′* and *5′*), and *NE*: novel exon. The *black bar* above each *mRNA* isoform delineates the specific sequence created by the splicing event. Exonic and junction PCR primers or microarray probes can be designed in those sequences to monitor the alternative isoforms

information as compared to a wild-type form. Such sequences could serve as the basis for the required specificity. Deletion events caused by exon skipping events (ES) or by the remaining uses of cryptic splice sites (3′ or 5′ ASD/ASA) will not provide such novel sequences. In this case, one can only rely on novel junction sequences to discriminate from the wild-type counterpart.

These general principles apply to the techniques that will now be described. They have been classified into two groups, low/medium throughput and high throughput.

3
Low/Medium Throughput Techniques

Until the introduction of PCR, the quantitative analysis of alternative transcripts was limited to technologies such as northern blotting or ribonuclease protection assays, both having severe limitations. Northern blotting is very time-consuming, requires a large amount of RNA, is only suitable for highly expressed transcripts, and may not be able to differentiate close isoforms (Streuli et al. 1987). RNAse protection assay is also a heavy technique, more efficient to monitor small sequence variations but very limiting for low abundant transcript quantification (Saccomanno et al. 1992). The introduction of amplification steps in quantification methods permitted one to overcome the limitations associated with these conventional methods.

3.1
RT-PCR-Based Platforms

Whether they are splice variants or not, the successful analysis of transcripts by qualitative, semi-quantitative, or quantitative RT-PCR is highly dependent on several parameters, including RNA quality, reverse transcription, reagent dispensing, and the selection of normalization methods/genes. Appropriate guidelines and processes to avoid potential pitfalls have been described in the following reviews (Wilhelm and Pingoud 2003, Bustin and Nolan 2004, Godfrey and Kelly 2005).

For the sake of simplicity, we will refer to a situation in which two isoforms are being expressed (often one of these isoforms will be considered the wild-type form). Irrespective of the type of splicing event, there will always be a “long” and a “short” isoform. For instance, for an exon-skipping event, the long form will be the wild-type and the short form the exon-skipped variant. For an intron retention event, the wild-type will this time be the “short” form and the “long” form will be the intron-retaining variant.

3.1.1 Semi-quantitative RT-PCR

Semi-quantitative PCR is an appropriate method to rapidly obtain a first set of data on the relative levels of expression between the two variants in one or more samples, or on the differential expression between two or more samples. PCR primers are either selected on both sides of the splice event (Fig. 2, primer set a) or selected in sequences specific to the long form (Fig. 2, primer set b) or common to both forms (Fig. 2, primer set c). PCR primers' efficacy and specificity are usually first verified on a gradient of annealing temperatures. End-point PCR at the selected temperature is next performed with a number of PCR cycles ensuring that the assay will stay in the exponential phase (usually between 20 and 30). PCR amplicons are next visualized by gel electrophoresis and can be further quantified by densitometry or via an Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto). Correction by internal normalization controls with comparable expression level is required. More sophisticated quantifications of the PCR amplicons have recently been described. First, pyrosequencing has been applied to the analysis of G protein Gαs subunit splice variants (Frey et al. 2005). This technology, initially developed for single nucleotide polymorphism (SNP) analysis relies on the differential incorporation of a deoxynucleotide by DNA Polymerase in primer extension assays, the PCR amplicons acting as

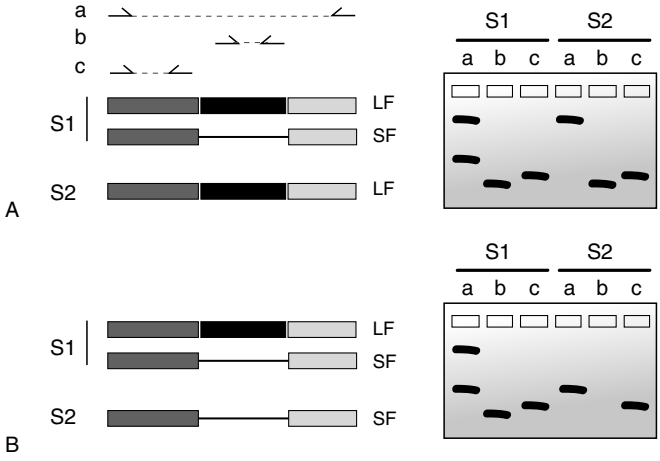

Fig. 2A, B. Semi-quantitative RT-PCR. **A**: Sample 1 *S1* contains both long and short forms while sample 2 *S2* contains only the long form. **B**: Sample 1 *S1* contains both long and short forms while sample 2 *S2* contains only the short form. Primers set *a* will amplify both variants and produce different size amplicons that can be resolved by gel electrophoresis. Primers set *b* will only amplify the long form. Primers set *c* will amplify both forms but produce one unique amplicon

templates. The pyrophosphate released during nucleotide incorporation produces a light signal proportional to the number of incorporated nucleotides. This approach was validated by using different ratios of plasmids expressing the Gαs splice variants as PCR templates. Second, primer extension with specific dideoxynucleotides coupled to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has also been used (McCullough et al. 2005). Splice variants can be differentiated as extension primers are designed to extend from one to a few bases into the variants' specific sequences, producing different mass oligonucleotides.

Regardless of the methodology used to quantify PCR products, one has to be very cautious in applying PCR to amplify more than one splice variant over more than 35 cycles. This is likely to introduce biases by reaching saturation levels and through competing templates. Results may then not appropriately reflect the absolute and relative levels of the splice variant RNAs (see more on competing templates in the following section).

3.1.2 Quantitative RT-PCR

Fluorescence-based real-time quantitative RT-PCR, combining amplification and detection, has constituted a major step forward in our ability to provide quantifiable measurements of transcripts with access to a large dynamic range, high accuracy, sensitivity, and no post-amplification manipulation. The accumulation of PCR amplicons is continuously monitored during cycle progression via different types of fluorescent-detection chemistries.

The quantification of the expression levels of the two splice variants is possible according to two different strategies. First, in the subtraction strategy, one primer pair located in the long form specific sequence detects and quantifies the long form only, whereas another primer pair, located within common sequences detects both the long and short transcripts (Fig. 2, primer pairs b and c). Subtraction of the former value from the latter one results in the quantification of short transcripts. Wherever possible, this strategy is the easiest to set up, since no junction-spanning primer or probe is necessary, which is a less flexible and time-consuming approach (see below). Unfortunately, this easier method seems to be efficient only when dealing with approximately equally abundant alternative transcripts, because the difference in expression level of both PCR amplicons must be significantly higher than the inherent inter-variations of PCR points. In the second, less flexible strategy, an assay is set up for each individual splice variant. It will always require the development of primers or probes that will be specific for a given "junction" sequence (Fig. 1). Designing specific junction primers or probes is not trivial. There are no well-defined rules, each case corresponding to a unique nucleic acid

environment. The required specificity is often difficult to achieve and proper controls will have to be evaluated in a time-consuming process. Specificity can be achieved at the level of the PCR amplification, at the level of detection, or at both. SYBR Green is a non specific DNA binding dye that will detect every produced PCR amplicon. Specific chemistries have been developed and tagged to oligonucleotide probes to provide an increased level of specificity, but with an increased cost.

As described by Vandenbroucke et al. (2001), the splice-junction-specific primer technique can be used both with SYBR Green or probe chemistry (Fig. 3A). In their work, the skipping of exon 37 of NF1 gene was detected at a level of 0.1% of the global transcript population using a primer encompassing the boundary between exons 36 and 38 combined with the SYBR Green chemistry. Nowadays, the specific amplification of an exon-skipping event by a junction-spanning primer is well documented (Brooks and Krahenbuhl 2001; Wellmann et al. 2001; Wong et al. 2002; Luther et al. 2003; Stiewe et al. 2004) and seems to be the method of choice, especially in instances where the short transcripts are expressed at low levels. The specificity of the junction primer ensures a selective amplification of the short variant without co-amplification of the long form. However, there are some constraints related to the lack of flexibility for the junction primer design. This strategy may be hard to apply in the case of inappropriate nucleotide composition at the junction region.

The boundary-spanning probe technique was proposed by Kafer et al. (1999) with the quantification of the human GM-CSF receptor β-subunit short alternative transcript (mut β-GMR; Fig. 3B). A Taqman probe (Roche Diagnostics, Basel) encompassing the spliced junction was used to detect the short isoform. In this case, the primers used to amplify the alternative short transcript could also amplify the longer wild-type β-GMR, but only the shorter isoform could be detected by the probe.

This technology was also described by Veistinen et al. (2002) using hybridization probes and the Fluorescence Resonance Energy Transfer (FRET) on the LightCycler instrument (Roche; Fig. 3C). In this model, a donor probe and an acceptor probe were designed for each side of the splice junction to detect the skipping of exon 3 in the Aiolos short variant (Aio-2). The transfer of fluorescence can only occur, when bound to the variant, the two probes are in close proximity.

Using the boundary-spanning probes, special attention should be given to the quantification of lowly expressed alternative transcripts. The PCR primers are not specific for the short variant and the co-amplification of both isoforms could affect the proper quantification through the depletion of primers or other reagents. Vandenbroucke et al. (2001) focused on the influence of excess full-length long transcript on the detection and quantification of the NF1 short transcript. They reported that interference could already be observed at a 32-fold molar excess of the long isoform resulting in a decreased amplification efficiency of the short variant. This competition issue was responsible for the failure in the amplification of alternative

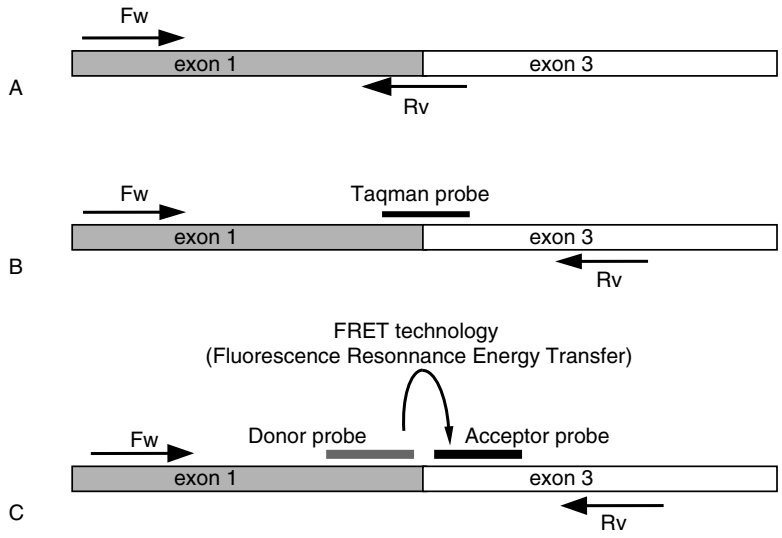

Fig. 3A–C. Real-Time Quantitative PCR. **A**: Boundary-spanning primer. **B**: Boundary-spanning Taqman probe. **C**: Boundary-spanning hybridization probe. Only the short isoform is represented. Fw: Forward; Rv: Reverse

splicing variants of the calpain 3 gene using a junction-spanning primer. This problem was solved by using different chemistries with boundary-spanning molecular beacon and scorpion probes and adjusting the amount of reagents (Taveau et al. 2002). Molecular beacons are probes attached to a hairpin loop that holds a fluorophore and a quencher in close proximity. When the probe binds specifically to its target the structure opens up and produces a fluorescent signal (Piatek and Atzori 1999). Scorpions are PCR primers flanked by a probe associated with both a fluorophore and a quencher (Whitcombe et al. 1999). The probe corresponds to a sequence in the vicinity of the primer and is held in a hairpin-loop configuration until the hairpin is opened up by hybridization during PCR amplification.

3.2 Alternative Technologies to RT-PCR

3.2.1 The Ligase Chain Reaction

The Ligase Chain Reaction (LCR) constitutes a first alternative to PCR platforms for specific splice isoforms' quantification. It is one of the many technologies developed in the last ten years for the detection of genetic disease, as well as for the detection of bacteria and viruses (Wiedmann et al. 1994) but it has also been evaluated for splice variants quantification

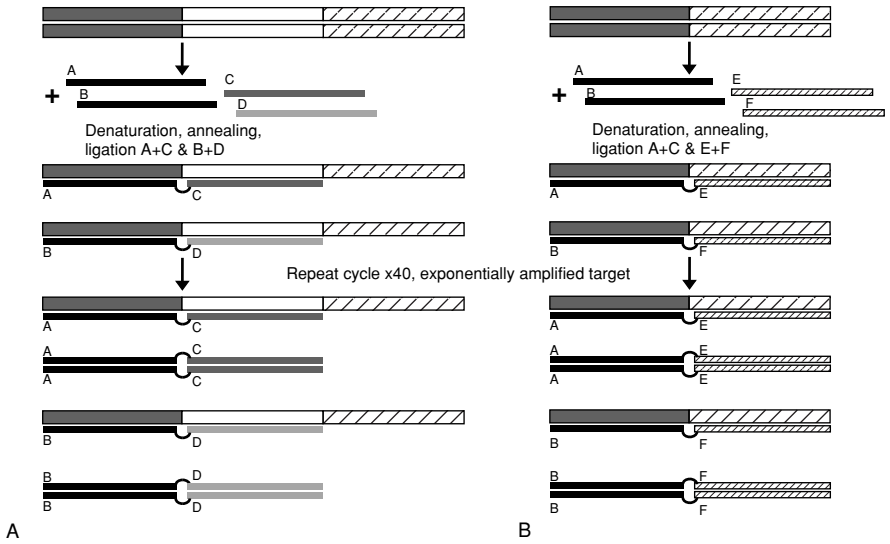

Fig. 4A, B. Ligase Chain Reaction (LCR). **A**: Specific detection of the long form. **B**: Specific detection of the short form. Open box corresponds to alternative exon. A and B probes match on 5'exon (dark box) C and D probes match on alternative exon E and F probes match on 3'exon (hatched box)

(Samowitz et al. 1995). LCR consists of two complementary oligonucleotide pairs homologous to adjacent sequences on the target DNA (Fig. 4). Upon hybridization, the adjacent pairs can be ligated to each other. The newly ligated oligonucleotides can next be specifically PCR amplified. In contrast, when the two adjacent oligonucleotides are separated by a splice event (intron retention, for example), ligation and thus amplification are impossible and the isoform is undetectable unless a specific oligonucleotide pair is designed inside the splice event. Amplification products will be detectable and quantifiable using devices such as radiolabels, fluorescent labels, immunologically detectable haptens, or immunocaptures of one probe. RASL (RNA-mediated Annealing, Selection, and Ligation) has also been applied to the study of splice variants (Yeakley et al. 2002). In this latter approach, oligonucleotides complementary to sequences located on both sides of a splice junction are ligated from an RNA template to be PCR amplified. Fluorescent amplicons are then applied to a microarray for detection.

3.2.2
The RNA-Invasive Cleavage Assay

Initially developed for genotyping applications, the RNA-invasive cleavage assay was applied to quantitatively measure the levels of expression of four

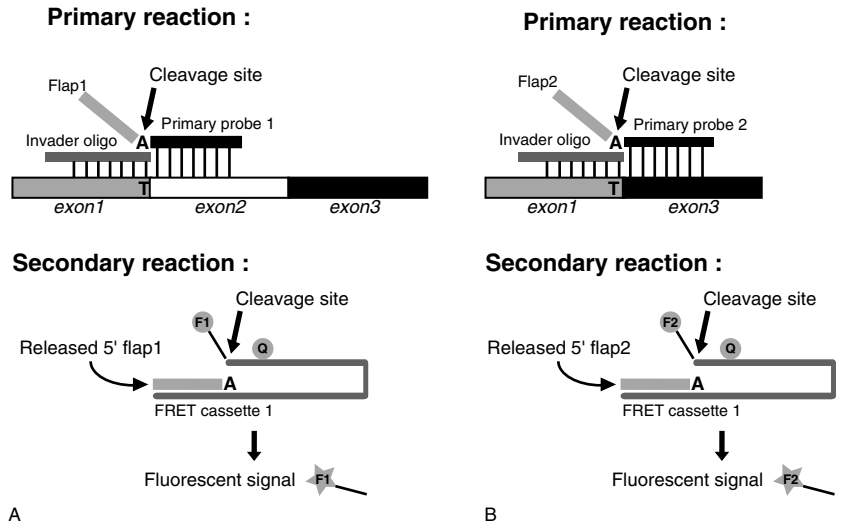

Fig. 5A, B. RNA Invasive Cleavage Assay. **A**: Specific detection of the long form. **B**: Specific detection of the short form

alternatively spliced FGFR2 transcripts (Wagner et al. 2003). Briefly, a three dimensional "invader" structure is formed by the hybridization of two overlapping oligonucleotides to the target sequence (Fig. 5). Next, a specific endonuclease (cleavase) recognizes and cleaves one of the oligonucleotides. This cleavage releases a flap that initiates a secondary cleavage reaction with a fluorescence resonance energy transfer (FRET) label. The selection of specific invader assay probe sets can be more complex than the selection of RT-PCR primers. Five invader RNA assay probe sets had to be designed to recognize unique splice junctions in each of the four FGFR2 variants with high sensitivity and specificity.

3.3 Conclusion

There is no unique or best strategy for the measurements of splice variants' expression among the above-described techniques. In quantitative PCR, the choices between junction probes or junction primers and between the different detection chemistries will mostly be driven by technical issues related to sequence composition of the junction region. Real-time PCR protocols using junction amplification primers is certainly the most frequently used as it represents the best compromise between accuracy, sensitivity, development time and price.

4
High-Throughput Analysis of Alternative Splicing Using Microarrays

4.1
Introduction

The techniques designed to monitor the structural alterations in mRNA generated through alternative splicing that have been reviewed so far do not have the capacity to investigate large numbers of genes and variants. DNA microarrays have become a widely used technology for large scale gene expression studies. Custom designs are becoming a routine, and cost-effective means to investigate a large number of genes within a single experiment. Several companies have now established versatile products that are robust and sensitive. They have been used successfully for determining general expression profiles, which, in turn, have been shown to contain information to classify tumors for their response to chemotherapeutics or for their ability to metastasize (Pomeroy et al. 2002; Hedenfalk et al. 2003; Brunet et al. 2004), predicting drug response, on-target or off-target responses (Clarke et al. 2004; Robert et al. 2004) or predicting the potential safety of compounds and the mechanisms of toxicity (Hamadeh et al. 2002; Liguori et al. 2005). However, the design of the microarrays used in all these studies contain one basic flaw: the majority of the probes are not specific for different products from the same gene. Probes are usually designed against the 3′ region of the gene, and in many cases do not even cover the coding region in genes with large 3′ untranslated regions.

Profiling alternative splicing on microarray platforms has been initially described in a limited number of studies (Hu et al. 2001; Clark et al. 2002; Modrek and Lee 2002; Yeakley et al. 2002; Castle et al. 2003; Johnson et al. 2003; Wang et al. 2003). Those describe the feasibility of monitoring splicing events on microarray platforms, but quantification methods for the absolute and relative levels of expression of splice variants have not been extensively developed. There are several aspects of microarray parameters that need special consideration when the platform is applied to the problem of detecting alternative splicing, including probe design, target labeling approaches, and analytical methods. These topics are considered in this section as they specifically apply to the identification of alternative splice events on microarrays.

4.2
Microarray Configuration and Probe Design

Microarrays originally came in several different flavors, depending on what was actually placed on the support. The early prototypes contained full length cDNAs, EST clones, or PCR products. These sequences were generally

long and had the potential to cross-hybridize with gene family members or genes encoding similar protein domains. Oligonucleotides offered the ability to carefully define the probe sequence for a more specific hybridization. Subsequently, oligonucleotides were spotted onto slides, and binding chemistries became very important. More efficient methods were developed to build oligonucleotides onto the substrate through in situ synthesis (Chee et al. 1996; Hughes et al. 2001) and constitute the basis for some of the major commercial products, such as the ones marketed by Affymetrix (Santa Clara, CA, USA) or Agilent Technologies. (Palo Alto, CA, USA).

There are potentially several strategies to design oligonucleotides for the detection of alternative splicing (Fig. 6). The traditional labeling protocols necessitated the design of the probes toward the end of the transcript. Samples labeled with oligo dT protocols produced fluorescent targets from the transcripts present in the RNA sample that were biased toward the 3′ end of the transcript. With this knowledge, the probes were also designed toward the 3′ end of target in order to optimize the match of the labeled targets with the probes (Fig. 6B) and thus were not at all suited to detect and monitor alternative splicing events. Another confounding factor was that the oligonucleotides were designed against EST sequence information. It is well established that the EST data is biased towards the 5′ and 3′ ends of the transcript (Strausberg et al. 1999).

One of the earliest attempts to gain information on alternative splicing was based on a microarray with a probe design consisting of 25-mer oligonucleotide designed against the 3′ end of the gene (Fig 6B). Twenty probes were designed with a companion single base mismatch as a control (for a total of forty probes for each gene) to monitor 1,600 rat genes (Hu et al. 2001). The standard Affymetrix analysis calculated the average difference between the perfect match probe and the mismatch probe to determine the expression level. An algorithm was developed to analyze individual oligonucleotide probes, rather than the collective set of twenty probes, for differential expression. Differences in expression values would indicate different levels of expression for different sections of the 3′ end of the gene, suggesting that an alternative splice event was present in the sequence monitored by the probes. Confirmation of the results indicated that 50% of the events detected by this method were confirmed by RT-PCR studies.

Subsequent designs were made that focused specifically on the process of splicing with probes designed against exons and specific exon-exon junctions at the splice sites. In one report, oligonucleotide probes were designed around the splice event to detect differences in splicing for intron-containing genes in yeast (Clark et al. 2002; Fig. 6A). Probe length was chosen as 40 nucleotides, and was designed against an exon, the intron, and at the junction of the two exons. Therefore, three probes were designed for each splice event. With specific interest in how splicing has been integrated into the genome through function, several yeast temperature-sensitive mutants with defects in splicing

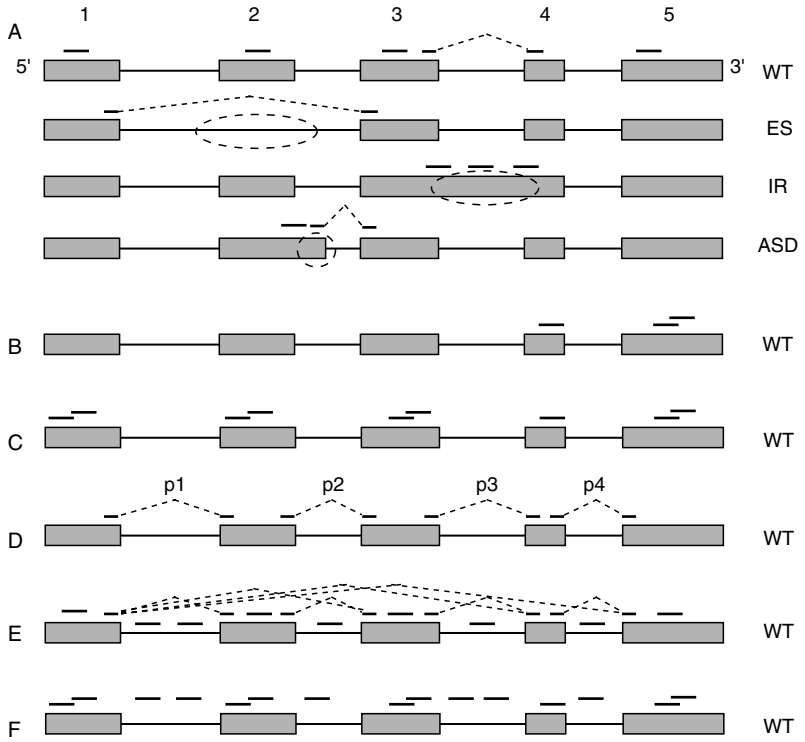

Fig. 6A–F. Comparison of existing microarray configurations and their ability to monitor splice variant. An example of probe location for a 5 exon transcript with 3 isoforms is shown (*WT*: wild-type; *ES*: exon skipping; *IR*: intron retention; *ASD*: alternative splice donor). **A**: Probes designed to specifically address splicing events. Note the presence of junction probes or exon probes to differentiate the wild-type and the known variants. **B**: Probes located on the 3′ end of the transcripts (could be a single probe or a set of tiling probes). Most commercial arrays or custom arrays fit with this design and will miss detection of ES, IR, and ASD. **C**: Probes are designed in all known exons (single or multiple probes/exon). This configuration would only detect exon-skipping events by absence of hybridization for probes designed to this exon. **D**: Probes are designed only over the known exon junctions. Skipping of exon 2 could be detected by absence or weak binding of probes *p1* and *p2*. ASD and IR would be more difficult to predict with this configuration. **E**: Exhaustive probe coverage of all exons and introns with all possible exon-exon junction. (For display purposes, only junction probes between exon 1 and all other exons are indicated) This approach virtually monitors any splicing event but requires huge number of probes for the analysis of a single gene. **F**: Tiling probes designed to scan the whole locus for a given gene (exons + introns)

factors were used to determine the effect of these mutations on splicing from a genomic viewpoint. Again, RT-PCR was used to confirm the findings that there are specific factors required for the removal of introns. This study also demonstrated that oligonucleotide probes can be used to detect and identify splicing alterations on a microarray.

More recently, several groups have reported using more extensively designed probes around splice events (Castle et al. 2003; Johnson et al. 2003; Wang et al. 2003; Pan et al. 2004; Le et al. 2004; Fehlbaum et al. 2005) (configurations corresponding to Figs. 6A, 6D, 6E). In one case, two types of probes, exon-specific and exon-exon junction-specific probes were designed for a set of 21 genes (Wang et al. 2003) or 316 human genes (Le et al. 2004). Several probes were designed over the same target sequence such that they overlapped each other by varying amounts of bases. In several other cases, single probes were designed across junctions or designed to exon or intron sequences for monitoring splice event on a genome-wide scale (Johnson et al. 2003; Pan et al. 2004). Johnson et al. (2003) used systematic exon-exon probes for more than 10,000 human genes while Pan et al (2004) combined information from exon and junction probes for 3,126 alternative splice events from 2,647 mouse genes. A common theme for all of these reports was that new analytical methods are required to analyze these new designs; they will be discussed in more detail below. In addition, these groups have found that there are significantly more alternatively spliced events than previously thought (about 70% of all genes appear to be susceptible to some form of alternative splicing).

Finally, several groups have used a tiling approach to monitor the expression of transcripts in the genome, in which probes have been designed against genomic regions linearly, over a predefined interval of bases (Shoemaker et al. 2001; Kapranov et al. 2002; Kampa et al. 2004; Bertone et al. 2004; Cheng et al. 2005). This approach requires very large number of probes (Fig. 6F) and can only be applied to selected loci. This strategy will be very informative with respect to "insertion" type splice events, such as intron retention, novel exon, or alternative usage of 3′ or 5′ splice sites creating exon extensions. It will not perform very well in characterizing "deletion"-type splice events such as exon skipping or alternative usage of 3′ or 5′ splice sites shortening exons.

Which configuration is best to monitor alternative splicing? The selected configuration should include probes that are specific for every type of splicing event (Fig. 1). Thus, exon-skipping events or other "deletion"-type events as just described above can only be monitored by junction probes. For instance, a probe spanning the second and fourth exon of a gene will monitor the skipping of exon 3 in that gene. Having a probe designed within exon 3 would not be sufficient in most instances where the wild-type isoform containing exon 3 will be co-expressed with the exon-3-deleted isoform. Some short insertional splice events (less than 30 nucleotides) such

as the NAG insertion-deletions at splice acceptor sites (Hiller et al. 2004) will also require junction probes. Monitoring constitutive exons and larger insertional events will be best achieved by standard probes optimized on the full length of the exonic sequence.

After the general design of the localization of the target sequences, there are other parameters that have been considered in selecting probes. Most methods have included a masking step where sequences have been identified for vector, interspersed repeats, low complexity sequences, and mitochondrial DNA contamination (Zhang et al. 2004; Schadt et al., 2004). Probes are next selected according to traditional parameters such as melting temperature, GC content, and secondary structure formation. The selected sequences are usually next aligned against genomic sequences and ESTs to identify and exclude potential cross-hybridizing probes. Several reports have looked at the constraints generated by the junction probes (Castle et al. 2003; Wang et al. 2003; Le et al. 2004; Fehlbaum et al. 2005). They concluded that short probes (24–36 mers) more or less centered on the splice junction would work best to provide the required specificities.

4.3 Labeling Protocols

It is generally acknowledged that standard labeling technologies are not sufficient to generate targets representative of complete transcripts. Current protocols utilize a 3′ biased labeling protocol, and the probes on the array are designed to account for this issue. Unfortunately, these approaches put severe limitation on the ability to detect alternative splicing events. One group specifically recognized this issue and developed a random primed protocol that produced amplified material from mRNA (Castle et al. 2003). Other groups utilized a non-amplified, random prime protocol that requires significantly more starting material (Zhang et al. 2004). Several commercial kits are now available or in development.

4.4 Data Analysis

Monitoring alternative splicing on microarrays presents specific challenges that are not present with microarray analysis as it has been performed so far. Probes for general expression studies are all considered equivalent, and are usually averaged to quantify overall gene expression. Assessment of alternative spliced events requires a higher resolution at the sequence level than previously attainable by standard format designs, as only a few oligonucleotides monitor the entire transcript. As described in the design section, oligonucleotides can be designed to detect small changes in sequence.

The detection of alternative splice variants in a sample is a difficult problem as the transcripts produced from a single gene locus are structurally different, but contain large amounts of common sequence. This requires that the oligonucleotide probes are focused on the sequence of the transcripts that are different among variants. These designs were detailed in the section above. In addition, specific data analysis processes and algorithms are required to determine the level of expression and/or differential expression of splice variants within different samples.

In analyzing alternative splicing, there is a need to define terminology for the issue of the comparison. Usually a RefSeq sequence is selected and comparisons of different RefSeqs, mRNAs or ESTs are made against the selected RefSeq, which will be named as the reference sequence for this discussion. Any sequence that contains a different sequence structure from the reference is termed the variant. Sequence differences between the reference and the variant are termed splice events. Variants can contain one or more splice events that differentiate the variant from the reference. A splice event is defined as a single difference of sequence between the reference and the variant. Thus, a splice event may take the form of a novel exon, exon skip, intron retention, or an alternative usage of a donor or acceptor site. Each one of these is a distinct event, and the variants or spliced isoforms consist of one or more splice events when the sequence is compared to the reference sequence. An important factor in the analysis of alternative spliced variants is that by using short oligonucleotides as probes, detection of only the splice event is possible and the presence of an isoform or variant must necessarily be inferred. In one approach, a matrix algorithm has been applied to determine the presence of variants from oligonucleotides' probe-expression data (Wang et al. 2003). However, the authors stated that there are limitations to the algorithm, particularly when the gene structure is not known or is incorrect. Importantly, they say that "the algorithm is intended for splice variant typing, not discovery." The matrix may also not yield a unique solution, in which case variant detection is not unique. The robustness of this method remains to be tested.

Ratio calculations have been employed to determine the extent of splicing detected by oligonucleotide probes on microarrays. In one instance, a ratio of two test samples was calculated for the junction probe and the exon probe. The log2 ratios were then subtracted to define a splicing index, which was used to determine that two yeast genes, Prp17p and Prp18p, are required for intron removal in cases in which short branchpoints to 3′ splice sites are present. In addition, a ratio method was used to calculate the relative amounts of different variants present between different samples to determine the relative abundance of the reference and variant (Fehlbaum et al. 2005). This was made possible by the design of both exon and junction probes, which represents the only configuration providing direct access to expression data related to the two molecular species generated by a splice event. Additional approaches have recently been applied to the detection of

alternative splicing. One report included the experimental protocol in designing the algorithm for the detection of qualitative changes in alternative splicing (Le et al. 2004). A theory is developed that discerns between general gene expression and changes in alternative splicing, uses log ratio correlation coefficients for the designed probes, and analyzes the group with clustering and graphical methods. Finally, using a similar probe design, a complex analysis was performed based on Bayesian inference and unsupervised learning algorithms (Pan et al. 2004). These last two reports clearly indicate the complexity of analyzing alternative splicing and the needs to develop specific tools.

4.5 Commercially Available Products

4.5.1 Custom Arrays

The investigators who have published on splicing-related microarrays have mostly used the custom services of major chip manufacturers. After selection of the splice events and of one of the probe configurations described previously, a probe design file was sent to the manufacturer. One recent study describing the function of four splicing regulators on annotated alternative splicing events in Drosophila made use of a 44K custom array produced by Agilent Technologies (Blanchette et al. 2005). Others reports that have already been cited in this chapter are also based on microarrays produced by the same company (Le et al. 2004; Pan et al. 2004; Johnson et al. 2003, Shoemaker et al. 2001). Custom microarrays are also produced by Affymetrix custom services (Hu et al. 2001; Wang et al. 2003; Kampa et al. 2004). Custom arrays and design services are also available from others companies.

4.5.2 Catalog Arrays

The two major array manufacturers mentioned just above have had an interest in alternative RNA splicing for some time now, and it is likely that this should be translated into commercial products in the near future. Besides its publications in the field (Wang et al. 2003; Kampa et al. 2004; Cline et al. 2005), Affymetrix is working on a next generation of whole-genome arrays dedicated to provide exon expression data (Blume 2005). Probe sets have been designed against every known and predicted human exon to produce microarrays taking advantage of a reduced feature size format of 5 microns. (www.affymetrix.com).

In addition to being the provider of custom microarrays to several industrial and academic groups (see 4.4.1 Custom Arrays), Agilent

Technologies has been collaborating with ExonHit Therapeutics (Paris) to produce splice arrays dedicated to monitor all transcripts within specific gene families, such as G-protein-coupled receptors, ion channels, and nuclear receptors, (www.splicearray.com). These arrays monitor the expression of the reference transcripts and of every known and potential splice event extracted from public databases via probe sets of exonic and junction probes specific for each event.

4.6 Conclusion

Microarrays are naturally evolving toward the inclusion of splice-related content. These products have the ambition of providing a global picture of the transcriptomes. This ambition comes with several underlying challenges with respect to probe design/configuration and data analysis. The tools are available today to address these challenges and to start providing more thorough pictures of transcriptomes. The combination of exon and junction probes will provide the most robust platforms to monitor known splice events. Microarrays could also be developed to identify novel splice variants. Tiling arrays fit in this category but suffers from the requirement of a large number of probes. A compromise may lie in a microarray that, in addition to probe sets designed against known splice events, would also include junction probes designed against every exon-intron region and against every neighboring exon. Such sets of probes could then identify intron retention events, single exon-skipping events, and exon extensions at the 5′ or 3′ ends via alternative splice site usages. The precise molecular structure of the novel splice events would then need to be determined by standard RT-PCR assays.

5 The Detection and Quantification of Splice Variants at the Protein Level

As with nucleic acid detection technologies, specificity is the key issue for an accurate measurement at the protein level. Different scenarios can be envisioned.

In some cases, the proteins encoded by two splice variants will be identical. This will happen when the splice event occurs upstream or downstream from the open reading frame without modifying it. This splice event may have a functional impact on the stability or the translatability of the mRNA and thus affect the steady-state level of the produced protein. However, as the two proteins are 100% homologous, it will not be able to differentiate them. This will only be possible at the RNA level.

Some splice events will rapidly incorporate a stop codon in the reading frame, resulting in truncated proteins. Although it is theoretically possible to generate antibodies that will be specific for the truncated variant if it displays a different three-dimensional structure or if a peptide epitope can elicit different responses whether it is internal or a free carboxy-terminal entity, in all likelihood an antibody will also recognize the full length protein as well. Qualitative data will be accessible if the two isoforms can be differentiated via another property, such as their respective sizes through electrophoresis.

In others instances, the splice event such as an exon skip will induce an in-frame deletion within the protein. The new variant will then display a novel junction from which could be derived a specific epitope. Several peptide antigens of varying lengths and more or less centered on the junction must be selected to increase the chances of producing a specific antibody.

The easiest scenario is encountered when an entirely novel peptidic sequence is incorporated in the variant. This will occur when a novel exon is added or when a splice event induces a shift in the reading frame and does not introduce a stop codon too soon. Provided the novel amino-acid content displays antigenic regions, specific antibodies are likely to be generated as exemplified for a MUC1 splice variant (Levitin et al. 2005) or for CD44 isoforms (Bennett et al. 1995).

In any event, the ability to generate robust, quantitative measurements at the protein level necessitates the development of immunoassays such as ELISA that will require at least one antibody specific for the splice variant.

6 Conclusion

The tools and platforms are present to appropriately take into account expression data coming from splice variants. Several alternatives exist to quantify limited numbers of genes and variants. They all, however, require careful design and adequate controls to ensure that specificity is achieved.

With the advent of microarray platforms to monitor and quantify splice variant expression levels, one is likely to be in a position to provide concrete answers to some of the issues that are often associated with alternative splicing, such as how frequently does it occur, what are the levels of expression of splice variants, what cis- and trans-acting factors control their expression, which variants are subject to nuclear-mediated decay, to what extent is alternative splicing deregulated in human diseases, how often are mutations or single nucleotide polymorphisms affecting RNA splicing, and which splice variants are species-specific? The availability of whole-genome sequences, of large databases of ESTs, and of improved algorithms has enabled bioinformaticians to address some of

these issues, draw hypotheses and generate *in silico* results (Yeo et al. 2005; Wang et al. 2004; Fairbrother et al. 2004; Lee and Wang 2005). When possible, these results were confirmed in vitro with model recombinant plasmids or with a limited number of endogenous mRNAs by semi-quantitative RT-PCR. The use of microarrays will render possible large scale validations of bioinformatics data. More generally, microarray based profiling assays will generate a selection of splice events that are expressed and/or deregulated in given biological settings. It should be remembered that most alternative splicing expression monitoring technologies analyze splice events rather than splice variants. Such events can then be further validated via one of the lower throughput techniques described in this chapter. Careful designs at this stage should allow, in translating splice event expression, validation of splice variant expression. In the end, many scientists will be primarily interested in the functional validation of selected splice variants. Drug discovery scientists profiling disease tissues could directly position the characterized splicing deregulations within signaling pathways and test their relevance via pharmacological tools in subsequent disease models. In addition, the functional relevance of splice variants will be eased via the advances of siRNA technologies in which isoform specific knock-down could be achieved (Kisielow et al. 2002, Celotto et al 2005). This would allow, for instance, study of the impact of expressing a given splice variant within settings in which the associated wild-type RNA would be expressed or not. The human transcriptome is undoubtedly more complex than once thought. Some of the major promised applications of genomics for human health will rely in our capacity to describe it in more depth and accuracy.

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Pre-mRNA Missplicing as a Cause of Human Disease

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

1 Importance of Alternative Splicing for Gene Regulation

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

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

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

1.1 Splice Sites are Selected Through Combinatorial Control

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

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

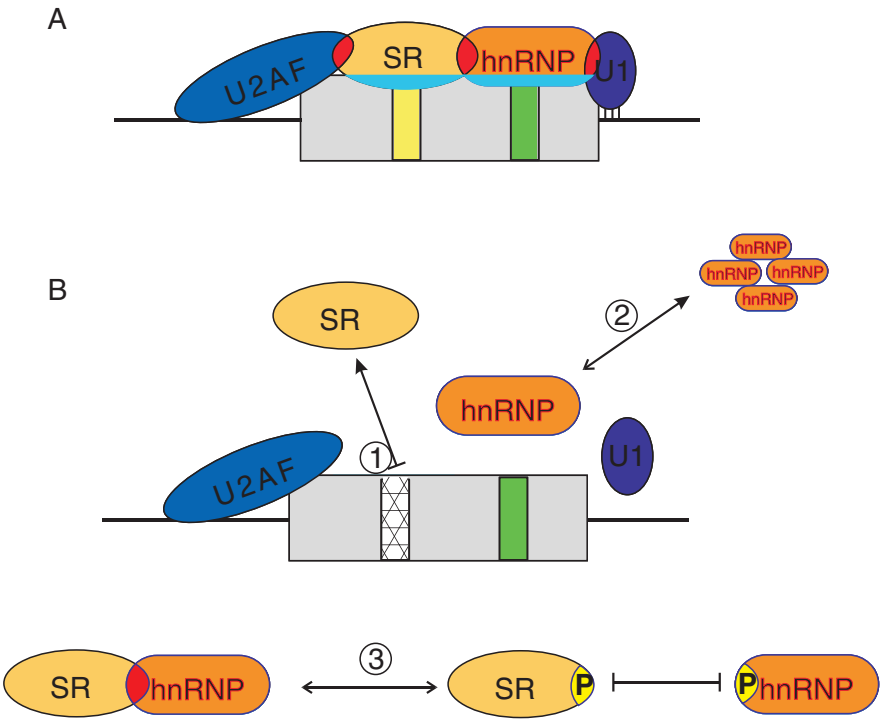

Fig. 1A, B. Change of splice site selection during disease. **A**: Formation of RNP complexes to recognize splice sites. The exon is shown as a *gray square*, the intron as *lines*. The formation of a complex between SR proteins and hnRNPs on two exonic enhancers (*small boxes* in the exons) is shown.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

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

2
Human Diseases Caused by Mutation in Splicing Signals

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

2.1
Mutation of Cis-acting Elements

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

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

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

2.2 Examples of Diseases

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

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

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

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

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

(*Continued*)

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

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

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

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

3
Changes of Trans Factors Associated with Diseases

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

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

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

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

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

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

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

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

5
Treatment of Diseases Caused by Missplicing

5.1
Gene Transfer Methods

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

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

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

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

5.2 Low Molecular Weight Drugs

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

5.3 Diagnostics

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

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

6 Conclusions

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

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Alternative Splicing: Therapeutic Target and Tool

Mariano A. Garcia-Blanco

Abstract. Alternative splicing swells the coding capacity of the human genome, expanding the pharmacoproteome, the proteome that provides targets for therapy. Splicing, both constitutive and regulated forms, can itself be targeted by conventional and molecular therapies. This review focuses on splicing as a therapeutic target with a particular emphasis on molecular approaches. The review looks at the use of antisense oligonucleotides, which can be employed to promote skipping of constitutive exons, inhibit inappropriately activated exons, or stimulate exons weakened by mutations. Additionally this manuscript evaluates methods that reprogram RNAs using reactions that recombine RNA molecules in trans. Preliminary, but exciting, results in these areas of investigation suggest that these methods could eventually lead to treatments in heretofore intractable ailments.

1 Introduction

Therapies targeted to specific genes or gene products promise unrivaled therapeutic index. Recently, transcript-specific therapies have been designed to revise RNAs using trans-splicing reactions or to modulate both constitutive and alternative splicing. This review will discuss RNA splicing and alternative splicing, briefly cover conventional therapeutic approaches to alter splicing, focus on the use of oligonucleotides as tools to mend splicing, and end with a discussion of trans-splicing technologies in the context of modulating splicing. For a comprehensive review on splicing and alternative splicing see (Black 2003) and for more extensive overviews of the interface between splicing, disease, and therapy (Caceres and Kornblihtt 2002; Cartegni, Chew et al. 2002; Faustino and Cooper 2003; Musunuru 2003; Roca, Sachidanandam et al. 2003; Garcia-Blanco, Baraniak et al. 2004; Garcia Blanco 2005; Kalnina, Zayakin et al. 2005).

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Alternative Splicing and Disease

1.1 Pre-messenger RNA Splicing and Disease

A typical human primary transcript, or pre-messenger RNA (pre-mRNA) contains eight introns, or intervening sequences, which account for about 90% of its mass, and nine exons, which are linked to form the mRNA. Recognition of exon–intron junctions, ligation of the exons and removal of introns (RNA splicing) is carried out by spliceosomes, which are dynamic and likely heterogeneous macromolecular machines. Spliceosomes recognize small exons in the context of much larger introns in a process that is mediated by conserved cis-acting sequence elements in the pre-mRNA. These elements are found at the exon–intron junctions, denoted as the 5′ and 3′ splice sites, and also dispersed throughout exons and introns. The latter are diverse array of sequence elements that can be divided into four functional categories: exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE; also known as intronic activators of splicing: IAS), and intronic splicing silencers (ISS; Black 2003; Garcia-Blanco, Baraniak et al. 2004). These sequence elements, and the factors that recognize them, are employed for the constitutive definition of introns and exons and can be engaged to regulate alternative splicing.

Alternative splicing is the process by which one primary transcript yields different mRNAs, which can lead to the translation of proteins of different and even antagonistic function. Current genome annotation reveals that the bulk of intron-containing transcripts are alternatively spliced (Johnson, Castle et al. 2003; Modrek and Lee 2003; Resch, Xing et al. 2004; Sorek, Shamir et al. 2004). Alternatively spliced exons generally have 5′ and 3′ splice site motifs that deviate from the consensus, and the regulated use of these exons depends on the balance between the activity of silencers (ESSs and ISSs) and enhancers (ESEs and ISEs). These *cis*-elements mediate their function by interacting, directly or indirectly, with *trans*-acting activators or repressors of splicing.

Both constitutive and alternative splicing can be disrupted by mutations in any of the aforementioned cis-elements or by abnormal structure, expression, or activity of the trans-acting factors that recognize them. These disruptions can be inherited or acquired. Many common acquired diseases, such as cancer (Gunthert, Stauder et al. 1995; Venables 2004; Kalnina, Zayakin et al. 2005) and cardiovascular disease (Daoud, Mies et al. 2002; Cooper 2005), have associated alterations in alternative splicing programs. These alterations can be critical components of the pathology. Many disruptions of splicing can also be inherited. Inspection of the Human Gene Mutation Database (update of 18 March 2005), reveals that out of 25,994 micro-lesions in 1,766 annotated genes, 4,299 impinge on splice sites (Stenson, Ball et al. 2003) – close to 17% of micro-lesions. These figures, however, are estimates that very likely undervalue the number of human mutations that affect splicing. First, there are probably many mutations that activate cryptic splicing in ways

that until recently could not be computationally predicted. Recent use of information-theory-based software, which can more powerfully identify exons (Nalla and Rogan 2005), is likely to reveal a much greater number of mutations that alter splicing patterns. Furthermore, alterations in ESS, ISS, ESE, and ISE elements, which are not included in the aforementioned database, will also disrupt splicing. One clear example of how mutations to different splicing elements can alter splicing of one gene product is the dominant condition known as familial isolated growth hormone deficiency. This syndrome is caused by mutations that map either to splice sites or to ISE and ESE elements (Faustino and Cooper 2003; Millar, Lewis et al. 2003; Ryther, McGuinness et al. 2003). Mutations that disrupt splicing trans-acting factors can result in global splicing defects. Obviously those profoundly affecting spliceosome components required for the processing of all or most pre-mRNAs are usually lethal. Some forms of retinitis pigmentosa (RP), which results from the loss of retinal rod photoreceptor cells, are caused by mutations in the constitutive splicing factors PRPF3, PRPF8, and PRPF31 (Faustino and Cooper 2003).

Many disruptions of splicing regulation lead to loss of splicing function (e.g., disruption of a splice site or of an ESE), however, alterations of *cis*-elements (such as creation of a cryptic splice site or of an ISS) can lead to gain of splicing function. Equally, mutations in genes encoding splicing factors can either reduce or increase the use of certain exons. In some disease states the result is an inappropriate alternative splicing choice. One example of this has been noted during progression of prostate cancer from androgen-dependent and well-differentiated tumors to androgen-independent undifferentiated tumors. This progression is often accompanied by a switch in the regulated splicing of *FGFR2* transcripts (Yan, Fukabori et al. 1993; Carstens, Eaton et al. 1997) and this change may be functionally significant for progression to a more aggressive tumor type (Yasumoto, Matsubara et al. 2004). The altered regulation of alternative splicing is likely to represent part of a global alteration of gene expression that parallels the evolution of aggressive disease.

2
Conventional Therapies that Have Impact on Splicing

Alternative splicing is perhaps the most potent engine that leads to the formation of many diverse protein isoforms with unique properties (Black 2000; Maniatis and Tasic 2002). An important property of proteins in the realm of medicine is their ability to be drug targets. It is not surprising, therefore, that protein isoforms created by alternative splicing are differentially affected by pharmacological agents(Bracco and Kearsey 2003). This leads to the inexorable conclusion that alternative splicing can profoundly impact

the development of isoform-specific conventional drugs. A salient example of isoform-specific pharmacology is provided by the nonsteroidal anti-inflammatory drugs (NSAIDs) and the realization that the cyclooxygenase inhibited by acetaminophen is the product of an alternative transcript of the *COX-1* gene (Chandrasekharan, Dai et al. 2002; Simmons 2003; Cui, Kuroda et al. 2004; Fig 1A).

Conventional drugs (i.e., small molecules) can affect the process of splicing directly and in some cases this can be used to alter alternative splicing choices. This strategy has been employed in the treatment of spinal muscular atrophy (SMA). SMA is an autosomal recessive disorder, which is caused by homozygous loss of function of the survival of motor neurons 1 (*SMN1*) gene. The severity of SMA is modified by the action of a paralog gene, *SMN2*, that encodes an identical SMN protein (Helmken, Hofmann et al. 2003). Although the *SMN2* gene is nearly identical to *SMN1*, there is a C to T transition at position 6 of exon 7 (Lorson, Hahnen et al. 1999). This change has been postulated to either create an ESS or inactivate an ESE (Cartegni, Chew et al. 2002; Kashima and Manley 2003). More recent studies using in vivo selection suggest that exon 7 may contain an extended silencing context (Singh, Androphy et al. 2004a; Singh, Androphy et al. 2004b; Singh, Androphy et al. 2004c; reviewed in Buratti and Baralle 2005), which may be similar to the zones of silencing we predicted for other exons (Wagner and Garcia-Blanco 2001). The functional consequence of the C to T transition is that *SMN2* transcripts predominantly skip exon 7, and these do not encode a functional SMN protein (Fig 1B). The greater the skipping of *SMN2* exon 7 the more severe the symptoms among SMA patients are. The goal of therapy is to increase levels of exon 7+ *SMN2* transcripts.

Given data that suggest a beneficial effect of increased *SMN2* gene dosage, a possible therapeutic approach is to increase transcription of the *SMN2* gene. This has been accomplished in tissue culture conditions interferons (IFNs; Baron-Delage, Abadie et al. 2000) and valproic acid (Brichta, Hofmann et al. 2003; Sumner, Huynh et al. 2003), and in mice by treating with sodium butyrate(Chang, Hsieh-Li et al. 2001; Fig 1B). Indeed there have been two small clinical trials using orally administered phenylbutyrate in patients with SMA. In one trial the authors show increased expression of SMN protein in leukocytes of SMA patients given phenylbutyrate (Brahe, Vitali et al. 2005) and in the other the authors concluded that phenylbutyrate improved motor function in confirmed SMA type II patients (Mercuri, Bertini et al. 2004). Both of these were pilot studies and much more extensive clinical trials will have to be carried out in order to test the effectiveness of these therapies on the course of SMA.

SMA could also be alleviated by directly increasing the inclusion of *SMN2* exon 7. An increase in exon 7 use was observed in SMA type I patients fibroblasts treated with the topoisomerase inhibitor aclarubicin (Andreassi, Jarecki et al. 2001). The increased inclusion of exon 7 led to

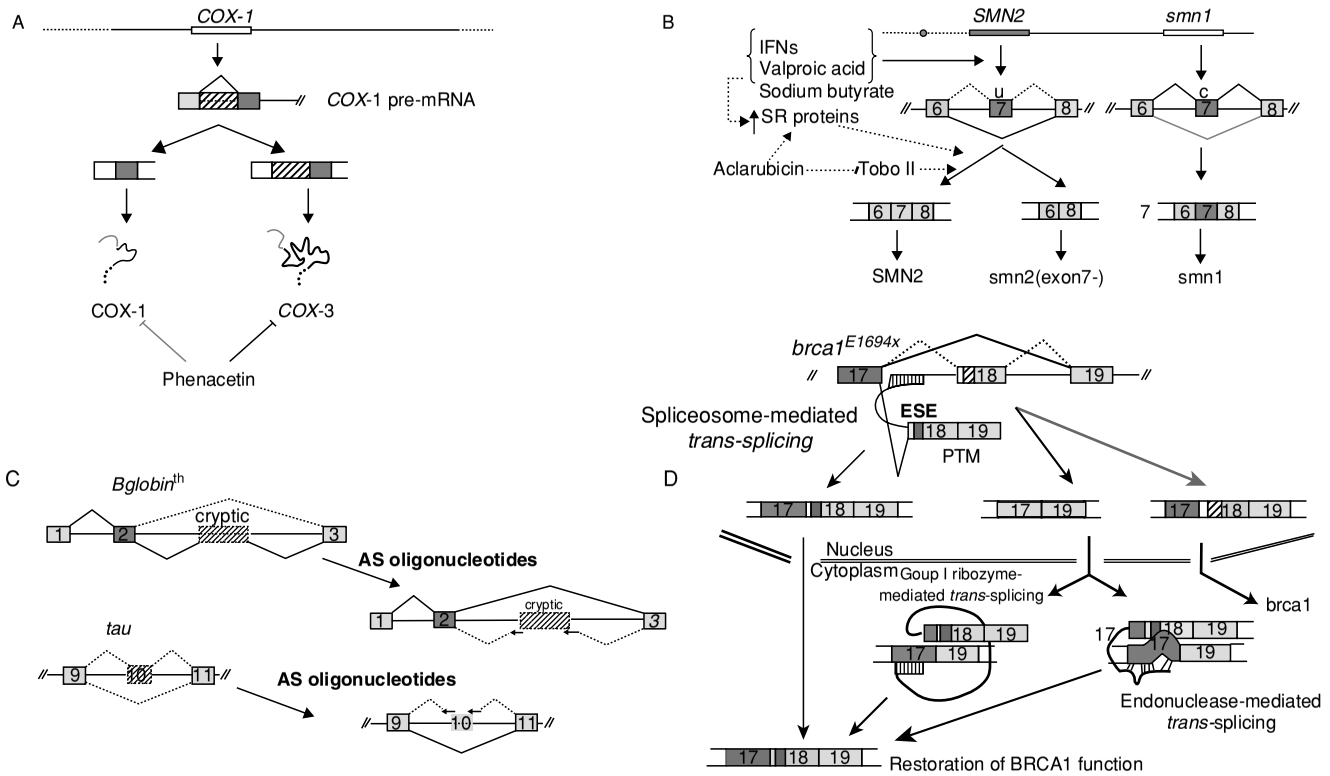

Fig. 1A–D. Alternative splicing and therapy. **A**: A schematic showing the alternative splicing of *COX-1* transcripts that leads to the synthesis of COX-1 and COX-3 isoforms. The latter appears to be abundant in the central nervous system and is more sensitive to phenacetin. **B**: A schematic of the paralogs *SMN2* and *smn1*, which latter is mutated in individuals with SMA. The regions spanning exons 6 through 8 of the primary transcripts is shown. The C to U difference in exon 7, which results in the skipping of this exon in SMN2 RNAs, is indicated. **C**: A thalassemic allele of human βglobin leads to production of a primary transcript that contains a cryptic exon, which is included in the majority of the βglobin mRNAs. Antisense oligonucleotides (*arrows*) can block the splice sites flanking the cryptic exon leading to a reduction in the inclusion of the cryptic exon. Similarly antisense oligonucleotides can be used to reduce the ratio of exon 10+ to exon 10-tau transcripts. **D**: The *panel* shows three distinct trans-splicing reactions that can be used to alter the ratio of BRCA1 isoforms. The goal of the therapeutic intervention is to increase the production of exon 18+ wt BRCA1. The figure was modified from Garcia-Blanco, Baraniak et al. (2004)

higher levels of SMN protein and gems, the intranuclear bodies shown to accumulate SMN (Ibid). Treatment with valproic acid had a similar effect on exon 7 use, and this effect was likely mediated by the induction of SR proteins (Brichta, Hofmann et al. 2003; Fig 1B). Both aclarubicin and valproic acid have been previously approved for other conditions and have been shown to be relatively safe, nonetheless the effects of these compounds on *SMN2* transcription and splicing are not well understood and very likely not specific. The development of more specific compounds that effectively inhibit or activate specific splicing factors, however, is beginning and these offer significant therapeutic promise (Muraki, Ohkawara et al. 2004).

3
Oligonucleotide Drugs and Splicing

Gene and transcript-specific therapies can be mediated by oligonucleotides or oligonucleotide-like compounds (Crooke 2004a; Crooke 2004b). These oligonucleotides are targeted to a specific RNA via base-pairing interactions. Their use was pioneered in the 1970s by Stephenson and Zamecnik (then at Massachusetts General Hospital) to inhibit Rous Sarcoma virus propagation in tissue culture cells (Stephenson and Zamecnik 1978; Zamecnik and Stephenson 1978). Today one such antisense drug (Vitravene) is currently in clinical use to treat cytomegalovirus retinitis in HIV infected patients (Vitarene Study Group 2002). Many more of these drugs are in clinical trials (Crooke 2004a; Crooke 2004b).

Antisense oligonucleotides can target many different types of RNAs and were first used to target pre-mRNAs by Kole and colleagues (Univ. North Carolina at Chapel Hill; Dominski and Kole 1993; Sierakowska, Sambade et al. 1996). In the last decade these investigators and others in the field have shown that these antisense oligonucleotides can impact splicing and this can be used to accomplish a therapeutic outcome. The antisense oligonucleotides have been chemical modified to stabilize them, enhance cellular entry, and prevent degradation of the bound transcripts by RNase H (usually achieved by replacing the 2′-*OH* with a 2′-*OMe*). For a comprehensive review of different chemical formulations for oligonucleotides or oligonucleotide-like polymers see Sazani, Astriab-Fischer et al. (2003) and Crooke (2004b)). Oligonucleotide based splicing therapies have evolved beyond conventional antisense approaches, which usually inhibit untoward splicing outcomes, and are now being used to activate desired splicing events. While this review will cover some of the more important developments in this area, the reader can go to Kole, Vacek et al. (2004) and Kole, Williams et al. (2004) for more comprehensive reviews of the literature.

3.1 Making Antisense of Splicing

Antisense oligonucleotides have been used to correct cryptic splicing in primary transcripts encoded by mutated human genes. A well-studied group of mutations caused by cryptic splicing is found in patients with β-thalassemias. These are caused by mutations within intron 2 of *βglobin* that activate cryptic splice sites and lead to expression of abnormal *βglobin* mRNA and result in low levels of hemoglobin A (Hb A; Fig 1C). The use of these cryptic splice sites in erythroid cells in culture has been abrogated by blocking antisense oligonucleotides (Lacerra, Sierakowska et al. 2000; Vacek, Sazani et al. 2003). In a similar approach Hb A restoration was achieved using larger antisense RNA molecules synthesized in the targeted cells (Gorman, Suter et al. 1998). These RNAs were engineered to contain small nuclear structural features of RNAs (snRNAs), which presumably help in localizing the RNAs to the nucleus and may also enhance interactions with splicing factors (Gorman, Suter et al. 1998). The antisense oligonucleotides and RNAs exert their effect by binding to the cryptic splice sites and sterically blocking the binding of splicing factors (e.g., binding of U1 snRNP to the cryptic 5′ splice site).

Antisense oligonucleotides directed at splice sites have also been used to alter the splicing of cystic fibrosis transmembrane conductance regulator (CFTR; Friedman, Kole et al. 1999), dystrophin (Pramono, Takeshima et al. 1996; Dunckley, Manoharan et al. 1998; Wilton, Lloyd et al. 1999; De Angelis, Sthandier et al. 2002), and cyclophilin transcripts (Liu, Asparuhova et al. 2004). Recently this technology has been applied to Hutchinson-Gilford Progeria Syndrome (HGPS), a disease that results in accelerated aging (Scaffidi and Misteli 2005). A common HSPG-causing mutation creates a cryptic 5′ splice site in exon 11 of the gene-encoding lamin A/C (LMNA). Use of this cryptic site leads to formation of a dominant negative form of lamin A, 50 lamin A, which in turn results in profound cellular abnormalities (De Sandre-Giovannoli, Bernard et al. 2003; Eriksson, Brown et al. 2003). The molecular and cellular defects observed in fibroblasts of HSPG patients were reversed by blocking the use of the exon 11 cryptic 5′ splice site with an antisense morpholino oligonucleotide (Scaffidi and Misteli 2005). Oligonucleotides targeting splice sites of alternatively used exons have been employed to alter splicing choices in *Bcl-x* (Taylor, Zhang et al. 1999), interleukin-5 receptor-alpha (Karras, Maier et al. 2001), and *tau* pre-mRNAs (Kalbfuss, Mabon et al. 2001; Fig 1C).

The studies described above target pre-mRNA sequences at or near the splice sites, however antisense oligonucleotides can be used to block splicing enhancers or silencers, which can be found in introns or exons quite far away from the splice sites (Takeshima, Wada et al. 2001; Bruno, Jin et al. 2004). Inhibition of splicing silencers (ESSs or ISSs) is of particular interest since it provides a way to activate otherwise repressed exons. This was achieved for the α exon of fibroblast growth factor receptor-1 (FGFR1) transcripts

(Bruno, Jin et al. 2004). The FGFR1 α exon is silenced by the action of two ISSs, and this silencing leads to the formation of an isoform of the receptor that predominates in glioblastomas. Bruno et al used antisense morpholino oligonucleotides to block these silencer elements in glioblastoma cells in culture promoting the inclusion of the α exon (Bruno, Jin et al. 2004).

While all of the work described above was carried out in tissue culture, proof of principle for splicing disruption by antisense oligonucleotides in animals had also been obtained. The first such system used transgenic mice harboring an EGFP reporter that was interrupted with the second intron from the βglobin *thalassemia-654* gene. This thalassemic mutation leads to cryptic splicing, interruption of the EGFP open reading frame, and low EGFP production (Sazani, Gemignani et al. 2002). Cryptic splicing was reduced using antisense oligonucleotides that blocked the splice sites and this resulted in increased levels of EGFP fluorescence (Sazani, Gemignani et al. 2002). This animal model has been very useful to study the effectiveness of different oligonucleotides.

Perhaps the most striking use of therapeutic oligonucleotides to alter splicing has been achieved in mouse models for Duchenne muscular dystrophy (DMD), which is an X-linked recessive disorder that leads to muscle wasting and weakness (OMIM # 310200). Severe DMD is usually associated with nonsense mutations in the dystrophin gene that lead to the complete absence of the protein, whereas milder allelic variants of DMD, such as Becker muscular dystrophy (BMD), are usually caused by internal truncations of the protein that retain partial function (Muntoni, Torelli et al. 2003). The milder phenotype of these truncations suggested that forcing the skipping of internal exons, which harbored nonsense codons, could be advantageous (Pramono, Takeshima et al. 1996; Dunckley, Manoharan et al. 1998; Wilton, Lloyd et al. 1999; reviewed in Aartsma-Rus, Janson et al. 2004). These potential therapies have been tested in *mdx* mice, which have a nonsense mutation in exon 23 in the *dystrophin* gene and are an animal model of human DMD (Sicinski, Geng et al. 1989). Dystrophin transcripts that include exon 23 encode a non-functional truncated dystrophin (and are also subject to nonsense mediated decay), whereas those that are missing exon 23 lead to the production of partially active dystrophin protein. *Mdx* mice were treated by intramuscular injection or transfection with 2′-*OMe* antisense oligonucleotides that blocked the splice sites of exon 23 to induce skipping of this exon (Mann, Honeyman et al. 2001; Lu, Mann et al. 2003; Wells, Fletcher et al. 2003). The oligonucleotide treated *mdx* mice show normal levels of dystrophin production in many muscle fibers and improved muscle function (Mann, Honeyman et al. 2001; Lu, Mann et al. 2003); however, the effects were restricted to a limited area and were short-lived. Recently two groups have attempted to deal with these issues. In one case a U7 snRNA was modified to include antisense sequences targeting the branchpoint sequence upstream of exon 23 and 5′ splice site of this exon and was transduced effectively to a high number of muscle fibers by intramuscular or intra-arterial delivery of an adeno-associated viral (AAV)

vector (Goyenvalle, Vulin et al. 2004). This resulted in the skipping of exon 23 and a concomitant rescue of dystrophin expression and function (Goyenvalle, Vulin et al. 2004). This delivery method also led to sustained expression of the exon 23 dystrophin up to 13 weeks after injection of the AAV encoding the antisense U7 snRNA (Goyenvalle, Vulin et al. 2004). Similar recovery of expression and function in *mdx* mice was observed by repeated systemic intravenous administration of *2′-OMe* oligonucleotides that block the 5′ splice site of exon 23 (Lu, Rabinowitz et al. 2005). This study did not examine expression or function beyond two weeks after the last oligonucleotide injection, and thus the persistence of the effect could not be gleaned from the data shown. The authors examined the levels of dystrophin expression and noted significant differences among different muscles with almost complete lack of expression in the heart (Lu, Rabinowitz et al. 2005).

These impressive studies give cause for optimism as they demonstrate the potential for the antisense approach to alter splicing in a therapeutic manner. Although rigorous determination of specificity for these methods is still lacking the studies did not reveal any significant toxicity (Lu, Rabinowitz et al. 2005) and it is very likely that we will see clinical trials in the very near future. Given sequence variation between mouse and human DMD genes the *mdx* mice cannot be used to test the sequence dependence of antisense formulations. To overcome this problem a second mouse model, the *hDMD* mouse, was engineered to harbor a single full-length human *wt* DMD gene, and this mouse was used to show exon skipping mediated by *2′-OMe* oligonucleotides (Bremmer-Bout, Aartsma-Rus et al. 2004). Obviously, diseases such as DMD are complex and correcting the skeletal muscle defects may only partially alleviate the plight of the patients, however, more sophisticated vehicles could eventually bring the therapeutics to most if not all the tissues affected.

3.2 Enhanced Antisense

The antisense approach has been enhanced by the use of bifunctional reagents that combine an antisense-targeting domain and an effector domain; see recent reviews by Garcia-Blanco, Baraniak et al. (2004) and Garcia-Blanco (2005). The effector domain can either silence (Villemaire, Dion et al. 2003) or activate (Cartegni and Krainer 2003; Skordis, Dunckley et al. 2003) an exon. One embodiment of this idea uses a hybrid between protein nucleic acid (PNA) and a peptide, where the PNA targets a specific exon (Cartegni and Krainer 2003). The effector domain of these oligomers is a peptide with 5, 10 or 15 arg-ser (RS) repeats. The targeted PNA-$(SR)_n$ polymers can activate the inclusion of the weak exons in vitro, while non-targeted PNA-$(SR)_n$ do not(Cartegni and Krainer 2003). A second incarnation of bifunctional oligomers uses a *2′-Ome*-modified binding domain and an effector domain, which is composed of RNA, that contains binding sites for known splicing *trans*-acting factors (Skordis,

Dunckley et al. 2003; Villemaire, Dion et al. 2003). The recruited factors mediate activation or silencing of the exon targeted. An example of this type of bifunctional oligonucleotide acts as an ESE and promotes inclusion of the *SMN2* exon 7 fibroblasts from SMA patients leading to partial restoration of SMN function (Eperon and Muntoni 2003; Skordis, Dunckley et al. 2003). The degree of SMN recovery achieved is expected to ameliorate disease severity and improve motor function to levels seen in milder forms of SMA (Coovert, Le et al. 1997). A similar approach was used to alter the alternative splicing of endogenous *Bcl-x* (*BCL2L1*) transcripts in order to diminish the Bcl-xL/Bcl-xS ratio in cancer cells in culture. In this case, the bifunctional oligonucleotide tethered an ESS and silenced the use of the alternative 5′ splice site used to create the Bcl-xL mRNA (Villemaire, Dion et al. 2003) thus lowering the Bcl-xL/Bcl-xS ratio, which is predicted to tilt the balance towards apoptosis.

These intriguing studies suggest that bifunctional antisense oligonucleotides can be efficacious in living cells and have potential as drugs that can alter splicing. Several questions about these clever formulations must be carefully evaluated: most importantly, will these reagents work in animals, what is the mechanism of action, are these methods exon-specific, and will these reagents present unique problems for delivery? These questions notwithstanding, the technologies that enhance the use of antisense oligonucleotides could have therapeutic potential. See also discussions in Buratti, Baralle et al. (2003), Eperon and Muntoni (2003), Khoo, Akker et al. (2003), and Garcia-Blanco, Baraniak et al. (2004).

4
Exon-Specific RNA Interference

A variation on the theme of short oligonucleotides that can alter ratios of splicing isoforms is exon-specific RNA interference (RNAi). Because RNAi and its clinical potential have been extensively reviewed (Dykxhoorn, Novina et al. 2003; Hommel, Sears et al. 2003; Wall and Shi 2003; Stevenson 2004; Shankar, Manjunath et al. 2005), these matters will not be described here. Suffice it to say that given the specificity of RNAi and the possibility of harnessing it to knockdown-specific mRNA isoforms (Celotto and Graveley 2002), we and others have proposed that exon-specific RNAi could be used therapeutically to alter splicing patterns (Garcia-Blanco, Baraniak et al. 2004).

5
RNA-Based Corrective Therapy

Methods that have been developed to reprogram RNAs can be used to modify alternative splicing patterns. RNA reprogramming can be achieved at many stages during the process of gene expression, and depending on the

method will take place in the cytoplasm or the nucleus (Fig. 1D; Sullenger and Gilboa 2002; Garcia-Blanco 2003; Garcia-Blanco, Baraniak et al. 2004; Mansfield, Chao et al. 2004; Pergolizzi and Crystal 2004).

The earliest target for RNA revision is the nascent primary transcript, where alternative splicing choices can be redirected to preferentially express certain isoforms over others. The nascent pre-mRNA is the target for spliceosome-mediated RNA *trans*-splicing (SmaRT; Puttaraju, Jamison et al. 1999; Garcia-Blanco 2003), which utilizes endogenous spliceosomes to catalyze splicing of an exon in the target transcript with an exon in an engineered pre-*trans*-splicing molecule (PTM; Fig. 1D; Garcia-Blanco 2003). The *trans*-splicing of a pre-mRNA and a PTM is possible because the 5′ splice site and the branchpoint sequence come together via a three-dimensional diffusion mechanism, which does not require the spliceosome to scan the intron (Pasman and Garcia-Blanco 1996). In the hypothetical example presented in Fig. 1D, the trans-splicing reaction insures that the mRNA products include exon 18, which would otherwise be mostly skipped. The targeting of a PTM to a specific pre-mRNA is accomplished via base-pairing interactions (Puttaraju, DiPasquale et al. 2001). After successful *trans*-splicing, the reprogrammed mRNA is transported to the cytoplasm and translated to produce the desired protein isoform.

SMaRT has been successfully used to restore CFTR function in human bronchial cystic fibrosis xenografts (Liu, Jiang et al. 2002). Furthermore, this method has been used to produce functional Factor VIII and correct the bleeding phenotype in a mouse model of hemophilia A (Factor VIII deficiency; Chao, Mansfield et al. 2003). More recently a mouse model of an X-linked immunodeficiency caused by loss of CD40L function was successfully treated using SMaRT (Tahara, Pergolizzi et al. 2004). While these studies merit cautious optimism, there are many potential limitations to this methodology in reprogramming alternative splicing. The first application of SMaRT to modify alternative splicing was the redirection of the alternative splicing exon 10 in tau transcripts in neuroblastoma cells in culture (Rodriguez-Martin et al, manuscript submitted). The alteration of exon 10 inclusion may have important clinical implications since mutations that increase or decrease exon 10 inclusion, leading to the development of a neurodegenerative disease known as fronto-temporal dementia with Parkinsonism, was linked to chromosome 17 (FTDP-17; Hong, Zhukareva et al. 1998; Hutton, Lendon et al. 1998; Spillantini, Murrell et al. 1998).

The specificity of spliceosome-mediated *trans*-splicing has not yet been completely ascertained, and competing reactions such as non-specific *trans*-splicing may lead to expression of toxic or immunogenic products. Moreover, this and the two technologies described below depend on distribution of genes into cells and tissues in living organisms, and thus share with all gene-therapy approaches the complex issue of delivery.

Two other *trans*-splicing technologies can alter splicing patterns post facto by modifying mature mRNAs in the cytoplasm (Fig. 1D). Group I

intron ribozyme mediated *trans*-splicing reaction, which provided the first example of RNA correction (Sullenger and Cech 1994), requires the expression of an RNA that encodes both a *trans*-splicing ribozyme and the coding sequence to be included in the revised RNA. Group I-ribozyme-mediated *trans*-splicing has been used to effect mRNA repair in cells in culture (Watanabe and Sullenger 2000; Rogers, Vanoye et al. 2002), but has yet to restore function in animals. Questions remain about specificity for Group I *trans*-splicing mRNA reprogramming.

A third and significantly different method to revise messages involves a *trans*-splicing reaction catalyzed by the archeon *Methanococcus jannaschii* tRNA-splicing endonuclease. If introduced into NIH3T3 cells with the appropriate RNA substrates, this endonuclease can catalyze *cis*- and *trans*-splicing reactions (Deidda, Rossi et al. 2003; Fig. 1D). Expressing the endonuclease and a targeting RNA lead to the reprogramming of exogenous luciferase mRNA and of endogenous carnitine acetyl transferase (*Crat*) mRNA. Unlike the two other methods described above, functional targeting depends not only on base pairing but also on the formation of a bulge (3 nt) – helix (4 nt) – bulge (3 nt) structure between the mRNA and the targeting RNA (Fig. 1D). The endonuclease precisely cleaves the target and the targeting RNAs in the bulges and endogenous mammalian enzymes (of unknown identity) ligate the cleaved RNAs. These *trans*-splicing reactions result in the production of an RNA that contains the 5′ end of the targeting RNA ligated to the mutant 3′ portion of the target (not shown in Fig. 1) and a reprogrammed target mRNA where the 5′ part of the target mRNA has been ligated to the 3′ portion of the targeting RNA. The requirement for a specific bulge–helix–bulge structure predicts that the specificity of this *trans*-splicing reaction should be higher than that achieved by Group I and spliceosome mediated *trans*-splicing reactions. On the other hand, the efficiency of the archeal endonuclease may be limited in the mammalian cell milieu – this issue has not been addressed and awaits careful quantification of reprogrammed mRNA levels and protein function.

6 Conclusion

Alternative splicing multiplies the number of targets for conventional as well as molecular therapies. Both acquired and inherited alternative splicing errors also provide critical objectives at which to aim therapeutic intervention. Molecular approaches to therapy can target the pre-mRNA, the splicing factors, or can alter isoform ratios by reprogramming abnormal mRNAs. The intersection between therapy and alternative splicing promises to be a busy crossing that hopefully will result in new and effective treatments for heretofore intractable diseases.

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SR Proteins as Potential Targets for Therapy

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Abstract. Serine- and arginine-rich (SR) proteins constitute a highly conserved family of pre-mRNA splicing factors that play key roles in the regulation of splice site selection, and thereby in the control of alternative splicing processes. In addition to conserved sequences at the splice junctions, splice site selection also depends upon different sets of auxiliary cis regulatory elements known as exonic and intronic splicing enhancers (ESEs and ISEs) or exonic and intronic silencers (ESSs and ISSs). Specific binding of SR proteins to their cognate splicing enhancers as well as binding of splicing repressor to silencer sequences serve to enhance or inhibit recognition of weak splice sites by the splicing machinery. Given that the vast majority of human genes contain introns and that most pre-mRNAs containing multiple exons undergo alternative splicing, mutations disrupting or creating such auxiliary elements can result in aberrant splicing events at the origin of various human diseases. In the past few years, numerous studies have reported several approaches allowing correction of such aberrant splicing events by targeting either the mutated sequences or the splicing regulators whose binding is affected by the mutation. The aim of the present review is to highlight the different means by which it is possible to modulate the activity of SR splicing factors and to bring out those holding the greatest promises for the development of therapeutic treatments.

1 Introduction

Recent genomic analyses have revealed that proteomic expansion is achieved with a limited number of genes, underscoring thereby the importance of transcriptional and post-transcriptional mechanisms in the increase of protein diversity. Among these mechanisms, which include the use of multiple transcription start sites (Quelle et al. 1995), polyadenylation (Gautheret et al. 1998), pre-mRNA editing (Keegan et al. 2001), and post-translational protein modifications (Banks et al. 2000), alternative splicing is considered to be the most important source of protein diversity in vertebrates (Black 2000; Graveley 2001; Maniatis and Tasic 2002). The most striking example is the Drosophila Dscam gene which contains 95 alternatively spliced exons and has the potential to generate over 38,000 different protein isoforms (Schmucker et al. 2000).

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1.1
The role of SR Proteins in Constitutive and Alternative Splicing

Pre-mRNA splicing takes place within the spliceosome, a large complex composed of four small nuclear ribonucleoproteins (U1, U2, U4/U6, and U5 snRNPs) and 50–100 non-snRNP splicing factors (Kramer 1996; Zhou et al. 2002; Makarov et al. 2002). Among them are the heterogeneous nuclear ribonucleoproteins (hnRNP), which associate with nascent transcripts and are implicated in splicing repression (Dreyfuss et al. 1996; Smith and Valcarcel 2000) as well as SR proteins which are required for constitutive splicing and also influence alternative splicing regulation (Fu 1995; Graveley 2000; Caceres and Kornblihtt 2002; Black 2003; Bourgeois et al. 2004).

SR proteins have a modular structure consisting of one or two copies of an N-terminal RNA recognition motif (RRM) and a C-terminal domain rich in alternating arginine and serine residues (RS domain). The RRMs mediate sequence-specific binding to the RNA, and thereby determine the substrate specificity, whereas the RS is mainly involved in protein–protein interactions that are thought to be essential for the recruitment of the splicing machinery and for splice-site pairing (Wu and Maniatis 1993; Tacke and Manley 1999). Very recently, it has been proposed that the RS domain also mediates sequential recognition of splice sites during spliceosome assembly (Shen and Green 2004).

In constitutive splicing, SR proteins intervene very early during the spliceosome assembly by favoring both the recruitment and stabilization of the U1 snRNP and U2 snRNP auxiliary factor (U2AF) at the 5′ and 3′ splice sites, respectively (Kohtz et al. 1994; Staknis and Reed 1994; Zuo and Maniatis 1996), these interactions being essential to define the limits between exons and introns. In the exon definition model (Berget 1995) SR proteins bind to multiple RNA sequences and interact simultaneously with factors associated with the 5′ splice site (U1–70 K) and to the 3′ region of the intron (U2AF) through a network of protein–protein contacts involving the RS domains of these proteins.

The most characterized function of SR proteins in the regulation of alternative splicing is the activation of weak 3′ splice sites, mostly characterized by an imperfect polypyrimidine tract poorly recognized by U2AF. Upon binding to exonic splicing enhancers (ESEs), SR proteins improve the recruitment of U2AF to the polypyrimidine tract and, subsequently, that of U2 snRNP (Zuo and Maniatis 1996; Zhu and Krainer 2000; Graveley et al. 2001; Guth et al. 2001). Alternatively, binding of a SR protein to an ESE element can counteract the effect of a juxtaposed splicing silencer prior to the recruitment of U2AF and U2 snRNP (Kan and Green 1999; Zhu et al. 2001).

1.2 SR Proteins Regulate Alternative Splicing in a Dose-Dependent Way

The notion that alternative splicing depends upon the relative concentrations of SR proteins and their antagonists has emerged when different studies have reported that an increased concentration of individual SR proteins promotes the use of the proximal alternative 5′ splice site in pre-mRNA substrates containing two or more splice donor sites. Conversely, increasing the concentration of the splicing repressor hnRNP A/B has the opposite effect and results in the selection of distal 5′ splice site (Mayeda and Krainer 1992; Mayeda et al. 1993; Caceres et al. 1994; Yang et al. 1994; Wang and Manley 1995). This general activity of SR and hnRNP proteins on 5′splice site selection does not appear to require the recognition of specific target sequences. By contrast, binding to specific sites is required when these splicing factors behave as enhancers or silencers, and three models have been proposed to explain the function of SR protein in enhancer-dependent splicing (Sanford et al. 2003).

In the first one, SR proteins bound to ESEs can promote U2AF recruitment via protein–protein interactions mediated by the RS domains, and activate an adjacent 3′ splice site. Alternatively, ESE-bound SR proteins could interact with a splicing coactivator like SRm160 (Blencowe 2000) and establish a set of interactions distinct from those required to recruit U2AF. Finally, SR proteins bound to splicing enhancers can counteract the activity of hnRNP proteins recognizing ESS elements, again depending on their relative abundance (Graveley 2000; Hastings and Krainer 2001).

Taken together, these observations indicate that a differential regulation of SR protein and splicing-repressor expression levels could constitute a key element to control the patterns of alternative splicing in a tissue-specific or developmentally regulated manner. In good agreement with this, tissue-specific variations in the total and relative amounts of SR proteins have been observed (Zahler et al. 1993) and the molar ratio of the SR protein ASF/SF2 to its antagonist hnRNP A1 was reported to vary significantly in different rat tissues (Hanamura et al. 1998).

1.3 SR Protein Activity is Regulated by Phosphorylation of Their RS Domain

Previous studies using ATP analogues and specific inhibitors of protein phosphatases PP1 and PP2A have revealed that a cycle of protein phosphorylation-dephosphorylation controls the activity of splicing factors during the two transesterification steps of the splicing reaction (Soret and Tazi 2003). Although the mechanism by which this cycle affects pre-mRNA splicing is not fully understood, it is clear that specific phosphorylation of serine residues

within the RS domain of SR proteins may be one of the key determinants regulating splicing events because it modulates homophilic and heterophilic interactions between RS domain-containing proteins (Soret and Tazi 2003). Inasmuch as RNA–protein interactions can also be affected by phosphorylation of the RS domain, these observations raise the intriguing possibility that phosphorylation of SR proteins may act as a regulatory switch whereby initial interactions with constitutive splicing factors like U1 snRNP, U2AF, and specific RNA sequences are strengthened to allow selection of an authentic splice site. Conversely, dephosphorylation of SR proteins weakens them allowing subsequent steps of spliceosome assembly to proceed (Soret and Tazi 2003; Bourgeois et al. 2004). Accordingly, both hyper- and hypophosphorylation of SR proteins inhibit splicing, demonstrating the critical importance of SR protein phosphorylation.

Several mammalian kinases specific for the phosphorylation of the serine/arginine-rich domains of SR proteins have already been identified: SRPK1 and SRPK2, the DNA topoisomerase I, and a family of four Cdc2-like kinases (Clk1/Sty and Clk2–4; Soret and Tazi 2003; Bourgeois et al. 2004). Although they have all been directly implicated in splicing, it has also been shown that shuttling of SR proteins between the nucleus and cytoplasm is critically dependent on the phosphorylation status of the RS domain. Indeed, phosphorylation releases SR proteins from storage/ assembly loci (nuclear speckles) and recruits them to the sites of active transcription. Thus the differential effects of phosphorylation on SR protein interactions have the potential to affect both splicing activity and subcellular trafficking (Soret and Tazi 2003).

1.4 Involvement of SR Proteins in Human Diseases

Inasmuch as recent studies predict that the majority of human exons contain splicing enhancers (Liu et al. 2001; Fairbrother et al. 2002) the corresponding genes represent an equal number of targets whose expression can be altered at the splicing level by exonic mutations. Accordingly, a growing number of reports indicate that the primary mechanism of disease in a significant fraction of disease-causing exonic mutations is a dramatic splicing abnormality rather than a direct effect on coding potential (Cooper and Mattox 1997; Caceres and Kornblihtt 2002; Cartegni et al. 2002; Faustino and Cooper 2003). These mutations can either disrupt or weaken a splicing enhancer and result in exon skipping. Such a situation naturally occurs in the case of the human survival motor neuron 2 (SMN2) gene which can not compensate for homozygous loss of SMN1 causing spinal muscular atrophy (SMA) because of a single C/T transition that prevents efficient splicing of the SMN2 exon 7. This transition appears to simultaneously disrupt an ASF/SF2 splicing enhancer and create a splicing silencer binding

hnRNP A1 (Cartegni and Krainer 2002; Kashima and Manley 2003). Conversely, the disease-causing mutation can create new positive regulatory sequences leading to activation of cryptic splice sites, thereby changing the overall splicing pattern of the mutant transcript. Such a mutation that strengthens a splicing enhancer specific for the SC35 SR protein has been recently described in a case of Leigh syndrome resulting from aberrant splicing of the pyruvate dehydrogenase E1α gene (Gabut et al. 2005). In both situations, means to increase or decrease the expression or activity of the SR protein recognizing the ESE altered or created by the mutation will turn out to be very useful to restore a wild type splicing pattern.

Aberrant splicing responsible for or tightly associated with human disease can also occur in the complete absence of mutation within the gene whose splicing is affected when the expression level or the activity of key splicing regulators is altered. Indeed, dramatic changes in alternative splicing patterns of many genes are associated with neoplasia and metastasis (Philips and Cooper 2000; Nissim-Rafinia and Kerem 2002; Venables 2004). The observation that these genes can be either regulators of apoptosis, tumor suppressors, or receptors mediating cell–cell and cell–matrix interactions has raised the question of whether aberrant splicing contributes to malignancy. In the case of CD44, which functions in cell adhesion, migration, and cell–matrix interactions, an alternative splicing switch has been clearly correlated with the acquisition of a metastatic potential in a rat adenocarcinoma cell line (Gunthert et al. 1991). Later on, specific CD44 variants have been found to be associated with many human cancers (Sneath and Mangham 1998). Interestingly, the relative abundance of specific SR proteins is increased during progression from preneoplasia to metastasis in a mouse model of mammary tumor and, again, increased SR protein levels correlate with increased complexity of CD44 isoforms (Stickeler et al. 1999).

Altogether, these observations support the idea that deregulation of alternative splicing could constitute a key determinant of tumor progression. Although relevant correlations between altered expression levels of a subset of SR protein and development of different cancer types have still to be established, one can reasonably consider that approaches allowing the correction of splicing abnormalities hold great therapeutic promise in the field of human cancer.

1.5 Unforeseen Modulation of Alternative Splicing by Various Molecules

For almost two decades alterations of alternative splicing have been observed for many different genes in response to various chemical compounds and hormones. Indeed, sodium butyrate was shown to modulate the splicing pattern of the calcitonin gene in human thyroid carcinoma cells (Nakagawa et al. 1988)

while ethanol and DMSO treatment changed the expression profile of the calcium channel subunits- and the neural cell adhesion molecule (NCAM)-encoding splice variants, respectively (Walter et al. 2000; Bolduc et al. 2001). In neurons, glutamate was reported to modify the splicing pattern of the beta-amyloid precursor protein, the NMDA receptor, and the cyclin L ania-6 mRNAs (Willoughby et al. 1995; Meshul et al. 1996; Sgambato et al. 2003). More recently, the splicing defect caused by mutations in the I-κ-B kinase (IKK) complex-associated protein (IKBKAP) encoding gene and responsible for familial dysautonomia (FD) has been rescued in patients' lymphoblast cell lines by treatment with the plant cytokinin kinetin (Slaugenhaupt et al. 2004). Except regarding sodium butyrate, which is discussed below, the mechanism of action of the other compounds is not yet clearly established.

Glucocorticoids and steroid hormones can also modulate alternative splicing events, sometimes in opposite ways. Indeed, dexamethasone regulates alternative splicing of the insulin receptor mRNA and decreases inclusion of the STRess-axis-regulated EXon (STREX) in the Slo mRNA, whereas testosterone promotes STREX inclusion (Kosaki and Webster 1993; Lai and McCobb 2002). Recent studies concerning the role of steroid hormones in alternative splicing have revealed that corresponding receptors can simultaneously control gene transcription activity and exon content of the mRNA by recruiting coregulators involved in both processes (Auboeuf et al. 2002, 2004; Dowhan et al. 2005).

Recently, inhibitors specific for topoisomerases I and II were shown to modulate Caspase-2 pre-mRNA splicing in human cells (Solier et al. 2004). If the relationship between topoisomerase I inhibition and splicing alteration is now clear (see below) the role of topoisomerase II in the control of alternative splicing remains to be established.

With the exception of sodium butyrate and topoisomerase I inhibitors, there is no clear evidence that the above-mentioned compounds regulate alternative splicing through alteration of SR proteins expression and/or activity. In the next paragraphs, we will review the different means by which targeting SR proteins in a more or less specific way allows manipulation of alternative splicing.

2
General Modulation of SR Proteins' Expression Level

2.1
Modulation of Splicing by Histone Deacetylase Inhibitors

The ability of histone deacetylase inhibitors (HDACs) to correct aberrant splicing has been essentially demonstrated by using the SMN2 gene. Since spinal muscular atrophy and the corresponding therapeutic prospects are extensively reviewed in another chapter of this volume, we

will only mention how HDACs can regulate splicing and whether they are suitable for therapeutic approaches.

The first HDAC inhibitor shown to restore inclusion of exon 7 in the SMN2 mRNA is sodium butyrate (NaBu; Chang et al. 2001). Similar observations were subsequently reported for valproic acid (VPA; Brichta et al. 2003; Sumner et al. 2003) and phenylbutyrate (PBA; Andreassi et al. 2004; Brahe et al. 2005). In the case of VPA and PBA, treatment was shown to induce transcriptional up-regulation of SMN2 as well as an increase of exon 7 inclusion, a feature most likely attributed to increased levels of Htra2-β1, which facilitates the correct splicing of SMN2 RNA. As already reported for NaBu, VPA also induced the expression of further SR proteins. Since VPA is known to regulate about 2% of genes (Pazin and Kadonaga 1997) one can easily speculate that some SR-protein-encoding genes belong to this fraction.

If the striking disadvantage of NaBu is its very short half-life of only 6 min in human serum, making it inadequate for therapy. VPA, as well as other HDACs, is an FDA approved drug with a half-life of 8–10 h and which has been in clinical use for more than 3 decades. Beyond the fact that the exact mechanism of SR-protein induction remains to be elucidated, this property may prove to be very important for the treatment of many disorders affected by alternative splicing.

2.2 Modulation of Splicing by Aclarubicin

Increased retention of SMN2 exon 7 has also been observed in type 1 SMA fibroblasts treated with the antibiotic aclarubicin (Andreassi et al. 2001). Anthracycline antibiotics such as aclarubicin and doxorubicin are widely used in chemotherapy against solid tumors and leukemia. Anthracyclines have also been shown to be potent differentiation inducers when used at subtoxic concentrations (Chenais et al. 2000). In this pathway, aclarubicin seems to specifically increase the expression of transcription factors, whereas doxorubicin appears to act at the post transcriptional level by modulating mRNAs half-lives (Morceau et al. 1996). Along this line, the regulation of alternative splicing by aclarubicin could result from increased levels of transcription factors controlling SR protein expression.

Immunofluorescence experiments have also shown that aclarubicin treatment induces a redistribution of SR proteins, likely related to a modification of their phosphorylation level. Whether aclarubicin modifies the expression level or interferes with the activity of kinases or phosphatases involved in the regulation of SR protein phosphorylation remains to be established.

As a therapeutic agent used in cancer treatment, the side effects and known toxicity of aclarubicin make it unsuitable for consideration in the treatment of SMA or other diseases resulting from splicing alterations. However, neither doxorubicin nor any other tetracycline derivatives tested

show any activity on SMN2 splicing, suggesting that the way aclarubicin modulates splicing is different from other activities of this drug. If true, it could then be possible to design related compounds with less toxic effects.

3
General Modulation of SR Proteins' Phosphorylation Level

3.1
Modulation of Phosphatase Activity

The first compound identified that has been shown to stimulate exon 7 inclusion in transcripts derived from the SMN2 gene is the phosphatase inhibitor sodium vanadate (Zhang et al. 2001). Inasmuch as sodium vanadate inhibits ATPase, alkaline, tyrosine, and multiple other phosphatases, SR proteins are among the most likely proteins whose phosphorylation level is influenced by this drug. However, due to its toxicity, sodium vanadate is not a candidate for patient treatment.

Conversely, activation of protein phosphatase 1 (PP1) for which SR proteins constitute specific substrates, has been observed in response to de novo ceramide (Chalfant et al. 2001). Accordingly, endogenous ceramide modulates the phosphorylation status of SR proteins in a PP1-dependent manner (Chalfant et al. 2001) and dephosphorylation of SR proteins stimulated by PP1 induces alternative 5′ splice site selection in adenovirus pre-mRNA in vitro (Cardinali et al. 1994). More recently, ceramide-induced activation of PP1 has been correlated to the regulation of alternative 5′ splice site selection of Bcl-x pre-mRNA toward the pro-apoptotic Bcl-x(s) isoform (Chalfant et al. 2002; Massiello et al. 2004).

Ceramide is an important regulator of various stress responses and growth mechanisms, and its formation has been observed in response to agonists such as tumor necrosis factor-α, γ-interferon, UV light, heat, and chemotherapeutic agents (Pettus et al. 2004). Given that SR proteins are up-regulated in different cancer types and possibly involved in their progression, chemotherapeutic agents inducing the formation of ceramide could function by interfering with their activity in alternative splicing through activation of the PP1 phosphatase.

3.2
Modulation of Kinase Activity

The first evidence that chemical compounds targeting enzymes involved in the phosphorylation of the SR proteins' RS domain could constitute precious tools for interfering with alternative splicing regulation came from

the study of a glycosylated indolocarbazole derivative (NB-506), a potent inhibitor of DNA topoisomerase I (Pilch et al. 2001; Soret and Tazi 2003).

DNA topoisomerase I is a nuclear target of a number of anticancer agents derived from the plant alkaloid camptothecin (Pommier et al. 1998) and for indolocarbazole derivatives (Bailly et al. 1999a; 1999b) such as the antibiotic rebeccamycin and the antitumor agent NB-506, which is under-going phase II clinical trials (Meng et al. 2003). While NB-506 is structurally analogous to the specific protein kinase C (PKC) inhibitor staurosporine (Anizon et al. 1998), it has no significant effect on PKC (Anizon et al. 1998; Pommier et al. 1998; Bailly et al. 1999b; Labourier et al. 1999) but inhibits both relaxation (Soret and Tazi 2003) and kinase activities of topoisomerase I (Pilch et al. 2001). Indeed, NB-506 has been shown in vitro to block spliceosome assembly and splicing through inhibition of SR protein phosphorylation. NB-506 also leads to specific inhibition of SR protein phosphorylation in cultured cells, suggesting that the drug could modulate gene expression by changing the splicing pattern of protein encoding genes. Consistent with this suggestion, NB-506 induces dramatic changes of the mRNAs' distribution in P388 leukemic sensitive cells but not in P388CPT5 cells, which are resistant to the drug. NB-506 treatment also leads to pronounced changes in alternative splicing pattern of several genes, among which *Bcl-X* and *CD 44* gene products are known to affect apoptosis and tumor progression (Pilch et al. 2001).

Recently, we have taken advantage of cell lines highly resistant to camptothecin and that were described to have hardly any detectable DNA topoisomerase I (Pourquier et al. 2000). Analyses of splicing patterns of pre-mRNAs and phosphorylation status of SR proteins in these cells lines allowed us to establish that the kinase activity of topoisomerase I is required for ESE-dependent splicing (Soret et al. 2003).

Altogether, these observations raised the amazing possibility that alterations of regulated splicing constitute a key step accounting for the remarkable antineoplastic activities exhibited by the NB-506 drug both in cell culture systems and in xenograft models of human tumors (Pommier et al. 1998). Targeting the kinase activity of topoisomerase I could then offer opportunities to develop novel anticancer drugs interfering with the expression of specific genes involved in cell proliferation and/or apoptosis.

We have also exploited the role of DNA topoisomerase I in ESE-dependent splicing to develop entirely novel diospyrin derivatives targeting its protein kinase activity and thereby modulating pre-mRNA splicing. Diospyrin is a natural bisnaphthoquinoid extracted from the stem bark of the *Diospyros montana* Roxb (Hazra et al. 1984) an indigenous medicinal plant of the Ebenaceae genus, common in India. This compound and its derivatives have revealed interesting antitumor activities (Hazra et al. 1984, 1994; Pal et al. 1996; Chakrabarty et al. 2002) and were shown to induce apoptosis of different human tumor cells (Chakrabarty et al. 2002) but the mechanism of action of this compound remained essentially unknown. Nevertheless, selective

inhibition of topoisomerase I from *Leishmania donovani* has been reported with diospyrin itself (Ray et al. 1998) and very recently, the related compound isodiospyrin, extracted from *Diospyros morrisiana*, was shown to inhibit human topoisomerase I (Ting et al. 2003). Most interestingly, it was reported that isodiospyrin antagonizes camptothecin-induced DNA cleavage mediated by topoisomerase I and strongly inhibits the kinase activity of the enzyme toward SF2/ASF (Ting et al. 2003).

We have observed that, while some derivatives indeed inhibit kinase activity of topoisomerase I, they did not block reactions of topoisomerase I on DNA. However, these drugs interfere with camptothecin-dependent topoisomerase I-mediated DNA cleavage, implying that diospyrin derivatives mediate a conformational change of topoisomerase I. Importantly, in vitro splicing reactions revealed that diospyrin derivatives alter various steps of splicing. Some diospyrin derivatives inhibit either the first or the second catalytic step of splicing but not spliceosome assembly, while diospyrin itself prevents the formation of full spliceosome. Diospyrin derivatives therefore represent a novel class of drugs that alter steps occurring after the spliceosome assembly. Along this line, identification of target proteins within purified spliceosomes will not only shed light on the role of topoisomerase I in splicing but also on the mechanisms responsible for catalytic activation of the spliceosome.

Drug-mediated manipulation of alternative splicing has also been obtained with a newly developed inhibitor of two members of the Clk (cdc-2-like kinase) family (Muraki et al. 2004). Clk/Sty, the prototypic member of this family, has the unusual property of phosphorylating both serine/threonine and tyrosine residues and has been shown to autophosphorylate on all three hydroxy-aminoacids (Ben-David et al. 1991; Howell et al. 1991; Lee et al. 1996). The C-terminal catalytic kinase domain of Clk/Sty carries the conserved EHLAMMERILG motif common to all members of this family, which were hence classified as LAMMER kinases (Yun et al. 1994; Nayler et al. 1997). When overexpressed, a catalytically active form of Clk/Sty causes a redistribution of SR proteins in the nucleoplasm of transformed cells (Colwill et al. 1996) and may even result in the accumulation of SF2/ASF in the cytoplasm. Conversely, a catalytically inactive form co-localized with SR proteins but did not induce their redistribution (Colwill et al. 1996). Due to mislocalization of splicing factors following overexpression of Clk in transfected cells, it is possible that the observed effects on splicing could be indirectly caused by depletion of specific SR proteins from the nucleus. Along this line, overexpression of Clks and particularly Clk2 has been shown to regulate alternative splicing of human Tau pre-mRNA and to favor exon 10 skipping, likely by releasing specific SR proteins from nuclear storage sites (Hartmann et al. 2001). Note that Tau alternative splicing deregulation in neurodegenerative diseases is reviewed in another chapter of this volume.

Through extensive screening of a chemical library, a benzothiazole compound (TG003) has been found to be a potent inhibitor of Clk/Sty activity. This drug, a competitive inhibitor of ATP acting specifically on Clk/Sty and Clk4, suppressed Clk/Sty-dependent alternative splicing in mammalian cells and during Xenopus development (Muraki et al. 2004). Since the inhibitory effects of the drug appear to be reversible and not toxic in two different cell lines treated for a few days, TG003 may rescue splicing alterations produced by exon skipping such as that observed in some amyotrophic lateral sclerosis (ALS) patients, where exon 9 of the EAAT2 (excitatory amino acid transporters 2) pre-mRNA is aberrantly skipped without any mutation in the gene (Lin et al. 1998).

Splicing modulation and alteration of subnuclear localization of an SR protein has recently been observed following inhibition of glycogen synthase kinase-3 (GSK-3; Hernandez et al. 2004). GSK-3, which has emerged as a key kinase able to interact with many of the proteins involved in the etiology of Alzheimer's disease, is a serine/threonine kinase involved in glycogen metabolism and mostly expressed in the central nervous system (Grimes and Jope 2001; Woodgett 1990). Overexpression of GSK-3β in the brain of conditional transgenic mice resulted in tau hyperphosphorylation and somatodendritic localization (Lucas et al. 2001).

Specific inhibition of GSK-3 activity by lithium chloride (Klein and Melton 1996) or the thiazole derivative AR-18 (Bhat et al. 2003) was found to modulate tau splicing toward inclusion of exon 10 and to induce nuclear relocalization of SC35 in speckles (Hernandez et al. 2004). Since GSK-3 colocalizes with SC35 in nuclear speckles after inhibition and is able to phosphorylate SC35 in vitro, this kinase likely plays a novel role in the regulation of alternative splicing through phosphorylation of SC35 and probably other SR proteins. GSK-3 inhibitors such as AR-18 could be useful in the treatment of tauopathies such as Pick's disease in which tau isoforms are predominantly found with three R repeats, both by their ability to induce exon 10 inclusion in tau mRNA and reduce tau protein phosphorylation.

4
Modulation of Specific SR Proteins' Expression Level

4.1
Overexpression of SR Proteins

The first evidence that modifying the relative abundance of SR proteins and their molar ratio to antagonist factors could determine the alternative splicing patterns of many genes was observed more than ten years ago in HeLa cells overexpressing either SF2/ASF or hnRNP A1 (Caceres et al. 1994).

The subsequent findings that a significant number of genetic diseases result from mutations that weaken the efficiency of splicing enhancers recognized by SR proteins or even create splicing silencers interfering with the normal activity of these factors (Cartegni et al. 2002; Faustino and Cooper 2003) has led to consider SR protein overexpression as a potential therapeutic approach to correct disease-causing aberrant splicing.

The first gene for which the effect of overexpression of splicing factors on the level of correctly spliced transcripts was studied is the cystic fibrosis transmembrane conductance regulator (CFTR) gene whose mutations are involved in cystic fibrosis (CF). Initial experiments, performed with minigene constructs carrying CFTR splicing mutations (Nissim-Rafinia et al. 2000; Pagani et al. 2000, 2003; Aznarez et al. 2003) revealed that overexpression of splicing factors such as Htra-α was sufficient to counteract the effect of the mutations. More recently, overexpression of Htra2-β1 and SC35 in cystic-fibrosis-derived epithelial cells carrying the 3849+10 kb C>T splicing mutation was shown to increase the level of normal CFTR transcripts, to activate the CFTR channel, and to restore its function (Nissim-Rafinia et al. 2004).

As observed for cystic fibrosis, patients who carry splicing mutations causing spinal muscular atrophy (SMA) show extensive variability in disease severity, inversely correlated with the level of correctly spliced SMN2 mRNA containing exon 7 (Gavrilov et al. 1998). Again, overexpression of the splicing factor Htra2-β1 and, later on, of the hnRNP G protein were found to promote exon 7 inclusion and to increase the level of full-length SMN2 transcript (Hofmann et al. 2000; Hofmann and Wirth 2002).

4.2 Down-Regulation of SR Proteins' Expression

Inducing decrease of a specific splicing factor expression has proved to be efficient at correcting aberrant splicing events of several genes involved in genetic diseases resulting from mutations strengthening or creating splicing enhancer elements.

In the case of frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) splicing mutations, including all intronic mutations and several exonic mutations, are associated with changes in the ratio between the Tau 3R and 4R isoforms (Goedert and Jakes 2005). For one of the exonic mutations (N279 K), a T to G change was shown to improve an exonic enhancer recognized by the Htra2-β and to stimulate tau exon 10 inclusion, increasing thereby the Tau 4R/3R ratio. As expected, down-regulation of Htra2-β by RNA interference in cells transfected with a tau minigene construct led to a reduction of exon 10 inclusion (Jiang et al. 2003).

We have recently reported the RNAi-mediated correction of an aberrant splicing event in primary cells from a patient in a case of pyruvate dehydro-

genase (PDH) complex deficiency explained by an intronic mutation of the E1α PDH gene (Gabut et al. 2005). This mutation, located downstream from the normal exon 7 5′-splice site, leads to the major expression of an aberrantly spliced E1α PDH mRNA, which results from the activation of a cryptic 5′-splice site and retains 45 nucleotides of intronic sequences (Mine et al. 2003). Both in vitro and in vivo approaches have allowed us to demonstrate that this mutation results in an increased binding of the SC35 splicing factor to a pre-existing suboptimal enhancer element. By transfecting the patient's primary fibroblasts with siRNAs targeting the SC35 coding sequences, we have shown that in vivo depletion of this splicing factor efficiently restores the use of the PDH authentic splice site (Gabut et al. 2005).

5
Selective Modulation of SR Proteins' Activity

Experiments mentioned above have revealed that drugs interfering with the phosphorylation level of SR proteins can modify the alternative splicing pattern of several genes. Such drugs that target most, if not all SR proteins, likely exhibit a significant cytotoxicity and are therefore not suitable for long term treatment. Conversely, compounds inactivating SR proteins with a higher selectivity should prove to be less toxic and more adapted to treat diseases in which the SR protein to be inactivated is well characterized.

In a search for such molecules, we have recently screened a large collection of known compounds for their ability to selectively inhibit in vitro splicing of reporters containing splicing enhancer sequences specific for different SR proteins (Soret et al. 2005). Among the 220 indole derivatives tested in this assay, several compounds exhibit general inhibitory properties while some others are highly selective for splicing events mediated by different classes of ESE sequences. By taking advantage of the intrinsic fluorescence of several of these drugs, we have observed that the specificity of a drug for a given SR protein is tightly correlated to its ability to interact with this splicing factor, mainly through its RS domain.

While the exact mechanism responsible for the selective action of the indole derivatives remains to be elucidated, they might alter post-translational modifications and/or interaction of SR proteins with specific and/or constitutive splicing factors. Consistent with this hypothesis, several indole derivatives prevent phosphorylation of the RS domain by topoisomerase I and to a lower extent by Clk/Sty kinase, a modification shown to be required for ESE-dependent splicing (Soret et al. 2003). It is therefore possible that modulation of the phosphorylation status of specific SR proteins could be relevant to the drug effect.

Despite the fact that the drugs were selected by in vitro experiments performed with very simple splicing substrates, these molecules are also

capable of inhibiting splicing events in vivo with good specificity. Indeed, some of them were shown to prevent viral RNA splicing in cells transfected with a HIV-1-derived splicing reporter, and to be potent inhibitors of HIV-1 production in cells chronically infected by the virus. HIV-1, like other human viruses, uses alternative splicing to produce the large number of proteins required for its multiplication. Since HIV-1 alternative splicing events were shown to be regulated by several members of the SR protein family (Ropers et al. 2004) inhibition of splicing by indole derivatives is a likely explanation for the inhibition of viral production in chronically infected cells and may well be a key mechanism for the remarkable antiviral activities exhibited by these molecules in cell culture systems (Ducrocq et al. 1980).

6
Concluding Remarks

In the past few years, novel therapeutics strategies aimed at correcting or circumventing splicing alterations have been actively studied. In addition to those reported in this review, these approaches include the use of oligonucleotides or modified snRNA linked to antisense sequences to block use of aberrant splice sites (Kalbfuss et al. 2001; Mercatante and Kole 2002; Sazani and Kole 2003; Goyenvalle et al. 2004) the design of peptide-nucleic acids (PNA) or bifunctional oligos mimicking or recruiting SR proteins at specific sites (Cartegni and Krainer 2003; Skordis et al. 2003) as well as trans-splicing strategy allowing the replacement of mutated exons by their wild-type counterparts (Liu et al. 2002).

Among the various approaches reviewed in this chapter, those leading to a general alteration of SR protein activity will likely be accompanied by severe side effects and therefore be reserved for use in cancer therapy. Along this line, it is tempting to speculate that alterations of regulated splicing constitute a key step accounting for the remarkable antineoplastic activities exhibited by topoisomerase I inhibitors.

Concerning the strategies aimed at modifying the expression level of a given SR protein, the one relying on RNA interference appears particularly interesting. Indeed, siRNAs are not only an exciting new tool in molecular biology, but also represent the next frontier in molecular medicine (Ryther et al. 2005). Guaranteeing specificity and finding safe delivery systems will need further work but the therapeutic promises of small RNAs to solve genetic diseases, cancers and neurologic disorders still remain important.

On the other hand, our discovery that several indole derivatives specifically inhibit ESE-dependent splicing through their direct and selective interaction with members of the serine-arginine rich (SR) protein family provides an attractive alternative to the use of siRNAs. Furthermore, their

specificity for a subset and possibly a single member of the SR protein family suggests that they could exhibit a low toxicity, therefore allowing their development as clinically usable drugs. Along this line, it is noteworthy that most of the drugs selected in our in vitro splicing inhibition screen were not previously considered as good candidates for use in cancer therapy because of their low cytotoxicity.

Our current studies are now aimed at confirming in vivo this potency and lack of deleterious side effects of indoles derivatives, but these observations already open exciting prospects for causal therapies, not only for genetic diseases resulting from aberrant splicing, but also for cancer or viral infections where splicing regulators are essential for the pathological process.

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Misregulation of Tau Alternative Splicing in Neurodegeneration and Dementia

Athena Andreadis

Abstract. Tau is a microtubule-associated protein that fulfills several functions critical for neuronal formation and health. Tau discharges its functions by producing multiple isoforms via intricately regulated alternative splicing. These isoforms modulate tau function in normal brain by altering the domains of the protein, thereby influencing its conformation and post-translational modifications and hence its affinity for microtubules and other ligands. Disturbances in tau expression result in disruption of the neuronal cytoskeleton and formation of pathological tau structures (neurofibrillary tangles) found in brains of dementia sufferers. More specifically, aberrations in tau splicing regulation directly cause several neurodegenerative diseases that lead to dementia. This review briefly presents our cumulative knowledge of tau splicing regulation in connection with the alterations in tau splicing seen in neurodegeneration.

1 Brief Overview of Constitutive and Alternative Splicing

In the genomes of all organisms except eubacteria, mRNA-encoding segments of the DNA (exons) are interrupted by non-coding segments (introns). To produce the mRNA, the noncoding segments are precisely excised by a nuclear process called splicing which is executed by the spliceosome, a ribonuclear particle with a dynamic and fluid cast of participants (Sharp 1994). During alternative splicing, splice sites are either utilized or bypassed when the primary transcript is processed. Invariably, alternative splicing is temporally and spatially regulated.

Alternative splicing is a versatile and widespread mechanism for generating multiple yet precisely regulated mRNAs from a single transcript (Grabowski and Black 2001; Graveley 2001). In turn, the ensuing mRNAs produce functionally diverse proteins (Black 2003; Graveley 2001). At least 60% of human genes are alternatively spliced (Herbert 2004), making this mechanism the major contributor to proteomic complexity both directly (generation of many isoforms from a single gene) and indirectly (modulation of domains that are modified post-translationally).

Numerous studies of cis determinants of alternative splicing have shown that splice site selection occurs via hierarchies defined by complementarity of splice sites, branch points, and their associated polypyrimidine tracts to

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their cognate snRNPs (small nuclear ribonucleoproteins; Grabowski and Black 2001; Smith and Valcárcel 2000). In an increasing number of systems, exon inclusion is achieved by use of exonic enhancers (Blencowe 2000). Exonic enhancers act by binding splicing factors that activate splicing of otherwise weak exons, and their sequences usually are either purine rich (GAR) or A/C rich (ACE; Coulter et al. 1997; Liu et al. 1998). However, regulation is sometimes conferred by intronic enhancers or exonic or intronic silencers. The silencers can act by binding factors which either inhibit docking of the spliceosome or compete with activators for binding (reviewed by Zheng 2004).

On the trans side of regulation, mammalian splicing regulators mostly belong to two superfamilies, the serine/arginine rich (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) proteins, neither of which is involved exclusively in alternative splicing (Dreyfuss et al. 2002; Graveley 2000). The former are also components of the spliceosome, whereas the latter are also involved in pre-mRNA transport, RNA stability, and transla-tional regulation.

Several mammalian splicing factors are enhanced in or restricted to specific cell types (reviewed by Musunuru 2003). Nevertheless, it appears that the exquisite calibration of mammalian alternative splicing is primarily achieved by SR and hnRNP proteins, which show distinct ratios in tissues and during development, despite their ubiquitous distribution (Dreyfuss et al. 2002). Splicing factors can exert their influence by binding directly to the pre-mRNA or to factors bound to it (thereby facilitating or hindering spliceosome entry), by titrating other factors via sequestration, or by modifying the basal spliceosome. Mutations of cis elements and alterations of trans factors can result in human disease by causing aberrant splicing (Faustino and Cooper 2003; Stoilov et al. 2002; Ranum and Day 2004).

2
Structure, Transcripts, and Alternative Splicing of the Tau Gene

Microtubules are versatile polymers whose primary function is to generate specific cell morphologies and organize intracellular components. Microtubule-associated proteins (MAPs) are a disparate group of proteins that regulate the microtubule polymer state and also interact with other cytoskeletal and subcellular components (reviewed by Ramirez et al. 1999).

Tau is a microtubule-associated protein enriched in axons of mature and growing neurons (Kempf et al. 1996). Tau is also found in the cell nucleus associated with the nucleolus (Thurston et al. 1997), in the distal ends of growing neurons (Black et al. 1996; DiTella et al. 1994), in oligodendrocytes (Gorath et al. 2001), and in muscle (Wei and Andreadis 1998). Tau fulfills several functions, which include neurite extension, establishment of neuronal

polarity, axonal microtubule assembly, stabilization and spacing, and interaction with the plasma membrane, possibly as part of signal transduction pathways (reviewed by Shahani and Brandt 2002). Tau undergoes extensive phosphorylation on Ser/Thr and Tyr residues in vivo (reviewed by Stoothoff and Johnson 2005). Phosphorylation affects the conformation and increases rigidity of the tau molecule, decreasing its affinity for microtubules.

Hyperphosphorylated, MT-dissociated tau protein is the major component of intracellular neurofibrillary tangles (NFTs), a hallmark of almost all types of neurodegeneration including Alzheimer's disease and the adult dementia that accompanies Down syndrome (Billingsley and Kincaid 1997; Lovestone and Reynolds 1997). NFTs, in the absence of extracellular amyloid deposits, define several neurodegenerative diseases grouped under the term "tangle-only tauopathies" (Ingram and Spillantini 2002; Sergeant et al. 2005). Among them are progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), argyrophilic grain disease, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Hyperphosphorylated tau is also found in patients suffering from myopathies: NFTs form in the brains of sufferers from myotonic dystrophy type 1 (DM 1), a pleiotropic disorder whose symptoms include dementia (Modoni et al. 2004; Sergeant et al. 2001) and NFT-like aggregates are present in the muscles of people suffering from inclusion-body myositis (reviewed by Askanas and Engel 2002).

Tau is encoded by a single-copy gene located on chromosome 17q21.1 in humans (Himmler 1989; Neve et al. 1986). It produces three transcripts of 2, 6, and 9 kb which are differentially expressed in the nervous system, depending upon stage of neuronal maturation and neuron type (reviewed in Andreadis 2005). Figure 1A shows the exon structure and splicing patterns of the tau gene and Fig. 1B shows the effects of splicing decisions on the molecule's function. Eight of the sixteen tau exons (2, 3, 4A, 6, 8, 10, 13, 14) are alternatively spliced (reviewed in Andreadis 2005). The resulting protein isoforms are a series of closely spaced bands from 58 kD to 66 kD and, in some neuronal tissues, a 110 kD isoform arising from the 9 kb mRNA (Drubin et al. 1988; Oblinger et al. 1991).

Figure 1C shows the tau isoforms that are prevalent in brain. Their relative abundance is spatially and temporally regulated: Exons 2, 3, and 10 are adult-specific, but their ratios differ in various CNS compartments. The exact function and ligands of exons 2 and 3 are unknown, although the N-terminal domain of tau interacts with the plasma membrane (Brandt et al. 1995) and undergoes phosphorylation by fyn at residue Tyr18 (Lee et al. 2004). Exon 10 increases tau affinity for microtubules, by introducing an additional "repeat" sequence of 31 amino acids (cassette exon 10) into the microtubule-binding domain of tau (the other microtubule-binding repeats are in exons 9, 11, and 12; Himmler et al. 1989; Lee et al. 1989).

All six possible product combinations of the 2/3/10 splicing events have been observed (Goedert et al 1989a; Himmler 1989; Kosik et al 1989),

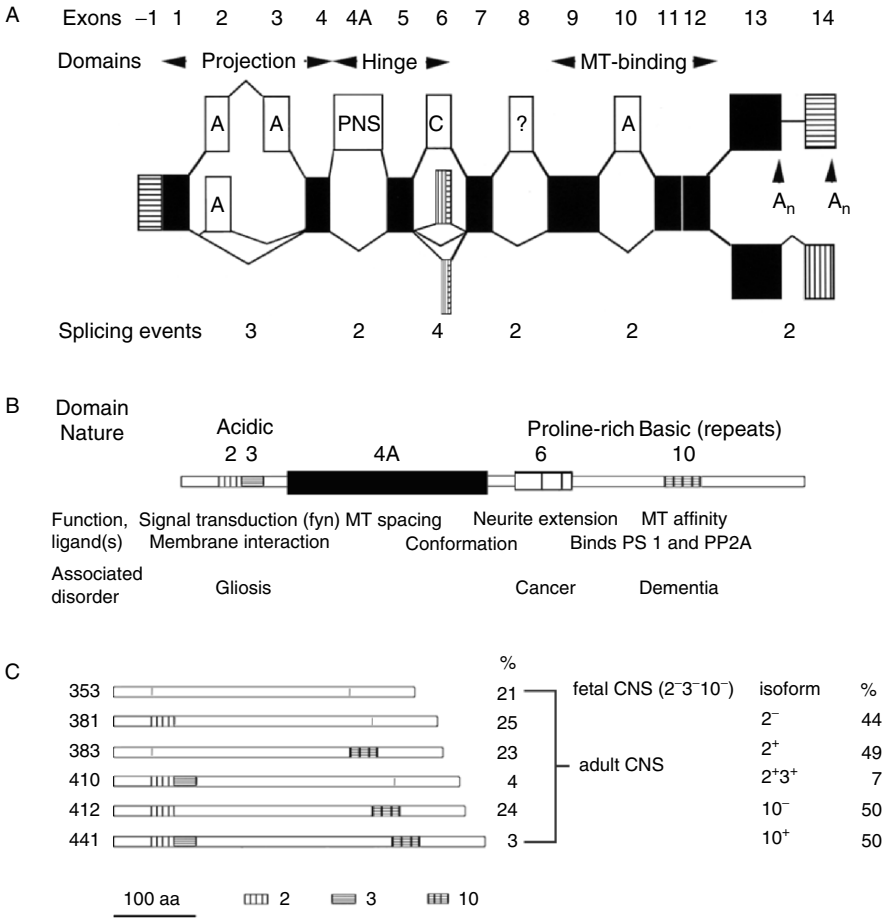

Fig. 1A–C. Tau mRNA species and the functions of the ensuing domains. **A**: Schematic representation of exons and splicing pathways in the tau gene. *Black* = constitutive; *white* = regulated (*A* = adult-specific, *PNS* = specific to the peripheral nervous system, *C* = complex, *?* = unknown); *horizontal stripes* = transcribed, untranslated regions; *vertical stripes* = alternative/additional reading frames. A_n indicate polyadenylation sites. The *numbers* underneath the exons indicate possible outcomes from each alternatively spliced region within the tau transcript. **B**: Diagram of the longest tau isoform. *Above the diagram* the general nature of the domain is noted. *Below the diagram* is a list of domain functions and of diseases in which the splicing of that particular region is or may be altered. **C**: Schematic depictions of tau isoforms abundant in the CNS. On the *left* is the length of each isoform in amino acids, on the *right* its relative abundance in adult CNS. *Below the diagrams* is a *scale bar* (*aa* = amino acids) and the *drawing conventions* for exons 2, 3, and 10

indicating that separate factors govern their splicing – a conclusion confirmed by extensive studies of splicing-factor effects on these exons (Arikan et al. 2002; Gao et al. 2000; Li et al. 2003; Wang et al. 2004; Wang et al. 2005). The expression pattern of exon 10 shows a crucial difference between rodent and human, which becomes relevant in neurodegeneration: exon 10 becomes constitutive in adult rodents (Kosik et al. 1989) whereas it remains regulated in the CNS of adult humans (Gao et al. 2000; Goedert et al. 1989b).

3
Tau Splicing and Neurodegeneration, Prologue

The connections between tau and dementia were long known and tantalizing, yet elusive. Several types of dementia exclusively show tangles in the brain (in neurons and/or glia) without amyloid plaques (Brandt et al. 2005). Also, in all dementias the number of NFTs correlates with the clinical severity of the disease (Arriagada et al. 1992; Bierer et al. 1995). Last but decidedly not least, many familial dementia pedigrees were mapped to 17q21, the tau locus. Despite these observations, tau was placed at the back of the neurodegeneration bus until 1998 for a simple reason: no mutations had yet been discovered in tau from dementia pedigrees. The researchers who were looking for such mutations were not "splicers" and naturally enough they originally focused on discovering missense or nonsense mutations in the tau protein.

In 1998, the characterization of several FTDP-17 pedigrees firmly placed tau and, specifically, its splicing directly upstream of the process that causes dementia (Clark et al. 1998; Hutton et al. 1998). Several experts believe that tangle-only tauopathies are the second most common type of dementia after Alzheimer's disease (Wilhelmsen 1998). Although these tauopathies show such clinical variability that they have often been misdiagnosed, their molecular causes are remarkably uniform: the afflicted pedigrees analyzed thus far predominantly show mutations in tau exon 10, although several pedigrees carry missense mutations in tau exons 1, 9, 11, 12, and 13 that influence microtubule binding and assembly, tau protein conformation, and tau translation (Ingram and Spillantini, 2002). Some of the exon 10 mutations are missense that influence microtubule binding, such as P301L. However, the majority are silent at the protein level but alter the ratio of exon 10 isoforms (Fig. 2).

A few years after the FTDP discoveries, a second connection was discovered between tau and an odd kind of neurodegeneration. Myotonic dystrophy 1 (DM 1) is the most common disease of its kind in adults, a multisystemic, dominantly inherited disorder whose outcome includes dementia classified as an atypical tauopathy (Ranum and Day 2004). DM 1 brains show tau hyperphosphorylation, formation of intraneuronal

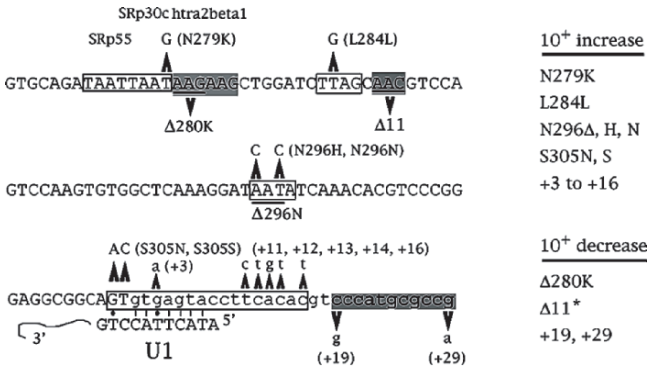

Fig. 2. Mutations of exon 10 found in pedigrees of neurodegenerative diseases (the exception is Δ11, designated by an *asterisk*, which was defined by the behavior of deletion constructs; D'Souza and Schellenberg 2000; Wang et al. 2004). The sequence of exon 10 and its proximal downstream intron is shown. Exonic sequences are in *uppercase*, intronic in *lowercase*. Point mutations are indicated, as well as their resulting missense mutations, if any. Deletions are *underlined*. The only mutation not shown is P301L/S, which does not affect splicing. The *boxed regions* define enhancers (*gray*) or silencers (*white*). Factors whose interactions have been confirmed are listed *above the elements* they recognize. Also shown is the complementarity of the 5′ splice site with the U1 snRNA. *Lines* are regular Crick/Watson pairs, *dots* are G-T pairs. The effect of each mutation on exon 10 splicing is listed on the *right*

aggregates, and significant reduction of tau isoforms containing exon 2 and 10 (Jiang et al. 2004; Sergeant et al. 2001). The disease, as discussed below, arises from titration of splicing regulators, which explains its pleiotropic phenotype.

This behavior makes tau a unique system for two reasons. Tau belongs to a tiny category of genes in which disturbances of alternative splicing cause disease despite production of wild-type protein. Additionally, tau is the only system documented so far in which changes in isoform ratios arising from splicing misregulation can cause neurodegeneration both directly (by cis mutations in tau exons) and indirectly (by variations in levels of trans factors that regulate tau exons).

As a result of these findings, it is not surprising that a significant interest arose in understanding the splicing regulation of tau exon 10 – although the DM 1 observations point to a possible pathogenic connection that involves exon 2. The body of this article will review what is known about the splicing regulation of exons 2 and 10 under normal and abnormal circumstances.

4
Commonalities in the Splicing Regulation of Tau Exons 2 and 10

Tau exons 2 and 10 show intriguing similarities in their expression patterns and behavior. Both are adult-specific, their relative levels of expression are congruent across different nervous tissues (Gao et al. 2000; Wei and Andreadis 1998) and the levels of isoforms containing them are roughly equal in adult CNS (Zhu et al. 2003; Fig. 1C). Also, the default splicing behavior of both exons is inclusion (Andreadis et al. 1995, Gao et al. 2000), yet most SR and hnRNP proteins inhibit their splicing (Li et al. 2003; Wang et al. 2004). This collective behavior is unusual, though consistent with the hypothesis (now confirmed by experimental data) that exons 2 and 10 must be primarily regulated through inhibition effected by silencers. Not surprisingly, the two share common points in their splicing regulation, as enlarged below: both contain exonic silencers at their 5′ end that are regulated by a complex consisting of splicing factors SRp30c, SRp55, and human transformer 2 beta 1 (htra2beta1).

Speculative models for the splicing regulation of exons 2 and 10 are shown in Fig. 3. These models come from all data available on the system and from cumulative knowledge of how cis elements and trans factors interact in other alternatively spliced systems.

4.1
Exon 2 – Splicing Regulation and the Possible Connection to DM 1

The default splicing pattern of exon 2 is inclusion, and the length of the flanking exons correlates inversely with its inclusion (Andreadis et al. 1995; Li et al. 2003). The intron-length effect is a general phenomenon seen with regulated exons, and probably reflects the availability of sequences that can act as low-affinity sinks for splicing factors. Exon 2 contains a pyrimidine-rich exonic silencer at its 5′ end, a purine-rich enhancer at its middle, and an intronic silencer ~300 nucleotides beyond the exon (Li et al. 2003; Wang et al. 2005). The intronic element is conserved across species and contains binding motifs for the intronic regulators PTB (polypyrimidine tract binding protein), nPTB (neuronal PTB), MBNL1 (muscleblind 1), and the CELF (CUG-BP and ETR-like factor) proteins, all of which regulate splicing of exon 2. Splicing regulators SRp30c, SRp55, htra2eta1, PTB, and CELF5 significantly inhibit exon 2 splicing, whereas CELF4, Nova1, SLM1 (Sam68-like mammalian protein 1), SLM2, and nPTB activate it (Li et al. 2003).

Recent work in our laboratory showed that the exonic silencer in exon 2 regulates splicing of the exon by directly binding SRp55 and SRp30c, which in turn bind the arginine-rich N-terminal domain (RS1) of

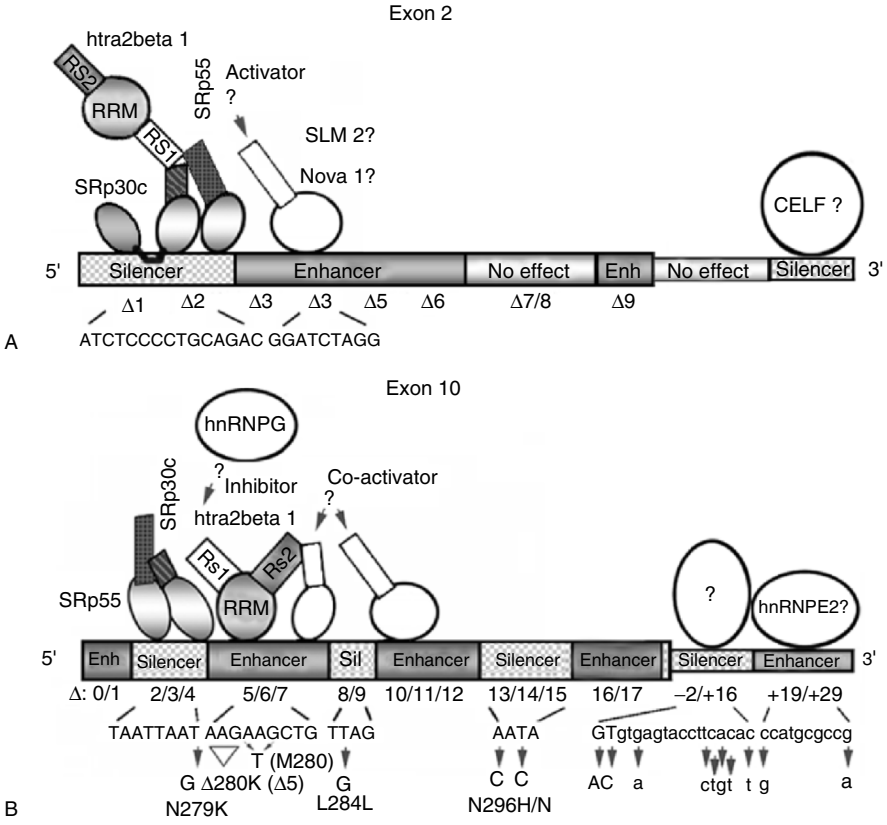

Fig. 3A, B. Models of regulation for tau exons **A** 2 and **B** 10. Cis elements which act as silencers (*gray*) and enhancers (*striped*) are indicated (*not to scale*). For the trans factors, binding to an enhancer implies that the factor activates splicing of that exon, whereas binding to a silencer implies that the factor inhibits it. For the factors, *circles* depict RNA recognition (RRM) domains, *rectangles* protein interaction (RS) domains. Factors whose identity and recognition site have been confirmed are *shaded*, whereas factors whose binding site and details of action are speculative are *white*. The *notations* for the deletions are those used by D'Souza and Schellenberg 2000 and Wang et al. 2005. In exon 10, the FTDP mutations are indicated as well as a double mutation (M280), which weakens the GAR enhancer

htra2beta1 (Wang et al. 2005). The inhibitory heterotrimer may interfere with the U2 snRNP, whose binding near the 3′ splice site is an early step in the splicing process. We also have preliminary evidence that CELF and MBNL proteins may bind to the silencer in the downstream intron.

The latter observation strongly suggests that members of these two splicing factor families almost certainly are native regulators of exon 2 splicing

in human brain, because exon 2 inclusion is suppressed in the brains of people who suffer from DM 1 (Sergeant et al. 2001). This is a peculiar tauopathy characterized by an unstable CTG repeat extension at the 3′-UTR of the DM protein kinase gene, which almost certainly sequesters members of the CELF and MBNL splicing families (Charlet et al. 2002; Ranum and Day 2004). In DM 1, inclusion of tau exon 2 drops tenfold, from 50% to 5%, whereas the change in exon 10 is twofold, from 50% to 25% (Jiang et al. 2004; Sergeant et al. 2001).

No mutations have yet been found in tau exon 2 in human disease pedigrees. Hence, it is not clear if the dementia that accompanies DM 1 is due to decreased levels of tau exon 2 or tau exon 10. Decrease of the 10^+ isoform is the obvious candidate by analogy with the outcome in tauopathies caused by cis changes in exon 10, in which a shift of even 10% in exon 10 expression is sufficient to cause FTDP (discussed below). However, the N-terminal tau domain interacts with the plasma membrane (Brandt et al. 1995), undergoes phosphorylation by fyn (Lee et al. 2004), and alterations in it – particularly truncations recently documented by conformation-sensitive tau antibodies – predispose tau to aggregation and tangle formation (Guillozet-Bongaarts et al. 2005). Additionally, Fe65, an adaptor protein which interacts with the cytoplasmic domain of amyloid precursor protein (Ermekova et al. 1998), also interacts with the N-terminal region of tau (Barbato et al. 2005). Thus, tau exon 2 and its splicing regulation must still be considered potential actors in neurodegeneration.

4.2 Exon 10 – Splicing Regulation and the Established Connection to Tauopathies

Most of the attention directed toward tau splicing regulation has been lavished on exon 10. This is not surprising, since pedigrees of several neurodegenerative diseases (FTDP-17, PiD, PSP, CBD) pinpoint exon 10 as one of the major causes of the malfunction – the other being missense mutations that affect microtubule binding (Ingram and Spillantini 2002).

Exon 10, like exon 2, shows a default splicing pattern of inclusion (Gao et al. 2000), again suggesting that its absence in fetal neurons may be due to a fetal-specific inhibitor. However, unlike exon 2, exon 10 splicing is influenced by the identity of its flanking exons: upstream exon 9 promotes its inclusion, whereas downstream exon 11 competes with it. As is the case with exon 2, the length of flanking introns also correlates inversely with exon 10 inclusion, regardless of trans context or the identity of flanking exons (Gao et al. 2000; Yu et al. 2004). However, there may exist specific regulatory elements in the introns flanking exon 10 – one such putative element is located ~177 nucleotides upstream of

the exon (Sobrido et al. 2003) and its downstream intron contains islands of conserved sequences (Wang et al. 2004), always a signal for the presence of regulatory elements.

All the exon 10 mutations found in dementia pedigrees are shown in Fig. 2. Many FTDP mutations cluster around the 5′ splice site of exon 10, engendering the hypothesis that its splicing is partly modulated by a putative hairpin loop that hinders interaction with the U1 snRNP (Clark et al. 1998; Grover et al. 1999; Hutton et al. 1998). However, all mutations that weaken the putative loop also increase complementarity to the 5′ end of U1, so the regulation can be explained by a suboptimal 5′ splice site without need to invoke the existence of a hairpin loop. Additionally, the proposed stem/loop structures are marginal: by the Tinoco rules, the free energies are –5.8 kcal/mol for the standard proposed stem/loop (Hutton et al. 1998) and –7.9 kcal/mol for the U1-exon 10 hybrid. Thus, the 5′ half of the proposed stem has higher affinity for U1 than for its proposed 3′ half. Independently of that, structures with such free energies are just stable enough to form in vitro but do not form in vivo (Solnick and Lee 1987).

An alternative explanation is that this region may inhibit exon 10 splicing by binding a factor (D'Souza et al. 1999; D'Souza and Schellenberg 2000; D'Souza and Schellenberg 2002). Several results argue in favor of the second theory: Compensatory mutations for positions +3 and +16 of the downstream intron do not restore the wild type splicing ratio (D'Souza et al. 1999; Grover et al. 1999); mutants at positions +11 to +16 increase exon 10 inclusion yet lie outside the range of U1 interaction (Fig. 2); and findings from both constructs and FTDP pedigrees indicate that there is a splicing enhancer located directly downstream of the silencer, which overlaps the 5′ splice site of exon 10 (D'Souza and Schellenberg, 2000; Stanford et al. 2003).

In contrast to the human scenario, tau exon 10 becomes constitutive in adult rats and mice (Kosik et al. 1989). In this connection, it is interesting that the region around the 5′ splice site of exon 10 is conserved only partially among human and mouse, diverging past position +8 (Grover et al. 1999). Given the strong effects of mutants in positions +11 to +29 (D' Souza et al. 1999; D'Souza and Schellenberg, 2000; D'Souza and Schellenberg 2002; Gao et al. 2000; Hutton et al. 1998; Stanford et al. 2003; Wang et al 2004), this must be at least one region where regulation of this exon differs among species. This crucial difference in the behavior of exon 10 in rodents may well correlate with the repeated failure to find neurofibrillary tangles in aged rodents as well as the difficulty of inducing them artificially in transgenic models (reviewed by Brandt et al. 2005).

Besides the involvement of the 5′ splice site, exon 10 splicing is affected by additional exonic sequences identified by FTDP mutations and/or systematic deletions (D'Souza et al. 1999; D'Souza and Schellenberg 2000;

D'Souza and Schellenberg 2002; Gao et al. 2000; Jiang et al. 2004; Wang et al. 2004; Wang et al. 2005). It contains at least two enhancers, one a GAR and one an ACE motif. The former corresponds to FTDP mutants N279K and Δ280K. Alteration or deletion of these two enhancers completely abolishes exon 10 inclusion. Exon 10 also contains three silencers, two of which overlap respectively with FTDP-17 mutants L284L and Δ296N/N296N/N296H (Grover et al. 2002; Wang et al. 2004). The former is a TTAG motif that acts as a splicing silencer in other systems, including tau exon 3 (Arikan et al. 2002; Del Gatto et al. 1996).

As with exon 2, although the default splicing behavior of exon 10 is inclusion, almost of the SR and hnRNP proteins tested inhibit its inclusion to some extent. Again, this collective behavior is unusual, although consistent with the hypothesis that exon 10 must be primarily regulated through inhibition. Splicing regulators SRp30c, SRp55, SRp75, 9G8, U2AF (U2 small nuclear ribonucleoprotein auxiliary factor), PTB, and hnRNPG strongly inhibit exon 10 inclusion, whereas htra2eta1, CELF3, and CELF4 activate it (Gao et al. 2000; Wang et al. 2004).

Htra2beta1 activates exon 10 splicing by binding to the GAR enhancer within exon 10, which overlaps mutants N279K and Δ280K (Jiang et al. 2003; Wang et al. 2005). Htra2beta1 may need a co-activator to exert its full effect on exon 10, since it is a weak activator by itself (Wang et al. 2005). HnRNPG, a known antagonist of htra2beta1 (Nasim et al. 2003), may inhibit exon 10 splicing by titrating out htra2beta1 – a conclusion strengthened by the finding that hnRNPG does not require its RNA recognition domain to exert its influence on exon 10 (Wang et al. 2004).

In contrast to the trans action of hnRNPG, SRp55 and SRp30c inhibit exon 10 splicing by binding directly to an AT-rich silencer upstream of the GAR enhancer, just as they do in exon 2. The heterodimer almost certainly interferes sterically with htra2beta1 through its N-terminal arginine-rich (RS1) domain (Wang et al. 2005). Extending the GAR enhancer increases splicing of exon 10, whereas altering its Gs to Ts decreases it (Jiang et al. 2003; Wang et al. 2005). However, mutant N279K appears to promote inclusion of exon 10 not by strengthening binding of activator htra2beta1, but by weakening the binding of inhibitor SRp30c (Wang et al. 2005).

Surprisingly, hnRNPA1 shows practically no influence on exon 10 (Gao et al. 2000; Wang et al. 2004). This is puzzling, because hnRNPA1 is invariably involved in cases of regulation via 5′ splice site selection (Chabot et al. 1997; Del Gatto et al. 1999), a mode indisputably operating on exon 10. Besides hnRNPG, another hnRNP protein influences exon 10: hnRNPE2, also known as poly(C) binding protein 2, moderately activates exon 10 inclusion (Broderick et al. 2004). Given its binding site preference, its effect on exon 10 and the usual location of hnRNP protein binding sites, hnRNPE2 may be influencing the enhancer in the downstream intron of exon 10 (Fig. 3B). Inclusion of exon 10 is also inhibited by hyperphosphorylation of splicing factors (Hartmann et al. 2001).

Exon 10 causes neurodegeneration through a relatively rare mechanism (only encountered so far in one other disease, Frasier syndrome, which is caused by altered ratios of a miniexon in the Wilms' tumor gene; Barbeaux et al. 1997; Klamt et al. 1998). Specifically, the dementia pedigrees in which splicing of exon 10 is affected produce wild-type tau, but its ratio has shifted from the normal 1:1 balance between the two exon 10 isoforms. Neurodegeneration occurs regardless of whether the ratio shifts towards 10^- (mutations Δ280K, +19, +29) or 10^+ (all the others) and regardless of how much the ratio changes. Interestingly, the tangles that form differ in terms of exactly how the tau in them aggregates, depending on which isoform (10^- or 10^+) is overexpressed (reviewed by Goedert and Jakes 2005).

5
Tau Connection to Disease, Epilogue

The actors and events which dictate the ratios of tau exons 2 and 10 are in the process of being defined. In contrast to the rapid increase in understanding at the molecular level, there is still or no clear sense of how tau deposition results in neurodegeneration and cognitive decline. Results from animal, cellular, and in vitro models give inconsistent results: some suggest that tau aggregated in tangles is the toxic species, while others indicate that aggregated tau is in fact an inert pool and a safe "warehouse" of otherwise toxic soluble tau species (reviewed by Brandt et al. 2005; Feinstein and Wilson 2005; King 2005). The latter theory gains support from both invertebrate and vertebrate animal models that overexpress tau, in which neurodegeneration occurs without obvious tangle formation.

Tau transgenic mice only recapture a subset of the FTDP symptoms, and the details of the pathology that they develop depend on the specifics of their transgene (Brandt et al. 2005). However, although the causal chain between the tau molecule and the neuronal death that heralds dementia is still unclear, it is certain that the tau splicing mutations are sufficient to cause neurodegeneration by altering the ratio of tau isoforms.

It is intriguing that disturbance in the relative tau isoform abundance should result in tangle formation and neuronal death. This correlates with the finding that mice which overexpress tau develop severe neuropathies or gliopathies regardless of transgene details (Brandt et al. 2005). This stands in stark contrast to tau null mice, which are viable though defective in neuronal maturation, muscular strength, and cognition (Dawson et al. 2001; Harada et al. 1994; Ikegami et al. 2000). These results suggest that tauopathies are variants of a dosage disease, like chromosomal trisomies.

The tau splicing variants affect specific functions of the protein: exon 2 modulates interactions with the membrane (Brandt et al. 1995) and may be involved in signal transduction mechanisms and growth cone dynamics

(Liu et al 1999). In this connection, it is interesting that the tau-homozygotes have morphologically altered small-caliber neurons, which have the highest proportion of microtubules close to the membrane (Harada et al 1994). Exon 10 increases affinity to microtubules (Lovestone and Reynolds 1997) and its presence may affect cytoskeletal fluidity. The two regulated domains also include potential sites for in vivo phosphorylation. Inclusion of these domains can alter tau affinity for microtubules, and influence its interactions with other cytoskeletal or membrane components, including regulatory kinases and phosphatases.

Skewed ratios of tau isoforms clearly influence the activity and effects of tau: besides the well-documented results of altered exon 10 inclusion, excess inclusion of exons 2 and 3 causes gliopathy and spinal cord degeneration (Higuchi et al. 2002) and tau is cleaved to its N-terminal fragment very early in neuronal apoptosis, in turn becoming an effector of the process (Canu et al. 1998; Fasulo et al. 2000). Moreover, tau transcription decreases greatly in neuronal cells vulnerable to apoptosis (Esclaire et al. 1998), whereas it increases in sufferers of Down syndrome, who often develop early-onset dementia (Mehta et al. 1999; Oyama et al. 1994).

One theory that would accommodate the behavior of all tau mutations is that tau polymerization (whether with itself or with other ligands) may require a precise stoichiometry of isoforms to correctly discharge one or more functions, which extend beyond just interaction with microtubules. Any disturbance in tau ratios may lead to the accumulation of tau unassociated with microtubules and its eventual precipitation into NFTs. Incorrect levels or species of soluble tau may sequester other cytoskeletal or membrane components, thereby disturbing axonal transport or architecture. Also, since tau in NFTs is irreversibly withdrawn from circulation, increase of NFTs may decrease tau levels below a limit critical to neuronal viability. Thus, altering the tau isoform ratio may correspond to either loss of function or gain of toxic function. At the cellular level, toxicity is defined by the final outcome of axonal disarrangement and eventual neuronal death. If enough neurons die, the brain can no longer rewire and reroute local functions, eventually resulting in the clinical presentations of dementia. Short-lived mammals such as rodents appear largely immune to such diseases, which require chronic accumulation. Disease onset is accelerated in pedigrees bearing mutations that make them more susceptible to deposition of insoluble proteins. Formation of aggregates has been proposed as the underlying unifying cause of neurodegenerative diseases (Singleton et al. 2004).

Increasing knowledge of the specific functions of the tau isoforms, as well as identification of the factors that regulate their splicing, will give significant insights into the normal and abnormal operation of the cooperative networks that establish and maintain neuronal morphology. Results from such work may reveal the processes common to types of dementia in which NFTs are the sole or major pathological manifestation and in the long term give us

a handle for ameliorating, preventing, or even reversing dementia – a specter that looms ever darker as the human lifespan lengthens.

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Spinal Muscular Atrophy and Therapeutic Prospects

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Abstract. The molecular genetic basis of spinal muscular atrophy (SMA), an autosomal recessive neuromuscular disorder, is the loss of function of the *survival motor neuron gene* (*SMN1*). The *SMN2* gene, a nearly identical copy of *SMN1*, has been detected as a promising target for SMA therapy. Both genes are ubiquitously expressed and encode identical proteins, but markedly differ in their splicing patterns: While *SMN1* produces full-length (FL)-*SMN* transcripts only, the majority of *SMN2* transcripts lacks exon 7. Transcriptional *SMN2* activation or modulation of its splicing pattern to increase FL-*SMN* levels is believed to be clinically beneficial and therefore a crucial challenge in SMA research. Drugs such as valproic acid, phenylbutyrate, sodium butyrate, M344 and SAHA that mainly act as histone deacetylase inhibitors can mediate both: they stimulate the *SMN2* gene transcription and/or restore the splicing pattern, thereby elevating the levels of FL-SMN2 protein. Preliminary phase II clinical trials and individual experimental curative approaches SMA patients show promising results. However, phase III double-blind placebo controlled clinical trials have to finally prove the efficacy of these drugs.

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Clinical Picture of Spinal Muscular Atrophy (SMA)

Spinal muscular atrophies are a genetically heterogeneous group of neuromuscular disorders with an autosomal or X-linked, recessive or dominant mode of inheritance. The majority of patients presents autosomal recessive inheritance with proximal manifestation of muscle weakness and atrophy, defined as autosomal recessive proximal spinal muscular atrophy (SMA). With an incidence of about 1:6,000 to 1:10,000, SMA is the second most frequent autosomal recessive disorder in humans (Pearn 1978; Czeizel and Hamula 1989). The carrier frequency, determined by direct molecular genetic testing, is 1:35 (Feldkötter et al. 2002; Cusin et al. 2003).

The clinical features of the disease are basically caused by the progressive loss of alpha motor neurons in the anterior horns of the spinal cord, which leads to symmetrical weakness and atrophy of the proximal voluntary muscles of legs, arms, and even of the entire trunk during disease progression. Electromyographic investigations in patients show a pattern of denervation, typically without sensory involvement or marked decrease in nerve conduction velocities. Muscle biopsy provides evidence of skeletal

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Alternative Splicing and Disease

muscle denervation, with groups of atrophic and hypertrophic fibers or fiber-type grouping most often found in chronic cases.

Since the disease severity of SMA is highly variable, the International SMA Consortium defined four clinical groups, depending on the age of onset and achieved motor abilities (Munsat and Davies 1992; Zerres and Rudnik-Schoneborn 1995).

Type I SMA (acute form, Werdnig-Hoffmann disease, MIM #253300) is the most severe form with generalized muscle weakness and hypotonia ("floppy infant") and a disease onset within the first six months of life. The children are never able to sit or walk and usually die within the first 2 years.

Type II SMA (intermediate form, MIM #253550) patients are able to sit but never able to walk unaided, usually present first symptoms after the first six months of life, and survive beyond 2 years.

Type III SMA (juvenile SMA, Kugelberg-Welander disease, MIM #253400) patients are able to sit and walk, and the lifespan is not reduced. Disease onset before the age of 3 years is classified as type IIIa, whereas an age of onset beyond 3 years is classified as type IIIb SMA.

Type IV SMA (adult form, MIM #271150) patients are comparatively mildly affected with an age of onset later than 30 years; they have a normal life expectancy.

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Molecular Basis of SMA

In 1990, types I, II, and III SMAs were mapped by linkage analysis to a region of about 10 cM on chromosome 5q11.2–13.3 (Brzustowicz et al. 1990; Gilliam et al. 1990; Melki et al. 1990) . The subsequent development of many new polymorphic markers allowed the critical region to be refined to less than 1 Mb (Melki et al. 1993; Soares et al. 1993; DiDonato et al. 1994; Melki et al. 1994; Wirth et al. 1994; Wirth et al. 1995). This region was shown to contain a highly complex genomic structure with an inverted and duplicated DNA segment of about 500 kb (Lefebvre et al. 1995), which considerably hampered the construction of a uniform physical map (Thompson et al. 1993; Melki et al. 1994; Lefebvre et al. 1995; Roy et al. 1995a; Roy et al. 1995b). As we know nowadays, this critical region is prone to de novo genomic rearrangements, including unequal crossovers, intrachromosomal rearrangements, and gene conversions (Melki et al. 1994; Wirth et al. 1997). Each 500 kb segment, which can be present in 0 to 4 copies per chromosome, contains five genes: the survival of motor neuron gene (*SMN*); the *BIRC1* (baculoviral IAP repeat-containing 1) gene, also known as *NAIP* (neuronal apoptosis inhibitory protein); the *SERF1* (small EDRK-rich factor 1) gene, also known as *H4F5,* the *GTF2H2* (general transcription factor IIH) gene or *p44*; and the *OCLN* (occludin) gene (Lefebvre et al. 1995; Schmutz et al. 2004). The polymorphic

region is proximally flanked by the unique gene *RAD17* and distally flanked by *TFNR* (Deimling von et al. 1999; Kelter et al. 2000).

2.1 SMN, the SMA Determining Gene

In 1995, Lefebvre and colleagues identified the survival of motor neuron gene 1 (*SMN1*) as the SMA disease-determining gene (Lefebvre et al. 1995). The *SMN* gene exists in two copies, *SMN1* and *SMN2*, which are almost identical except for five nucleotide differences: one each in exon 7 (840C→T, codon 280, nt position 27141), exon 8 (nt position 27869) and intron 6 (nt position 27092) and another two in intron 7 (nt positions 27289 and 27404), respectively (Fig. 1; Lefebvre et al. 1995; Burglen et al. 1996). All other variants described so far are found in both *SMN1* and *SMN2* and do not allow the *SMN* gene copies to be distinguished (Brahe et al. 1996; Hahnen and Wirth 1996; Monani et al. 1999). The ~1.5 kb *SMN* transcripts encode a 294 amino acid protein of 38 kDa (Lefebvre et al. 1995) that is ubiquitously expressed but markedly increased (~50-fold to 100-fold) in spinal cord as compared to other tissues (Lefebvre et al. 1995; Coovert

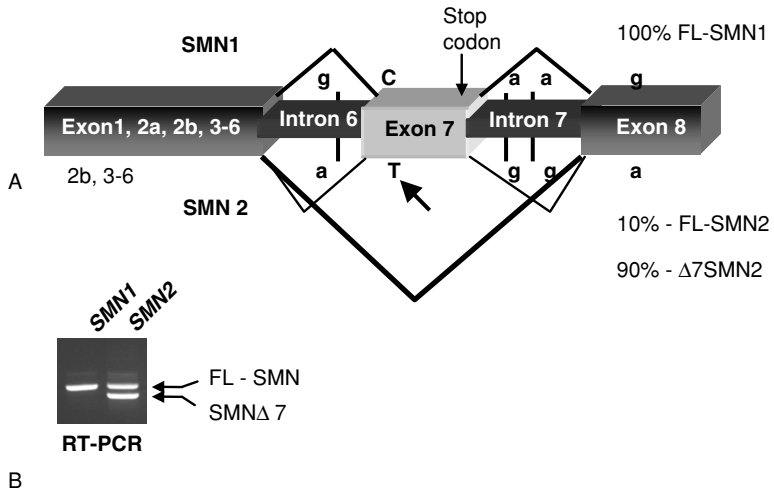

Fig. 1A, B. Genomic structure, nucleotide and splicing differences between *SMN1* and *SMN2*. **A**: The *SMN* gene copies can be distinguished by 5 nucleotide exchanges of which only the C → T transition in exon 7 lies within the coding region. The nucleotide exchange in exon 7 is a translationally silent mutation. Therefore full-length *SMN1* and *SMN2* mRNA encode identical proteins of 294 amino acids. However, the C→ T transition affects an exonic splicing enhancer and/or silencer causing alternative splicing of *SMN2* pre-mRNA. *SMN2*Δ7 transcripts produce a truncated and unstable protein. **B**: RT-PCR of *SMN1* and *SMN2* transcripts

et al. 1997; Lefebvre et al. 1997). Homozygous *SMN1* mutations are causative for SMA, while the SMA phenotype mainly depends on the number of *SMN2* copies (Lefebvre et al. 1995; Wirth 2000). The existence of two different *SMN* genes that are differently spliced (see below) is specific to humans. Mice and rats carry one *Smn* gene only, while primates also have several *SMN* gene copies, which, however, are not subject to alternative splicing (DiDonato et al. 1997a; Rochette et al. 2001) and are therefore orthologs of *SMN1*.

2.2 Alternative Splicing of SMN Transcripts

The two *SMN* genes, *SMN1* and *SMN2*, show a striking difference in the processing of their primary transcripts (Fig. 1). The disease-determining *SMN1* gene mainly produces full-length mRNA (FL-*SMN1*) including all exons (1, 2a, 2b to 8) whereas *SMN2* produces only very low amounts (~10%) of full-length mRNA (FL-*SMN2*) and predominantly alternatively spliced transcripts lacking exon 7 (*SMN2*Δ7). FL-*SMN* transcripts derived from both *SMN* copies encode an identical FL-SMN protein composed of 294 amino acids, with a stop codon located in exon 7. In comparison, the *SMN2*Δ7 transcripts encode a truncated SMN protein of only 282 amino acids, with a C-terminus that differs from the FL-SMN protein by the last 4 amino acids. The truncated protein is unstable and shows a reduced oligomerization capacity which has been shown to be essential for proper SMN function (Lorson and Androphy 1998; Lorson et al. 1998). Additionally, both *SMN* genes produce very low amounts of alternatively spliced transcripts lacking either exon 3, exon 5, or both (Gennarelli et al. 1995). Skipping of these exons produces in-frame proteins lacking the respective encoded domains. The loss of exon 3 is of particular interest, since the corresponding region contains a so called Tudor domain that is essential for the interaction of SMN with Sm proteins (see below). Absence or missense mutations within the Tudor domain either abolish or reduce the ability of SMN to interact with Sm proteins (Buhler et al. 1999; Mohaghegh et al. 1999; Sun et al. 2005).

2.2.1 Splicing Regulation of SMN Exon 7

SMN exon 7 spans 54 nt and harbors a stop codon at nt position 49 to 51. The last position of exon 7 is an adenosine residue, which places exon 7 into the minor group of internal exons lacking a 3′-end G residue (Burge et al. 1999). Exon 7 is characterized by a weak 3′splice site due to a suboptimal polypyrimidine tract (Lim and Hertel 2001). The C-to-T transition at position +6 of exon 7 (840C→T) is the only difference between the two *SMN* genes localized within the coding region. However, it is a translationally

silent mutation and consequently FL-*SMN1* and FL-*SMN2* transcripts encode identical proteins.

Correct splicing of exon 7 depends on various cis-acting elements (enhancers and silencers) localized within exon 7 as well as within the introns 6 and 7 (Lorson et al. 1999; Hofmann et al. 2000; Lorson and Androphy 2000; Cartegni and Krainer 2002; Hofmann and Wirth 2002; Miyajima et al. 2002; Young et al. 2002a; Kashima and Manley 2003; Miyaso et al. 2003). They are recognized by various splicing factors, SR and SR-like proteins as well as hnRNPs (Hofmann et al. 2000; Cartegni and Krainer 2002; Hofmann and Wirth 2002; Young et al. 2002a; Kashima and Manley 2003). Most of the elements seem to be highly conserved and are also involved in pre-mRNA splicing of the murine *Smn* gene (DiDonato et al. 2001). There is no tissue specificity observed concerning the ratio of FL-*SMN* vs *SMN*7 in a certain individual as demonstrated by quantitative RT-PCR (Lorson et al. 1999; Helmken et al. 2003).

In 1999, Lorson and colleagues demonstrated that the C→T transition in exon 7 is sufficient to cause skipping of this exon of the *SMNΔ2* gene. They constructed wildtype *SMN1* and *SMN2* minigenes (exon 6 to exon 8) as well as hybrid minigenes in which the five nucleotide differences were exchanged one by one either on the *SMN1* background with *SMN2* specific nucleotides or vice-versa, followed by in vivo splicing and analysis of *SMN* transcripts (Lorson et al. 1999). They demonstrated that an exonic splicing enhancer (ESE) is disrupted by the C→T exchange within the *SMN2* gene, thus being responsible for the alternative splicing (Lorson et al. 1999; Lorson and Androphy 2000). Later, Cartegni and Krainer stated that the C→T exchange lies within a conserved heptamer motif of an ESE, CAGA-CAA, which is directly recognized by the SR-rich splicing factor SF2/ASF in *SMN1*, but disrupted in *SMN2* derived transcripts. UV-cross-linking experiments showed specific interaction of SF2/ASF with exon 7 of *SMN1* but not with *SMN2,* thus promoting exon 7 inclusion (Cartegni and Krainer 2002).

In contrast to these findings, Manley's group demonstrated that the C→T exchange in *SMN2* creates a new exonic splicing silencer rather than disrupting an ESE, which finally functions as a binding site for the repressor protein hnRNP A1. It has been shown that the reduction of hnRNP A1 in HeLa cells by RNA interference promotes exon 7 inclusion in *SMN2*. Using in vitro UV-cross-linking, hnRNP A1 was found to bind exon 7 of *SMN2* but not of *SMN1* (Kashima and Manley 2003).

A debate is still alive and further experiments have to prove which of the hypotheses is correct. Meanwhile, Singh and colleagues showed that the 5′end of exon 7 contains an extended inhibitory context composed of several overlapping sequence motifs. Together, they regulate a larger sequence than the hnRNP A1 binding site (Singh et al. 2004b; Singh et al. 2004a).

In addition to the 5′end ESE and/or ESS, another ESE is found in the center of exon 7 that binds the SR-like splicing factor Htra2-β1 (Hofmann et al. 2000). Htra2-β1 is the ortholog of *Drosophila melanogaster* transformer-2

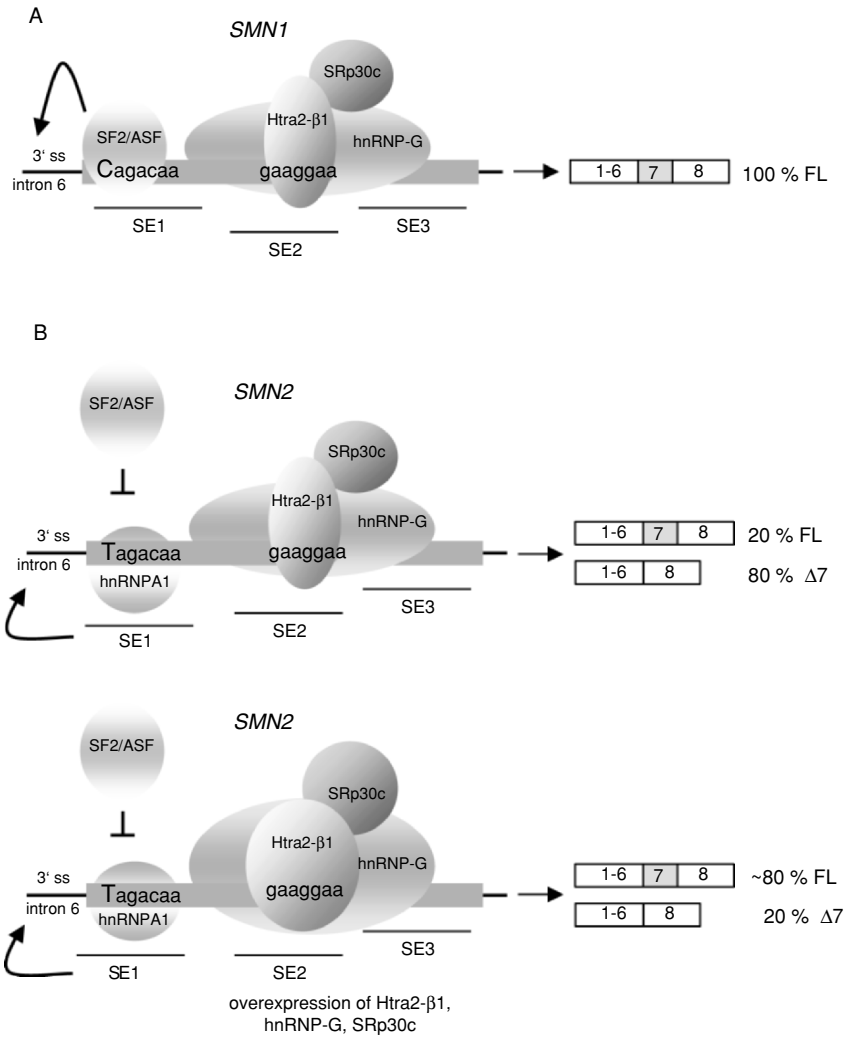

Fig. 2A, B. Model for an *SMN* exon 7 specific spliceosomal complex. **A**: The SR-protein SF2/ASF recognizes and binds to the critical heptamer sequence within the SE1 element of *SMN1* exon 7 and facilitates exon 7 inclusion (Cartegni and Krainer 2002). However, full-length *SMN1* transcripts are only produced when the SE2-domain is intact (Lorson and Androphy 2000). **B**: The C→T transition in *SMN2* disrupts the critical heptamer sequence within SE1, and the splicing factor SF2/ASF cannot bind to the 3′ splice site of nascent *SMN2* transcripts. Thus SF2/ASF completely fails to facilitate inclusion of exon 7 (Cartegni and Krainer 2002). Another paper shows that the C→T transition in exon 7 creates a new exonic splicing silencer which is bound by hnRNP A1 (Kashima and Manley 2003). The impaired splicing of exon 7 can partly be overcome by the AG-rich exonic splicing

(Tra2), a gene essential for sex differentiation that is regulated by alternative splicing (Baker 1989). Protein–RNA binding experiments showed a strong interaction of Htra2-β1 with the GA-rich region localized in the center of exon 7 of *SMN1* and *SMN2*. Mutations in this region abolish the capacity of both *SMN1* and *SMN2* to produce correctly spliced transcripts (Hofmann et al. 2000; Hofmann and Wirth 2002). In addition, the splicing factors SRp30c (an SR-rich splicing factor) as well as hnRNP-G and RBM (belonging to the group of hnRNPs) directly bind Htra2-β1 and further enhance the inclusion of exon 7 (Hofmann and Wirth 2002; Young et al. 2002a). This network of splicing factors binding to the central ESE in exon 7 is most likely responsible for the 10–15% full length mRNA generated by *SMN2*. Overexpression of these splicing factors, either separately or in combination, restores the splicing capacity of *SMN2* minigenes up to 80% and substantially increases endogenous SMN protein levels (Fig. 2; Hofmann et al. 2000; Hofmann and Wirth 2002; Brichta et al. 2003). A potential therapy of SMA based on the modulation of the *SMN2* splicing pattern that increases FL-SMN levels has been hypothesized (Hofmann et al. 2000).

Exon 7 skipping in *SMN2* is furthermore facilitated by two intronic splicing silencers localized in intron 6 from –112 to –68 bp (element 1) and intron 7 from +59 to +72 bp (element 2; Miyajima et al. 2002; Miyaso et al. 2003). However, mutations or deletions within these regions do not affect the correct splicing of wild-type *SMN1* pre-mRNA. A 33 kD protein has been shown to interact with element 1 of *SMN2* but not of *SMN1*. Element 2 possesses a characteristic stem–loop structure, in which the correct matching of the nucleotides within the stem is essential. In these papers, other genes with a complete matching of the nucleotides within the stem–loop have been identified, although the experimental proof for the role in the regulation of splicing still has to be presented (Miyajima et al. 2002; Miyaso et al. 2003).

Fig. 2A, B. (*Continued*)
enhancer within the SE2 element and the recruitment of several SE2-dependent *trans*-acting splicing factors: Htra2-β1, hnRNP-G, SRp30c (Hofmann et al. 2000; Hofmann and Wirth 2002; Young et al. 2002a). So far, Htra2-β1 is the most important SE2-dependent splicing factor identified, since it binds directly and specifically to the ESE within the SE2 element of *SMN* RNA and facilitates exon 7 inclusion. The protein–RNA interaction of Htra2-β1 and exon 7 is stabilized and Htra2-β1 function is enhanced by simultaneous direct protein–protein interaction of Htra2-β1with hnRNP-G which for its part binds very efficiently although nonspecifically to *SMN* RNA. SRp30c binds Htra2-β1 but barely *SMN* RNA (Young et al. 2002b). However, since hnRNP-G and SRp30c are attached to one another very efficiently (Venables et al. 2000) the whole complex is further stabilized. Overexpression of those SE2-dependent splicing factors can convert the *SMN2* splicing pattern and produce some 80% of *SMN2* full-length transcript

2.3 The SMN Protein Function

The SMN proteins encoded by FL-*SMN1* and FL-*SMN2* transcripts are identical. Both genes are ubiquitously expressed. The SMN protein is present in the cytoplasm as well as in the nucleus, where it is localized in the so-called gemini of coiled bodies (gems) as well as in the coiled (Cajal) bodies (Lefebvre et al. 1995; Liu et al. 1997; Young et al. 2000). SMN is part of a multiprotein complex containing numerous SMN-interacting proteins (sometimes referred to as Gemin 2–7) and the Sm class of spliceosomal U snRNPs (Liu and Dreyfuss 1996; Liu et al. 1997; Charroux et al. 1999; Charroux et al. 2000; Baccon et al. 2002; Gubitz et al. 2002; Meister et al. 2002; Pellizzoni et al. 2002). In addition the SMN complex has been shown to transiently interact with spliceosomal snRNPs U1, U2, U4, and U5 (Meister et al. 2002). The SMN protein has a major housekeeping function in the assembly and disassembly of the diverse small nuclear ribonucleoproteins (snRNP) and in pre-mRNA splicing (Fischer et al. 1997; Pellizzoni et al. 1998). Interestingly, functional studies indicated that the SMN complex mediates the formation of spliceosomal U snRNPs in an ATP-dependent manner and thus functions as an RNP chaperone (Liu et al. 1997; Meister et al. 2001). Although the molecular basis of this assisted RNP assembly is poorly understood so far, some important interactions of the SMN complex have been revealed by these studies.

Despite the vast knowledge concerning the SMN biochemistry, it remains unclear how an impaired ubiquitous SMN function in spliceosomal biogenesis specifically causes alpha motor neuron degeneration. This raises the question of whether SMN fulfills an additional function exclusively in alpha motor neurons. Immunocytochemical studies have localized SMN in dendrites and axons and suggest a role in transport of RNA along the axons (Bechade et al. 1999; Pagliardini et al. 2000). Zhang and colleagues showed that the SMN protein is localized in granules present in neurites and growth cones of cultured neuronal cells. SMN-containing granules exhibited rapid, bidirectional movements depending on both microtubules and microfilaments (Rossoll et al. 2003; Zhang et al. 2003).

SMN is highly expressed during embryogenesis, but levels decline rapidly after birth. In SMA-transgenic mice, the length of the dendrites is significantly reduced, whereas the number of motor neurons is not significantly affected as compared to controls (Monani et al. 2000). Conditional neuronal knock-out mice (SmnΔ7) lack axonal sprouting (Cifuentes-Diaz et al. 2002).

Consistent with these findings, knock-down of the Smn protein by antisense morpholinos in zebrafish embryos has revealed a significant axonal dysmorphology. These fail to reach motor neuron endplates due to early branching and truncation, which suggests an important role of SMN in the pathfinding of axons (McWhorter et al. 2003).

2.4 SMN1 Gene Mutations in SMA Patients

The vast majority of SMA patients (94%) shows a homozygous absence of *SMN1* exon 7 and 8 or exon 7 only due to deletions of *SMN1* or gene conversion of *SMN1* into *SMN2* (Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995; Velasco et al. 1996; Simard et al. 1997). Based on this relatively uniform mutational spectrum found in SMA patients, a fast and reliable molecular genetic PCR-based testing is available (van der Steege et al. 1995; Wirth et al. 1999). About 90% of SMA patients presenting homozygous absence of exon 7 also show homozygous absence of exon 8, while less than 10% of the patients present homozygous absence of exon 7 but not of exon 8 (Wirth 2000). The molecular basis for this phenomenon is gene conversion, a common mutational mechanism in the SMA region that causes either conversion of *SMN1* into *SMN2* or vice-versa (Lefebvre et al. 1995; Hahnen et al. 1996; van der Steege et al. 1996). Gene conversion has been described as a de novo event in rare cases (Raclin et al. 1997; Wirth et al. 1997; Wirth et al. 1999). The region of conversion can encompass the complete *SMN* gene as well as only a part of it. The smallest conversion event described in the literature affected only exon 7, which was the ultimate proof for the importance of *SMN* exon 7 in SMA pathogenesis (Lefebvre et al. 1995; Lorson et al. 1999).

Besides homozygous absence of *SMN1*, a minority of SMA patients (~4%) exhibit intragenic *SMN1* mutations. Typically, they are compound heterozygous with a deletion on one and a subtle mutation on the other chromosome 5 (Bussaglia et al. 1995; Hahnen et al. 1995; Lefebvre et al. 1995; Rodrigues et al. 1995; Velasco et al. 1996; Simard et al. 1997; Wirth et al. 1999; Wirth 2000; Ogino and Wilson 2002; Clermont et al. 2004; Sun et al. 2005). About 35 subtle mutations have been identified so far, many of them being missense mutations shown to disturb the proper function of the SMN1 protein (Lorson et al. 1998; Buhler et al. 1999; Sun et al. 2005). About 4% of patients showing a clear SMA phenotype indistinguishable from proximal SMA fail to show any mutation within the *SMN1* gene, pointing toward genetic heterogeneity (Wirth et al. 1999).

Homozygous absence of *SMN2*, a genotype found in about 3–5% of control individuals, has no apparent phenotypical consequences (Lefebvre et al. 1995). Thus, the presence of at least one fully functional *SMN1* gene is sufficient to protect from SMA.

2.5 Influence of SMN2 Copy Number on the SMA Phenotype

Despite apparently similar mutations, e.g. homozygous absence of *SMN1*, SMA patients present considerably different severities of the disease.

The main cause for this fact is the variable number of *SMN2* copies which directly correlates with the disease severity (Burghes 1997; Brahe 2000; Feldkötter et al. 2002). According to this observation, the majority of type I SMA patients carry two *SMN2* copies, type II SMA patients three *SMN2* copies, type III SMA patients three or four copies, and type IV four to six *SMN2* copies (Feldkötter et al. 2002; Wirth et al. 2006). Analysis of FL-*SMN2* versus *SMN2*Δ7 transcripts of type I-III SMA patients showed a ratio of about 20:80 in type I, 30:70 in type II, and 40:60 in type III SMA patients (Helmken et al. 2003). On the protein level, there are significant differences, especially between type I and II SMA patients versus type III patients and controls (Coovert et al. 1997; Lefebvre et al. 1997; Helmken et al. 2003). Individuals carrying 5–6 *SMN2* copies develop very mild SMA symptoms (type IV SMA; Wirth, unpublished results) whereas 8 *SMN2* copies fully protect from developing SMA (Vitali et al. 1999). Similar phenotypical differences have been observed in transgenic SMA mice carrying 2 to 8 copies of the human *SMN2* gene on an *Smn*-knockout background (Hsieh-Li et al. 2000; Monani et al. 2000).

2.6 Evidence for Further Genes Modifying the Disease Severity

In rare cases, siblings with identical *SMN1* mutations and identical *SMN2* copy number reveal marked phenotypical discrepancies, reaching from affected to SMA-unaffected, suggesting the existence of modifying genes not linked to chromosome 5q (Cobben et al. 1995; Hahnen et al. 1995; Wang et al. 1996; DiDonato et al. 1997b). In this context, the gender is of particular interest: except for a few cases, most unaffected but *SMN1*-deleted persons are females. Furthermore, there is a significant deviation from the expected 25% segregation ratio observed among females in type III SMA families (Rudnik-Schöneborn et al. 1994). More recently, Helmken and colleagues have been able to demonstrate that the modifying factor is regulating either the translation rate or the stability of the SMN protein, since unaffected persons with homozygous absence of *SMN1* reveal significantly more SMN protein compared to their affected siblings (Helmken et al. 2003). In contrast, phenotypically discordant siblings neither present differences on DNA level in the 5q13-region (identical *SMN1* mutations, identical *SMN2* copy numbers, identical 5q13 haplotypes) nor on RNA level (identical ratios of FL-*SMN2* vs. *SMN2*Δ7 transcripts; Helmken et al. 2003). This phenomenon was tissue specific and has been shown only in lymphoblastoid cell lines, not in primary fibroblasts derived from phenotypically discordant SMA siblings. In addition to the marked discrepancy concerning the SMN protein level, similar differences were found for SMN1-interacting proteins (Gemin 2, Gemin 3, ZPR1, hnRNP-G) and for Htra2-β-1. Especially the correlation between SMN and Htra2-β is highly

interesting, since Htra2-β1 is involved in regulation of pre-mRNA processing of exon 7. Unfortunately, the feedback mechanism controlling the amount of Htra2-β1 through SMN is yet unknown. SMN and Htra2-β1 proteins do not interact as shown by Co-IP analysis (Helmken et al. 2003). The identification of the modifying gene could open a new therapeutic strategy to cure SMA, since that biological pathway would point out a possibility for preventing SMA in case of homozygous absence of *SMN1*.

3 Therapeutic Prospects for SMA

Development of a therapy for spinal muscular atrophy is an exceptional challenge for the scientific community. Meanwhile, SMA seems destined to become one of the first inherited diseases in humans that may be cured by transcriptional activation and correction of the splicing of a copy gene. Disclosure of the molecular cause of SMA and the molecular basis of the alternative splicing of *SMN2* exon 7 presents the opportunity to develop therapeutic strategies that modulate transcription, splicing, and translation regulation of *SMN2*. Various therapeutic strategies have been considered so far:

(a) Elevation of the endogenous FL-SMN protein level encoded by *SMN2*
   - Transcriptional *SMN2* activation via the gene promotor
   - Restoration of the correct splicing of *SMN2* pre-mRNA
   - Taking advantage of the pathway being responsible for the translation regulation of *SMN2* in some unaffected individuals carrying homozygous *SMN1* mutations

(b) Compensation of the lack of sufficient SMN protein by:
   - Stem cell therapy
   - Gene therapy

(c) Improvement of motor neuron viability through alternative pathways

In the following section strategy (a) will be discussed in more detail.

3.1 Drugs that Increase the SMN Protein Level

Since demonstration that each SMA patient lacking *SMN1* carries 1 to 4 or sometimes even more *SMN2* gene copies (Burghes 1997; Brahe 2000; Feldkötter et al. 2002; Wirth et al. 2006), researchers worldwide have eagerly been searching for substances that increase *SMN2*-derived SMN protein levels in order to identify candidate drugs for SMA therapy. Several compounds have been described to increase SMN protein levels in fibroblasts and/or lymphoblastoid cell lines derived from SMA patients, including: the histone deacetylase (HDAC) inhibitors sodium butyrate (Chang et al. 2001); valproic

acid (Brichta et al. 2003; Sumner et al. 2003; Brichta et al. 2006); phenylbutyrate (Andreassi et al. 2004); SAHA and M344 (Hahnen et al. 2005); as well as interferon (Baron-Delage et al. 2000); hydroxyurea, a cell cycle inhibitor (Grzeschik2005); aclarubicin, an anthracycline antibiotic (Andreassi et al. 2001); sodium vanadate, a phosphatase inhibitor (Zhang et al. 2001); and indoprofen, a nonsteroidal anti-inflammatory drug (Lunn et al. 2004). HDAC inhibitors act on both transcriptional activation and/or splicing correction of *SMN2* pre-mRNA; interferon activates the transcription of *SMN2* only; indoprofen increases the amount of SMN2 protein without an elevation on RNA level whereas aclarubicine and sodium vanadate only facilitate the inclusion of exon 7 into the *SMN2* pre-mRNA. While most of these substances are not suitable for SMA therapy due to unfavorable toxicity profiles, some HDAC inhibitors are already FDA-approved drugs and used in the therapy of various diseases. These drugs will be highlighted in more detail.

3.1.1 Histone Deacetylase (HDAC) Inhibitors that Increase the SMN-RNA/Protein

One of the most exciting findings in SMA research was the identification of HDAC inhibitors as a group of drugs that increase the SMN protein levels in vitro and in vivo by activating the transcription and/or correcting the splicing of *SMN2*. Activation and repression of gene transcription largely depends on chromatin structure. Chromatin consists of DNA, histones, and non-histone proteins. Approximately two superhelical turns of DNA containing 146 base pairs are wrapped around an octamere of core histones H4, H3, H2A, and H2B, forming the basic unit named nucleosome (Luger et al. 1997). Nucleosomes are repeating units building up the chromatin and showing a dynamic structure that can be more condensed or relaxed depending on the balance of posttranslational histone modifications. Posttranslational modifications occurring at the amino-terminal tails of histones include acetylation, methylation, and phosphorylation. The enzymes responsible for acetylation are histone acetyltransferases (HATs). They add acetyl groups to N-terminal lysines of H3 and H4 histones. Thus, the chromatin structure becomes more relaxed and transcription factor complexes have better access to promotor regions in order to activate gene transcription. In contrast, histone deacetylases (HDAC) remove acetyl groups, the chromatin structure becomes more compact and gene transcription is repressed. Histone modifications are reversible (Zhang and Reinberg 2001; Marks et al. 2004). In humans there are three classes of HDAC enzymes. Class I includes HDAC 1, 2, 3, and 8; these are small molecules of 22–55 kDa localized exclusively in the nucleus. Class II includes HDAC 4, 5, 6, 7, 9, and 10; these are larger molecules of 120–135 kDA shuttling between nucleus and cytoplasm in response to certain cellular signals. Class III HDACs are most likely not acting on histones, but rather on transcription factors such as p53 (Marks et al. 2004).

HDAC inhibitors belong to three categories of drugs:

1. Hydroxamic acids: trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), pyroxamide, oxamflatin
2. Short chain fatty acids: sodium butyrate (NaBu), phenylbutyrate (PB), valproic acid (VPA)
3. Benzamides: MS275, M344, CI-994

Hydroxamic acids and benzamides are the most potent HDAC inhibitors, being active at nanomolar concentrations. The short-chain fatty acids are less potent and micromolar amounts are required to inhibit HDAC activity.

VPA mainly inhibits HDAC2. Besides reversible binding to the catalytic center, it also facilitates its effect on HDAC2 through increased ubiquitination of HDAC2 followed by elevated proteasomal degradation (Kramer et al. 2003).

The first HDAC inhibitor shown to increase SMN levels in EBV transformed lymphoblastoid cell lines was sodium butyrate. SMN transgenic mice treated with NaBu survived twice as long as untreated mice (Chang et al. 2001). NaBu is characterized by a very short terminal half-life of only a few minutes in human serum and is therefore inadequate for SMA therapy.

VPA, another short chain fatty acid with a much longer half-life of about 8–10 h in human serum, significantly increases SMN protein levels in cultured fibroblast cell lines. Treatment of cultured fibroblasts with 0.5 μM to 500 μM VPA increased the *SMN2* mRNA and protein level about 2–4-fold depending on the *SMN2* copy number. An exceptionally interesting finding was that VPA not only activates the transcription of the *SMN* gene but also restores the correct splicing of *SMN2* transcripts, most likely through Htra2-β1, which showed similarly increased values of about 2–4-fold in VPA-treated cells (Brichta et al. 2003). These findings suggest a double mechanism of action on *SMN2* in humans. Additionally, VPA activates the transcription of *SMN* in neuroectodermal tissue such as organotypic hippocampal brain slices from rat and humans (obtained from epilepsy surgery) as well as rat motor neurons (Brichta et al. 2003; Hahnen et al. 2006). The effect of VPA (1–10 mM) on SMN expression in cultured fibroblasts derived from SMA patients has been confirmed by Sumner and colleagues (Sumner et al. 2003).

Phenylbutyrate, a third fatty acid with a relatively short half-life in human serum (2–4 h), induced the *SMN2* RNA and protein level by 50 to 160% in type I and by 80–400% in type II and III SMA patients (Andreassi et al. 2004).

Among the second generation HDAC inhibitors, M344 and SAHA evolved as potent candidate drugs by increasing SMN2 protein levels at low micromolar doses in primary fibroblast cell lines from patients with SMA, in human organotypic hippocampal brain slices, as well as in rat motor neurons (Hahnen et al. 2006; Riessland et al., 2006).

3.2 Correction of Exon Skipping by Synthetic Small Molecules

Independently, two research groups showed that small synthetic molecules are able to restore the correct splicing of exon 7 leading to significantly elevated levels of FL-*SMN2* transcripts (Cartegni and Krainer 2003; Skordis et al. 2003). Cartegni and Krainer developed so called antisense peptide nucleic acids (PNAs), which are peptide chimeric molecules. The PNAs have a natural peptide-like backbone and standard nucleobases that form highly specific and stable complexes with the target RNA. At the C terminus, the PNA is bound to a peptide composed of 10 serine/arginine (SR) repeats. These molecules are nuclease resistant, form stable PNA–RNA complexes and can cross the cell membrane (Cartegni and Krainer 2003). Skordis and colleagues designed tailed oligoribonucleotides that were complementary to *SMN2* exon 7 and contained additional noncomplementary sequences (tails) that were predicted to mimic ESE sequences (GAA repeats, known to efficiently recruit Htra2-β1; Skordis et al. 2003).

Although these molecules act very efficiently in experiments in vitro, it is not yet known if a systemic administration can be successful and, most importantly, if they will pass the blood-brain barrier.

3.3 A First Causal Therapy for SMA Patients?

VPA has been shown to significantly increase SMN protein levels in fibroblast cell lines treated with drug amounts ranging from 0.5 μM to 50 μM (Brichta et al. 2003). VPA is an FDA-approved drug that has been used in the therapy of epilepsy for more than three decades (Zaccara et al. 1988). More recently, VPA has also gained importance as anticonvulsant in manic depression, migraine, and dementia (Papatheodorou et al. 1995; Mathew et al. 2000; Lonergan et al. 2004). Although it is known that VPA up- or downregulates about 2% of genes (Pazin and Kadonaga 1997), severe side effects are relatively rare. Based on these data, a pilot trial with VPA in 10 parents of SMA patients, each of them carrying one *SMN1* and one to four *SMN2* copies, has been initiated for a period of four months (VPA serum level 80 μg/ml). In 6 of the 10 SMA parents an increase in FL-*SMN*2 mRNA levels of 40 to 300% was determined. Particularly significant was the increase of SMN protein levels in 7/9 carriers raising from 1.8-fold to 13.8-fold under VPA treatment (Brichta et al. 2004).

Individual experimental curative approaches in 20 patients with type I, II and III SMA treated with VPA (serum level 70–80 μg/ml) revealed elevated FL-*SMN2* mRNA levels in 7 patients and unchanged or even decreased levels in 13 patients. (Brichta et al. 2006). An improvement of the

clinical picture after 5–6 months of treatment was observed in about 50% of the patients (Wirth, unpublished data). Some patients presented decreased L-carnitin levels which were compensated by substitution with acetyl-carnitin. Observation of patients with unchanged/decreased FL-*SMN2* transcript levels underscores the need for a biomarker. However, so far we do not know if *SMN* expression in blood reflects *SMN* expression in α-motor neurons and correlates with muscle strength. Therefore, long-term clinical trials in SMA patients that correlate *SMN* expression in blood with individual motor function tests are required. Two large trials with VPA/L-carnitin are in progress: in type I SMA patients in Germany (www.initiative-sma.de), and in type II and III SMA patients in the United States (www.FSMA.org).

A pilot trial with PBA in SMA patients already revealed promising results (Brahe et al. 2005). However, only phase III placebo-controlled clinical trials will provide the final proof for a potential use of HDAC inhibitors in SMA therapy.

In summary, VPA and PBA seem thus far to be promising compounds due to their excellent bioavailability, good penetration of the blood-brain barrier, and comparatively rare side effects at therapeutic doses. Clinical phase II and III trials are ongoing to ascertain whether the drugs are suitable for SMA therapy (Brahe et al. 2005; www.fSMA.org and www.initiative-sma.de). However, searching for further compounds is necessary due to the fact that in rare cases VPA and PBA cause severe liver and pancreas damage, interdicting further treatment. Out of the second generation HDAC inhibitors, SAHA is already under clinical phase II investigation for cancer treatment and recently has been shown to cross the blood-brain barrier with no observation of dose-limiting toxicity (Kelly et al. 2003).

4
Conclusions and Perspectives

What makes SMA a human genetic disease that may be cured in the future:

- Ninety-six percent of all SMA patients carry the same mutation in *SMN1* that enables early postnatal molecular genetic testing
- The severity of SMA correlates with the *SMN2* copy number that can easily be determined by quantitative PCR before treatment
- *SMN2* and *SMN1* produce identical proteins
- Htra2-β1, a nonessential splicing factor which promotes exon 7 inclusion can be upregulated by HDAC inhibitors
- The correct splicing of *SMN2* can be restored by exogenous factors (chemical drugs and small molecules)

- VPA and PBA are FDA-approved drugs. VPA has been used in long term therapy of epilepsy for more than three decades and rarely shows severe side effects
- First pilot trials in SMA patients treated with VPA or PBA show functional improvements after several months of treatment

SMA may become one of the first human inherited disorders in which our knowledge about the molecular basis of the disease and the regulation of transcription and splicing of the *SMN2* copy gene smoothes the way toward a causal therapy. Once phase III clinical trials have proven the drug efficacy in SMA patients, a newborn screening program will be a further important step in quick and early identification of the kids carrying *SMN1* homozygous deletions and treating them before the first symptoms appear.

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Misregulation of Alternative Splicing Causes Pathogenesis in Myotonic Dystrophy

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Abstract. Myotonic dystrophy (DM), the most common form of adult onset muscular dystrophy, affects skeletal muscle, heart, and the central nervous system (CNS). Mortality results primarily from muscle wasting and cardiac arrhythmias. There are two forms of the disease: DM1 and DM2. DM1, which constitutes 98% of cases, is caused by a CTG expansion in the 3′ untranslated region (UTR) of the *DMPK* gene. DM2 is caused by a CCTG expansion in the first intron of the *ZNF9* gene. RNA containing CUG- or CCUG-expanded repeats are transcribed but are retained in the nucleus in foci. Disease pathogenesis results primarily from a gain of function of the expanded RNAs, which alter developmentally regulated alternative splicing as well as pathways of muscle differentiation. The toxic RNA has been implicated in sequestration of splicing regulators and transcription factors thereby causing specific symptoms of the disease. Here we review the proposed mechanisms for the toxic effects of the expanded repeats and discuss the molecular mechanisms of splicing misregulation and disease pathogenesis.

1 Myotonic Dystrophy

DM is a multisystemic, autosomal dominant disorder that is the most common form of adult onset muscular dystrophy. Manifestations of the disease are highly variable, consisting of muscular, neuronal, and endocrine features, each of which may vary in severity. Muscle dysfunction is the most common symptom including muscle weakness, pain, and myotonia (difficulty relaxing muscle after voluntary contraction). Cardiac symptoms include conduction defects and arrhythmias, potentially resulting in sudden death. Endocrine abnormalities result in glucose intolerance. Testicular failure is common and is associated with sterility (Harper 2001).

Myotonic dystrophy type I (DM1) is the most common form, accounting for approximately 98% of DM cases. DM1 is caused by a CTG expansion in the 3′UTR of the myotonic dystrophy protein kinase (*DMPK*) gene on chromosome 19q13.3 (Brook et al. 1992; Fu et al. 1992; Mahadevan et al. 1992). While *DMPK* alleles in unaffected individuals contain 5–34 repeats, expanded alleles can reach 50–2000 repeats in individuals with

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DM1. The prevalence of DM1 is estimated to be 1 in 8,000 worldwide (Harper 2001).

Longer repeat lengths correlate with a younger age of onset and increased severity of the disease (Tsilfidis et al. 1992). The most severe form of DM1 is congenital myotonic dystrophy (CDM) in which repeat sizes are >1,400. CDM is characterized by neonatal hypotonia, respiratory failure, which is often fatal, facial diplegia, and mental retardation (Harper 2001). The hypotonia in infants with CDM is thought to involve a developmental defect in skeletal muscle differentiation and maturation. The survival rate for individuals with CDM is approximately 50%. Those that survive improve but then later develop adult DM symptoms in their second or third decade (Harper 2001). These observations suggest that CDM and adult onset disease significantly differ in that CDM represents a developmental abnormality while adult onset disease represents a degenerative process.

DM type 2 (DM2), also called proximal myotonic myopathy (PROMM), makes up 2–3% of myotonic dystrophy cases (Harper 2001). The mutation that causes DM2 is a CCTG expansion in the first intron of the *ZNF9* gene located on chromosome 3q21 (Liquori et al. 2001). DM2 appears to be a milder disease than DM1 but DM2 expansions are larger ranging from 75 to 11,000 CCTG repeats. The clinical presentation of DM1 and DM2 are strikingly similar, however, there are important differences, potentially reflecting mechanistic differences in disease pathology. A congenital form of DM2 has not been identified. In addition, DM1 is associated with atrophy of type 1 skeletal muscle fibers in contrast to DM2, in which atrophy of type 2 fibers is observed (Tohgi et al. 1994).

Recently, a novel multisystemic myotonic disorder has been identified in a large French pedigree, associated with frontotemporal dementia (Le Ber et al. 2004). Histological features in muscle and brain were similar to histological features of DM and the causative region was mapped within chromosome 15 (15q21–24). Therefore, the disease was designated as DM type 3 (DM3). The causative mutation has not been identified.

2
Repeat Instability

DM is typified by anticipation in which disease severity increases in successive generations. The molecular basis for anticipation is germline instability in which repeats expand. In addition to germ line instability, the repeat sizes in DM1 alleles expand in somatic tissues during aging (Ashizawa et al. 1993; Martorell et al. 2004; Wong and Ashizawa 1997). The molecular mechanisms for the genetic instability of triplet repeat expansions have been extensively investigated in bacteria, yeast, and mice (Cummings and Zoghbi 2000; Gomes-Pereira et al. 2004;

Gourdon et al. 1997; Pearson et al. 1997; Savouret et al. 2003; Wells 1996). One proposed mechanisms for genetic instability is the DNA polymerase slippage model, which predicts that repeat size variability arises during DNA replication in a cell-division dependent manner (Richards and Sutherland 1994). Folding of expanded CTG repeats into hairpin or alternative non-B DNA structures is thought to cause slippage of DNA polymerase during replication due to mismatched base pairs (Gacy et al. 1995; Gellibolian R 1997). In support to this, NMR studies showed that CTG repeats can form three different types of hairpin structures generating mismatched base pairs, which allows expansion of repeats during DNA replication (Chi and Lam 2005).

Transgenic mouse models have been used to establish a strong link between mismatch repair (MMR) proteins and repeat instability. For example, transgenic mice containing the entire human *DMPK* gene with 55-CTG repeats obtained from a mildly affected patient showed both intergenerational and somatic repeat instability as observed in individuals with DM (Gourdon et al. 1997). When transgenic mice expressing expanded CTG repeats in the mouse *Dmpk* gene were mated with mice lacking individual MMR proteins, the instability of expanded CTG repeats was altered. In mice lacking *Msh3*, the instability of CTG repeats was corrected. Conversely, the instability of repeats worsened in mice lacking *Msh6* (Pearson et al. 1997). The absence of *Msh2* in transgenic mice expressing >300 unstable CTG repeats favored contractions of the repeats, both in tissues and through generations (Savouret et al. 2003). When transgenic mice deficient for *Pms 2* were mated with mice expressing CAG/CTG repeats, the rate of somatic expansions were reduced by 50% and a higher frequency of large deletions was detected (Gomes-Pereira et al. 2004). On the other hand, deletion of genes involved in various DNA repair pathways like *Rad52*, *Rad54* (homologous recombination) and *DNA-PKc*s (non-homologous end-joining) did not affect repeat instability (Savouret et al. 2003). These results indicate that MMR proteins are involved in different aspects of triplet repeat instability. In addition to the MMR pathway, methylation is found to be important for repeat instability. Expansions of CTG repeats were destabilized in DM1 cells in the presence of DNA methyltransferase inhibitors (Gorbunova et al. 2004). These results indicate that there is more than one mechanism involved in CAG/CTG repeat instability.

3
Mechanism of DM Pathogenesis

The mutant alleles containing CTG or CCTG expansions are transcribed and processed normally into polyadenylated and spliced mRNAs. The mature *DMPK* mRNAs containing the expanded CUG repeats are not

exported to the cytoplasm but rather accumulate in nuclear foci detectable by in situ hybridization (Davis et al. 1997; Fardaei et al. 2001; Fardaei et al. 2002; Taneja et al. 1995). Similarly, the excised intron from the expanded *ZNF9* allele also accumulates in nuclear foci (Liquori et al. 2001; Ranum and Day 2002). However, it is unclear how the CTG and CCTG expansions in noncoding regions cause a multisystemic disease.

Three hypotheses have been proposed for the molecular mechanisms of DM pathogenesis: (1) loss of function of *DMPK*, (2) loss of function of surrounding genes, and (3) RNA gain of function. Knockout mouse models generated to test the loss of function of DMPK and surrounding genes were only mildly related to a DM phenotype. In contrast, the transgenic animal models with expanded CTG repeats strongly supported the RNA "gain of function" hypothesis.

3.1 Loss of Function of DMPK

DMPK is a serine–threonine kinase expressed in skeletal muscle, heart, and to a lesser extent in brain and testes (Lam et al. 2000; Ueda et al. 2000). *DMPK* transcripts are subject to cell-type-dependent alternative splicing (Groenen et al. 2000; Wansink et al. 2003). All isoforms contain CTG repeats in the 3′ UTR except one isoform that splices out the repeats using an alternative splice acceptor site in exon 15 (Tiscornia and Mahadevan 2000). The biological function of *DMPK* is unknown, however, data suggest that DMPK protein might be involved in regulation of actin cytoskeleton (Jin et al. 2000) and in calcium homeostasis (Kaliman et al. 2005). In addition, specific *DMPK* splice variants localize to endoplasmic reticulum and mitochondrial membranes, and their presence causes ER and mitochondrial clustering (van Herpen et al. 2005). In individuals with DM1, nuclear retention of *DMPK* mRNA from the expanded allele results in reduced DMPK protein levels (Ueda et al. 1999). Thus, it was proposed that low levels of DMPK protein contribute to disease pathogenesis. To understand the function of *DMPK* and its role in DM pathogenesis, *Dmpk* knockout mice were generated (Reddy et al. 1996). These mice developed cardiac conduction abnormalities (Berul et al. 1999; Reddy et al. 1996; Saba et al. 1999), altered calcium homeostasis (Benders et al. 1997), abnormal sodium channel gating (Mounsey et al. 2000), and reduced skeletal muscle force (Reddy et al. 1996), suggesting that *Dmpk* may be involved in maintenance of muscle fiber. While a *Dmpk* knockout mouse displayed the relatively mild symptoms observed in DM1 patients, it did not reproduce the most characteristic and severe features of the disease like myotonia or muscle wasting.

Transgenic mice overexpressing *DMPK* developed hypertrophic cardiomyopathy and increased neonatal mortality (Jansen et al. 1996), however these transgenic mice did not show prominent features of DM.

3.2 Loss of Function of Surrounding Genes

Expanded CTG repeats were shown to alter chromatin structure and have regional effects on gene expression (Otten and Tapscott 1995; Wang et al. 1994). The CTG expansion in the *DMPK* 3′UTR are located immediately upstream of the *SIX5* promoter region and were shown to lower *SIX5* expression (Gennarelli et al. 1999; Inukai et al. 2000; Klesert et al. 1997; Thornton et al. 1997). Six5 is a transcription factor required for eye development in *Drosophila*, and the mouse homologue is implicated in distal limb muscle development (Harris et al. 2000). *Six5* knockout mice develop ocular cataracts and infertility resembling some features of DM1 (Klesert et al. 2000; Sarkar et al. 2000). Cardiac conduction abnormalities were also noted in *Six5* knockout mice (Wakimoto et al. 2002). However, the most common symptoms of DM1 such as muscle weakness, wasting, and myotonia were not reproduced in *Six5* knockout mice (Klesert et al. 2000; Sarkar et al. 2000). The identification of a second locus causing DM2 reduced the likelihood that loss of function of *DMPK* or flanking genes was the determinative mechanism for at least the symptoms common for DM1 and DM2.

3.3 RNA "Gain of Function" Hypothesis

Transgenic mice expressing 250 CTG in the final exon of the human skeletal alpha actin gene (HSA_{250}) displayed characteristics of the DM phenotype (Mankodi et al. 2000). Specifically, HSA_{250} mice but not mice expressing transgenes containing five repeats (HSA_5) developed myotonia, a classical feature of DM (Mankodi et al. 2000). Muscle histology showed increased central nuclei, ringed fibers in muscle, and variability in fiber size similar to histological features observed in individuals with DM1. Nuclear foci were detected by in situ hybridization. These mice had a higher mortality rate than normal controls. The reason for increased mortality was unclear (Mankodi et al. 2000). These results suggested that CTG repeats in the absence of *DMPK* mRNA are sufficient to cause several DM symptoms and strongly supported a hypothesis proposing an RNA gain of function (Timchenko et al. 1996a; Wang et al. 1995). On the other hand, HSA_{250} mice did not develop muscle weakness or wasting, indicating that there are likely to be other determinants involved in disease pathogenesis.

A second set of transgenic mice expressing 300 CUG repeats in the natural context of the human *DMPK* mRNA developed mild muscle and brain abnormalities consistent with DM1 (Seznec et al. 2001). Histological abnormalities in muscle included central nuclei, mild muscle regeneration, degeneration, and altered mitochondrial morphology without significant muscle

weakness and wasting. The authors detected myotonia in transgenic mice by EMG (Seznec et al. 2001). Unlike HSA_{250}, expression of RNA containing expanded CUG repeats was not limited to skeletal muscle. These transgenic mice showed abnormal tau protein expression in the brain similar to DM1 patients, providing evidence for toxic effects of CUG expansion in CNS.

Here we summarize the additional evidence for an RNA gain-of-function hypothesis. First, the fact that two different loci containing similar expanded repeats cause strikingly similar diseases strongly suggest that DM1 and DM2 pathogenesis is independent of a loss of function of the affected loci. Second, only the repeats and no other mutations within the DM1 or DM2 locus cause DM, indicating that the expanded repeats themselves rather than a loss of function of the mutant alleles are determinative for the disease. Third, the RNA transcribed from the mutated allele containing expanded repeats (CUG/CCUG) accumulates in discrete nuclear foci detectable by in situ hybridization (Liquori et al. 2001; Taneja et al. 1995). Fourth, *Dmpk* and *Six5* knockout mice do not reproduce a strong DM phenotype (Benders et al. 1997; Berul et al. 1999; Klesert et al. 2000; Reddy et al. 1996; Saba et al. 1999; Sarkar et al. 2000). These results indicate that expression of expanded CUG or CCUG repeats independent of the loci is sufficient to induce the major features of the disease.

The expression of CUG or CCUG repeat containing RNAs is proposed to induce pathogenesis by at least three mechanisms: (1) misregulation of pre-mRNA alternative splicing, (2) interference with muscle differentiation, and (3) transcriptional interference. Each of these potential mechanisms will be discussed below.

3.3.1 Misregulation of Alternative Splicing

Alternative splicing is a process by which multiple mRNA isoforms are generated from individual genes. The majority of human genes undergo alternative splicing explaining, in part, the disparity between the relatively small number of genes and the complexity of the human proteome (Modrek and Lee 2002; Xu et al. 2002). Alternative splicing gives rise to protein isoforms that significantly differ in their functions (Black 2003). Alternative splicing is often regulated according to cell type or developmental stage. Regulation involves binding of regulatory factors to intronic or exonic elements (Black 2003). The regulation of alternative splicing can have an enormous impact on multiple aspects of cell and tissue physiology (Lopez 1998). Aberrant regulation of alternative splicing has been implicated in several human diseases (Faustino and Cooper 2003; Lopez 1998). Ten misregulated alternative splicing events that have been identified in DM1 heart, skeletal muscle, and central nervous system are summarized in Table 1.

IR splicing has been shown to be misregulated in DM2 skeletal muscle consistent with a similar pathogenic mechanism as in DM1 (Savkur et al.

Table 1. Summary of alternative splicing events misregulated in DM1

Pre-mRNA	Mis-regulated exon/intron	Reference
Cardiac troponin T (*TNNT2* or *cTNT*)	exon 5	Philips et al. (1998)
Insulin receptor (*IR*)	exon 11	Savkur et al. (2001)
Chloride channel (*CLCN-1*)	intron 2 and exon 7a	Charlet-B. et al. (2002b); Mankodi et al. (2002)
Microtubule-associated protein tau (*MAPT*)	exon 2 and 10	Sergeant et al. (2001); Jiang et al. (2004)
Myotubularin-related protein 1 *MTMR1*	exons 2.1 and 2.3	Buj-Bello et al. (2002)
Fast skeletal troponin T (*TNNT3*)	fetal exon	Kanadia et al. (2003a)
N-methyl-D-aspartate receptor (*NMDAR1*)	exon 5	Jiang et al. (2004)
Amyloid precursor protein (*APP*)	exon 7	Jiang et al. (2004)

2004). In all cases, the regulation of alternative splicing is disrupted such that normal mRNA variants are expressed, but in inappropriate tissues or developmental stages. Alternative splicing of only a subset of genes is misregulated in DM indicating that most genes are unaffected (Jiang et al. 2004; Philips et al. 1998). Interestingly, all pre-mRNAs misregulated in DM1 normally undergo a developmentally regulated splicing switch. In DM adult tissues, the embryonic or fetal splicing patterns for these genes are retained. Misexpression of the early developmental isoforms for *IR* and *CLCN-1* has been shown to directly correlate with disease symptoms such as insulin resistance and myotonia, respectively (Charlet-B. et al. 2002b; Mankodi et al. 2002; Savkur et al. 2001). The next section summarizes all the pre-mRNAs that are misspliced in individuals with DM.

3.3.1.1 TNNT2 (*cTNT*) Contraction of striated muscle is regulated by binding of calcium to the troponin complex located on the actin-based thin filament. This complex consists of troponin T, troponin I, and troponin C (TNT, TNI, and TNC, respectively). This complex regulates the calcium-dependent interaction of actin and myosin that results in muscle contraction (Cullen et al. 2004).

TNNT2 is the cardiac isoform of TNT, which is the gene expressed in embryonic heart, embryonic skeletal muscle, and adult cardiac muscle

(Anderson et al. 1991). Alternative splicing of exon 5 is regulated such that the exon is included in mRNAs produced during early development of heart and skeletal muscle but the exon is skipped in adult heart (Anderson et al. 1995). The two major TNNT2 isoforms generated by alternative splicing of exon 5 confer different calcium sensitivity to the myofilament, affecting the contractile properties of maturing muscle (Godt et al. 1993; McAuliffe et al. 1990). *TNNT2* alternative splicing is disrupted in DM1 such that exon 5 is inappropriately included in adult cardiac muscle (Philips et al. 1998). Mutations in *TNNT2* and *cTNI* genes are associated with inherited heart diseases including hypertrophic and dilated cardiomyopathies (Lu et al. 2003). Specifically, mutations in *TNNT2* gene are implicated in dominantly inherited familial cardiomyopathies (Forissier et al. 1996; Nakajima-Taniguchi et al. 1997; Thierfelder et al. 1994; Watkins et al. 1995). Thus, the expression of fetal *TNNT2* isoform in DM1 patients might contribute to the reduced myocardial function and conduction abnormalities seen in DM patients.

3.3.1.2 TNNT3 The *TNNT3* gene encodes the TNT isoform expressed in fast-twitch skeletal muscle myofibers. A fetal exon is located between exons 8 and 9 of *TNNT3* gene and this fetal exon is inappropriately included in adult DM1 skeletal muscle (Kanadia et al. 2003a). The functional consequences of the inappropriate isoform are unknown.

3.3.1.3 IR IR is a tetrameric complex with two alpha and two beta subunits. Binding of insulin to the extracellular alpha subunits causes autophoshorylation of intracellular beta subunits (Joost 1995; Kellerer et al. 1992). Alternative splicing of exon 11 of the alpha subunit generates two isoforms: IR-A, which lacks exon 11, and IR-B, which includes exon 11 (Mosthaf et al. 1990; Seino and Bell 1989). Expression of the two isoforms is regulated in a tissue-specific manner such that IR-B is expressed predominantly in tissues responsible for glucose homeostasis such as liver, adipose tissue, and skeletal muscle (Condorelli et al. 1994). IR-A has a higher affinity for insulin with lower signaling capacity and is expressed at low levels in these tissues (Kosaki et al. 1995; Vogt et al. 1991). The inappropriate expression of IR-A in skeletal muscle directly correlates with the insulin resistance seen in DM1 and DM2 patients (Savkur et al. 2001; Savkur et al. 2004).

3.3.1.4 ClC-1 The muscle-specific chloride channel (ClC-1), encoded by *CLCN-1* gene, is the predominant chloride channel in adult skeletal muscle (Bardouille et al. 1996; Pusch 2002), and loss of function mutations in this gene results in inherited myotonias in humans and other mammals (Beck et al. 1996; Koch et al. 1992; Rhodes et al. 1999; Zhang et al. 2000). Aberrant splicing of the *CLCN-1* pre-mRNA results in the loss of CIC-1 protein in skeletal muscle of individuals with DM1 or

DM2 due to introduction of premature termination codons, which is thought to trigger nonsense mediated decay, resulting in degradation of *CLCN-1* mRNA (Charlet-B. et al. 2002b; Mankodi et al. 2002). The loss of CIC-1 correlates well with the myotonia observed in individuals with DM1 and DM2.

3.3.1.5 Tau Tau (encoded by the *MAPT* gene) is a microtubule-associated protein that is required for polymerization and stability of microtubules involved in axonal transport (Buee et al. 2000; Goedert et al. 1992). Exons 2, 3 and 10 are alternative exons that are developmentally regulated giving rise to six different isoforms (Andreadis et al. 1992). Exon 10 encodes an additional microtubule-binding domain, which increases its affinity to microtubules (Hartmann et al. 2001; Varani et al. 2000). Exon 2 alters the structure and function of the membrane-binding domain of tau allowing connection of microtubules to the axonal membrane (Brandt et al. 1995; Li et al. 2003). Exon 10 is not included in fetal brain but is included in 50% of the transcripts in adult brain. Similar to exon 10, exons 2 and 3 are excluded in fetus but included in adult. Two independent studies demonstrated that fetal forms of *MAPT* (excluding exons 2, 3 and 10) were inappropriately expressed in adult brain of individuals with DM1 (Jiang et al. 2004; Sergeant et al. 2001). Expression of human fetal tau isoforms in transgenic mice results in neurofibrillary tangles which are also seen in other neurological diseases (Andreadis 2005; Gotz et al. 2001; Ishihara et al. 2001). In addition, neurofibrillary tangles can be detected in brains of individuals with DM1 (Kiuchi et al. 1991; Vermersch et al. 1996). These observations raise the possibility that expression of a fetal tau isoform might be involved in production of neurofibrillary tangles affecting behavioral and cognitive functions in individuals with DM1.

3.3.1.6 APP The characteristic features of Alzheimer's disease are senile plaques and neurofibrillary tangles in the brains of affected individuals. The major component of senile plaques is amyloid, a peptide derived from proteolysis of a large beta-amyloid precursor protein (APP). APP is a type I trans-membrane glycoprotein existing in eight isoforms generated by alternative splicing of exons 7, 8, and 15 (Sandbrink et al. 1996). Exon 7 is believed to encode a serine protease inhibitor domain (Ponte et al. 1988). The *APP* mRNA, which excludes exon 2 and 7 is fetus-specific (Tang et al. 2003). Fetal forms of *APP* excluding exon 7 are inappropriately expressed in brains of individuals with DM (Jiang et al. 2004). The consequences for the loss of this protease inhibitory domain by exclusion of exon 7 in DM1 are unclear.

3.3.1.7 NMDAR1 N-methyl-D-aspartate receptors (NMDAR) are involved in excitatory transmission in the mammalian brain and are crucial for brain development, learning, and memory (Sato et al. 2000; Tsien et al.

1996). *NMDAR1* pre-mRNA has three alternative exons: 5, 21, and 22. Differential usage of these exons generates at least seven mRNA isoforms that encode proteins with different physiological properties and subcellular distribution (Durand et al. 1993; Zukin and Bennett 1995). The protein segment encoded by exon 21 is believed to be important for localization of NMDAR1 to the post-synaptic plasma membrane (Ehlers et al. 1995). The *NMDAR1* isoform including exon 21 was increased in brain tissues of individuals with DM1 (Jiang et al. 2004). Similarly, the inclusion of exon 5 of *NMDAR1* is thought to affect the intracellular distribution of NMDAR1 (Pal et al. 2003; Traynelis et al. 1995). Increased exon 5 and exon 21 inclusion was detected in brain tissues of DM1 patients (Jiang et al. 2004), implicating a possible correlation between a change in the distribution of NMDAR1 and the CNS symptoms of DM.

3.3.1.8 MTMR1 The *MTMR1* gene belongs to a conserved family of phosphatidylinositol 3-phosphate [PI (3)P] phosphatases (Laporte et al. 2001) involved in regulation of intracellular vesicular trafficking and membrane transport (Simonsen et al. 2001). Alternative splicing of exons 2.1, 2.2, and 2.3 generates the muscle-specific protein isoforms A, B, and C (Buj-Bello et al. 2002). Exons 2.1, 2.2, and 2.3 encode 8, 9, and 17 amino acids, respectively (Buj-Bello et al. 2002). A switch from isoform A to C is detected in individuals with CDM. It is unclear whether there is a change in protein function due to the isoform switch since phosphatase activities seems to be similar (Buj-Bello et al. 2002). However, there are two independent reports suggesting that point mutations or deletions in the *MTMR1* gene are associated with myotubular myopathy, a disease associated with hypotonia and respiratory insufficiency resembling some features of CDM (Copley et al. 2002; Zanoteli et al. 2005). In addition, the loss of *MTM1*, a gene closely related to *MTMR1*, is implicated in a congenital muscular disorder called X–linked myotubular myopathy, exhibiting some similarities to CDM such as hypotonia, muscle weakness, and muscle fibers with central nuclei (Wallgren-Pettersson et al. 1995).

3.4 Mechanisms of Misregulated Alternative Splicing

The specific mechanism by which expression of CUG- or CCUG-repeat RNA induces splicing misregulation is unclear. However, there is substantial evidence linking the misregulation of alternative splicing observed in DM tissues with two families of RNA binding proteins: CUG-BP and ETR-3-Like Factors (CELF) and muscleblind-like (MBNL). Members of both the CELF and MBNL families were first identified based on their binding to CUG-repeat RNA in vitro (Lu et al. 1999; Michalowski et al. 1999; Miller et al. 2000; Timchenko et al. 1996b). Members of both protein

families have been demonstrated to bind RNA and to directly regulate alternative splicing of multiple pre-mRNAs including several that undergo misregulated alternative splicing in DM (Charlet-B. et al. 2002b; Ho et al. 2004; Philips et al. 1998; Savkur et al. 2001). Interestingly, CELF and MBNL proteins have been shown to antagonistically regulate two splicing events that are misregulated in DM tissues (*TNNT2* and *IR*), and the splicing patterns of at least these two pre-mRNAs are consistent with either loss of MBNL activity and/or a gain of CELF activity (Ho et al. 2004). There is evidence strongly supporting both increased CELF activity and reduced MBNL activity as determinative factors in misregulated splicing in DM. Each of these families will be described as well as their potential role in misregulated alternative splicing.

3.4.1 Increased CUG-BP1 Splicing Activity

There are six CELF paralogues in humans: ETR-3 (CUG-BP2/BRUNOL3/NAPOR), CELF3 (BRUNOL1), CELF4 (BRUNOL4), CELF5 (BRUNOL5), CELF6 (BRUNOL6), and CUG-BP1 (BRUNOL2/CUG-BP1). The CELF paralogues are 43–78% identical and all six have three RNA recognition motifs (RRMs) and a 160–230 amino acid divergent domain separated by RRMs 2 and 3 (Ladd et al. 2001). CELF proteins are involved in both nuclear and cytoplasmic events such as alternative splicing, RNA editing, and mRNA stability and translation (Anant et al. 2001; Ladd et al. 2001; Ladd et al. 2004; Mukhopadhyay et al. 2003; Timchenko et al. 1999). With regard to alternative splicing, CELF proteins have been shown to regulate a number of pre-mRNAs by directly binding to U/G-rich motifs within introns (Charlet-B. et al. 2002a; Charlet-B. et al. 2002b; Faustino and Cooper 2005; Philips et al. 1998). Alternatively spliced genes that are regulated by CELF proteins include *TNNT2* exon 5, *IR* exon 11, *CLCN-1* intron 2, *NMDAR1* exons 5 and 21, actinin muscle-specific exon, and *MTMR1* exon 2.1 and 2.2 (Charlet-B. et al. 2002a; Charlet-B. et al. 2002b; Faustino and Cooper 2005; Gromak et al. 2003; Philips et al. 1998; Savkur et al. 2001; Zhang et al. 2002).

CUG-BP1 is the most studied member of the CELF protein family. CUG-BP1 has been demonstrated to directly regulate three alternative splicing events that are misregulated in DM: *CLCN-1* intron 2, *TNNT2* exon 5, and *IR* exon 11 (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). In tissue culture, the misregulated splicing patterns observed for these three genes in DM1 tissues can be recapitulated by overexpression of CUG-BP1, suggesting that DM cells exhibit an increased activity of CUG-BP1 or other members of the CELF family (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). Consistent with increased CUG-BP1 splicing activity, CUG-BP1 protein levels are increased in DM1 skeletal muscle tissue (Savkur et al. 2001), DM1 skeletal muscle cultures

(Dansithong et al. 2005; Savkur et al. 2001) and in DM1 heart tissue (Timchenko et al. 2001a). In addition, transgenic mice expressing CUG-BP1 eight to ten fold above endogenous levels inhibited muscle differentiation and resulted in neonatal lethality (Timchenko et al. 2004). Histological features were variably consistent with what is observed in CDM patients including centrally positioned nuclei suggestive of immature skeletal muscle. However, type 1 slow myofiber numbers were increased while these are decreased in DM skeletal muscle. The mechanism of muscle immaturity is thought to be due to altered translation of p21 and myogenin mRNAs by CUG-BP1. In another line of transgenic mice overexpressing CUG-BP1 in heart and skeletal muscle using the mouse creatine kinase promoter (MCKCUG-BP), transgene expression was associated with neonatal lethality (Ho et al. 2005a). Histological changes were consistent with CDM, as well as splicing changes observed for *TNNT2* exon 5 and *MTMR1* exons 2.1 and 2.2 in cardiac muscle, and *MTMR1* exons 2.1 and 2.2 and *CLCN-1* exon 7a in skeletal muscle tissue (Ho et al. 2005a).

3.4.2 Sequestration of MBNL Proteins

The three human MBNL paralogues are homologues of *Drosophila* muscleblind (*mbl*), which is required for *Drosophila* photoreceptor and muscle differentiation (Artero et al. 1998; Begemann et al. 1997). *MBNL1*, *MBNL2*, and *MBNL3* are located on chromosomes 3, 13, and X, respectively (Fardaei et al. 2002; Miller et al. 2000). MBNL1 was identified based on its ability to bind double-stranded CUG-repeat RNA in HeLa cell nuclear extracts (Miller et al. 2000). All three MBNL proteins colocalize with expanded CUG and CCUG RNA nuclear foci in cultured cells as detected by immunofluorescence (Fardaei et al. 2002; Mankodi et al. 2001; Miller et al. 2000). In addition, MBNL can bind to expanded (up to 50) CCUG, CUG, and CAG repeats as detected by a yeast three-hybrid assay (Kino et al. 2004). Both MBNL1 and MBNL2 are expressed in skeletal muscle and heart, two tissues that are prominently affected in DM (Kanadia et al. 2003b). MBNL3 expression is restricted to the placenta in the adult mice and is more widely expressed in the embryo (Fardaei et al. 2002; Miller et al. 2000).

The observations that muscleblind proteins colocalize with the expanded CUG and CCUG repeats strongly suggests that loss of MBNL function due to sequestration on CUG-repeat RNA plays a major role in DM pathogenesis (Dansithong et al. 2005; Fardaei et al. 2002; Jiang et al. 2004; Mankodi et al. 2001; Miller et al. 2000). Results from *Mbnl* knockout mice strongly support this hypothesis (Kanadia et al. 2003a). Targeted deletion of exon 3 in mice ($MBNL1^{\Delta E/\Delta E}$) to eliminate the MBNL1 isoforms that bind expanded CUG/CCUG RNA repeats resulted in myotonia, cataracts, and RNA splicing defects that are striking characteristics of DM.

Histological analysis of muscle revealed increased central nuclei and splitting of myofibers. MBNL1ΔE/ΔE mice showed abnormal retention of the *TNNT3* fetal exon and *CLCN-1* exon7a in skeletal muscle and *TNNT2* exon 5 in heart consistent with splicing changes seen in individuals with DM1 and without changes in steady state levels of CUG-BP1 (Kanadia et al. 2003a).

The MBNL family was recently identified as direct regulators of alternative splicing (Ho et al. 2004). Specifically, MBNL proteins regulate splicing of *TNNT2* exon 5 and *IR* exon 11 via direct binding to adjacent intronic elements (Ho et al. 2004). MBNL and CELF proteins have antagonistic effects on the splicing patterns of these two pre-mRNAs, however, MBNL and CELF proteins bind to different sites within the pre-mRNAs indicating that the antagonism is not due to a competition for a common binding site. In addition, regulation by CELF and MBNL appeared to be completely independent, as TNNT2 minigenes containing mutant CUG-BP1 binding sites still responded to MBNL1, and vice versa (Ho et al. 2004).

The patterns of misregulation for *TNNT2*, *IR*, and *CLCN-1* are consistent with increased CUG-BP1 activity and with decreased MBNL1 activity. Whether the splicing effects in DM are due primarily to loss of MBNL activity or a gain of CELF protein activity remains an open question, with evidence supporting both. Several pieces of evidence, some of which was noted above, support a role for MBNL depletion. First, MBNL proteins colocalize with CUG- and CCUG-repeat RNA foci, which is consistent with a loss of function due to sequestration (Fardaei et al. 2002; Mankodi et al. 2001; Miller et al. 2000). Second, misregulated splicing patterns and striking phenotypic similarities to DM are observed in *Mbnl* knockout mice (Kanadia et al. 2003a). Third, a recent study showed that loss of MBNL1 function was the critical event in aberrant splicing of IR in DM1 cultured cells (Dansithong et al. 2005). Fourth, of the two pre-mRNAs directly regulated by MBNL proteins (TNNT2 and IR), the splicing patterns of both alternative exons in DM tissues are consistent with a loss of MBNL activity (Ho et al. 2004).

There are also several results that support a role for increased CELF activity. First, CUG-BP1 steady state levels are increased in DM skeletal muscle and heart tissues as well as in DM cell cultures (Dansithong et al. 2005; Savkur et al. 2001; Timchenko et al. 2001a). Second, the splicing patterns of all of the three pre-mRNAs (*TNNT2*, *IR*, and *CLCN-1*) shown to be directly regulated by CUG-BP1 are consistent in DM tissues with increased CUG-BP1 activity (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). Third, a TNNT2 minigene expressed in DM cell cultures reproduces the splicing pattern observed for the endogenous *TNNT2* pre-mRNA in DM tissues (Philips et al. 1998). Similarly, the "DM" splicing pattern for both TNNT2 and IR minigenes can be induced in normal cells by co-expression of a plasmid containing expanded CTG repeats (Ho et al. 2004; Philips et al. 1998; Savkur et al. 2001). Importantly,

minigenes that contain mutations in the CUG-BP1 binding site are no longer responsive to CUG-BP1, to the effects in DM cell cultures, or to co-expression of CUG-repeat RNA (Philips et al. 1998; Savkur et al. 2001). These results indicate that the effects of the repeats on splicing require the CUG-BP1 binding site and suggest a direct role for CUG-BP1.

There is also evidence suggesting that sequestration of MBNL proteins is not sufficient to explain the *trans*-acting effects of CUG-repeat RNA on splicing. First, the mutated TNNT2 minigene that is not responsive to CUG-repeat RNA remains responsive to depletion of MBNL1 using siRNAs (Ho et al. 2004). The finding that a minigene that does not respond to co-expression of CUG-repeat RNA still responds to MBNL1 depletion indicates that the effects of CUG RNA on splicing involves more than MBNL depletion (Ho et al. 2004). Second, recent results indicate that MBNL colocalizes with nuclear RNA foci containing either CUG- or CAG-repeat RNA from transiently transfected plasmids. Expanded CUG-repeat RNA induces splicing changes of TNNT2 and IR minigenes when coexpressed with the minigenes but CAG repeats of equal length and expressed at comparable levels have little effect on splicing (Ho et al. 2005b). When FRAP analysis was used to determine the relative affinity of a MBNL1-GFP fusion protein for CUG- and CAG-repeat RNA foci in vivo; no differences were detected (Ho et al. 2005b). Therefore, CUG and CAG RNA appear to have similar abilities to sequester MBNL while only CUG repeats have a *trans*-dominant effect on splicing. Finally, MBNL3, also called CHCR (Cys3His CCG1-Required), was identified as an inhibitor of muscle differentiation in C2C12 cells (Squillace et al. 2002). Muscle differentiation defects observed in DM is not consistent with loss of MBNL3 function since MBNL3 sequestration in nuclear foci should favor differentiation. These results suggest that while loss of MBNL activity is likely to play a role in the splicing abnormalities observed in DM, the effects of the repeats on alternative splicing regulation appear to involve a mechanism more complex than sequestration of MBNL alone.

Accumulation of RNA foci is an important hallmark of DM (Davis et al. 1997; Jiang et al. 2004; Liquori et al. 2001; Mankodi et al. 2003; Miller et al. 2000; Taneja et al. 1995). The formation of the foci was recently shown to require MBNL as RNAi-mediated depletion of MBNL in DM1 myoblasts reduced the number of foci by 70% (Dansithong et al. 2005). It is clear that the repeat-containing RNA is pathogenic but it is not clear whether foci contain the pathogenic form of the RNA. The finding that CAG-repeat RNA forms foci, colocalizes with MBNL but does not alter splicing strongly suggest that foci formation and the potential to sequester MBNL alone is not sufficient for misregulated alternative splicing. Additional support for the inconsistency between toxicity and foci formation comes from a recent report showing that expression of 162 CTG repeats in the 3′ UTR of a reporter gene formed foci in *Drosophila* tissues without inducing pathology, suggesting that foci formation was not toxic to *Drosophila*

(Houseley et al. 2005). Similarly, foci formation by RNAs containing only CUG repeats is not sufficient to induce muscle-differentiation defects in the C2C12 cell line (Amack and Mahadevan 2001). In summary, these results strongly suggest that foci formation alone is not pathogenic.

3.4.3 Sequestration of Other RNA Binding Proteins

In addition to MBNL proteins, the splicing regulators hnRNP H and F colocalize with CUG foci in neurons of DM1 patient brain samples (Jiang et al. 2004). Neuron-specific c-*src N1* exon is regulated by hnRNP F (Min et al. 1995) and hnRNP H regulates *NF-1* exon 3, thyroid stimulating hormone beta subunit (*TSH beta*) genes (Buratti et al. 2004), HIV-1 tev-specific exon 6D (Caputi and Zahler 2002) and beta tropomyosin (Chen et al. 1999). The relevance of hnRNP H and hnRNP F colocalization with RNA foci is not clear since splicing of *c-src* is not disrupted in neurons (Jiang et al. 2004).

Double-stranded-RNA-dependent protein kinase R (PKR), is activated by double-stranded RNA as a response to viral infections (Williams 2001). Activation of PKR inhibits translation by phosphorylation of translation initiation factor eIF2 alpha (Clemens 2001). PKR was identified as one of the RNA-binding proteins that bind to double-stranded CUG repeats, and PKR is activated by CUG-repeat expression in vitro (Tian et al. 2000). Further studies using mouse models; however, indicated that PKR is not crucial to disease pathogenesis. Neither myotonia nor histological changes were altered in HSA_{250} mice on a PKR–/– or PKR–/+ background, suggesting that PKR is unlikely to be relevant to DM pathogenesis (Mankodi et al. 2003).

3.4.4 Transcriptional Interference

The toxicity of expanded CUG repeats is proposed to result from sequestering transcription factors similar to a mechanism of pathogenesis for polyglutamine expansions (Ebralidze et al. 2004). The transcription factors Sp1 and retinoic acid receptor gamma were found to be recruited to the expanded CUG repeats and depleted from the active chromatin correlating with reduced expression of several genes including *CLCN-1* (encodes for ClC-1 protein) detected by real time RT-PCR analysis (Ebralidze et al. 2004). In addition to alternative splicing misregulation and likely downregulation by NMD, reduced transcription of *CLCN-1* mRNA might also contribute to loss of ClC-1 protein and myotonia. In contrast to the expectation that transcription factors are sequestered with CUG-repeat RNA, however, Jiang and colleagues could not detect Sp1 or retinoic acid receptor gamma associated with RNA foci in brain tissues of DM1 patient cells by immunofluorescence (Jiang et al. 2004).

3.4.5 Muscle Differentiation Defects and Altered Translation Regulation

Muscle weakness and wasting are the major causes of mortality in individuals with DM1 (Harper 2001). Delays or defects in muscle differentiation have been proposed as the major factors that lead to muscle weakness and wasting. In culture, normal muscle cells proliferate in growth medium; upon removal of growth factors, the cell cycle is inhibited and the cells enter the differentiation pathway. Differentiating cells fuse into multi-nucleated myotubes and express muscle-specific genes (Olson 1992). A defect in muscle differentiation has been observed both in individuals with congenital and adult onset DM1 (Furling et al. 2001; Timchenko et al. 2001b). Morphological and histochemical studies revealed developmental defects in satellite cells from individuals with CDM expressing 2300 CTG repeats. Myoblast fusion was less complete in cells with nuclear RNA foci suggesting a defect in myogenic differentiation associated with CUG-repeat RNA. In addition to poor muscle differentiation, satellite cells had a reduced life span and proliferation capacity in culture (Furling et al. 2001).

Myoblasts from individuals with DM1 were unable to withdraw from the cell cycle when stimulated to differentiate (Timchenko et al. 2001b). In C2C12 cells, four- to ten-fold constitutive overexpression of the human *DMPK* 3′UTR inhibited muscle differentiation. The inhibitory activity was mapped to a 239-nucleotide region located upstream of the CTG repeats (Sabourin et al. 1997). Recently, the toxic effects of *DMPK* 3′ UTR was reproduced in transgenic mice overexpressing *DMPK* 3′-UTR with wild type (11) or expanded (91) CTG repeats (Storbeck et al. 2004). Both expanded and wild-type CTG-repeat-expressing mice displayed muscle atrophy supporting the previous findings that mainly *DMPK* 3′ UTR is responsible for muscle differentiation defects (Storbeck et al. 2004). Myoblast cultures from these animals showed reduced fusion, but disruption of muscle differentiation was worse in the presence of expanded CTG repeats. These results suggest that the *DMPK* 3′UTR was sufficient for defects in muscle differentiation. On the other hand, in C2C12 cell lines stably expressing the normal *DMPK* 3′UTR, muscle differentiation was not disrupted. Only *DMPK* 3′UTR expressing 200 CTG repeats inhibited C2C12 myoblast differentiation (Amack et al. 1999). These results suggest that repeats are necessary for inhibition of muscle differentiation. MyoD was identified as a target for the inhibitory effects of *DMPK* 3′UTR with expanded CTG repeats during C2C12 differentiation as well as during DM1 myoblast differentiation (Amack et al. 2002; Timchenko et al. 2001b). Specifically, expression of the *DMPK* 3′UTR with 200 CTG repeats severely reduced MyoD levels (Amack et al. 2002) and reduced levels of MyoD were detected in myoblasts from individuals with DM1 (Timchenko et al. 2001b).

The p21 protein, which is an important regulator of cell cycle progression and muscle differentiation, was identified as a downstream target for the muscle differentiation defects induced by expanded CTG repeats (Timchenko et al. 2001b). CUG-BP1 was shown to enhance p21 translation by binding to GCN repeats in the 5′ UTR of p21 mRNA (Timchenko et al. 2001b). Accumulation of CUG-BP1 in the nuclei of DM cells resulted in reduced levels of p21 translation leading to reduced differentiation of these cells. These results suggest that reduced MyoD and p21 are directly involved in muscle development abnormalities seen in CDM or defects in muscle regeneration seen in DM1.

The molecular mechanisms for muscle weakness and wasting in DM2 patients are not clear since there is no involvement of *DMPK* 3′UTR in DM2. To date, none of the transgenic or knockout mouse models have reproduced a clear progressive muscular dystrophy phenotype as observed in individuals with DM1 (Berul et al. 2000; Kanadia et al. 2003a; Klesert et al. 2000; Mankodi et al. 2000; Mounsey et al. 2000; Reddy et al. 1996; Sarkar et al. 2000; Seznec et al. 2001; Timchenko et al. 2004). There are several possibilities for the lack of this phenotype in mouse models. First, mice might not show the same phenotype as humans due to physiological differences. Second, there is no mouse model that expresses more than 300 CTG repeats. Longer repeats might have more severe effects in muscle development. Alternatively, higher levels of expression of shorter repeats could also show a more severe phenotype. Finally, the mouse life span might not be long enough for the disease to worsen. In the future, it will be important to have animal models that could represent dystrophy seen in DM to better understand the molecular mechanisms involved and find better ways to treat patients.

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Redirecting Splicing to Address Dystrophin Mutations: Molecular By-pass Surgery

Stephen D. Wilton, Susan Fletcher

Abstract. Mutations in the dystrophin gene that prevent synthesis of a functional protein lead to Duchenne muscular dystrophy (DMD), the most common serious childhood muscular dystrophy. The major isoform is produced in skeletal muscle and the size of the dystrophin gene and complexity of expression have posed great challenges to the development of a therapy for DMD. Considerable progress has been made in the areas of gene and cell replacement, yet it appears that any potential therapy for DMD is still some years away. Other approaches are being considered, and one that has generated substantial interest over the last few years is induced exon skipping. Antisense oligonucleotides have been used to block abnormal splice sites and force pre-mRNA processing back to the normal patterns. This approach is re-interpreted to address the more common dystrophin mutations, where normal splice sites are targeted to induce abnormal splicing, resulting in specific exon exclusion. Selected exon removal during processing of the dystrophin pre-mRNA can by-pass nonsense mutations or restore a disrupted reading frame arising from genomic deletions or duplications. Attributes of the dystrophin gene that have hampered gene replacement therapy may be regarded as positive features for induced exon skipping, which may be regarded as a form of by-pass surgery at the molecular level. In humans, antisense oligonucleotides have been more generally applied to down-regulate specific gene expression, for the treatment of acquired conditions such as malignancies and viral infections. From interesting in vitro experiments several years ago, the dystrophin exon-skipping field has progressed to the stage of planning for clinical trials.

1
The Dystrophin Gene and Duchenne Muscular Dystrophy

1.1
The Dystrophin Gene and Products

Consisting of 79 exons and spanning some 2.4 Mb, the dystrophin gene is under the control of 7 promoters and is the largest known gene (Ahn and Kunkel 1993; Byers et al. 1993; Chelly et al. 1990b). A diagrammatic representation of the gene is shown in Fig 1. Depicting the three main promoters for the muscle, cortical and Purkinje isoforms with the internal promoters within introns 29, 44, 55, and 71, this figure does not do justice to the magnitude and complexity of the gene. The average exon length is

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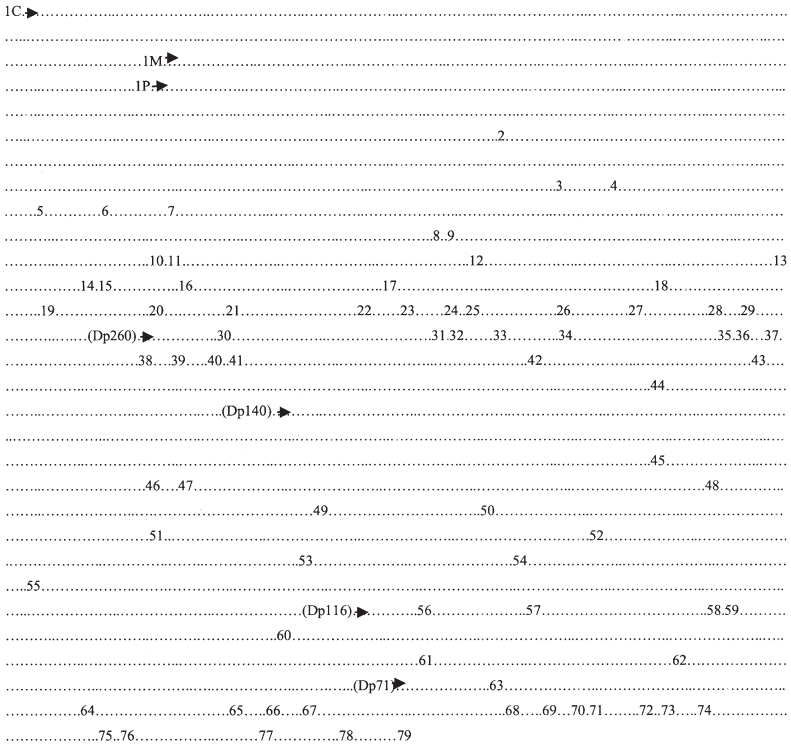


Fig. 1. Diagrammatic representation of the exon and intron arrangement in the dystrophin gene. Exons vary in length from 32 bases to 2,703 bases (encoding the 3′ untranslated region) with an average length of 177 nucleotides. Each *dot* represents ~400 bases of intronic sequence. The *arrows* (➔) indicate promoters that generate the various isoforms with *1C*, *1M*, and *1P* representing the cortical, muscle, and Purkinje promoters, respectively, while *Dp260*, *Dp140*, *Dp116*, and *Dp71* indicate other isoform promoters

about 170 bases, with each dot representing about 400 nucleotides of intronic sequence. Other genes are considered 'large'. Nebulin for example consists of 183 exons and has a mature mRNA in excess of 20 kb, but this gene only spans 249 kb of genomic sequence (Donner et al. 2004), making the entire nebulin gene shorter than dystrophin intron 44. Several of the dystrophin introns are longer than 100 kb with the largest, intron 44, in excess of 250 kb.

Why the dystrophin gene is so large remains unresolved. It has been estimated that RNA polymerase II would take approximately 16 hours to transcribe the entire dystrophin gene, and then 99.4% of the primary transcript is discarded during splicing (Tennyson et al. 1995). The presence of introns in excess of 100 kilobases implies that there must be some role for these huge non-coding tracts of sequence, to justify the additional burden of

their maintenance and processing. There is now considerable interest in non-coding RNAs as an additional source of control of gene expression (Mattick 2004a; Mattick 2004b). It is possible that motifs in some of the dystrophin introns regulate expression of dystrophin isoforms or of other genes. Intronic sequences cannot be dismissed as 'junk' as there are internal promoters and alternative polyadenylation sites within dystrophin introns.

The 427kD dystrophin gene product consists of 3,685 amino acids and plays an integral role as a mechanical link between the actin cytoskeleton and the extracellular matrix (Blake et al. 2002). The full-length muscle-specific protein has four distinct domains (Fig. 2). The amino terminus contains two actin binding sites, followed by a central rod domain consisting of 24 spectrin-like repeats, interrupted with four proline-rich hinges (Jarrett and Foster 1995; Koenig and Kunkel 1990; Koenig et al. 1988). The cysteine-rich domain includes 15 cysteine residues, two EF hand motifs, and a ZZ domain, with the following carboxyl terminal domain composed of two regions predicted to form α-helical-coiled coils, which have been implicated in other protein–protein interactions (Koenig et al. 1988).

In skeletal muscle, dystrophin binds to a transmembrane protein, β-dystroglycan, through interactions with the EF hand motifs and the ZZ domain. Another member of the dystroglycan complex, α-dystroglycan, binds to the extracellular matrix component, laminin-2 (Rentschler et al. 1999). The sarcoglycan:sarcospan complex, comprised of α-, β-, γ- and δ–sarcoglycan and sarcospan, is presumed to have a role in membrane stabilization (Araishi et al. 1999). Other proteins associating with dystrophin include α-dystrobrevin and α-syntrophin, suggesting that dystrophin has roles in addition to mechanical support, as α-syntrophin recruits the enzyme nNOS to the sarcolemma (Ahn et al. 1996).

There are at least seven different promoters that drive the expression of a variety of dystrophin isoforms. The major isoform expressed in muscle is Dp427m. Two other full-length isoforms are expressed in brain, Dp427c in cortical neurons and the hippocampus, and Dp427p in Purkinje neurons

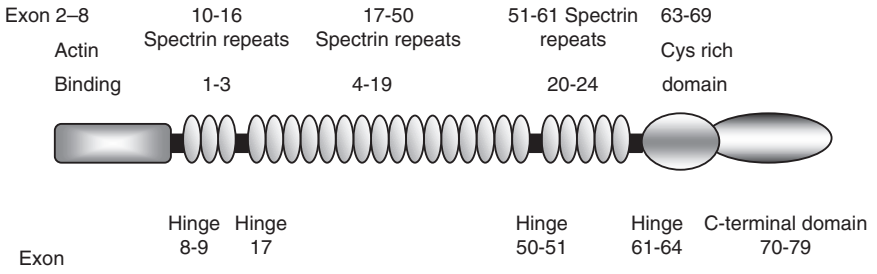


Fig. 2. Diagrammatic representation of the full length protein gene product adapted from Koenig et al. (1988)

and also at lower levels in skeletal muscle (Gorecki et al. 1992). These products have unique first exons, which are spliced to exon 2 of the major isoform.

Four internal promoters direct the production of shorter isoforms, classified according to the molecular weights, Dp260 (D'Souza et al. 1995), Dp140 (Lidov et al. 1995), Dp116, Dp71, and Dp40 (Blake et al. 1992; Tinsley et al. 1993; Lederfein 1992).

The functional significance of the different dystrophins remains unclear. Although it is presumed that the Dp427c and Dp427p isoforms offer structural support similar to Dp427m, the shorter isoforms lack the actin-binding domains. Associations could still occur through the carboxyl domain, allowing these shorter isoforms to have roles in stabilization, and possibly enzyme localisation to a non-muscle-dystrophin-associated glycoprotein complex (Blake et al. 1992; Blake et al. 2002). Additional diversity of dystrophin isoforms arises from alternative splicing to produce multiple novel transcripts that show a tissue-specific distribution (Sironi et al. 2002).

1.2 Duchenne and Becker Muscular Dystrophy

Arising from nonsense or frame-shifting mutations in the dystrophin gene that typically preclude synthesis of a functional gene product, Duchenne muscular dystrophy (DMD) is the most common, serious form of childhood muscular dystrophy (Koenig et al. 1987; Koenig et al. 1988; Monaco 1989). This is an X-linked recessive condition that occurs at a cited frequency of about 1 in 3,800 live male births (Emery 1991 ; Mostacciuolo et al. 1987), with one in three cases arising from a de novo mutation. Becker muscular dystrophy, a milder allelic disorder has a much lower cited incidence of 1 in 14,000 (Mostacciuolo et al. 1993). It has been postulated that the lower frequency of BMD may be due in part to undiagnosed, asymptomatic cases.

The normal dystrophin gene product is thought to function much like a girder, with a primary role in stabilizing myotube structure. Although affected males appear normal at birth, the muscle fibres are compromised by the absence of a functional dystrophin. In these young patients, muscle repair and regeneration keeps pace with the loss of damaged fibres, which occurs in a relentless, progressive, and predictable manner. Eventually the regenerative capacity is overwhelmed and DMD patients begin to show signs of muscle weakness between the ages of 3 and 5 years. Affected individuals may appear clumsy, are unable to keep up with their peers, and are typically restricted to a wheelchair by 12 years. Recent improvements in care management include steroid treatment, physiotherapy, and surgery. Substantial benefit is provided by nocturnal mechanical assisted ventilation,

and with combined interventions DMD patients are now living into their mid to late twenties (Eagle et al. 2002).

Becker muscular dystrophy also arises from mutations in the dystrophin gene, but these are typically in-frame deletions producing internally deleted proteins, with intact amino and carboxyl domains. A study of Italian BMD patients characterised mutations in detail and found 35 out of 37 cases were in-frame and the majority of the mutations occurred in the distal part of the rod domain (exons 45–60; Comi et al. 1994). These authors noted that BMD patients with mutations towards the 5′ end of the gene tended to have a more severe clinical phenotype, with faster progression and lower dystrophin expression, leading to earlier presentation. Depending upon the nature and position of the mutations, BMD may manifest from borderline DMD (intermediate) to almost asymptomatic. Some BMD patients have remarkably mild symptoms, despite deletions involving almost half the gene encoding the central rod domain (England et al. 1990; Winnard et al. 1993). Deletions in the dystrophin gene involving exon 45–47 and 45–48 have been reported to be consistently associated with very mild BMD (Bushby 1992).

There are reports of dystrophin deletions being identified indirectly. Melis and colleagues (Melis et al. 1998) reported three unrelated cases, after more detailed dystrophin molecular studies were undertaken, subsequent to an incidental finding of elevated serum creatine kinase levels. Dystrophin exon deletions of 32–44, 48–51, and 48–53 were identified, and adult male relatives from each family found to carry one of these deletions were either normal, or had very mild muscle involvement. In another chance diagnosis, a four-year-old female was found to be a carrier of a dystrophin exon 48 deletion, after she was originally referred for evaluation for a persistent elevation of aspartate aminotransferase (Morrone et al. 1997). Four male relatives, aged between 8 and 58 years, were found to carry the same mutation and as yet had exhibited no clinical symptoms.

1.3 Dystrophin Mutations

The dystrophin gene appears particularly susceptible to inactivation through a wide range of DNA perturbations. There is a high de novo mutation rate, with one in three cases of DMD presenting with no prior family history (Emery 1980). The new mutations may have occurred one or two generations back and would not be evident until an affected male is born. The incidence of gonadal mosaicism has been reported to be as high as 14% in new cases of DMD (Bakker et al. 1989).

The unusually large size of the dystrophin gene is believed to contribute to the high mutation rate, particularly involving the two deletion hotspots around exons 3–7 and 45–55 (Galvagni et al. 1994; Koenig et al. 1987; McNaughton et al. 1997). The dystrophin introns are rich in mobile

elements that are able to self-replicate, move to another chromosomal location, and hence accumulate in the genome (McNaughton et al. 1997). Although the dystrophin gene spans some 2.4 Mb, the recombination rate across this gene has been estimated to be 4 times higher than the anticipated rate across that expanse of DNA, with hotspots of recombination correlating to the deletion-prone regions (Oudet et al. 1992).

Considering the size and nature of the dystrophin gene, it is not surprising that most of the different types of mutations that could potentially inactivate a gene product have been identified in this gene. Examples of mutations that can disrupt the reading frame are shown in Fig. 3. The most common type of mutation in the dystrophin gene are genomic deletions of one or more exons, and these collectively account for approximately 60% of cases (Fig. 3b). Duplications are less common and are involved in less than 10% of cases (Fig. 3c).

Nonsense mutations occur at a frequency of about 15%, with the remainder of subtle DNA disease-causing mutations being microinsertions or deletions, splice motif defects (Figs. 3d, 3e and 3f), and a few missense mutations. Since dystrophin is presumed to have a primarily structural role, missense mutations are relatively rare (<2%) and would be restricted to those domains involved in binding to actin, β-dystroglycan, syntrophin, or dystrobrevin. No obvious hotspots for minor DNA base changes/mutations have been reported, although the Leiden database (www.dmd.nl) does indicate that some exons (19, 44, 50, 62/63, and 70) contain more mutations than the others. An examination of dystrophin mutation databases indicates that splice mutations account for some 10–15% of DMD cases, although the precise consequences of the splicing defect may vary.

Genomic deletions or duplications of exons that disrupt the reading frame have an obvious mechanism of precluding synthesis of a functional protein. Mutations that affect splicing, by either inducing complete or partial loss of an exon, or retention of intronic sequences, either flanking the exon or as a pseudoexon, also have the potential to disrupt the reading frame and will be equally catastrophic (Fig. 3d–f). However, it can be more difficult to predict the molecular consequences of some DNA changes on the splicing pattern. Furthermore, caution is required when classifying certain DNA changes as missense or nonsense mutations, or even silent polymorphisms that do not alter the encoded protein. An increasing number of genes are being reported to be inactivated by apparently innocuous DNA changes. Silent polymorphisms in the myophosphorylase and Lamin A/C genes have been reported to induce abnormal splicing patterns that result in McArdle's disease (Fernandez-Cadenas et al. 2003; Gamez et al. 2003) and Hutchinson-Guilford progeria, respectively (Cao and Hegele 2003; Eriksson et al. 2003; Huang et al. 2005; Pollex and Hegele 2004). These silent polymorphisms, which do not alter the encoded amino acid, could easily be overlooked and emphasize the importance of RNA-based screening protocols.

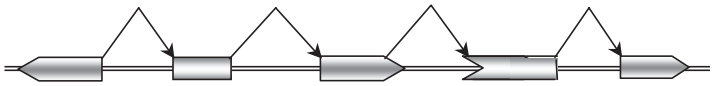


Fig. 3a. Diagrammatic representation of normal splicing patterns. Exons are depicted to interlock precisely in order to maintain the reading frame (not to scale)

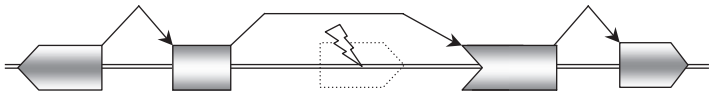


Fig. 3b. Splicing around a genomic deletion. This is the most common type of mutation in the dystrophin gene; in this example the reading frame of the processed transcript will be disrupted

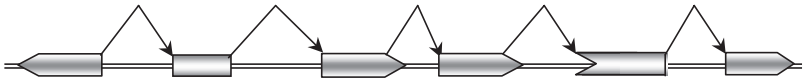


Fig. 3c. Splicing involving a genomic duplication. This mutation has compromised normal splicing and in this example the reading frame has been disrupted

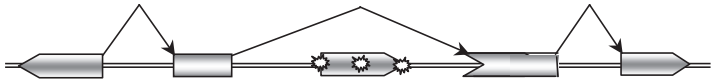


Fig. 3d. Abnormal splicing arising from mutations (✡) in either acceptor, within the exon itself or donor splice site. These mutations have compromised normal splicing so the exon is omitted from the mature mRNA. In this example the reading frame has been disrupted

The most common consequence of a splice motif mutation is loss of recognition of the exon, so that it is removed with the flanking intronic domains during pre-mRNA processing (Fig. 3d). The golden retriever model of muscular dystrophy (GRMD) arises from an A to G mutation at the penultimate base of intron 6, which disrupts that acceptor splice site. This mutation leads to exon 7 omission from the mature mRNA transcript, with subsequent disruption of the reading frame (Sharp et al. 1992).

DNA mutations can induce activation of cryptic splice sites that either result in the loss of some exonic sequence or the retention of some intron in the mature mRNA (Fig. 3e and 3f). Activation of cryptic splice sites can lead to inclusion of intronic sequences on either side of the exon, loss of some exon bases or, depending upon the context of nearby sequences, the retention of an entire block or pseudo-exon. A splice site mutation in the human dystrophin gene at the second base of intron 26 (donor site), led to the complete inactivation of that splice site, with subsequent activation of a downstream cryptic splice site. The inclusion of 117 bases of intronic sequence into the mature dystrophin transcript did not disrupt the reading frame, but contained an in-frame stop codon 30 nucleotides downstream of exon 26 (Wilton et al. 1994).

Another feature that should be considered is that the nature and position of disease-causing mutations will influence which isoforms are affected. Mutations 5′ to dystrophin exon 29 will be restricted to the full length Dp427 isoforms, while gene lesions downstream will progressively affect more of the shorter isoforms. The most common DMD mutations involving deletions around exons 45–55 will also affect Dp260 and Dp140. Although the functions of the shorter isoforms are not known, all gene replacement studies to date have concentrated on re-introducing the muscle isoform, either as a full-length (Liang et al. 2004), mini (Dunckley et al. 1993), or micro dystrophin (Gregorevic et al. 2004; Roberts et al. 2002; Scott et al. 2002), (for review see (Bogdanovich et al. 2004; Morgan 1994). This is not unreasonable, considering that skeletal and cardiac muscle are severely compromised (for review see Emery 1989). However, as DMD is a multisystem

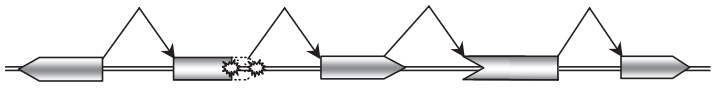


Fig. 3e. Activation of a cryptic splice site after either mutation of the normal donor splice site, or an intronic mutation that has created a stronger than normal splice site. This pattern of processing results in the inclusion of some intronic sequence in the mature mRNA, with potential disruption of the reading frame

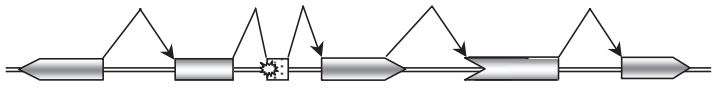


Fig. 3f. Activation of cryptic splice sites with subsequent recognition and incorporation of a pseudo-exon in the mature transcript. In this example the reading frame is maintained

disorder, any treatment that could address mutations in all the multiple isoforms must have greater potential.

1.4 Atypical Mutations: Exceptions to the Reading Frame Hypothesis

The reading frame hypothesis holds true for over 90% of cases of DMD, where nonsense or frame-shifting mutations are typically associated with the severe form of the disease (Monaco et al. 1988). Although nonsense or frame-shifting mutations in the dystrophin gene are expected to inactivate the gene product and result in DMD, some of these mutations can also present as milder Becker muscular dystrophy (BMD). It has been proposed that alternative splicing patterns, induced as a consequence of the primary gene lesion, could overcome the primary defect (Winnard et al. 1995). Subsequent studies have revealed that some disease-causing lesions reduced the splicing efficiency and facilitated removal of the exon carrying the nonsense mutation, without disrupting the reading frame (Fajkusova et al. 2001; Shiga et al. 1997; Flanigan K, Howard M, Salt Lake City, personal communication).

Individuals with frame-shifting genomic deletions involving the 5′ hotspot of dystrophin exons 3–7, 5–7, and 3–6 generally present with an intermediate phenotype (Muntoni et al. 1994). Rather than being restricted to a wheelchair by the age of 12 years, intermediate muscular dystrophy patients may be wheel-chair bound at ages 13 to 15. Several theories have been put forward to explain this variation in severity, including re-initiation of translation (Malhotra et al. 1988) and alternative splicing that results in restoration of the reading frame (Chelly et al. 1990a). In support of the latter possibility, naturally occurring alternatively spliced transcripts have been detected in patients with exon 3 to 7 deletions, where exon 1 was joined to exon 10 and exon 2 was spliced to exon 8 (Chelly et al. 1990a; Surono et al. 1999). The mechanism through which these in-frame transcripts are generated has not yet been established. It is possible that the genomic deletions have compromised normal splicing in that region of the dystrophin pre-mRNA, causing a low level of natural exon skipping. High fidelity splicing requires the appropriate splicing motifs to be organised within a particular spatial context. Although introns 3, 5, and 6 may be considered of typical size for the average gene at 4.8, 6.6, and 6.8 kb, respectively, introns 2, 4, and 7 are much larger at 170.3, 21.4, and 110.2 kb. Should the deletion breakpoints involved in the loss of exons 3–7 occur in the middle of introns 2 and 7, the loss of some 179 kb of intronic sequence would substantially alter the spatial arrangements and could easily disrupt pre-mRNA processing involving the remaining splice sites. Consequently, mutations in this region of the dystrophin gene transcript may be regarded as ‘leaky’ with respect to dystrophin expression.

In ‘an exception to the reading frame exceptions’, a recent report was published describing an asymptomatic boy with a nonsense mutation that

left only 1.6% of the dystrophin gene product untranslated (Suminaga et al. 2004). There was no evidence of alternative splicing around this mutation and the mild phenotype is most likely due to some redundancy of the extreme carboxyl domain of the dystrophin protein.

One of the first splice site mutations identified to cause BMD occurred within the donor splice site of exon 19 (Wilton et al. 1993). The A to C change in the third base of intron 19 reduced splicing efficiency so that only a low level of normal transcript was produced, while the majority of the dystrophin mRNA from this patient was out-of-frame and could not be translated into a functional protein. Low levels of normal dystrophin were detected by immunohistochemical staining and by western blotting, consistent with a diagnosis of BMD, and providing unequivocal proof that even low levels of dystrophin can be beneficial.

1.5 Revertant Fibres

Revertant fibres are dystrophin-positive fibres that have been detected immunohistochemically in dystrophic muscle. These dystrophin-positive fibres are found in most animal models of muscular dystrophy, and at least 50% of DMD patients (Hoffman et al. 1990; Klein et al. 1992; Schatzberg et al. 1998; Sherratt et al. 1993; Wallgren-Pettersson et al. 1993; Wilton et al. 1997). The dystrophin in revertant fibres has been shown to be internally deleted, with RNA and protein studies involving epitope mapping indicating that many exons have been omitted from the mature mRNA (Klein et al. 1992; Lu et al. 2000; Sherratt et al. 1993; Wallgren-Pettersson et al. 1993; Wilton et al. 1997). The natural exon skipping by-passed either a nonsense mutation or a frame-shifting re-arrangement and resulted in a transcript that could be translated into a Becker–muscular–dystrophy-type protein. It should be noted that the revertant fibres in the animal models or the human cases are not uniform, but rather represent a variety of exonic combinations (Lu et al. 2000; Wallgren-Pettersson et al. 1993; Wilton et al. 1997).

The incidence of revertant fibres is too low to be of any clinical benefit (Fanin et al. 1995) but these dystrophin-positive fibres demonstrate that some natural, albeit low level, exon skipping mechanism can by-pass mutations that would have otherwise induced the premature truncation of dystrophin synthesis. The origin of dystrophin in revertant fibres is not known but could be due to either a secondary genomic deletion to remove the primary gene lesion and restore the reading frame, or some alternative splicing mechanism allowing multiple exons to be skipped from the mature dystrophin transcript. Genomic deletions are common in the dystrophin gene and provide some indirect evidence for a somatic mutation origin for revertant fibres. However, in situ

hybridization studies using an *mdx* mouse intron 23 genomic probe indicated that there were no genomic deletions in the majority of dystrophin-positive fibres, suggesting some alternative processing of the dystrophin pre-mRNA (Lu et al. 2000). Regardless of their origin, an important feature of revertant fibres is that exon skipping in the dystrophin gene does occur naturally, and since these dystrophin-positive fibres persist, the dystrophin is at least partially functional, and the immune system should be tolerant to some dystrophin.

2 Splicing

2.1 Mechanism of Pre-mRNA Processing

The splicing of pre-mRNA involves the precise removal of the intervening intronic sequences from the coding exonic domains. Processing of a nascent pre-mRNA does not occur in isolation, but as one step within a coordinated network coupling transcription, 5′ capping, splicing, polyadenylation, and subsequent nuclear transport (Kornblihtt et al. 2004; Maniatis and Reed 2002). During transcription, the carboxy-terminal domain of RNA Pol II acts as an assembly platform and recruits factors required for nascent gene transcript processing (Fong and Zhou 2001). As illustrated in Fig. 4a, consensus sequences at the exon:intron (donor) and intron:exon (acceptor) splice sites are shown and indicate invariant and less conserved bases. Using dystrophin exon 16 as an example, splice motifs predicted by ESEfinder (Release 2.0; Cartegni, et al. 2003; http://rulai.cshl.edu/tools/ESE) to recruit other splicing factors are shown (Fig. 4b and Table 1).

Eukaryotic cells have developed a complex system where 145 distinct proteins and 5 small nuclear RNAs assemble to form the spliceosome on

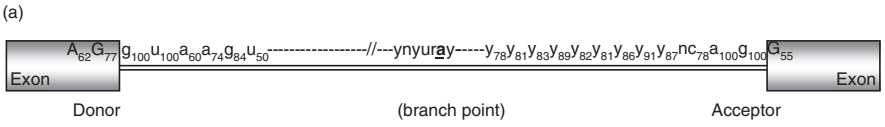


Fig. 4a. The three main elements identified for spliceosome assembly at the exon: intron junctions, not shown to scale. The first and last two bases of the intron are almost invariant, as shown by the *subscript numbers* representing base frequency. *Upper case letters* represent exonic sequence while *lower case letters* are intronic. The *branch point* is found between 18 and 40 bases, 5′ to the acceptor splice site with y and r representing pyrimidine and purine residues respectively (Krawczak et al., 1992)

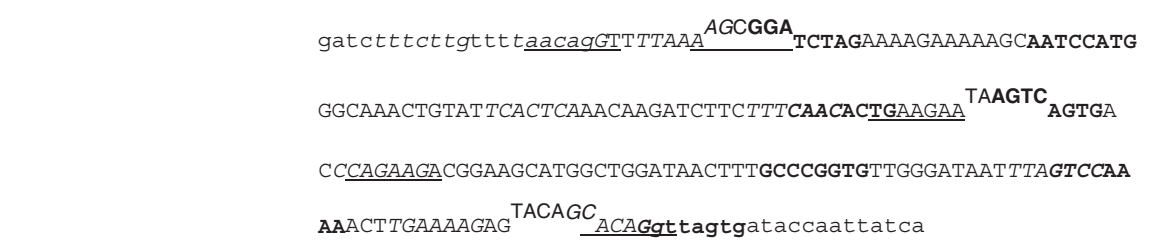


Fig. 4b. Nucleotide sequence of dystrophin exon 16 in capital letters with flanking intronic sequence shown in lower case letters. Binding domains predicted by ESE finder (Cartegni et al. 2003; http:// rulai.cshl.edu/tools/ESE) are shown with SF2/ASF sites underlined, SC35 in *bold*, SRp40 in *italics* and SRp55 as *superscript letters*

the pre-mRNA in a highly orchestrated fashion (Zhou et al. 2002). The fundamental mechanism involves two coordinated trans-esterification reactions at the exon:intron (5′ or donor) and intron:exon (3′ or acceptor) splice sites, with subsequent ligation of the exons that flanked the excised intron. The pre-mRNA processing occurs as a consequence of the coordinated assembly of the huge 3-MDa spliceosome complex (Hastings and Krainer 2001). The adenosine residue in the branch point attacks the 5′ phosphodiester bond at the beginning of the intron. The intron is then folded into a lariat structure, while during the second part of the reaction, the nucleoside at the 3′ end of the upstream exon attacks the phosphodiester bond at the 3′ end of the intron. The phosphodiester bond formed between the 2 exons results in the release of the intronic lariat.

2.2 Alternative Splicing

It has been estimated that only 1.5% of the human genome is committed to protein coding sequences, with the human genome containing less than 25,000 genes (International Human Genome Sequencing 2004). Despite the lower than expected gene number, the protein coding component of the genome is composed of over 231,600 exons, which are spread across an estimated 1.7 Gb of transcribed RNA. The magnitude and complexity of the processes involved to accurately combine the appropriate exons to produce the hundreds of thousands of mature gene transcripts represents an amazing accomplishment of coordinated biological engineering. To further add to the complexity of this process, it has also been estimated that 40–75% of all human genes are alternatively spliced in a tissue specific and/or developmental manner (Herbert 2004).

Table 1. Predicted weight of splice factor binding can be represented in a table format to estimate most likely exonic domains involved in recruiting various splice factors

	SF2/ASF			SC35			SRp40			SRp55	
	Thr = 1.956			Thr = 2.383			Thr = 2.67			Thr = 2.676	
Pos	Motif	Score	Pos	Motif	Score	Pos	Motif	Score	Pos	Motif	Score
16	AACAGGT	3.126	32	GGATCTAG	2.458	5	TTTCTTG	2.696	29	AGCGGA	3.069
28	AAGCGGA	2.240	52	AATCCATG	2.493	15	TAACAGG	4.057	107	TAAGTC	3.387
99	CTGAAGA	2.696	94	CAACACTG	2.579	24	TTAAAAG	3.875	191	TACAGC	4.104
120	CAGAAGA	3.819	109	AGTCAGTG	2.742	72	TCACTCA	3.391			
123	AAGACGG	3.110	150	GCCCGGTG	2.602	91	TTTCAAC	3.554			
196	CACAGGT	5.631	171	GTCCAAAA	2.581	119	CCAGAAG	3.675			
			200	GGTTAGTG	4.264	168	TTAGTCC	2.894			
						182	TGAAAAG	3.366			
						195	GCACAGG	3.820			

"Thr" = Threshold

2.3 Antisense Oligonucleotide Modification of Splicing Patterns

Antisense oligodeoxynucleotides have been referred to as 'informational drugs' (Cohen 1991) where the specificity of the compound is determined by the nucleotide sequence. Antisense oligonucleotides were first used to down-regulate gene expression through blockade of translation, or induction of RNaseH action to degrade the target gene transcript (Zamecnik and Stephenson 1978). Newer chemistries and more reliable and consistent synthesis protocols have resulted in oligonucleotides which have greater nuclease resistance (see Section 3). These antisense oligonucleotides can exert a more sustained biological effect but also do not induce RNaseH activity when bound to an RNA transcript. Unlike gene down-regulation applications, the efficacy of antisense oligonucleotide redirected splicing can be monitored by the appearance of a novel product. In this manner, there can be no doubt as to the effect of the antisense oligonucleotides on the target gene transcript.

Many spliceosome components must be localised directly to the single stranded motifs in the pre-mRNA, or indirectly to other components that are recruited to the donor, acceptor, branch point and ESEs, or intronic silencers. The original concept of antisense oligonucleotide-induced redirection of splicing was that these compounds would anneal to single-stranded splicing motifs and prevent formation of the spliceosome in that region. The spliceosome is assembled for the removal of each intron, and then after exon ligation the components are disassembled for use in another splicing event. As such, the interactions of the splicing factors and the pre-mRNA are dynamic, and it is more likely that the antisense oligonucleotides prevent assembly rather than displace already bound splicing factors.

Since the most common consequences of a splice site mutation are either exon skipping or the activation of a cryptic splice site, the application of antisense oligonucleotides to a particular splicing motif should also result in either exon skipping or activation of a cryptic splice site. Now, with some experience in this area, several investigators have found the most likely consequence to be exon removal (Aartsma-Rus et al. 2004b; Errington et al. 2003; Gebski et al. 2003; Mann et al. 2001; Mann et al. 2002; Pramono et al. 1996; Takeshima et al. 2001; van Deutekom et al. 2001; Wilton et al. 1999). Activation of cryptic splice sites in the dystrophin gene has only occurred in a few cases, and these cryptic splice sites could be suppressed by a second antisense oligonucleotide (Dominski and Kole 1993). Alternately, we have found that redesigning the first antisense oligonucleotide to a different motif within the same exon resulted in precise exon skipping (Wilton, Fletcher, and McClorey, unpublished observations).

2.4 Antisense Oligonucleotide Chemistries

An antisense oligonucleotide should possess the following properties for application to targeted exon skipping:

1. It should induce no RNaseH activity when annealed to an RNA target.
2. It should be resistant to degradation by nucleases, so that it can persist and exert a biological effect for as long as possible.
3. It should be readily taken up by the cell and localised within the nucleus where pre-mRNA processing occurs.
4. It should anneal to the target motif with sufficient strength to block assembly of spliceosome complexes, but not with such an avidity that there is the possibility of cross annealing to non-target sequences.
5. Side-effects associated with the backbone chemistry should be minimal.
6. Sequence-specific side effects should be avoided.
7. Large scale synthesis capacity for clinical grade production should be possible.

The phosphorothioate backbone is formed by the incorporation of a sulphur atom instead of the non-bridging oxygen in the phosphodiester backbone. This conferred some protection against nuclease degradation, while maintaining a structure similar to the natural molecule and was an important feature of second generation antisense oligonucleotides. Deoxyribonucleotides on a phosphorothioate backbone were more stable than the natural phosphodiester backbone and are able to induce degradation of specific gene transcripts, through the induction of the ubiquitous enzyme RNaseH (Furdon et al. 1989).

Several different chemistries have been used to induce exon skipping in the dystrophin gene, including oligodeoxynucleotides (Pramono et al. 1996), 2′-*O*-methyl and 2′-*O*-(2-Methoxy)ethyl modified bases (Wilton, unpublished data), all on phosphorothioate backbones, chimeric 2′-*O*-methyl/ethylene bridged nucleic acids (Surono et al. 2004; Yagi et al. 2004), locked nucleic acids (Aartsma-Rus et al. 2004b), morpholino oligonucleotides (Aartsma-Rus et al. 2004b; Gebski et al. 2003), and peptide nucleic acids (Aartsma-Rus et al. 2004b).

2.5 Candidate Genes for Splicing Intervention

Specific removal of one or more exons from a gene transcript would be catastrophic in many cases, either because of disruption of the reading frame or the loss of a crucial protein coding domain. However, there are

some situations in which induced exon removal could be employed to by-pass a more serious gene defect.

Several criteria should be met for a gene and its product to be regarded as amenable to antisense oligonucleotide redirection of splicing. The candidate gene should consist of many exons, some of which can be deleted without seriously compromising the gene product. Ideally, the induced gene transcript and the translated protein should have relatively long half-lives, so that readministration would not have to be frequent. The tissue or cells in which the gene is expressed should have a slow turnover, so that once synthesised, the induced product would persist. Finally, the induced protein should not elicit an immune response.

As discussed previously, the dystrophin gene meets all these conditions and offers one of the more amenable targets for induced exon skipping. Extensive pre-mRNA processing is required to combine the 79 exons found in the mature full length Dp427 isoform. Some in-frame dystrophin deletions result in almost asymptomatic BMD, and provide unequivocal proof that parts of the dystrophin protein can be lost with minimal consequences (England et al. 1990; Heald et al. 1994). The dystrophin protein has an extremely slow turnover, as do the mature muscle fibres. The naturally occurring revertant fibres detected in dystrophic tissue arise as the result of an exon skipping event (Klein et al. 1992; Lu et al. 2000; Sherratt et al. 1993; Wallgren-Pettersson et al. 1993; Wilton et al. 1997) and not only does the revertant dystrophin stabilize the muscle, but also does not illicit an immune reaction (Ferrer et al. 2000).

3
Molecular By-pass Surgery for Duchenne Muscular Dystrophy

3.1
The Dystrophin Gene and Redirected Splicing

The principle of antisense oligonucleotide-induced exon skipping to by-pass mutations in dystrophin pre-mRNA transcripts is illustrated in Fig. 5. This mode of action is similar to the use of antisense oligonucleotides to prevent translation by blocking motifs at, or slightly upstream of, the AUG initiation codon. Antisense oligonucleotides directed downstream of the initiation codon are not efficient at blocking translation, presumably because they are displaced by the RNA helicases and ribosomes during the translation elongation (Summerton 1999). This feature would imply that the chances of translation blockade of a homologous gene after chance cross hybridisation should be minimal, as the translation initiation and splicing motifs constitute a small proportion of the pre-mRNA transcript. We have found that considerable

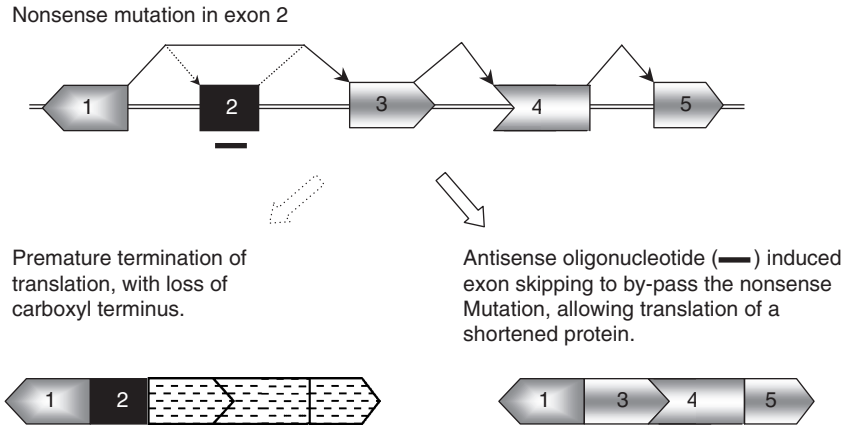


Fig. 5a. Induction of specific exon skipping by antisense oligonucleotide blockade of normal splicing motifs. Transcripts of mRNA carrying nonsense mutations cannot direct protein translation downstream of the defect, so the genetic information is lost (indicated by *dashed fill*) and the carboxyl terminal domain is not produced. Exclusion of an exon that carries a premature termination codon allows synthesis of a shorter, internally deleted protein with intact amino and carboxyl domains

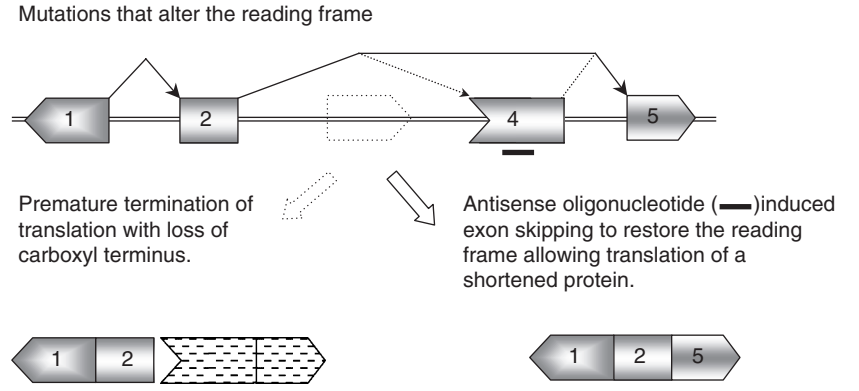


Fig. 5b. Induction of abnormal splicing by antisense oligonucleotides masking normal splicing motifs. The reading frame is represented by interlocking exon junctions. Transcripts of mRNA inactivated by frame-shifting genomic deletions cannot direct protein translation downstream of the mutation, so the genetic information is lost (indicated by *dashed fill*) and the carboxyl terminal domain is not produced. Exclusion of one or more flanking exons can restore the reading frame, allowing synthesis of a shorter, internally deleted protein with intact amino and carboxyl domains

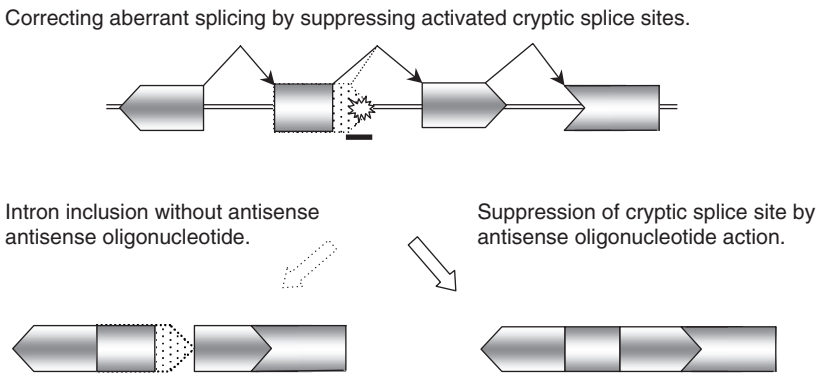


Fig. 5c. Restoration of normal splicing by antisense oligonucleotides masking aberrant cryptic splice sites. The same approach could be applied to mask exonic cryptic splice sites, or prevent incorporation of pseudo-exons, as shown in Figs. 3e and 3f

optimisation, generally through empirical experimentation, is needed to design antisense oligonucleotides that can induce efficient redirection of splicing (Errington et al. 2003; Mann et al. 2001; Mann et al. 2002).

The first antisense oligodeoxyribonucleotide (ODN) reported to alter splicing of the dystrophin gene transcript was a 31mer directed to an exon 19 motif implicated in exon recognition (Pramono et al. 1996). Despite the fact that both exon 19 acceptor and donor splice sites were intact, an intra-exonic deletion led to the exclusion of the entire exon (Matsuo et al. 1991). Transfection of the antisense ODN induced low levels of exon 19 skipping in cell culture. Our laboratory has found that antisense oligodeoxyribonucleotides can redirect splicing to a limited extent, but only when directed to some dystrophin targets. Furthermore, when compared to other chemistries with greater nuclease resistance and which do not induce RNase H activity, the ODNs were found to be very inefficient (Gebski et al. 2005).

Chemistries that are better suited to re-directing splicing patterns include those with modifications at the 2′ ribose, including 2′-*O*-methyl (2OMe) or 2′-*O*-(2-methoxy)ethyl (2MOE) groups (Altmann et al. 1996; Verma and Eckstein 1998) and the 2′-*O*,4′-C-methylene-linked nucleotides (locked nucleic acids) (Orum and Wengel 2001; Wahlestedt et al. 2000).

The suitability of an oligonucleotide chemistry cannot be judged only on its resistance to degradation, as locked nucleic acids have been reported to exhibit even greater nuclease resistance and stronger annealing to the target sequences (Braasch et al. 2002). These compounds proved extremely effective at redirecting the processing of the target gene transcript, but Aartsma-Rus et al. (Aartsma-Rus et al. 2004b) concluded that the strength of annealing could lead to a lack of specificity. The high affinity would allow

mis-annealing to related sequences, as the 14mer used in this study had a calculated Tm of 131°C. Locked nucleic acids containing 3 mismatched nucleotides were still able to redirect splicing in an in vitro assay, indicating the potential to cross hybridise to moderately related gene transcripts (Aartsma-Rus et al. 2004b).

Peptide nucleic acids and morpholino oligonucleotides have uncharged backbones, are highly resistant to degradation, and as such can persist for extended periods once taken up by the cell. Kole and colleagues, using a transgenic reporter mouse found that both peptide nucleic acids and morpholino oligonucleotides were able to redirect splicing after systemic administration (Sazani et al. 2002). Our laboratory has evaluated several peptide nucleic acids directed at the donor splice site of the *mdx* dystrophin exon 23 and found that those without a lysine tail were ineffective. The addition of a 4-lysine tail to a peptide nucleic acid has been reported to enhance cellular uptake (Sazani et al. 2002), however, this was not the case in our laboratory (Harding, Fletcher, and Wilton, unpublished observations). Exon 23 deleted transcripts were sporadically detected in cultures treated with antisense peptide nucleic acid (4 lysine), but the efficiency was well below that achieved with other chemistries. Other researchers have reported similar findings. A peptide nucleic acid with a 4 lysine tail was ineffective at inducing exon 46 skipping in the human dystrophin gene transcript (Aartsma-Rus et al. 2004b).

We have also examined the ability of morpholino oligonucleotides to induce specific exon skipping in the dystrophin gene in vitro and in vivo, with very promising results (Gebski 2003; Fletcher et al., manuscript in preparation). This was in contrast to a report by Aartsma-Rus and colleagues who found minimal exon skipping when a morpholino oligonucleotide was directed against human dystrophin exon 46 (Aartsma-Rus et al. 2004b). We are currently investigating whether these inconsistencies in morpholino oligonucleotide action are due to targeting (design) or delivery.

Unlike gene down-regulation to suppress a disease-associated transcript, the ability of the antisense oligonucleotides to induce exon skipping may not need to be absolute to achieve therapeutic outcomes. If some pre-mRNAs are not suitably modified during one particular round of transcription and processing, it is possible that the antisense oligonucleotides could exert the desired effect during the production of the next gene transcript.

3.2 Antisense Studies in Animal Models of Muscular Dystrophy

The *mdx* mouse model of muscular dystrophy (Bulfield et al. 1984) offers a convenient molecular system to refine antisense oligonucleotide design and assess delivery to by-pass a dystrophin mutation. The absence of dystrophin in the *mdx* mouse arises from a nonsense mutation in exon 23

(Sicinski et al. 1989) and removal of this exon, which does not disrupt the reading frame, would by-pass the primary gene lesion. Dunckley and colleagues were the first to report exon skipping in the *mdx* dystrophin gene transcript using a 2′-O-methyl 12mer directed at the intron 22 acceptor splice site to induce skipping of exons 22 to 30 (Dunckley et al. 1998). Persistence of the shortened transcript or dose-related responses were not discussed, and the induced exon skipping was not as specific as anticipated.

Other studies targeting exon 23 for removal found that the intron 23 donor splice site was most amenable to induce consistent and reproducible exon skipping in a dose-dependant manner in vitro and in vivo (Lu et al. 2003; Lu et al. 2005; Mann et al. 2001; Wilton et al. 1999). Refinements in targeting achieved exon 23 skipping in transfected cell cultures with antisense oligonucleotide lipoplexes at concentrations as low as 10 nM (Mann et al. 2002). Targeting the exon 23 acceptor site, at the end of intron 22, with a series of antisense oligonucleotides from 20 to 30 nucleotides long was unable to induce any specific exon 23 removal (Mann et al. 2001; Wilton et al. 1999), or any multiple exon skipping as reported earlier (Dunckley et al. 1998). This result was somewhat surprising since it was assumed that masking crucial motifs involved in splicing would redirect the processing of the pre-mRNA, but as discussed in the next section, it appears there is no consistent target that may be used to reliably induce exon skipping.

Nucleic acid delivery is a crucial issue, as different delivery agents influenced levels of induced exon 23 skipping and uncomplexed oligonucleotide was essentially ineffective. It was noted that some transcripts were missing both exon 22 and 23 (Mann et al. 2001), although there is no obvious sequence homology between the antisense oligonucleotide and regions within exon 22. The concomitant removal of both exons indicated that the splicing of these exons is tightly linked and emphasizes the need for a screening assay that examines several exons on either side of the target.

Transfection of cultured cells with uncomplexed 2′-O-methyl antisense oligonucleotide yielded no consistent exon 23 skipping, likewise administration of the oligonucleotide alone in vivo appeared very inefficient. Lu et al. (2003) demonstrated efficient dystrophin exon 23 skipping in vivo after injection of the antisense oligonucleotide with the block copolymer F127. Recently, more widespread restoration of dystrophin synthesis has been reported after intravenous antisense oligonucleotide administration (Lu et al. 2005). Effective systemic delivery of antisense oligonucleotides will be essential before exon skipping becomes a viable therapy for DMD.

The *mdx* mouse offers only one target for antisense oligonucleotide evaluation. Interestingly, targeting the same coordinates in the human dystrophin gene transcript that induced exon skipping in the mouse (that is, the last 2 bases of exon 23 and the first 18 bases of intron 23), resulted in no detectable skipping of human exon 23 (Mann, Wilton, Fletcher, unpublished observations). Although the coordinates are the same, the target sequence is different and this suggests that each exon must be addressed on a case by case basis.

Another mouse model of relevance for dystrophin studies is the transgenic 'humanized' mouse carrying the entire human dystrophin gene (Bremmer-Bout et al. 2004). The development of this transgenic hDMD mouse could prove to be very useful for evaluating oligonucleotide design and delivery. This group also demonstrated that specific antisense oligonucleotides could induce human dystrophin exon skipping without any detectable disruption of the endogenous mouse dystrophin transcript (Bremmer-Bout et al. 2004). This clearly demonstrates the exquisite specificity of this approach, as there is strong homology between the mouse and human dystrophin genes. Indeed, the homology is absolute in some regions, such as exon 19 where the same oligonucleotides can induce exon 19 skipping in human and murine cells (Errington et al. 2003). In this report, some human specific antisense oligonucleotides were described as being able to induce low levels of exon skipping in murine cultured cells, despite several mismatches, but only after transfection at high concentrations.

The canine model of muscular dystrophy requires that exons 6 and 8 be removed to restore the frame-shift induced by exon 7 exclusion. Our laboratory has been able to induce efficient skipping of these exons in cultured cells and we have detected induced dystrophin by western blotting in vitro and in vivo (McClorey, Fletcher, and Wilton, unpublished observations). Cultured GRMD myoblasts are not robust, and the difficulty in detecting dystrophin may be a reflection of the culture and growth conditions (McClorey, Fletcher, and Wilton, unpublished observations). Other research groups have access to this canine model, and the lack of reports from them of dystrophin production after antisense treatment is justification for added concern, particularly in light of anticipated human trials.

3.3 Human Dystrophin Gene Studies

The first studies on induced exon skipping in the dystrophin gene transcript were undertaken by Matsuo and colleagues who identified a DMD patient with a deletion of 58 bases within exon 19 (Matsuo et al. 1990; Matsuo et al. 1991). Interestingly, these researchers used antisense oligodeoxynucleotides on a phosphorothioate backbone, a chemistry that is normally applied to gene down-regulation. Although this group has extended their studies to evaluate new antisense chemistries (Surono et al. 2004; Takeshima et al. 2001), a single patient trial using systemic administration of antisense oligodeoxynucleotides commenced at the end of 2003. This patient has a deletion of exon 20, which induces a frame-shift in the dystrophin mRNA, rendering it defective, but the removal of exon 19 is predicted to restore the reading frame, with the presumed benefit of inducing some functional dystrophin. Further results from this study are eagerly anticipated.

Vigorous efforts are underway to design antisense oligonucleotides directed at various motifs, but primarily exonic splicing enhancers in the human dystrophin gene (De Angelis et al. 2002; Takeshima et al. 2001; van Deutekom et al. 2001 Aartsma-Rus 2003). The most common type of mutation inactivating the dystrophin gene are genomic deletions of one or more exons, predominantly involving either of the two hotspots (Oudet et al. 1992). Other, more subtle DNA changes, such as nonsense mutations and splicing errors, occur relatively evenly distributed across the gene, and thus many different antisense oligonucleotides must eventually be developed to address the spectrum of disease-causing mutations.

As one example of choosing potential targets for antisense induced exon skipping, the nucleotide sequence of human dystrophin exon 16 is shown with flanking intronic nucleotides in Fig. 4b. Donor and acceptor splice sites are readily identified at the intron:exon and exon :intron junctions. Numerous predicted binding sites for proteins involved in exon recognition (SF2/ASF, SC35, SRp40, and SRp55) with weights of these sites shown in Table 1. There is a substantial cluster of potential binding sites just upstream of the donor splice site that may indicate an ideal target for antisense oligonucleotide masking. A panel of 6 antisense oligonucleotides were designed to mask these binding sites, as well as the acceptor and donor site; the annealing coordinates are shown in the legend of Fig. 6a.

Only some antisense oligonucelotides masking the acceptor splice site were able to induce consistent exon 16 excision from the mature mRNA

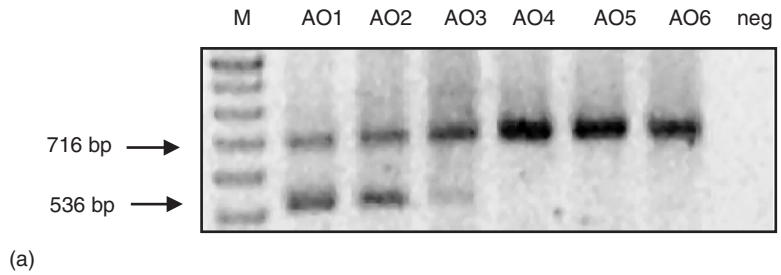
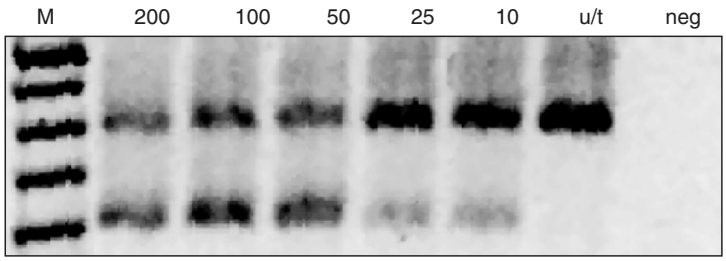


Fig. 6a. RT-PCR showing exon 16 removal from the dystrophin mRNA 24 hours after human myotubes were transfected with 200 nM antisense oligonucleotides:lipoplexes targeting 6 different domains of the target exon. The annealing coordinates of these 6 different antisense oligonucleotides are AO1: (–12+19), AO2: (–06+25), AO3: (–06+19), AO4: (–07+13), AO5: (+92+116), and AO6: (+12–11). Negative prefixes indicates intronic sequence while positive numbers represent exonic nucleotides from the 5′ end of the exon. Hence –X+Y indicates X nucleotides of the upstream intron and the first Y bases of exon (an acceptor site), +X+Y represents an intra-exonic target (an ESE) and +X–Y designates the last X bases of the exon and the first Y bases of the downstream intron (a donor splice site; Harding, Fletcher, and Wilton, manuscript in preparation)

after transfection into human primary myotube cultures (Fig. 6a). Despite masking the donor splice site and several predicted ESE motifs of considerable weight, some antisense oligonucleotides were found to be unable to induce exon 16 skipping. Other antisense oligonucleotides directed at ESEs within exon 16 also appeared inactive, but compounds directed at the acceptor splice site were found to induce strong and consistent exon skipping (Fig. 6a). Some of the antisense oligonucleotides that masked the acceptor site induced substantial exon-skipping in transfected human cultured cells, and subsequent titration experiments confirmed a dose-dependant response (Fig. 6b).

We have now designed antisense oligonucleotides that induce reliable exon skipping in the many parts of the human dystrophin mRNA that could respond to exon skipping. In some cases, targeting acceptor, donor, or ESEs can induce efficient exon skipping (Errington et al. 2003), while other exons appear to be efficiently removed only if the donor, or the acceptor, or a particular ESE is masked.

A panel of antisense oligonucleotides that can restore the reading frame in the more commonly encountered DMD deletions has been developed in Leiden, and it is estimated that 20 antisense oligonucleotides would be able to address the mutations in 50% of patients with deletions and 22% with duplications listed in the extensive Leiden database (Aartsma-Rus et al. 2002). It appears that the majority of mutations in the dystrophin gene should be ameliorated to varying extents by selected exon exclusion. It has been estimated that more than 75% of all DMD patients would benefit from dystrophin gene transcript manipulation so that some functional gene product is restored (Aartsma-Rus et al. 2004a). Dystrophin mutations that could not respond to an exon-skipping therapy include those located in the promoter, those with extensive genomic deletions, or those with deletions involving crucial binding domains at the amino or carboxyl terminus.



(b)

Fig. 6b. RT-PCR showing exon 16 removal from the dystrophin mRNA 24 hours after human myotubes were transfected with varying amounts of an antisense oligonucleotide:lipoplex targeting the last 12 bases of intron 15 and the first 19 bases of exon 16 (Harding, Fletcher, and Wilton, manuscript in preparation)

Each antisense oligonucleotide targeting a different region of the dystrophin transcript must be regarded as a new drug, and will therefore require appropriate safety studies before approval can be granted for human administration. In an effort to minimize the number of antisense oligonucleotides required to address all amenable dystrophin mutations, combinations of antisense oligonucleotides could be used to address mutations within a defined region of the dystrophin transcript (Aartsma-Rus et al. 2004a). These researchers also examined bi-functional antisense oligonucleotides that had one half directed at human dystrophin exon 45 and the other half annealed to exon 51. This compound effectively restored the reading frame in a DMD cell line with a deletion of exons 46 to 50. Interestingly, low levels of exon 45 to 51 skipping were detected in a normal cell line, raising the possibility of bi-functional antisense oligonucleotides addressing a variety of mutations. If exons 45 to 51 could be efficiently removed, this one compound should be able to by-pass nonsense mutations, splice-site mutations, deletions, and duplications in this area.

In a another approach, we have developed a cocktail of three antisense oligonucleotides directed at exons 6, 7, and 8 of the human dystrophin transcript (McClorey, Fletcher, and Wilton, unpublished work). The efficiency of the skipping of these exons was surprising, considering that exons 6 and 8 are separated by over 110 kb of intronic sequence.

3.4 Antisense Oligonucleotide Delivery

Although intramuscular injections of antisense oligonucleotides in animal models (Bremmer-Bout et al. 2004; Lu et al. 2003; Mann et al. 2001; Mann et al. 2002) provide proof of the principle that dystrophin expression can be restored, it is apparent that such a regimen would be of limited use in a clinical setting. The spread of dystrophin from the injection site is restricted even in small muscles, and repeated application of hundreds of injections to treat major muscles should not be considered. A localised treatment of selected muscles in the hand, wrist, and forearm may preserve remaining hand muscle function in older patients, for whom a systemic treatment would be of little benefit. For 'molecular by-pass surgery' to provide a substantial benefit to the patient, it would be necessary for systemic administration to commence in young patients. With this approach, it may be possible to slow the progression of muscle damage before it becomes overtly established and may also address the other dystrophin isoforms.

Systemic distribution would be essential to potentially address the more common dystrophin deletions involving exons 45–55 as these would not only affect expression of the full length isoforms Dp427p and Dp427c but also Dp260, Dp140 and possibly Dp116. Although the function of these

isoforms is unknown, uptake of the appropriate antisense oligonucleotide could restore the reading frame. The loss of these isoforms is not addressed by conventional dystrophin cDNA replacement by viral or plasmid vectors.

Animal studies have conclusively shown that antisense oligonucleotides can be administered by intramuscular injections and induce substantial amounts of dystrophin (Lu et al. 2003). The induced dystrophin was sufficient to allow detectable changes in force of contraction in the treated muscle after a single injection. The efficacy of the antisense oligonucleotide was greatly enhanced when the therapeutic compound was administered with either cationic liposome preparations such as Lipofectin (Invitrogen, Carlsbad, CA, USA; Mann et al. 2001) or a block co-polymer such as F127 (Lu et al. 2003).

Weekly systemic administration of a 2′-O-methyl antisense oligonucleotide with F127 into the *mdx* mouse demonstrated that such an approach could restore dystrophin expression in all skeletal muscles (Lu et al. 2005). Interestingly, dystrophin expression in cardiac tissue was not detected.

Although systemic administration appears daunting, several clinical trials are currently underway. Intravenous administration of antisense oligonucleotides are being used in anticancer trials and should provide a useful baseline for dystrophin trials (Desai et al. 2005). This work will only provide a starting point for the human trials as there are major differences between treating adult cancer patients and young children with a chronic condition.

4 Clinical Trials

Exploitation of synthetic antisense oligonucleotides to alter gene transcripts is in its infancy and cannot be compared to the elegant and highly specific alternative splicing that occurs naturally. Much of the early antisense work on redirecting splicing occurred in cell-free systems, however progress in some areas has been so rapid that clinical trials to address dystrophin mutations are being planned (Muntoni et al. 2005).

Several issues must be considered. Rather than one therapeutic compound, such as a recombinant plasmid, a virus, or a cell preparation that would aim to replace the defective dystrophin gene, the antisense approach must be tailored to each individual type of mutation. The first step in applying antisense oligonucleotides to redirect dystrophin splicing is to determine the precise gene defect, and then optimize manipulation of splicing to by-pass the protein-truncating mutation. This therapy should be regarded as more a personalised medical treatment than a general treatment for all muscular dystrophy patients.

4.1 Fine-Mapping Dystrophin Mutations

The majority of dystrophin deletions are readily detected using multiplex DNA screening of the most commonly involved exons (Abbs et al. 1991; Chamberlain et al. 1991). Although generally satisfactory as part of a molecular diagnosis of dystrophin involvement, more comprehensive screening will be necessary to define the exons involved in a genomic deletion. For example, in a multiplex PCR screen to scan for structural integrity of the dystrophin gene (Abbs et al. 1991; Chamberlain et al. 1991), loss of exon 6 is typical of deletions involving the 5′ hotspot in the dystrophin gene. Since exons 5 and 7 are not included in these multiplex sets, the extent of exon involvement cannot be established, as the dystrophin gene deletion could involve exons 5–6, exons 6–7, or even 5–7. Genomic deletions of exons 6–7 and 5–7 would require the removal of exon 8 to restore the reading frame, while a genomic deletion of exons 5–6 would need exons 7 and 8 to be removed to by-pass the frame-shift.

Nonsense mutations or small insertions/deletions within a single exon must be precisely characterised so that the target exon is identified for appropriate induced skipping during pre-mRNA processing. A superficial characterisation of the mutations in the dystrophin gene will no longer be adequate.

There are several more comprehensive screening protocols, including MLPA (multiplex ligation-dependent probe amplification (Janssen et al. 2005; Schwartz and Duno 2004), single condition amplification/internal primer (SCAIP) sequencing (Flanigan et al. 2003) and detection of virtually all mutations by SSCP (DOVAM-S; Feng et al. 2002). In all these procedures, every exon is examined so that precise rearrangements around genomic deletions of one or more exons can be identified. The identification of point mutations, microinsertions/deletions, nonsense mutations, and splice motif defects will require more effort, but many of these cases could be more amenable to induced exon skipping because the dystrophin gene is essentially intact. A recent report (Dent et al. 2005) indicated that 7% of a large cohort of unselected dystrophinopathy patients had mutations outside the protein coding regions.

4.2 Trial Design

The first trials involving the administration of antisense oligonucleotides to DMD patients will be designed to establish safety profiles and confirm proof of principle. The ages of participants will vary according to country and will depend upon recommendations by local ethics and safety committees. Ethical and safety concerns are likely to preclude the involvement of

very young patients in the early trials, even though these individuals should be most responsive, since extensive muscle degeneration would not have occurred. Instead, older and in most cases non-ambulant patients will be recruited, even though the muscle degeneration, scarring, and fibrosis will be more established.

Several factors must be taken into account for these first trials. Would it be preferable to target one particular region of the dystrophin gene commonly involved in DMD mutations, so that the antisense oligonucleotide could be applicable to many patients, or target another region more amenable to specific exon removal? Those exons to be targeted to restore the reading frame in the majority of patients may not necessarily be the same exons that can be most efficiently removed and conclusively demonstrate proof of principle. Should the first DMD mutations to be addressed be 'absolute', where there is no dystrophin produced, or 'leaky', where endogenous, natural exon skipping has allowed a low level of dystrophin expression? In this latter case, there should be no immune stimulation if the induced dystrophin is not novel to that individual. It would be essential that there is an unambiguous increase in dystrophin expression as a consequence of the antisense oligonucleotide treatment. One justification to commence trials with an 'absolute' mutation is the assurance that any dystrophin detected would be attributable to the antisense oligonucleotide. If revertant fibres could not be detected in a trial participant, it is possible that any induced dystrophin would be recognised as foreign and elicit an immune response. In some respects, it may be safer to commence trials with intermediate/severe BMD cases to ensure that there are no immunological consequences.

There are no right or wrong choices here. Many factors must be taken into account to separate the perceived risks from the potential benefits. While there is some discussion over the most appropriate exons to target in these early trials, there is also as much debate over the structure and nature of the trials.

No benefit would be conferred to the participants in these initial trials involving intramuscular injections. It is generally recognised that individual treatment of muscles by intramuscular injection is not a viable option, and systemic delivery will be essential for the antisense oligonucleotides to provide a clinical benefit. While direct intramuscular administration should provide some information on safety with respect to limited exposure and proof of concept, it cannot address fundamental issues like dosage, frequency of administration, levels of dystrophin expression, persistence, and any physiological changes.

The chemistry of the antisense oligonucleotides to be used is still being considered. Most work has been done with 2′-O-methyl phosphorothioate antisense oligonucleotides, but this particular chemistry has not been tested in humans. Considerable toxicology and safety profile data for these compounds will be required. On the other hand, the morpholino and 2′-O-(2-methoxy) ethyl oligonucleotides have already been used in several clinical trials.

It must be noted that the participants in antisense oligonucleotide clinical trials are generally adults receiving substantial doses over a relatively short period. This will not be applicable to DMD, as young patients may have to receive a particular dose for the course of their lives, unless some other treatment becomes available. The different antisense oligonucleotide chemistries offer some choices, but it remains to be established if an antisense oligonucleotide that exerts prolonged and sustained induced exon skipping would be the best compound to address a chronic condition. With increased stability and persistence comes the potential risk of long term adverse effects, or inadvertent cross-reaction with other gene transcripts. An antisense oligonucleotide with a shorter half-life of days or weeks may prove to be a better choice.

Any new therapeutic compound to be administered into humans requires rigorous and extensive safety and toxicology profiles to be undertaken. This will be particularly necessary for the first series of antisense oligonucleotides to address dystrophin mutations. If these antisense compounds prove efficacious, and there are no obvious deleterious effects, subsequent regulatory requirements for the release of new antisense oligonucleotides would need to be discussed. Although the most common mutations may be addressed by a few antisense oligonucleotides, this therapy may eventually require 50 or 60 different compounds for extensive coverage of the dystrophin gene.

New compounds must first address the risk:benefit equation before they can be considered. In DMD, there is a clear and relentless progression of muscle wasting, which has been well established, and for which there is no cure. Steroids have been recognised as being of some benefit, but even here, there are major side effects including obesity, cataracts, mood swings, and osteoporosis. Nevertheless, the risk of the side effects is out-weighed by the benefits and steroids continue to be prescribed.

5
The Future

One of the limitations of antisense oligonucleotide therapies is that the compounds must be re-administered as required. The frequency of application will be dependent on several factors, including efficiency of delivery, uptake and persistence of the antisense oligonucleotide, as well as the functionality of the induced dystrophin. The quality of the induced dystrophin would be expected to influence the rate of myotube turnover.

Approaches undertaken to address sustained exon skipping include the use of plasmid (Brun et al. 2003) or adeno-associated virus to introduce antisense sequences into myotubes (Goyenvalle et al. 2004). Goyenvalle et al. (2004) described substantial exon skipping in the *mdx* mouse with

widespread and near normal restoration of dystrophin synthesis. This work raises important questions that must be addressed. Viral-based dystrophin gene replacement relies on one construct replacing the defective gene, regardless of the type of gene lesion. Vector-based exon skipping would require many different constructs to address the numerous dystrophin mutations. Gaining regulatory approval for multiple viral constructs is likely to be a more onerous task than the same process for an equivalent number of antisense oligonucleotides.

As many different compounds will be required to address all cases of DMD, the first few targets chosen must be considered carefully. If this bypass surgery is shown to be safe and has clearly defined benefits and low risk to the patients, antisense oligonucleotides to other regions of the dystrophin gene should become available as quickly as possible.

The implementation of new antisense oligonucleotides is not technically difficult. Our laboratory and others have been optimising antisense oligonucleotide design to human motifs for some time and the majority of dystrophin exons have been targeted. Production of these compounds to clinical grade is an expensive procedure but well within current capabilities. The major challenge will be in addressing the safety and regulatory issues that would be associated with gaining approval to use many new drugs for one disorder.

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Altered Splicing in Prelamin A-Associated Premature Aging Phenotypes

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Abstract. Hutchinson-Gilford progeria (HGPS), a rare and severe developmental disorder characterized by features recalling premature aging, and restrictive dermopathy (RD), a neonatal lethal genodermatosis, have recently been identified as being primary or secondary "laminopathies." These are heterogeneous disorders due to altered function of lamins A/C or related proteins. In physiological conditions, mature lamin A is obtained through a series of post-translational processing steps performed on a protein precursor, prelamin A. The major pathophysiological mechanism involved in progeria is an aberrant splicing of pre-mRNAs issued from the *LMNA* gene, due to a de novo heterozygous point mutation, leading to the production and accumulation of truncated lamin A precursors. Aberrant splicing of prelamin A pre-mRNAs causing the production of more extensively truncated precursors is involved in the allelic disease restrictive dermopathy. Other restrictive dermopathy cases are due to the inactivation of a key enzyme involved in the maturation of lamin A precursors (ZMPSTE24). In functional terms, all these conditions share the same pathophysiological basis: intranuclear accumulation of lamin A precursors, which cannot be fully processed (due to primary or secondary events) and exert toxic, dominant negative effects on nuclear homeostasis. Most other laminopathies are due to autosomal dominant *LMNA* point mutations inferred to cause single amino acid substitutions. In any case, the impact of these mutations on pre-mRNA splicing has rarely been assessed. These disorders affect different tissues and organs, mainly including bone, skin, striated muscles, adipose tissue, vessels, and peripheral nerves in isolated or combined fashions, giving rise to syndromes whose severity ranges from mild to perinatally lethal. In this chapter we review the structure and functions of lamins A/C in physiological and pathological conditions, describe their known or putative roles, namely, in the pathogenesis of HGPS and RD in relation to existing animal models, and envisage possible targeted therapeutic strategies on the basis of recent research results.

1
Altered Lamin A/C Function and Disease: the "laminopathies"

The term "laminopathies" refers to a group of very heterogeneous dis-orders, from both a genetic and a clinical point of view. All these disorders share a primary or secondary altered function of lamins A/C, ubiquitous nuclear proteins with multiple fundamental functions, and their molecular partners.

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Alternative Splicing and Disease

In a general way, laminopathies cause an unsatisfactory quality of life, demand expensive medical care and, in many cases (namely, laminopathies affecting striated muscles and laminopathies characterized by premature and accelerated aging), lead to premature death.

This group of disorders is characterized by a very large phenotypic spectrum, which has continued to evolve since their first implication in the pathogenesis of Emery-Dreifuss muscular dystrophy, in 1999, to the more recent implication in progeria and restrictive dermopathy.

In this first part of the chapter, we will present the *LMNA* gene, lamins A/C, the proteins it encodes, together with the cellular context to which these proteins belong, and their known or putative roles. We will subsequently review the laminopathies discovered to date.

In the second part of the chapter we will more deeply describe the premature aging syndromes due to lamin A altered splicing or post-translational processing mechanisms.

In the third and last part we will describe the existing animal models of laminopathies, characterized by premature aging, together with the phenotype-correction approaches that have been proven useful in mouse and cellular models, in particular by correcting the most common splicing defect (Scaffidi and Misteli 2005), which will hopefully find a clinical application in humans in the near future.

1.1 *LMNA*-Encoding Lamins A/C, Fundamental Nuclear "Bricks"

The *LMNA* gene is localized at chromosome 1q21.2-q21.3 and is composed of 12 exons, spanning a genomic segment of ≈25.4 kb and including a coding sequence of 1992 base pairs. Through an alternative splicing mechanism, *LMNA* encodes lamins A/C (or A-type lamins): lamins A, AΔ10, C, and C2 of which two, lamins A and C, are major isoforms expressed in all vertebrates' differentiated cells (Rober et al. 1989; for review see Gruenbaum et al. 2005). Lamin AΔ10 is expressed in some tumors and physiologically in specific human tissues (Machiels et al. 1996), while lamin C2 has only been identified in mouse male germ line cells (Furukawa et al. 1994).

Mature lamin A is obtained from a precursor (prelamin A) through a series of post-translational processing steps, which will be described further on.

The alternative splice site used in pre-mRNAs for the alternative production of transcripts encoding lamins A and C is located inside exon 10: the message encoding lamin A comprises the first 90 base pairs (bp) of exon 10, followed by exons 11 and 12, while the message encoding only lamin C comprises the whole exon 10. Consequently, the last 21 bp of exon 10 are lamin C specific while exons 11 and 12 are lamin A specific. These aspects have to be underscored, since they have important pathophysiological consequences, to be discussed further on.

At the protein level, prelamin A (664 residues) and lamin C (572 residues) share the first 566 amino acids while they differ at their C-terminal tail, where they possess, respectively, 98 and 6 unique residues. The AΔ10 isoform does not contain the 37 amino acids encoded by exon 10. In the C2 isoform the 86 N-terminal amino acids are replaced by 6 unique amino acids.

The post-translational processing steps that lead to the production of mature lamin A (646 residues) take place at prelamin A's C-terminal tail. Indeed, prelamin A's four most C-terminal amino acids form a "CaaX" isoprenylation signal (CaaX = cysteine, aliphatic, aliphatic, any amino acid) on which the following sequential modifications are performed (Holtz et al. 1989; Sinensky et al. 1994; Fig. 1):

1. Farnesylation (Lutz et al. 1992) of the cysteine of the CaaX motif by a farnesyl transferase (Sinensky et al. 1994)
2. Cleavage of the last 3 amino acids (aaX) by a zinc metalloprotease called ZMPSTE24 (or FACE-1; Freije et al. 1999; Pendas et al. 2002)
3. Methylation of the farnesylated C-terminal cysteine, operated by the isoprenylcysteine carboxy-methyl transferase (ICMT; Bergo et al. 2002)
4. Proteolytic cleavage of the 15 most C-Terminal amino acids, operated again by the metalloprotease ZMPSTE24 (Corrigan et al. 2005)

B-type lamins include two major isoforms, lamins B1 and B2, ubiquitously expressed at all developmental stages (Moir et al. 2000a), and a minor one, lamin B3, expressed in mouse male germ cells (Furukawa et al. 1993).

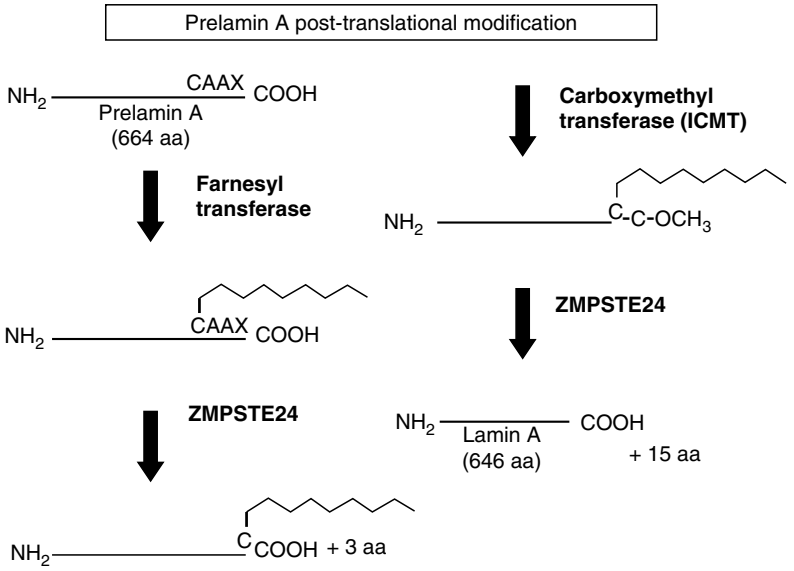

Fig. 1. Schema of the four prelamin A post-translational maturation steps through which lamin A is produced

lamin B1 is encoded by the *LMNB1* gene on chromosome 5q23.2–31.1 and lamins B2 and B3 are expressed through alternative splicing of the *LMNB2* gene on chromosome 19p13.3.

B-type lamins must be isoprenylated and carboxymethylated in order to be localized at the nuclear envelope (Kitten and Nigg 1991); lamin B1 is further cleaved by the metalloprotease Rce1 (Maske et al. 2003).

A and B type lamins differ in their expression patterns, their biochemical properties (Gerace and Blobel 1980), functions, and localizations during the cell cycle (for review, see Hutchison 2002).

Lamins constitute the group V of the intermediate filament (IF) family of proteins (Aebi et al. 1986). The unique viscoelastic properties of IFs make them more resistant than microtubules or microfilaments to physical strain. Although having these structuring functions, IFs are highly dynamic molecules (for review, see Chou et al. 2001).

Like the other IF proteins, they contain a central α coiled-coil (rod) domain, and two variable globular domains at their N- and C-terminal extremities.

Lamin A/C structure specifically contains, in comparison to other IF proteins:

– Six additional heptamers in the rod domain (which is thus composed of 352 instead of 310 amino acids)
– A nuclear localization signal (NLS, amino acids 417–422) and an Ig-like domain in the C-terminal region (amino acids 430–545, Krimm et al. 2002, Dhe-Paganon et al. 2002)
– A C-terminal CaaX motif (in lamin A precursors)

Lamins A and C respectively have molecular weights of 72 and 65 kDa, while the precursor prelamin A weighs about 74 kDa.

Together with B-type lamins, A-type lamins in all differentiated cells constitute the nuclear *lamina*, a fibrous meshwork underlying the nucleoplasmic face of the nuclear envelope. This last term refers to a physical and functional complex including outer and inner nuclear membranes (ONM, INM), the perinuclear space, and nuclear pore complexes (NPCs) that allow the bidirectional passage of ions and molecules from and to the nucleoplasm (for review, see Mounkes et al. 2003). The nuclear lamina is physically connected with the INMs through several integral INM proteins (more than 60 have been identified to date; Zastrow et al. 2004; Schirmer et al. 2005), interacting simultaneously with INMs, lamins and, in most cases, peripheral chromatin.

More than two thirds of the total nuclear lamins A/C are localized in the nucleoplasm, where they form structures of yet unidentified type, potentially playing a role in the control of several fundamental nuclear functions including DNA replication and repair, RNA transcription and splicing (Bridger et al. 1993; Hozak et al. 1995; Moir et al. 2000b).

The nuclear envelope and lamina thus not only allow a physical separation between nuclear and cytoplasmic phenomena, but are also involved in

the general regulation of gene expression profiles through the interaction of A-type lamins with a great number of partners involved in different signaling pathways and nuclear functions (Zastrow et al. 2004).

1.1.1 Involvement in Nuclear Structure, Morphology, and Resistance to Stress

Many studies have shown the role of the nuclear *lamina* in the determination of nuclear shapes. For example,

1. The expression in somatic cells of mouse male germ cell specific lamin B3, confers to these cells a hooked form, similar to that of spermatocytes, physiologically expressing lamin B3 (Furukawa et al. 1993)
2. The expression of dominant negative lamin B1 forms (B1Δ rod) in cells causes the massive deformation of nuclear envelopes (Schirmer et al. 2001)
3. Altered nuclear morphologies have been observed in knock-out *Lmna* mice (Sullivan et al. 1999) as well as in several disorders due to *LMNA* mutations (Vigouroux et al. 2001; Novelli et al. 2002; De Sandre-Giovannoli et al. 2003; Navarro et al. 2004)

Lamins control nuclear size also: indeed, *Xenopus* nuclear extracts depleted of lamins assemble in nuclei that have a much reduced size. (Meier et al. 1991).

1.1.2 Regulation of Gene Expression

The direct interaction of lamins A/C with chromatin as well as with several molecular partners as transcription factors, with transcriptional and splicing machineries, with the molecular apparatus responsible of replicating and repairing DNA, and with structural proteins of the nuclear matrix (Shumaker et al. 2003; Barboro et al. 2003), makes it not astonishing that a role is being demonstrated for them in an increasing number of functions related to the control of gene expression (for review, see Mounkes et al. 2003 and Shumaker et al. 2003).

Indeed, it is clear today that the gene expression patterns which are established and maintained during cell differentiation do not result only from the action of stage-specific transcription factors but also from the establishment of different chromatin structural formations. We know that chromatin can assume "open" (euchromatin) or "closed" (heterochromatin) formations that are, respectively, permissive or refractory to transcription (for review, see Gasser 2001) and that are transmitted to daughter cells (for review, see Pirrotta 1998).

Cytogenetic studies have shown that a large proportion of condensed heterochromatin, including telomeres and centromeres, is localized in proximity

to the nuclear envelope (for review, see Qumsiyeh 1999). Lamins as well as many INM integral proteins directly interact with chromatin (Stierlé et al. 2003, Ye and Worman 1996), being potentially able to affect its structure at the nuclear periphery. Moreover, lamins and some of their molecular partners can specifically bind the genomic sequences called MARS (matrix attachment regions) and SARS (scaffold attachment regions; Luderus et al. 1994; Zhao et al. 1996), very likely contributing to the higher-order organization and partitioning of chromatin into functional loops in the nuclear matrices. Several pieces of evidence suggest that nucleoplasmic lamins form an internal skeleton that could also support different nuclear activities, such as the replication and repair of DNA and the splicing of pre-mRNAs, through interactions with molecular partners such as nuclear actin, which could have motor functions in this cellular compartment as well (Sasseville and Langelier 1998; Shumaker et al. 2003; Amankwah and De Boni 1994), and with NuMa, the nuclear mitotic apparatus protein (Barboro et al. 2002, Barboro et al. 2003).

Besides these aspects, lamins play a role in gene expression regulation through interactions with different factors involved in transcription or signal transduction. That is for example, interactions with:

- Oct-1, which represses the collagenase gene when it colocalizes with B-type lamins at the nuclear lamina (Imai et al. 1997)
- p110Rb, a transcriptional repressor, whose active form colocalizes with lamins A/C at the nuclear periphery in vivo and interacts with them in vitro (Mancini et al. 1994)
- LAP2α, a nucleoplasmic interactor of A type lamins, which is necessary to retain Rb in the nuclear interior, within a complex with lamins A/C, (Markiewicz et al. 2002)
- hsMOK2, a transcription factor playing a role in neuronal development (Dreuillet et al. 2002)
- SREBP1, an adipocyte-specific differentiation factor (Lloyd et al. 2002), whose interaction with lamin A seems to be altered in lipodystrophic patients carrying specific lamin A mutations

1.2 The "Laminopathies"

Since 1999, when the involvement of the *LMNA* gene in the pathogenesis of autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD) was identified (Bonne et al. 1999), at least 14 other allelic or related diseases have been discovered to be due to defects in *LMNA* or functionally related genes. These disorders are referred to today, from a pathophysiological and molecular point of view, as "laminopathies" or "envelopathies" (Table 1).

Laminopathies are allelic disorders showing a very heterogeneous phenotypic spectrum, affecting different tissues in separate or combined

Table 1. Laminopathies identified to date, in alphabetical order

Disease short name	Disease name	Involved genes	OMIM #	References
AWS	Atypical Werner Syndrome	*LMNA*	150330	Chen (2003)
BOS	Osteopoikilosis, Buschke-Ollendorf syndrome, Melorheostosis	*MAN1*/ *LEMD3*	166700 155950	Hellemans (2004)
CMT2	Axonal autosomal recessive Charcot-Marie-Tooth disease (CMT2B1) and axonal autosomal dominant CMT	*LMNA*	605588	De Sandre-Giovannoli (2002); Goizet (2004)
CMD1A	Cardiomyopathy, dilated 1A	*LMNA*	115200	Fatkin (1999)
EDMD1	X linked Emery-Dreifuss dystrophy type 1	*STA* (*emerin*)	310300	Bione (1994)
EDMD2/3	Emery-Dreifuss muscular dystrophy types 2 (autosomal dominant) and 3 (autosomal recessive)	*LMNA*	181350	Bonne (1999)
FPLD	Dunnigan-type familial partial lipodystrophy	*LMNA*	151600	Cao and Hegele (2000); Shackleton (2000)
HEM/ Greenberg dysplasia	Hydrops-ectopic calcification moth-eaten skeletal dysplasia	*LBR*	215140	Waterham (2003)

(*Continued*)

Table 1. Laminopathies identified to date, in alphabetical order—(*Cont'd*)

Disease short name	Disease name	Involved genes	OMIM #	References
HPGS	Hutchinson-Gilford progeria syndrome	*LMNA*	176670	De Sandre-Giovannoli (2003); Eriksson (2003); Cao and Hegele (2003); Plasilova (2004)
LDHCP	Lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy, leukomelanodermic papules	*LMNA*	608056	Caux (2003)
LGMD1B	Limb-girdle muscular dystrophy type 1B	*LMNA*	159001	Muchir (2000)
MADA	Mandibuloacral dysplasia type A	*LMNA*	248370	Novelli (2002)
MADB	Mandibuloacral dysplasia type B	*ZMPSTE* 24	608612	Agarwal (2003)
PHA	Pelger Huët anomaly	*LBR*	169400	Hoffmann (2002)
RD	Restrictive dermopathy	*LMNA*	275210	Navarro (2004)
RD	Restrictive dermopathy	*ZMPSTE* 24	275210	Navarro (2004); Navarro (2005)
	Seip Syndrome	*LMNA*		Csoka (2004)

fashions, with variable severity, from mild to lethal in the perinatal period. Different laminopathies present with overlapping features.

"Tissue-specific" laminopathies due to *LMNA* mutations include:

1. Those affecting skeletal and cardiac muscles:
   – Autosomal dominant and recessive Emery-Dreifuss muscular dystrophy (respectively EDMD2 and EDMD3; Bonne et al. 1999; Raffaele di

Barletta et al. 2000); while X-linked EDMD is due to mutations in *STA*, encoding emerin, an INM integral protein directly interacting with lamins A/C (Bione et al. 1994; Clements et al. 2000; Sakaki et al. 2001)
   - Limb-girdle muscular dystrophy type 1B with cardiac conduction defects (LGMD1B; Muchir et al. 2000)
   - Autosomal dominant dilated cardiomyopathy with conduction defects type 1A (DCM 1A; Fatkin et al. 1999)
2. Those affecting adipose tissue:
   - Familial partial lipodystrophy, Dunnigan type (FPLD; Shackleton et al. 2000)
   - Seip syndrome (Csoka et al. 2004)
3. Those affecting peripheral axons:
   - Autosomal recessive axonal Charcot-Marie-Tooth disease type 2B1 (CMT2B1; De Sandre-Giovannoli et al. 2002; Chaouch et al. 2003) and autosomal dominant axonal Charcot-Marie-Tooth disease (Goizet et al. 2004)

Systemic laminopathies affecting several tissues (skeletal, cardiac, cutaneous, adipose) include:

- Mandibuloacral dysplasia types A and B (MADA), respectively due to *LMNA* and *ZMPSTE24* mutations, and clinically characterized by: hypoplastic clavicles and mandible, acro-osteolyses, retarded closure of fontanels, joint contractures, lipodystrophy, insulin-resistance, alopecia, hepatomegaly, and alteration of cutaneous pigmentation (acanthosis nigricans; Novelli et al. 2002; Agarwal et al. 2003)
- A syndrome combining lipodystrophy, insulin-resistant diabetes, leucomelanodermic papules, liver steatosis, cardiomyopathy (LDHCP; Caux et al. 2003)

Syndromes characterized by features typical of premature aging include:

- Hutchinson-Gilford progeria syndrome (HGPS; De Sandre-Giovannoli et al. 2003)
- Restrictive dermopathy (Navarro et al. 2004; Navarro et al. 2005)
- Overlapping phenotypes such as atypical Werner syndrome, with phenotypic criteria which might not completely satisfy the diagnosis of Werner syndrome (Chen et al. 2003)

Recently, loss-of-function mutations of *MAN1*/*LEMD3,* encoding another INM integral protein have been identified in patients carrying allelic disorders characterized by abnormal ossification: osteopoikilosis, Buschke-Ollendorf syndrome (BOS) and melorheostosis (Hellemans et al. 2004).

Laminopathies are thus a model of clinical heterogeneity, their clinical spectrum being extremely large, both in the wide range of tissues involved, and in the severity of the phenotype.

For some laminopathies (mainly affecting nerve, skeletal and cardiac muscles), a high intra-familial clinical variability has been observed as well

(Vytopil et al. 2002; Mercuri et al. 2004; Tazir et al. 2004; Mercuri et al. 2005; Benedetti et al. 2005).

Several pathophysiological mechanisms involved in this heterogeneous group of diseases have been postulated or proved:

1. In striated muscles, nuclei bearing altered or absent lamin A/C isoforms are more prone to structural damage secondary to repeated mechanical strain (Broers et al. 2004; Lammerding et al. 2004). There are several lines of evidence proving a direct connection between nuclear proteins and the actin cytoskeleton; defects in this network could result in impaired tissue integrity (Starr and Han 2003). The cytoplasmic intermediate filament desmin network has been shown to be progressively disrupted in *Lmna* null cardiomyocytes, with harmful consequences on muscle function (Nikolova et al. 2004)
   Lammerding and colleagues have also shown that NF-kappa B-regulated gene transcription in response to mechanical stimulation was reduced in *Lmna*–/– fibroblasts, leading to impaired viability of mechanically strained cells (Lammerding et al. 2004)
2. Certain mutant lamins might lose their ability to correctly interact with tissue-specific molecular partners, giving rise to tissue-specific laminopathies. This mechanism has been proposed for the adipose-tissue specific transcription factor SREBP1 in patients affected with Dunnigan type familial partial lipodystrophy carrying specific *LMNA* mutations (Lloyd et al. 2002)
   The tumor suppressor protein p110Rb binds the transcription factor E2F and represses transcription through the recruitment of histone deacetylases (Kaelin 1999). Hypophosphorylated Rb colocalizes and interacts with lamins A/C at the nuclear periphery, in particular in foci proximal to the nucleolus (Mancini et al. 1994; Kennedy et al. 2000): this subnuclear localization has proven to be dependent on lamin A/C expression (Johnson et al. 2004). Rb has been shown to play a major role in the maintenance of muscle and fat cell differentiation through studies on embryos and cells (Wu et al. 2003; Classon et al. 2000; Fajas et al. 2002). Furthermore, Favreau and colleagues have shown that myoblast-to-myotube differentiation is impaired in C2C12 clones overexpressing lamin A R453 W, one of the most frequent mutations observed in EDMD. The transfected cells showed low levels of the transcription factor myogenin and persistence of a large pool of hyperphosphorylated, inactive Rb (Favreau et al. 2004)
   Another tissue-specific interaction has been described between MOK2, a nerve-specific transcription factor, and lamins A/C (Dreuillet et al. 2002)
3. Other mutant lamins might lose their ability to interact with ubiquitous molecular partners (e.g., transcription factors) or to support/direct specific nuclear events, giving rise to systemic laminopathies. Indeed lamins A/C have been shown to interact directly with core histones

(Taniura et al. 1995) and chromatin (Stierlé et al. 2003), and might participate to the organization of subnuclear structural patterns necessary to regulate tissue-specific gene expression programs (Kosak and Groudine 2004). Notably, inactivated X chromosomes, developmentally regulated loci and gene-poor chromosomes have been shown to be localized at the nuclear periphery

Lamins A/C have been shown to participate in DNA replication events (Spann et al. 1997; Moir et al. 2000b) as well as to RNA transcription and splicing (Kumaran et al. 2002; Spann et al. 2002)

In the context of progeroid laminopathies, Rb might play a role as well, since, through its interaction with the tumor suppressor p53, this protein has a crucial importance in human cellular senescence (Hara et al. 1991, Shay et al. 1991). Furthermore, mice carrying "gain-of-function" mutated p53 alleles show overt progeroid features (Tyner et al. 2002)

4. As will be further discussed, it has been proved that specific laminopathies leading to premature and accelerated aging (i.e., Hutchinson-Gilford progeria syndrome and restrictive dermopathy) are due to aberrant accumulation of truncated or wild-type prelamin A precursors, exerting dominant negative toxic effects on cells (Goldman et al. 2004; Fong et al. 2004; Scaffidi and Misteli 2005)

2
Altered Lamin A Splicing and Progeroid Syndromes

The best known segmental progeroid syndromes are due to defects of nuclear DNA repair mechanisms. These include: Bloom syndrome, ataxia–telangiectasia (Louis-Bar syndrome), Fanconi's anemia, xeroderma pigmentosa, Cockayne's syndrome, trichothiodystrophy, Rothmund-Thomson syndrome, and other related disorders (for review see Woods 1998; Hasty et al. 2003).

In 2003 we identified the involvement of the *LMNA* gene in the pathogenesis of Hutchinson-Gilford progeria syndrome, one of the most emblematic segmental aging disorders (De Sandre-Giovannoli et al. 2003). These results were independently confirmed by Eriksson and colleagues (Eriksson et al. 2003).

If and how progeroid syndromes linked to A-type lamins have a functional correlation with progeroid disorders associated with DNA repair alterations remains to be established. Some hypothetical links can be identified, one of them relates to the interaction of lamins A/C with actin (Sasseville et al. 1998), emerin, also interacting directly with nuclear actin (Holaska et al. 2004), and nuclear spectrin molecules. Indeed, emerin increases the actin polymerization rate and is proposed to contribute to the formation and stabilization of an actin cortical network at the inner nuclear membrane (Holaska et al. 2004).

Spectrin heterodimers bind short actin filaments at the erythrocyte membrane and αII spectrin molecules have been identified in cell nuclei (McMahon et al. 2001). Furthermore, nuclear αII spectrin has been shown to interact with FANCA and XPF, factors involved in repair of DNA interstrand cross-links (Sridharan et al. 2003). It could be imagined that, through the aforementioned interactions, lamins A/C are involved as well in the DNA interstrand cross-links repair processes.

Another link between A-type lamins and DNA repair mechanisms is Rb, a critical mediator of DNA damage checkpoints that interacts directly with A-type lamins (Ozaki et al. 1994; Markiewicz et al. 2002; Johnson et al. 2004).

Expression of mutated lamin A lacking part of its N-terminal domain exerts dominant negative effects on nuclear assembly of Xenopus nuclear extracts, including the delocalization of replication factor complexes (RFCs) and proliferating cell nuclear antigen (PCNA), and is involved as well in DNA replication and repair (Spann et al. 1997; Mortusewicz et al. 2005).

2.1 Hutchinson-Gilford Progeria Syndrome

The Progeria syndrome, first described at the end of the 19th century by Jonathan Hutchinson (Hutchinson 1886) and later by Hastings Gilford (Gilford 1904), is a very rare and severe developmental disorder (its prevalence is about 1 in 4 to 8 million) characterized by the precocious appearance of pathologies which are typical of advanced age. The median age at diagnosis is 2 years. The clinical phenotype is characterized by an overall severe growth retardation (median final height = 100–110 cm; median final weight = 10–15 kg), usually associated to the following features:

- Skeletal alterations include: macrocephaly with craniofacial disproportion (frontal bossing, ectropion, small, low-set ears, micrognathism, long beaked nose, persistent fontanels, hypoplastic clavicles, generalized osteopenia/osteoporosis with repeated pathologic fractures (Khalifa 1989), acro-osteolysis of distal phalanges or clavicles, progressive joint stiffness, coxa valga with "horseback-riding" stance
- Generalized marked muscular dystrophy and atrophy is found, with pain of muscular origin
- Cutaneous changes include: generalized lipodystrophy with a thin, atrophic, dry and inelastic skin, presenting with sclerodermatous focal lesions (Jansen and Romiti 2000) and hyperpigmented zones. Cutaneous appendices become atrophic, giving rise to alopecia and absence of eyebrows. The venous superficial network is prominent, mainly on skull and thorax
- From a cardiovascular point of view, the patients present with precocious and extremely severe atherosclerosis, often cardiomyopathy, and

death occurs at a median age of 13.5 years, in most cases due to myocardial infarction. The longest lifespan reported is 45 years. Premature subintimal fibrosis of great arteries has been reported (Baker et al. 1981; Ackerman et al. 2002)
- A variety of immunological abnormalities have been reported as well (Harjacek et al. 1990)
- Some cases presenting with very severe skeletal pathology have been reported (de Paula Rodrigues et al. 2002)
- The cognitive functions of the affected children are completely conserved

Not all of the aging processes are advanced in affected children: notably there's often a delayed dentition, cancer incidence is not augmented, dementia, cataract, or deafness are usually not observed.

The observed sex ratio of progeria is: M:F=1.2:1. No ethnic-specific recurrence has been reported in these patients.

Most of the typical Hutchinson-Gilford progeria cases are due to a recurrent, de novo point mutation in *LMNA* exon 11: c.1824C>T. This mutation is localized in the part of the gene specifically encoding lamin A. Its predicted effect is a silent amino acid change at codon 608 (p.G608G). In fact, this sequence variation is not silent, in that it occurs in a probable exon splicing enhancer (ESE, for review, see Cartegni et al. 2002), and is predicted to cause a reduced affinity for the ASF/SF2 splicing factor. In consequence, a cryptic splice site is activated in transcripts issued from the mutated allele, which is located 5′ to the variation, between nucleotides 1818 and 1819 (Fig. 2a). Consequently, smaller, aberrant transcripts lacking the last 150 base pairs of exon 11 are produced, together with the wild type transcripts from the mutated allele. This phenomenon is described in the following way, according to standard international nomenclature: [r.1824C>T+r.1819_1968del] (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). A quantification of the relative amounts of transcripts and proteins issued from mutated and wild type alleles was estimated by Reddel and colleagues. They showed that abnormally spliced (progerin) transcripts constituted 84.5% of the total mRNA derived from the mutant allele and 40% of all lamin A transcripts obtained from both alleles (Reddel and Weiss 2004).

In the deleted transcript, the reading frame is conserved, causing the appearance of a truncated Prelamin A precursor, also called progerin or laminΔ50, lacking amino acids 607 to 656 (p.V607_Q656del; De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003).

This protein lacks the second cleavage site recognized by ZMPSTE24 during prelamin A post-translational processing, so that this truncated prelamin A precursor cannot undergo complete maturation. While it can undergo the first three processing steps, i.e., farnesylation, cleavage, and methylation, it cannot undergo the second cleavage, at the fourth processing step, likely maintaining farnesyl and methyl moieties on its C-terminal cysteine residue.

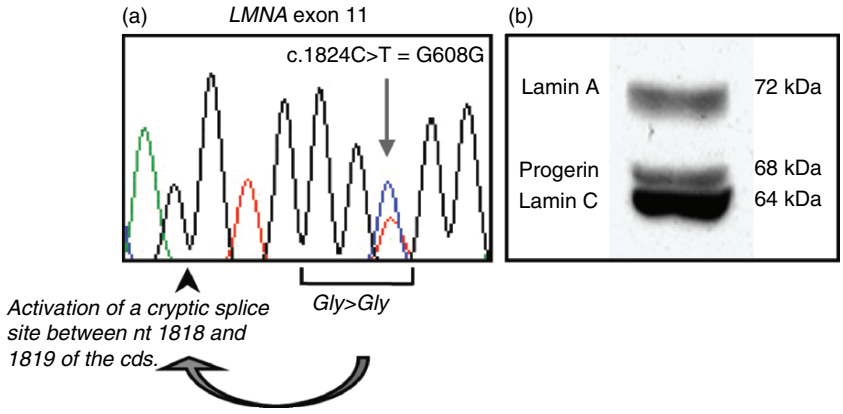

Fig. 2. **a**: Elecropherogram of *LMNA* exon 11 in a patient affected with Hutchinson-Gilford progeria, showing the heterozygous c.1824C>T transversion (p.608G). The schema shows the consequence of the mutation, consisting in the activation of a splice site located between coding nucleotides 1818 and 1819 in transcripts issued from the mutated allele. **b**: Western blot analysis with anti-human lamin A/C antibodies on fibroblast cultures issued from a patient affected with HGPS and carrying the heterozygous c.1824C>T transversion (p.G608G). Progerin, weighing about 68 kDa, migrates in proximity to lamin C

Progerin can be visualized in western blot studies from a patient's cultured cells (Fig. 2b) with antibodies directed against the N-terminal part of lamins A/C, as a 68 kDa protein migrating between lamins A (72 kDa) and C (64 kD).

It is of major importance to underscore here that the pathomechanism involved in progeria is thus not limited to an abnormal splicing event, which per se maintains in this case the reading frame and could potentially lead to the production of a partially functional protein product, but it is intimately linked to aberrant functional interactions of the truncated protein produced. Indeed, the deletion resulting from the aberrant splicing process prevents prelamin A precursors from interacting with one of its major post-translational processing enzymes, ZMPSTE24. Moreover, the truncated prelamin A produced from the deleted in-frame transcripts is not recognized by the cell as an aberrant product, so that its ubiquitination and proteasomal degradation are somehow prevented. It remains to be established if and how the probable farnesylated status of the protein is involved in the prevention of its degradation.

As we will discuss further on, it has subsequently been shown that:

1. The intranuclear, ubiquitous accumulation of this incompletely processed precursor exerts toxic, dominant negative effects on wild-type residual proteins (Goldman et al. 2004; Scaffidi and Misteli 2005; Fong et al. 2004)

2. These toxic effects are entirely reversible, under many measurable aspects, when the intranuclear amounts of precursors are reduced through different approaches (Scaffidi and Misteli 2005; Fong et al. 2004)

Indeed, the nuclei of HGPS patients' cultured cells show important alterations of morphology and composition, which increase with the number of passages, in correlation with an apparent increase of nuclear amounts of progerin (Bridger and Kill 2004; Goldman et al. 2004). Supporting the hypothesis of progerin's dominant negative effect, the same nuclear alterations can be induced by transfection of cDNAs encoding Progerin in wild-type cells (Goldman et al. 2004, Scaffidi and Misteli 2005).

Indirect immunofluorescence experiments with antibodies directed against lamins A/C or some of their molecular partners allow to observe nuclear blebs and herniations of the nuclear envelope, thickening of the nuclear lamina, loss of peripheral heterochromatin, and clustering of nuclear pores (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003; Goldman et al. 2004). Some of these nuclear alterations, in different extents and percentages of cells, are observed as well in other laminopathies such as FPLD, EDMD, and MADA (Vigouroux et al. 2001; Favreau et al. 2003; Novelli et al. 2002).

From a clinical point of view, in our experience, it should be underlined that: while on one hand in children affected with highly typical Hutchinson-Gilford progeria, the *LMNA* c.1824C>T mutation (p.G608G) is very probably identified by molecular screening, on the other hand the presence of this mutation should not be excluded in patients presenting with neonatal progeroid features.

"Neonatal progeria" cases had been described before a molecular diagnosis was made available and were controversial. It was indeed discussed to which nosologic groups these patients should belong: whether they represented neonatal HGPS cases, Wiedemann-Rautenstrauch syndrome cases, or neither (Rodriguez et al. 1999a; Faivre et al. 1999; Rodriguez et al. 1999b). Recently, two papers have shown that children carrying – at least – the *LMNA* p.G608G mutation can present with very severe neonatal progeroid phenotypes (Navarro et al. 2004; Sevenants et al. 2005)

These observations obviously raise the question of the very likely influence of yet unidentified modifier genes or possibly epigenetic factors increasing the clinical severity. Interpersonal variations of the efficacy of splicing (i.e., mutated transcripts' aberrant or correct splicing) and, consequently, of the rate of production and global amounts of progerin produced, might also be one of the factors modulating the severity *LMNA* p.G608G phenotypes, ranging from typical HGPS, to neonatal progeria, to restrictive dermopathy.

In most cases the recurrent point c.1824C>T mutation is thought to occur *de novo* in maternal or paternal germ lines or in zygotes at an early developmental stage. A paternal effect had been hypothesized on the basis

of advanced paternal age (DeBusk 1972; Jones et al. 1975) and has been reported by D'Apice and colleagues in 2004 (D'Apice et al. 2004). The description of sibs affected with HGPS, in the context of a majority of sporadic cases, had led to hypothesize the possibility of germinal mosaicism (DeBusk et al. 1972). One case of somatic and germinal mosaicism for the *LMNA* c.1824C>T mutation has recently been reported in the unaffected mother of a child affected with progeria (Wuyts et al. 2005).

At least 14 other lamin A/C mutations have been reported as causing progeroid phenotypes including mandibuloacral dysplasia (homozygous p.R527H; Novelli et al. 2002), progeria (heterozygous p.G608S, heterozygous p.E145K; Eriksson et al. 2003; compound heterozygous p.R471C and p.R527C; Cao and Hegele 2003; p.T623S, Fukuchi et al. 2004; homozygous p.K542N, Plasilova et al. 2004), atypical Werner syndrome (p.R133L, p.L140R, p.A57P, Chen et al. 2003; p.E578V, Csoka et al. 2004), atypical progeroid syndromes (heterozygous p.R644C, Csoka et al. 2004), Seip syndrome (heterozygous p.T10I, Csoka et al. 2004) and restrictive dermopathy (IVS11+1G>A, causing p.G567_Q656del, which we will discuss further on). These are mostly localized in the lamin-A-specific C-terminal tail and in the N-terminal region. Of these mutations, only three have been reported to specifically alter lamin A splicing and lead to the production of truncated protein products (p.G608G, p.T623S and IVS11+1G>A; Fig. 3).

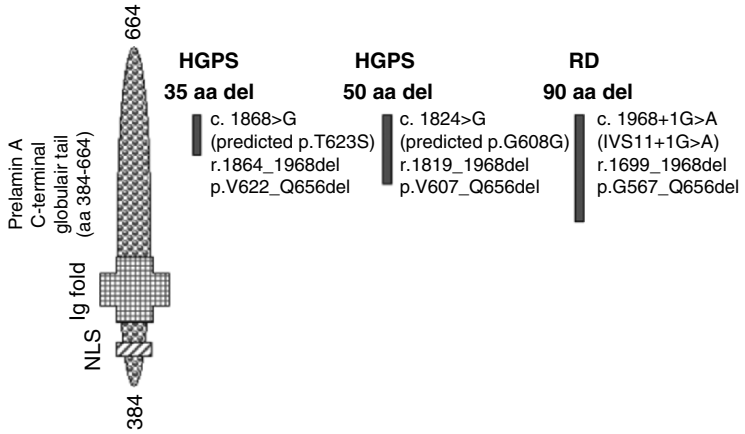

Fig. 3. Schema showing the three different truncated prelamin A forms that have been associated with progeroid disorders. All the deletions are localized in prelamin A Carboxy-terminal globular tail and are due to *LMNA* mutations affecting the splicing of exon 11. *NLS* = nuclear localization signal; *Ig fold* = immunoglobulin fold

A few remarks should be made regarding two of these mutations:

- The heterozygous p.R133L mutation had previously been described in a patient affected with generalized acquired lipoatrophy, insulin-resistant diabetes, hypertriglyceridemia, hepatic steatosis, hypertrophic cardiomyopathy with valvular involvement, and disseminated whitish papules (Caux et al. 2003)
- The heterozygous p.R644C mutation in lamin A/C had already been reported as being associated to a CMD1A phenotype (Genschel et al. 2001) and a LGMD1B phenotype, together with another variation affecting the same codon (R644H) (Mercuri et al. 2004)

In 2003, Agarwal and colleagues observed for the first time the involvement of *ZMPSTE24*/*FACE-1* in the pathogenesis of a progeroid disorder: severe mandibuloacral dysplasia (MADB; Agarwal et al. 2003). The reported patient carried two compound heterozygous mutations: p.Trp340Arg and p.Leu362PhefsX19, caused by a frame-shifting insertion between coding nucleotides 1085 and 1086 (c.1085_1086insT).

In addition, as will be further discussed, mice inactivated for the *Zmpste24* gene showed a series of phenotypic features recalling different laminopathies characterized by premature aging and death (Pendas et al. 2002; Bergo et al. 2002).

2.2 Restrictive Dermopathy

In 2004 a perinatal lethal genodermatosis, restrictive dermopathy (RD; Table 1), was linked to primary or secondary lamin A dysfunction (Navarro et al. 2004). Typical RD cases are characterized by intrauterine growth retardation, tight and rigid skin with erosions on flexure sites, prominent superficial vessels, epidermal hyperkeratosis, small "O" shaped mouth, small pinched nose, micrognathia, sparse/absent eyelashes and eyebrows, bone mineralization defects, thin and dysplastic clavicles, pulmonary hypoplasia, multiple joint contractures, and early neonatal death (Fig. 4).

2.2.1 Lamin-Linked RD

In 2004, a mutation involving altered lamin A pre-messenger splicing was shown to be involved as well in the pathogenesis of this very severe disorder. The observed female patient presented with typical RD signs and lived to 6 months of age, a longer lifespan than typical of RD cases. She carried a heterozygous c.1968+1G>A (IVS11+1G>A) transversion, leading to the skipping of the totality of exon 11 in transcripts. Since

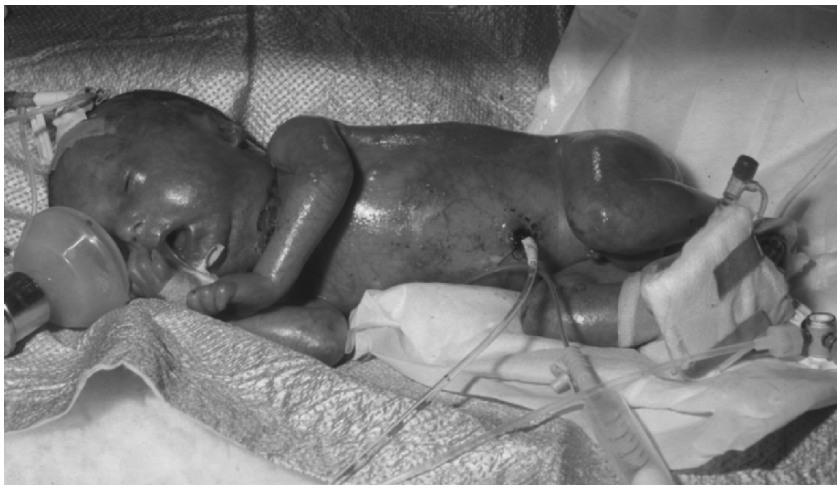

Fig. 4. Patient affected with restrictive dermopathy. The taut and thin, translucent skin, together with prominent superficial vessels and erosions, are evident. Joints are fixed in flexion due to generalized joint contracture. The expression is fixed with the mouth open in typical "O" position; microretrognathism as well as absence of eyelashes, eyebrows, and hair can be observed. (Courtesy of Oxford University Press)

exon 11 contains 270 base pairs, the resulting truncated transcript once again conserved an open reading frame, leading to the production of a truncated protein missing amino acids 567 to 656 (p.G567_Q656del). This protein was visualized by western blot studies with antibodies directed against lamin A/C N-terminal tail, showing, as expected, a molecular weight similar to lamin C (Navarro et al. 2004).

Both HGPS and *LMNA*-linked RD prelamin A precursors carry a deletion of the domain in which the second ZMPSTE24 cleavage takes place during the post-translational maturation process. Indeed, the RD-associated mutation c.1968+1G>A causes aberrant splicing and skipping of the whole exon 11 in transcripts, overlapping with and encompassing the smaller HGPS deletion due to the c.1824C>T mutation. In the two cases, the consequences are similar: the truncated transcripts maintain a reading frame so that more or less broadly truncated prelamin A precursors are produced; these are not recognized as pathological by the cells and accumulate inside the patient's nuclei, where they exert toxic effects. As we will discuss further, the same pathophysiological mechanism is observed in *ZMPSTE24*-linked RD: in these cases the absence of this fundamental prelamin A processing enzyme causes the accumulation of normal-length precursors that cannot be cleaved by other metalloproteases and thus also accumulate in cell nuclei.

Immunocytochemical analyses also showed, in the patient affected with *LMNA*-linked RD, important alterations of nuclear shape and composition – notably lamins A/C, lamin B1, and emerin are excluded from one nuclear pole, as observed in mice lacking lamins A/C (Sullivan et al. 1999) or, partially, in the L530P knock-in progeria mouse model (Mounkes et al. 2003). In particular, in that mouse model, a complex splicing mechanism has been proved to be responsible for the phenotype, given that the same predicted missense mutation in man causes an EDMD phenotype.

A second patient, affected with another milder form of RD, carried the c.1824C>T mutation (p.G608G) identified in most typical HGPS patients.

A large inter- and intra-familial clinical variability has been observed as well, given a lamin A/C mutation, in many other less severe laminopathies, underlying the likely impact on the phenotype of different genetic backgrounds or epigenetic factors (Fatkin et al. 1999; Raffaele di Barletta et al. 2000; Bonne et al. 2000; Brodsky et al. 2000; Brown et al. 2001; Vytopil et al. 2002).

2.2.2 ZMPSTE24-Linked RD

In 2004 and 2005, Navarro et al. identified as the genetic cause of "typical", autosomal recessive restrictive dermopathy, the complete inactivation of ZMPSTE24, due to different genomic alterations (Navarro et al. 2004, 2005). Indeed, out of 10 explored patients carrying a wild type *LMNA* sequence:

- Seven carried a homozygous frame-shifting insertion (c.1985–1986insT) in exon 9, leading to the apparition of a premature termination codon (p.Leu362PhefsX19)
- Three carried the same insertion at the heterozygous state, together with another, different "null" alteration on the opposite allele:

(a) A large genomic deletion encompassing 5.428 Kb and leading to the in-frame skipping of exons 3, 4, and 5 at the mRNA level (r.271_627del)
(b) A C>T transition at nucleotide 1249 (c.1249C>T) predicting a nonsense substitution from glutamine to stop in exon 10 (p.Gln417X).
(c) A cytosine deletion (c.295delC) in exon 3 leading to a shifted reading frame and the creation of a premature termination codon (p.Pro99LeufsX38)

Of note, the c.1085_1086insT insertion, identified to date at least at the heterozygous state in all patients carrying a *ZMPSTE24*-related disease, and transmitted from the parents in most cases, seems to occur in a mutational hotspot (Agarwal et al. 2003; Navarro et al. 2004; Navarro et al. 2005; Shackleton et al. 2005). Indeed, the thymine stretch located in *ZMPSTE24* exon 9 (nucleotides c.1077 to c.1085) had already been reported as being a

specific target for microsatellite instability (MSI) in colorectal tumors (Mori et al. 2001).

Western blot studies in six different patients showed that no ZMPSTE24 was detectable in different tissues. As expected, in all patients the presence of untransformed prelamin A precursors was also noted, weighing about 74 kDa, together with normal lamin C and the complete absence of lamin A.

Of note, RT-PCR studies of *ZMPSTE24* transcripts were always possible, indicating that the genomic alterations did not affect the production and stability of the transcripts, instead the altered enzymes are never detectable, either not being produced or rapidly degraded.

Immunocytochemical analyses performed with different antibodies directed against lamins A/C (detecting both prelamin A and lamin C), lamin A (detecting only prelamin A), lamin B1, and emerin showed numerous and major nuclear alterations of morphology and composition. Of note, blebs or herniations were numerous and shown to specifically contain lamin C and emerin, but not prelamin A and B1. A similar pattern of absence of lamin A/C partners from a nuclear pole was observed as well in nuclei of mouse embryonic fibroblasts (MEFs) issued from homozygous *Lmna* KO mice (Sullivan et al. 1999). Nucleoplasmic aggregates were observed to contain prelamin A and emerin but not lamin B1 (Navarro et al. 2005).

Restrictive dermopathy is thus another disorder due to abnormal accumulation of prelamin A precursors. In these patients, the precursor that accumulates is not truncated and probably remains farnesylated as well, not being able to undergo the successive ZMPSTE24 cleavage.

The identification of novel *LMNA* mutations affecting lamin A-specific splicing will help to bring new insights into the pathophysiology of prelamin-A-related disorders.

3 Hypothetical Pathophysiological Mechanisms Involved in Laminopathies and Progeroid Syndromes Linked to *LMNA* and *ZMPSTE24* in Relation to Animal Models Possible Therapeutic Strategies

While different, nonexclusive, pathophysiological mechanisms have been evoked and are under discussion for most non-progeroid laminopathies, progeroid syndromes linked to *LMNA* and *ZMPSTE24* defects have recently been shown to share a common pathophysiological pathway that could, in principle, be corrected by targeted therapeutic strategies.

In particular, *Lmna* and *Zmpste24* knock-out mouse models have been extremely helpful in the search of pathophysiological mechanisms involved in progeroid laminopathies and will be shortly presented hereafter.

In 1999, Sullivan and colleagues produced a murine model of *Lmna* inactivation (Sullivan et al. 1999). Homozygous knock-out mice completely lost lamin A/C expression and showed a phenotype that reproduced, in particular, some aspects of human laminopathies affecting skeletal muscles (i.e., EDMD2/3-LGMD1B-CMD1A) and nerves (CMT2B1) together with reduced postnatal growth and lifespan, which was shortened to about 8 weeks. In contrast to what happens in human, where a *LMNA* half gene dose does not seem to suffice to guarantee normal muscular function (Bonne et al. 1999), heterozygous mice were indistinguishable from wild-type mice.

Null *Lmna* mice were normal at birth but shortly after developed a muscular dystrophy in skeletal and cardiac muscles, similar to that observed in EDMD and CMD patients (Sullivan et al. 1999). They also developed an axonal peripheral neuropathy resembling CMT2B1 (De Sandre-Giovannoli et al. 2002).

Conversely, the observed lipoatrophy does not recapitulate the distribution pattern observed in lipodystrophic patients and has been interpreted as a consequence of stress. On the other hand, these mice have a growth retardation, die prematurely, and have immunocytochemical nuclear alterations that are partly similar to those observed in patients affected with *LMNA* and *ZMPSTE24*-related progeroid syndromes.

The knock-in mouse model *Lmna* L530P, a substitution that causes EDMD in human, seems to recapitulate more faithfully the cell and tissue alterations observed in HGPS patients (Mounkes et al. 2003). Homozygous mice show a severe growth retardation, several developmental abnormalities, and die prematurely (4–5 weeks after birth). Their phenotypic characteristics include: abnormal dentition, joint stiffness, epidermal hyperkeratosis, lipodystrophy, a reduction of hair follicles and sebaceous glands, muscular atrophy, generalized osteopenia, and scapular hypoplasia. Conversely, compared to human, these mice do not develop generalized atherosclerosis nor acro-osteolyses. In these mice, a complex splicing defect (either skipping of exon 9 or retention of part of intron 9) causing a reduction of about half of the lamin A/C wild-type content, has been shown to cause the phenotype, in contrast to human, in which the simple p.L530P substitution is supposed to occur. Indeed, this lamin A/C substitution has been reported as impeding the interaction with emerin (Raharjo et al. 2001).

Mice inactivated for *Zmpste24* (Pendas et al. 2002) show striking phenotypic similarities with patients affected with lamin-related progeroid syndromes in general, and restrictive dermopathy in particular.

Heterozygous mice are indistinguishable from wild-type mice, as expected, since the pathophysiological mechanism involved is supposed to be the same as in human, where half *ZMPSTE24* gene dosage is sufficient to guarantee prelamin A processing . Conversely, homozygous knock-out mice show a severe postnatal growth retardation, premature death (at about

20 weeks), abnormal dentition, joint stiffness, epidermal hyperkeratosis, lipodystrophy, a reduction of hair follicles and sebaceous glands, muscular dystrophy, dilated cardiomyopathy with interstitial fibrosis and atherosclerosis, generalized osteopenia, and scapular hypoplasia. Numerous spontaneous pathological fractures have been observed (Pendas et al. 2002, Bergo et al. 2002).

Immunocytochemistry and electronic microscopy experiences have shown altered nuclear morphologies with prelamin A accumulation (Pendas et al. 2002).

Patients affected with *ZMPSTE24*-linked RD represent the exact human counterpart of these mouse models. Indeed, the phenotypes are comparable, although more severe in human when ZMPSTE24 is completely inactivated, and the involved pathophysiological mechanisms are presumably the same, involving the aberrant intranuclear accumulation of prelamin A precursors.

In this context, and as an additional proof of the toxic effects of prelamin A accumulation, a recent report has shown that 50% reduction of prelamin A amounts can spectacularly improve the phenotype of *Zmpste24* knock-out mice (Fong et al. 2004). In this study, the authors showed that double knock-out mice carrying the *Zmpste24–/– Lmna*+/– genotype, expressing half the prelamin A of *Zmpste24–/– Lmna* +/+ mice, show a complete reversal of the clinical phenotype, including growth, muscular strength, bone and soft tissue development, and lifespan.

These studies indicate that when *Zmpste24* is inactivated a toxic prelamin A threshold exists, which if exceeded leads to the development of progeroid phenotypes. In this context, *ZMPSTE24*-linked RD phenotypes represent one extreme form, with total enzyme inactivation and extreme prelamin A accumulation, while several MAD phenotypes resulting from partial *ZMPSTE24* inactivation represent "intermediate" forms (Agarwal et al. 2003, Shackleton et al. 2005). Transposing the results obtained in mice to humans suggests that in principle, particularly in the latter cases, it could be possible to ameliorate the clinical phenotype by decreasing prelamin A or increasing ZMPSTE24 expression.

Accumulation of unprocessed truncated precursors, expressed in HGPS patients carrying the c.1824C>T mutation, is likewise toxic for cells. Presumably, the same is true for RD patients carrying an even larger truncation in prelamin A, due to the c.1968+1G>A (IVS11+1G>A) *LMNA* splicing mutation.

We do not know yet how prelamin A accumulation mediates these effects, whether it polymerizes with wild-type lamin A, hampering its localization and function, or whether it interacts with different molecular partners in an aberrant, permanent way. Whatever the mechanism(s) involved, these effects are dominant negative, in that the presence of the truncated precursors prevents even high amounts of normal lamin A (either endogenously expressed or overexpressed by transfection) from

rescuing the cellular phenotype (Goldman et al. 2004, Scaffidi and Misteli 2005).

On analogous bases, in cells issued from patients carrying the c.1824C>T heterozygous mutation, progerin levels have been experimentally reduced in vitro by transfection of an oligonucleotide targeted to the activated splicing site (Scaffidi and Misteli 2005). Through this experience, the authors have assisted at a spectacular reversion of the cellular pathological phenotype, which is a major advance and a ray of hope directed toward the development of molecular therapeutic strategies. Indeed, while it had been demonstrated by Fong and colleagues that double knock-out mice did not *develop* pathologic progeroid features (Fong et al. 2004), Scaffidi and Misteli demonstrate with this study that, even once established, the cellular defects characterizing progeria are *reversible*.

The reduction of progerin expression, assessed by RT-PCR and western blot studies, was proportional to the quantity of morpholino oligonucleotide transfected. Reversal of the cellular phenotype was evaluated with quantities of oligonucleotide sufficient to abolish progerin expression and included the re-establishment of normal nuclear morphology and composition (the expression levels of several lamin A/C functional partners and genes that were previously altered were measured, as well as the behavior of wild type lamin A proteins).

Furthermore, this phenotypic reversal was independent of cell division, occurring in cells not undergoing mitosis, observation having major implications for the development of therapeutic strategies.

Mandibuloacral dysplasia, atypical progeroid syndromes, atypical Werner syndrome, Hutchinson-Gilford progeria syndrome, neonatal progeria syndromes, and restrictive dermopathy linked to *LMNA* or *ZMPSTE24* alterations appear thus to constitute a series of allelic disorders whose phenotypic severity varies over a large spectrum, probably depending on the mutations involved, the remaining tissue-specific and ubiquitous molecular interactions, the absence/presence/quantity of unprocessed prelamin A precursors, and the residual ZMPSTE24 activity, in a fashion with intimate functional interconnections (Fig. 5).

4 Conclusions and Future Prospects

In order to deepen our knowledge of rare and complex diseases such as laminopathies, and envisage the first targeted therapeutic approaches, collaborative and coordinated efforts of multidisciplinary groups specializing in different domains are absolutely necessary. A close collaboration between researchers and medical practitioners in clinical genetics, molecular genetics, and cellular biology must take place.

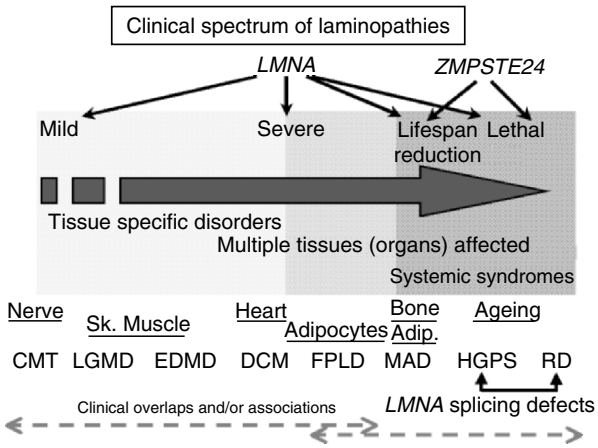

Fig. 5. Clinical spectrum of laminopathies associated with *LMNA* and *ZMPSTE24* point mutations and *LMNA* splicing defects

Fundamental and clinical research on laminopathies will allow us to: (a) enlarge the phenotypic spectrum of systemic laminopathies; (b) advance in establishment of genotype–phenotype correlations in order to improve the clinical and molecular diagnosis and follow-up of patients; (c) extend our comprehension of the pathophysiological mechanisms underlying laminopathies, consequently shed light on physiological functions of lamins and their molecular partners; (d) evaluate the similarities or the differences existing between the pathologies developed by patients carrying lamin A/C alterations and the same pathologies developed during "physiological" aging such as osteoporosis, atherosclerosis, sclerodermiform cutaneous lesions, and amyotrophy; (e) and finally, evaluate the possibility of taking advantage of different therapeutic strategies targeting the splicing defect observed in progeria in order to treat these young patients. In this respect, different therapeutic approaches have already proven useful, in vitro or in animal models, in correction of pathogenic splicing defects in different diseases, such as β-thalassemia, cystic fibrosis, Duchenne muscular dystrophy, to name a few (for a review, see Garcia-Blanco et al. 2004; Sazani and Kole 2003). The techniques used comprised delivery of snRNA structures, antisense oligonucleotide-like molecules, bifunctional antisense oligonucleotides (ESSENCE = exon-specific splicing enhancement by small chimeric effectors; TOES = targeted oligonucleotide enhancer of splicing; TOSS = targeted oligonucleotide silencing of splicing), spliceosome-mediated RNA *trans*-splicing (SMaRT), isoform-specific RNA interference, group I ribozyme-mediated *trans*-splicing, use of a tRNA archeal splicing endonuclease. For many of these systems, delivery and specificity have not yet been evaluated in animal models, a necessary step towards human

applications, but some of them have and appeared to prove effective (for a review, see Garcia-Blanco et al. 2004 and included references).

Progress in this field is moving very quickly, giving hope of effective clinical applications in the near future, as well in patients carrying accumulation of prelamin A precursors due to *LMNA* or *ZMPSTE24* defects. Indeed, it has been shown in cells and animal models that the severity of the phenotype correlates with the amount of transcripts aberrantly spliced and with the accumulation of prelamin A precursors, and that, in this context, the pathological phenotypes are largely reversible upon reduction of prelamin A dosages to below a threshold level. These observations make the search for therapeutic strategies a more easily reachable goal in prelamin-A-associated premature aging syndromes.

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Splicing Modulation as a Modifier of the CFTR Function

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Abstract. A significant fraction of CF-causing mutations affects pre-mRNA splicing. These mutations can generate both aberrant and correct transcripts, the level of which varies among different patients. An inverse correlation was found between this level and disease severity, suggesting a role for splicing regulation as a genetic modifier. Subsequent studies showed that overexpression of splicing factors modulated the level of correctly spliced RNA, transcribed from minigenes carrying CF-causing splicing mutations. Overexpression of splicing factors also modulated the level of normal CFTR transcripts, transcribed from the endogenous CFTR allele carrying splicing mutations, in CF-derived epithelial cells. Several of the factors promoted higher level of correct CFTR transcripts. The increased level of normal transcripts led to activation of the CFTR channel and restoration of its function. Restoration was also obtained by sodium butyrate, a histone deacetylase inhibitor, known to up-regulate the expression of splicing factors. These results highlight the role of the splicing machinery as a modifier of disease severity in patients carrying splicing mutations and shed a new light on the therapeutic potential of splicing modulation for genetic diseases caused by splicing mutations.

1
The CF Disease and the CFTR Gene

Cystic fibrosis (CF) is an autosomal recessive lethal disease affecting 1 in 2,500 newborns among Caucasians (though rare among Orientals at 1:90,000) (Collins 1992; Welsh 1995). The major clinical characteristics of CF are progressive lung disease, caused by thick and dehydrated airway mucus frequently infected with Pseudomonas and Staphylococcus, leading to respiratory failure and CF mortality, and exocrine pancreatic insufficiency, of which CF is the most common cause in childhood. In addition, most males are infertile, due to congenital bilateral absence of the vas deferens (CBAVD). Other CF characteristics include bile duct obstruction, reduced fertility in females, high sweat chloride, intestinal obstruction, nasal polyp formation, chronic sinusitis, liver disease, and diabetes (Collins 1992; Welsh 1995).

The cystic fibrosis transmembrane conductance regulator (CFTR) gene comprises 27 coding exons, spanning over 250 kb on human chromosome 7q31.2 (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989). The mRNA transcribed from the CFTR gene is 6.5 kb. The encoded protein,

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which comprises 1480 amino acids with a molecular weight of ~170 kDa, is a chloride (Cl^-) channel in the apical membrane of exocrine epithelial cells (Riordan et al. 1989). Phosphorylation of the CFTR by protein kinase A, regulated by cyclic adenosine monophosphate (cAMP), and ATP hydrolysis, are essential for activating the chloride channel (Collins 1992; Frizzel 1995; Gabriel et al. 1993; Li et al.1996; McIntosh and Cutting 1992; Welsh 1992). In addition to the CFTR function as a chloride channel, it appears to have an effect on a growing number of proteins. The CFTR modifies the function and properties of other ion transporters including chloride, sodium, and potassium channels and the $Cl^-/HCO3^-$ exchanger. Moreover, it has an effect on water permeability, ATP transport, and mucus secretion (reviewed in Greger et al. 2001; Kunzelmann et al. 2000).

2
Spectrum of Splicing Mutations in the CFTR Gene

All CFTR exons are required for generation of functional CFTR proteins. Nevertheless, several exons (3, 4, 9, 12, 14a, 16, 17b, and 22) were shown to undergo partial aberrant skipping that generates non-functional CFTR proteins, in CF patients and normal healthy individuals (Bienvenu et al. 1996). Over 1100 mutations have been identified so far along the entire CFTR gene (http://www.genet.sickkids.on.ca/cftr/). A significant fraction (15.7%) of the CFTR mutations affects the pre-mRNA splicing of the gene, by disrupting or generating intronic splicing motifs, required for exon recognition. These mutations can be divided into two subclasses according to their position and effect on the splicing pattern (Nissim-Rafinia and Kerem 2002). Subclass I (60% of the CFTR splicing mutations) includes mutations that disrupt the invariant intronic splice-site sequences, which completely abolish exon recognition. Subclass II includes intronic mutations in the variant motifs, which can lead to both aberrantly and correctly spliced CFTR transcripts, by either weakening or strengthening exon-recognition motifs. Subclass II also includes intronic mutations that generate cryptic donor or acceptor sites and can lead to partial inclusion of intronic sequences. In addition, there are mutations and polymorphisms that disrupt exonic splicing motifs, which also affect the CFTR splicing pattern.

2.1
Splicing Mutations Leading to Complete Aberrant Splicing

Subclass I includes mutations such as 621+1 G→T, 711+1 G→T, and 1525–1 G→A (Ramalho et al. 2003; Zielenski et al. 1993). Patients carrying subclass I mutations present a typical severe CF disease, including early age

at diagnosis, elevated sweat chloride levels, pancreatic insufficiency, and pulmonary disease. The severe disease is a result of complete absence of correctly spliced transcripts.

2.2 Splicing Mutations Leading to Partial Generation of Correct Splicing

Subclass II includes mutations that generate both aberrantly and correctly spliced transcripts, such as 3849+10 kb C→T, 2789+5 G→A, 3272–26 A→G, IVS8–5T (Pagani et al. 2003b; Ramalho et al. 2002) and reviewed in (Nissim-Rafinia and Kerem 2002). These mutations are associated with relatively milder form of CF, compared to ΔF508 homozygous and to homozygous for subclass I splicing mutations, due to the partial generation of normal transcripts, translated to normal proteins in the affected tissues. Nevertheless, there is a marked variability in disease severity among different patients in the same organ, and among different organs of the same patient (Pagani et al. 2003b; Ramalho et al. 2002) and reviewed in (Nissim-Rafinia and Kerem 2002). In this section we discuss the variability in disease severity among patients carrying several subclass II intronic splicing mutations, as well as exonic mutations that lead to aberrant splicing.

2.2.1 The 3849+10 kb C→T Mutation

The 3849+10 kb C→T is a nucleotide substitution, 10 kb downstream nucleotide 3849, the last nucleotide of CFTR exon 19 (Fig. 1a). This substitution generates a cryptic donor splice site that leads to partial inclusion of 84 bp "exon" between exon 19 and exon 20 (Highsmith et al. 1994). The 84 bp "exon" contains an in-frame stop codon and therefore the aberrantly spliced transcripts lead to the generation of a truncated protein. The 3849+10 kb C→T is the twelfth most common mutation worldwide with frequency of 0.2% (http://www.genet.sickkids.on.ca/cftr/), and is particularly frequent in several subpopulations, including Polish (4%) and Ashkenazi Jews (6%; Kerem et al. 1995; Orgad et al. 2001).

The 3849+10 kb C→T mutation was found to be associated with a relatively mild form of CF. The patients present better nutritional status, older age at diagnosis, and moderately elevated sweat chloride values (Augarten et al. 1993; Highsmith et al. 1994). Nevertheless, there is a marked variability in disease severity among different patients in the same organ, and among different organs of the same patient. Some patients have severe pulmonary disease, while others have normal pulmonary function. About 30% of the patients suffer from pancreatic insufficiency, while others have normal pancreatic function. Interestingly, several of the males carrying the 3849+10

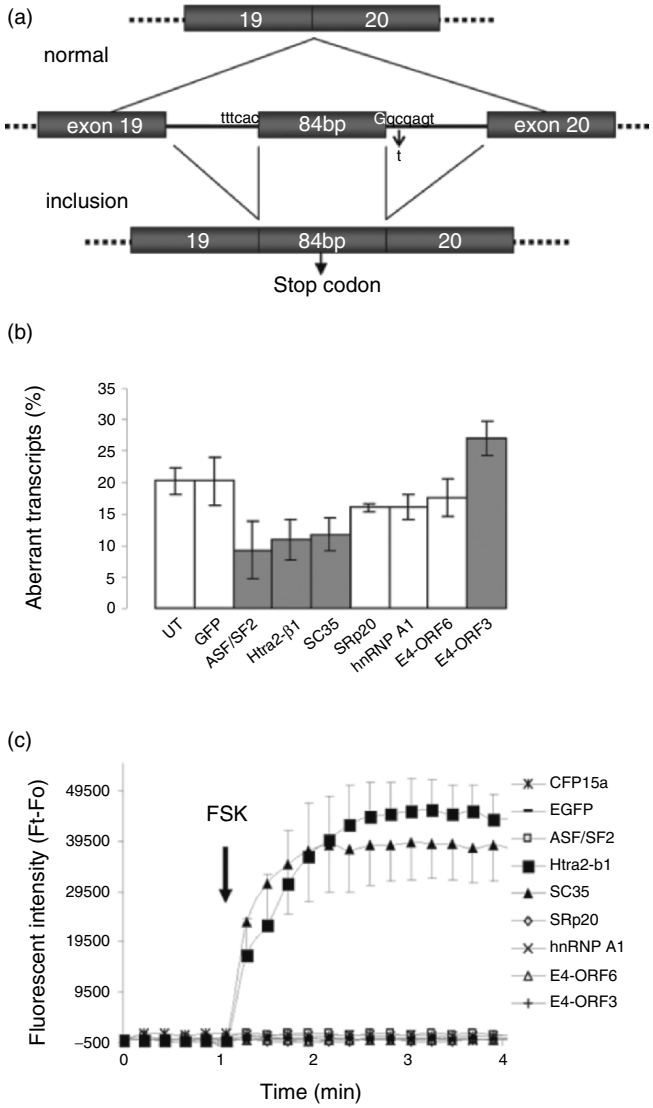

Fig. 1a–c. The effect of overexpression of splicing factors on the splicing pattern of CFTR exons and on CFTR Cl^- efflux, in CFP15a cells carrying the 3849+10 kb C→T splicing mutation (Nissim-Rafinia et al. 2004). **a**: The effect of the 3849+10 kb C→T mutation on the CFTR splicing. **b**: The effect of splicing factors on the level of aberrant CFTR transcripts. *Gray columns* represent a significant effect ($P<0.01$) using Mann-Whitney u test, adjusted with Bonferroni correction for 8 comparisons. *White columns* represent no effect. *Error bars* represent the standard deviations. *UT* = untransfected. **c**: The effect of splicing factors on the CFTR Cl^- efflux. *Black symbols* indicate activation of the CFTR Cl^- efflux. *Empty symbols* indicate no effect. An arrow indicates the time of forskolin administration

kb C→T mutation, are fertile, although the vast majority of male CF patients suffer from CBAVD (Augarten et al. 1993; Dreyfus et al. 1996; Gilbert et al. 1995; Hamosh and Corey 1996; Highsmith et al. 1994).

2.2.2 The 2789+5 G→A Mutation

The 2789+5 G→A mutation is a nucleotide substitution at position +5 of the splice donor site in intron 14b, leading to incomplete skipping of exon 14b, predicted to generate out-of-frame, truncated product (Highsmith et al. 1997). This mutation accounts for 0.1% of CF chromosomes worldwide, and appears with a higher frequency in Greece (4.5%) and in North France (3%; Claustres et al. 2000; Estivill et al. 1997). The 2789+5 G→A mutation is associated with a moderately mild form of CF. Nevertheless, there is variability in disease severity among different patients in their lung function and pancreatic disease; about 60% of the patients suffer from pancreatic insufficiency, while others have normal pancreatic function (Dugueperoux and De Braekeleer 2005; Highsmith et al. 1997).

2.2.3 The 3272–26 A→G Mutation

The 3272–26 A→G mutation leads to the creation of an alternative acceptor splice site that competes with the normal site of exon 17b, but still allows some correctly spliced transcripts to be produced. The aberrantly spliced transcripts include extra 25 nucleotides from intron 17a, which contain a stop codon (Beck et al. 1999). This mutation is widespread in Europe, with the highest frequency in Portugal (2%; Amaral et al. 2001). Patients carrying the 3272–26 A→G mutation show phenotypic variability in age at diagnosis, lung function, and pancreatic disease. The correctly spliced transcripts (~5%) were suggested to account for the milder and variable phenotype found in these patients (Ramalho et al. 2002).

2.2.4 The IVS8–5T Allele

Another well-studied splicing allele is the IVS8–5T, in the acceptor site of exon 9 (Chillon et al. 1995a; Jarvi et al. 1995). This allele was found to generate high levels of exon 9 skipping, due to a short polypyrimidine tract, which consists of only five thymidines. Aberrant transcripts, lacking exon 9, are translated into non-functional proteins (Chu et al. 1993; Strong et al. 1993). The IVS8–5T allele is frequent (20–25%) among infertile men due to CBAVD. This frequency is significantly higher than the frequency of the IVS8–5T allele in the general population (5%; Chillon et al. 1995a; Jarvi et al. 1995; Zielenski and Tsui 1995). Therefore, the IVS8–5T was

designated as a mutation causing CBAVD. The IVS8–5T allele was also found in several typical mild CF patients (Kerem et al. 1997; Noone et al. 2000). Thus, the IVS8–5T allele is associated with an extreme variability in clinical CF presentation: from normal healthy fertile individuals or males with CBAVD to typical clinical phenotype of CF.

In addition to the IVS8–5T two other IVS8-T alleles are found in the population, the IVS8-7T and IVS8-9T. Most of the population (~85%) carries the IVS8–7T, and ~10% carry the longest tract of IVS8–9T (Chillon et al. 1995a). All the IVS8 poly-T alleles are shorter than the minimal effective 11 pyrimidine tract required for efficient splicing (Coolidge et al. 1997). Indeed, each of these alleles generates both correctly and aberrantly spliced transcripts (Chu et al. 1993). An inverse correlation is found between the length of the poly-T and the level of exon 9 skipping. The longest allele (IVS8-9T) leads to low levels of exon 9 skipping (<10%), the IVS8-7T allele leads to higher levels of skipping (up to 50%), and the shortest IVS8-5T allele leads to very high levels (up to 90%; Chu et al. 1993; Larriba et al. 1998; Mak et al. 1997; Rave-Harel et al. 1997). It should be noted that although an inverse correlation is found between the length of the poly-T and the level of exon 9 skipping, this level varies considerably among individuals carrying each of the alleles (Chu et al. 1993; Larriba et al. 1998; Mak et al. 1997; Rave-Harel et al. 1997).

An additional cis element, which regulates the splicing pattern of exon 9, is a TG repeat, located in a close proximity upstream to the T tract. The number of the TG repeat varies in the population between 10 and 13. The level of correctly spliced exon 9 transcripts is in correlation with the length of the TG repeat, such that a longer repeat is associated with higher levels of correctly spliced transcripts (Buratti et al. 2001). Taken together, a shorter T tract and a longer TG repeat in the acceptor site of exon 9 lead to high levels of exon 9 skipping.

2.3
Exonic Point Mutations Leading to Aberrant Splicing

The identification of exonic splicing motifs revealed that mutations in coding regions might also affect the splicing pattern of their pre-mRNAs. Disruption of these splicing motifs, whether by nonsense, missense, or silent mutations, might promote skipping of an exon, or of part of an exon. This was shown for several human disease genes, such as ataxia-telangiectasia mutated (ATM), neurofibromatosis (NF)-1, breast cancer (BRCA)1, survival motor neuron (SMN), and CFTR (Aznarez et al. 2003; Caceres and Kornblihtt 2002; Cartegni et al. 2002; Faustino and Cooper 2003; Liu et al. 2001; Nissim-Rafinia and Kerem 2002; Pagani et al. 2003b). Since direct RNA analysis is not routinely performed for diagnosis, it is likely that the fraction of splicing mutations is larger than was anticipated.

Indeed, it was shown that 11–13% of mutations in the coding region disrupt exonic splicing motifs in ATM and NF-1 genes (Ars et al. 2000; Teraoka et al. 1999).

In the CFTR gene, mutations in exons 9 (Q414X, G424S, I444S, Q452P and A455E), 12 (D565G and G576A), and 13 (D651 N, G654S, E656X, 2108delA, E664X and T655S) were shown to affect the splicing pattern of these exons (Aznarez et al. 2003; Pagani et al. 2003a; Pagani et al. 2003b). One of these mutations, the A455E, is common worldwide, with frequency of 0.1%, while the rest of the mutations are rare and were found only in few individuals. The effect of these mutations on the disease severity depends on the level of aberrant splicing together with the type of mutation and its location within the CFTR gene. Exon 9 mutations are mostly associated with mild phenotypes (except for Q414X). Exon 12 mutations were found in atypical CF patients and carriers. The mutations in exon 13 have variable consequence, E656X and 2108delA result in a severe disease, while E664X, T655S, D651 N are associated with a milder phenotype and G654S is considered a nucleotide polymorphism (Aznarez et al. 2003; Pagani et al. 2003a; Pagani et al. 2003b; http://www.genet.sickkids.on.ca/cftr/).

3 Correlation Between Levels of Correctly Spliced RNA and Disease Severity

In order to determine whether there is a correlation between the level of correctly spliced RNA and pulmonary disease severity, several studies analyzed the splicing pattern of RNA transcribed from alleles carrying splicing mutations in the respiratory epithelium of patients, (Chiba-Falek et al. 1998; Chillon et al. 1995b; Highsmith et al. 1997; Ramalho et al. 2002; Rave-Harel et al. 1997). Two of the mutations the 3849+10 kb C→T and IVS8–5T were studied in large groups of patients. The analyses revealed that the level of correctly spliced transcripts varies considerably among patients carrying the same splicing mutation, (1–50% among patients carrying the 3849+10 kb C→T mutation and 6–37% among individuals carrying the IVS8–5T). Importantly, a significant inverse correlation is found between the level of correctly spliced transcripts and pulmonary disease severity, such that lower levels are associated with a severe disease, while higher levels are associated with a milder phenotype (Fig. 2). The RNA splicing pattern was also studied in a few patients carrying other CFTR splicing mutations (1811+1.6 kb A→G, 2789+5 G→A, and 3272–26 A→G) (Chillon et al. 1995b; Highsmith et al. 1997; Ramalho et al. 2002). The mutation 1811+1.6 kb A→G generated 1–3% of correctly spliced transcripts, leading to severe disease, whereas 2789+5 G→A and 3272–26 A→G generated 4% and 5% correctly spliced transcripts, respectively, leading to a

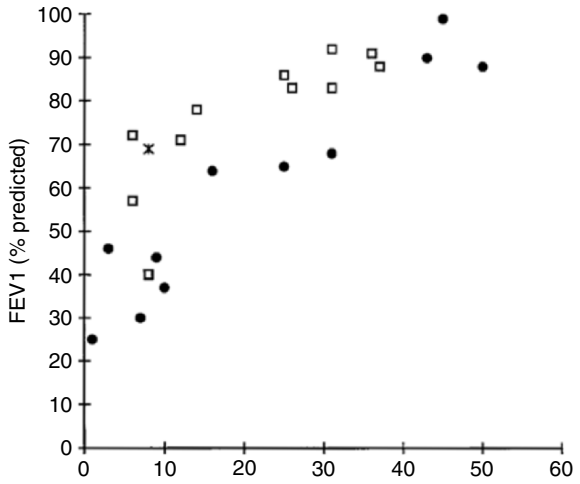

Fig. 2. Combined data correlating normal CFTR RNA, transcribed in nasal epithelium, and lung function, expressed as FEV1 values (Chiba-Falek et al. 1998). *Filled circles* represent the results from patients with the 3849+10 kb C→T allele, *open squares* represent the results from patients with the IVS8-5T allele. The *asterisk* represents the result from a patient homozygous for the 3849+10 kb C→T mutation reported by Highsmith et al. (1994). The correlation coefficient is r 5 0.75, P<0.0001

milder form of disease. Thus, the results of these studies show similar correlations, such that lower levels are associated with a severe disease, while higher levels are associated with a milder phenotype.

The levels of correctly spliced transcripts also differ between various organs of the same patient, in accordance to the severity of the disease in each organ (Chiba-Falek et al. 1999; Mak et al. 1997; Rave-Harel et al. 1997). Several groups studied the IVS8–5T allele in respiratory epithelium and epididymis of the same individual (Mak et al. 1997; Rave-Harel et al. 1997). These studies showed that in CBAVD males the level of correctly spliced transcripts is lower in the epididymis than in the respiratory epithelium (10–24% versus 26–37%), whereas in an infertile CF patient with severe lung disease the level of correctly spliced transcripts was low in both tissues (6% in both). Such an association was also shown for the 3849+10 kb C→T mutation (Chiba-Falek et al. 1999). An inverse correlation was found between the level of correctly spliced transcripts and disease severity in several organs of an aborted fetus. Higher levels of correctly spliced transcripts were found in the unaffected trachea, colon, and lung (17–26%) and a very low level was detected in the severely affected ileum of the fetus (1%).

Taken together, the level of correctly spliced transcripts inversely correlates with disease severity, among the same tissue of different patients and among different organs of the same patient. It should be noted that there is no clear threshold for the mild CF disease or between mild to severe

disease. The level of correctly spliced transcripts in patients with mild disease varies with a large range of 4–50%, whereas severe disease is caused by lower levels of correct transcripts (0–3%). Some carriers of CFTR mutations (which probably have high level of correct transcripts, of over 50%) may have increased susceptibility to certain pulmonary diseases, such as asthma.

4
Splicing Modulation by Splicing Factors

The correlation between levels of correctly spliced RNA and disease severity (Sect. 3) suggested that the level of correctly spliced RNA is the molecular basis for disease variability, among patients carrying splicing mutations. Splicing is regulated through the interaction of a complex repertoire of splicing factors with various splicing motifs (reviewed in Black 2003). Differences in the levels of functional splicing factors were found among different tissues, which have been suggested to regulate the tissue-specific levels of alternatively spliced transcripts. It was therefore hypothesized that splicing regulation plays a role as a genetic modifier of disease severity, in patients carrying splicing mutations (Nissim-Rafinia and Kerem 2002).

4.1
Splicing Modulation of CFTR Minigenes Carrying Splicing Mutations

As the first step in understanding the role of splicing regulation as a modifier of the CF disease, the effect of overexpression of splicing factors on the level of correctly spliced CFTR transcripts was studied in minigenes carrying CFTR splicing mutations (Aznarez et al. 2003; Nissim-Rafinia et al. 2000; Pagani et al. 2000; Pagani et al. 2003a; Pagani et al. 2003b). The studied mutations included the intronic 3849+10 kb C→T and IVS8–5T and the exonic mutations in exons 9, 12, and 13, as mentioned above (Sect. 2). The splicing factors used in these studies represent several major families. Included are the heterogeneous nuclear ribonucleoprotein A1 (from the hnRNP family), various factors from the SR protein family (ASF/SF2, SRp20, SRp30c, SRp40, SRp55, SRp75, and SC35), and Htra2-β1 and Htra2α from the SR-like family. Further information on these families of splicing factors can be found in Chap. 4 of this volume. Alternative splicing is also a major mechanism underlying gene expression of viral genes. There are viruses in which the alternative splicing process is controlled by the host cell splicing machinery, while others encode their own splicing factors. Among the latter is adenovirus-2, in which most

transcription units encode two or more alternatively spliced mRNA (Imperiale et al. 1995). The adenovirus-2 genome encodes two proteins that have antagonistic effects on alternatively spliced viral genes (Nordqvist et al. 1994). These proteins, E4-ORF3 and E4-ORF6, were shown to promote exon inclusion and skipping, respectively, on viral genes.

4.1.1 The 3849+10 kb C→T Minigene

Our group constructed minigenes carrying the cryptic 84 bp exon with the normal C or the mutant T in the 3849+10 kb position (Nissim-Rafinia et al. 2000). The normal minigene led to the generation of only correctly spliced transcripts (in which the 84 bp were excluded), as expected, whereas the mutant minigene led to aberrantly spliced transcripts only (in which the 84 bp were included), as seen in patients with a severe disease. The cellular splicing factors used to study the splicing modulation were ASF/SF2 and hnRNP A1. In addition, adenoviral splicing factors E4-ORF3 and E4-ORF6 were studied. Cotransfection of the cellular and viral splicing factors hnRNP A1 and E4-ORF6 with the mutant minigene promoted skipping of the 84 bp "exon." This led to the generation of correctly spliced transcripts that could not be detected without overexpression of the splicing factors. As expected, all splicing factors had no effect on the splicing pattern of the normal minigene.

4.1.2 The IVS8–5T Minigene

Several minigenes carrying the IVS8–5T were constructed to study the effect of cis (splicing motifs) and trans (splicing factors) elements on the splicing pattern of exon 9 (Hefferon et al. 2002; Nissim-Rafinia et al. 2000; Pagani et al. 2000). All the constructed minigenes led to the generation of both correctly and aberrantly spliced transcripts. However, their level varied with the length of the adjacent TG repeat, as found in patients and healthy individuals. Interestingly, the splicing pattern varied also among cell lines into which the minigenes were transfected (Nissim-Rafinia et al. 2000). The effect of splicing factors on the splicing pattern of the IVS8–5T minigenes was studied by overexpression of the cellular splicing factor hnRNP A1, various factors from the SR protein family (ASF/SF2, SRp20, SRp30c, SRp40, SRp55, SRp75, and SC35) and the viral factors E4-ORF3 and E4-ORF6. Each of the cellular splicing factors promoted skipping of exon 9, which resulted in an increase in the level of aberrantly spliced transcripts. The viral factor E4-ORF3 promoted inclusion of exon 9, which resulted in a decrease in the level of aberrantly spliced transcripts. The viral factor E4-ORF6 had no effect on the IVS8–5T (Nissim-Rafinia et al. 2000; Pagani et al. 2000).

In order to study the contribution of the various IVS8 poly-T alleles in regulating the effect of splicing factors on the splicing pattern of CFTR

exon 9, minigenes carrying the IVS8-7T and IVS8-9T were also studied (Nissim-Rafinia et al. 2000; Pagani et al. 2000). As seen in the population, the level of aberrantly spliced RNA transcribed from the IVS8-9T, 7T and 5T minigenes inversely correlated with the length of the poly-T tract. The effect of overexpression of splicing factors on the splicing pattern of exon 9 was correlated with the length of the poly-T tract. The splicing pattern of the 5T minigene was significantly modulated by most studied splicing factors. The 7T minigene was moderately modulated only by several splicing factors (E4-ORF3 and ASF/SF2), while no effect was detected on the 9T minigene with all the studied factors (Nissim-Rafinia et al. 2000). Moreover, the effect of overexpression of splicing factors on the splicing pattern of IVS8–5T minigenes inversely correlated with the TG length, such that longer repeats were modulated less efficiently than shorter repeats (Buratti et al. 2001).

Several splicing enhancers and silencers were shown to contribute to the determination of exon 9 splicing pattern. It includes an exonic splicing enhancer (ESE) and silencer (ESS) and an intronic splicing silencer (ISS) in exon 9 and its downstream intron (Pagani et al. 2000). The promotion of exon 9 skipping by several SR proteins (ASF/SF2, SRp55 and SRp75) was reduced upon deletion of the ESS and ISS elements. In addition, binding assays showed that SR proteins bind the ISS *in vitro*. Thus, the studied SR proteins affect the splicing pattern of exon 9 by binding to its inhibitory elements (Pagani et al. 2000). Binding assays also revealed a novel splicing factor, TDP-43, which binds the TG repeat, leading to exon 9 skipping (Buratti et al. 2001). However, Hefferon et al. showed that another mechanism might play a role in exon 9 splicing regulation. They have shown that the TG tract regulates exon 9 splicing by the formation of an RNA secondary structure (Hefferon et al. 2004).

Another study investigated whether in addition to the effect of the TG and the T tracts, the splicing pattern is affected by the exon 9 donor site and by acceptor sites of exons 8 and 10 (Hefferon et al. 2002). The results showed that exon 9 donor site deviates from the consensus sequence. Indeed, strengthening this donor site led to a significant decrease in the level of exon 9 skipping. In addition, the exon 8 donor site has an effect on the exon 9 splicing pattern. Strengthening the exon 8 donor site, which also deviates from the consensus, led to higher level of exon 9 skipping, suggesting that the exon 8 donor site competes with the exon 9 donor site as a splicing signal. This might result from a competition between exon 8 with exon 9 donor sites, as splicing signals.

4.1.3 Exon 9 Minigene Carrying the A455E Mutation

The effect of several exon 9 mutations on the splicing pattern of this exon has been studied in minigene systems (Pagani et al. 2003a). The results show that different mutations have various effects. Several mutations promote exon 9 skipping (Q414X, G424S, I444S, and A455E), while Q452P

promotes exon inclusion. As expected, not all studied mutations in exon 9 had an effect on its splicing pattern (N418S, D443Y, and V456F). SR-motif-score matrices were calculated to investigate whether the mutations that lead to aberrant splicing do so through disruption of SR-binding ESEs. However, none of the mutations changed any known SR motif. Interestingly, Q452P created a new SC35 motif, thus SC35 might have promoted inclusion through binding to this site. The effect of ASF/SF2 on the splicing pattern of a minigene carrying the A455E mutation was further studied. Overexpression of ASF/SF2 promoted exon 9 skipping and led to higher levels of aberrantly spliced mRNA transcribed from this minigene. It should be noted that ASF/SF2 had the same effect on a minigene carrying normal exon 9 (Pagani et al. 2003a) indicating that the A455E mutation does not disrupt the ASF/SF2 binding site. Hence, it is suggested that the mutation might disrupt the binding of another yet unidentified splicing factor that promotes exon 9 inclusion.

4.1.4
Exon 12 Minigenes Carrying the D565G and G576A Mutations

As mentioned in Sect. 2, exon 12 undergoes partial (5%–30%) aberrant skipping in both normal individuals and CF patients (Bienvenu et al. 1996; Bremer et al. 1992; Slomski et al. 1992). The molecular basis for this aberrant skipping could be the exon 12 donor site, which deviates from the consensus in position +4 (contains a T instead of the consensus A) (Pagani et al. 2003b). Indeed, a minigene carrying exon 12 generated low levels of exon skipping (~15%), while a minigene with the consensus A, led to a complete inclusion of exon 12 (Pagani et al. 2003b). Exonic regulatory elements (CERES) were found to reside within exon 12. The missense mutations D565G and G576A that reside within the regulatory elements, promote exon 12 skipping in individuals as well as in minigenes carrying these mutations (Pagani et al. 2003b). The D565G mutation promoted high levels (~60%) of exon 12 skipping, whereas the G576A mutation resulted in nearly 100% exon 12 skipping. Thus, the studied mutations disrupt elements that promote exon inclusion. Cotransfection of the cellular splicing factors hnRNP A1, ASF/SF2, SRp40, SRp55, and SRp75, either with the normal or the mutant minigenes, led to an increase in exon 12 skipping, whereas PTB had no effect on the splicing pattern of exon 12.

4.1.5
Exon 13 Minigenes

Skipping of the first 248 nucleotides of exon 13 was found among several CF patients, in which no mutations were detected (Aznarez et al. 2003; Hull et al. 1994). These aberrant spliced transcripts resulted from the selection

of a cryptic 3′ spliced site in exon 13. Mutagenesis of a minigene carrying exon 13 revealed an ESE motif between the cryptic and the correct 3′ spliced site (Aznarez et al. 2003). The effect of several CFTR mutations (D651N, E656X, 2108delA, E664X, and T665S), located within or near the ESE motif, on the splicing pattern of exon 13 was studied in this minigene system. It was found that these exonic mutations disrupt the correct splicing of exon 13. Overexpression of the cellular splicing factors ASF/SF2 and Htra2α resulted in modulation of the splicing pattern of exon 13. ASF/SF2 promoted skipping of the 248 bp in minigenes carrying the E664X and T665S, increasing the level of aberrant spliced transcripts, whereas Htra2α promoted inclusion of the 248 bp in the E656X and 2108delA minigenes, increasing the level of correctly spliced transcripts.

Taken together, the results of these studies show that all (n=10) the minigenes can be modulated by overexpression of splicing factors (Table 1). This splicing modulation depends on intronic and exonic cis elements, which are probably the binding sites of various splicing factors. Importantly, higher levels of correctly spliced transcripts were generated by several splicing factors (Table 1).

4.2 Splicing Modulation of Endogenous CFTR Allele Carrying 3849+10 Kb C→T Splicing Mutations and Restoration of the CFTR Function

In order to investigate whether higher levels of correctly spliced transcripts can restore the CFTR function, cells comprising the entire CFTR coding region carrying a splicing mutation were studied (Nissim-Rafinia et al. 2004). An epithelial cell-line (CFP15a) from a nasal polyp of a CF patient, carrying the 3849+10 kb C→T splicing mutation was established to analyze the effect of overexpression of splicing factors on the splicing pattern and protein function. Overexpression of Htra2-β1, SC35, and ASF/SF2 promoted skipping of the 84 bp exon and led to an increase in the level of correctly spliced transcripts, while SRp20, hnRNP A1, and E4-ORF6 had no significant effect (Fig. 1b). E4-ORF3 promoted inclusion and led to a significant increase in the relative level of aberrant transcripts. Thus, overexpression of splicing factors can modulate the splicing pattern of the cryptic 84 bp exon, in RNA transcribed from endogenous CFTR alleles carrying the 3849+10 kb C→T mutation. It should be noted that hnRNP A1 and E4-ORF6, which promoted skipping of the 84 bp in the minigene carrying the 3849+10 kb C→T mutation, also promoted skipping of the endogenous allele, but the effect was not statistically significant (Nissim-Rafinia et al. 2004). This difference might reflect previously identified variability among cell lines (Nissim-Rafinia et al. 2000; Pagani et al. 2003b). In addition, ASF/SF2, which had no effect on the splicing pattern of the 3849+10 kb

Table 1. Splicing modulation by overexpression of splicing factors

Mutation	Location	Affected Exon/ Intron	Splicing factor	Effect	Correction	Reference[a]
A445E	Exon 9	–exon 9	ASF/SF2	Skipping	–	Pagani (2003a)
IVS8-5T	Intron 8	–exon 9	ASF/SF2	Skipping	–	Nissim-Rafinia (2000); Pagani (2000)
			SRp20, SRp30c	Skipping	–	
			SRp40, SRp55	Skipping	–	
			SRp75, SC35	Skipping	–	
			hnRNP A1	Skipping	–	
			E4-ORF3	Inclusion	+	
			E4-ORF6	–	–	
D565G	Exon 12	–exon 12	ASF/SF2	Skipping	–	Pagani (2003b)
			SRp40, SRp55	Skipping	–	
			SRp75	Skipping	–	
			hnRNP A1	Skipping	–	
			PTB	–	–	
G576A	Exon 12	–exon 12	ASF/SF2	Skipping	–	Pagani (2003b)
			SRp40, SRp55	Skipping	–	
			SRp75	Skipping	–	
			hnRNP A1	Skipping	–	
			PTB	–	–	

D651 N	Exon 13	−248 bp	ASF/SF2	Skipping	−	Aznarez (2003)
E656X	Exon 13	−248 bp	Htra2α	Inclusion	+	Aznarez (2003)
2108delA	Exon 13	−248 bp	Htra2α	Inclusion	+	Aznarez (2003)
E664X	Exon 13	−248 bp	ASF/SF2	Skipping	−	Aznarez (2003)
T665S	Exon 13	−248 bp	ASF/SF2	Skipping	−	Aznarez (2003)
3849+10 kb C→T	Intron 19	+84 bp	hnRNP A1	Skipping	+	Nissim-Rafinia (2000)
			E4-ORF6	Skipping	+	
3849+10 kb C→T[b]	Intron 19	+84 bp	ASF/SF2	Skipping	+	Nissim-Rafinia (2004)
			SC35	Skipping	+	
			Htra2-β1	Skipping	+	
			E4-ORF3	Inclusion	−	

[a]Et al. in each case

[b]All the information in the table was obtained from minigene studies, except for this row. It was obtained from a cell line comprising the endogenous allele carrying the splicing mutation

C→T minigene, promoted skipping of the 84 bp in the endogenous allele. This can be either due to variation among different cell lines, or because of lack of ASF/SF2 binding sites in the minigene.

The CFTR protein is a cAMP-stimulated Cl^- channel that also regulates other transport proteins. To study the effect of splicing modulation on the CFTR function, cells carrying the 3849+10 kb C→T mutations (CFP15a) were analyzed (Nissim-Rafinia et al. 2004). The CFTR channel in these cells is inactive (Fig. 1c). Overexpression of the splicing factors Htra2-β1 and SC35, which increased the level of correctly spliced transcripts, activated the CFTR channel and restored its function (Fig. 1c). In contrast, splicing factors that did not increase the level of correctly spliced transcripts (SRp20, hnRNP A1, E4-ORF6, and E4-ORF3) did not restore the CFTR function, further indicating that the functional restoration resulted from the increase in the level of correctly spliced CFTR transcripts. Restoration of the CFTR function is expected to result from increasing the level of normal and full-length CFTR transcripts. Since several CFTR exons undergo in some individuals partial aberrant splicing, these exons might also be modulated by overexpression of splicing factors, leading to a decrease in the level of full-length CFTR transcripts. This was shown for overexpression of ASF/SF2, which promoted skipping of exon 9 and significantly increased the level of aberrantly spliced transcripts. Taken together, Htra2-β1 and SC35 increased the level of normal and full-length transcripts sufficiently for activation of the CFTR function, whereas ASF/SF2, which promoted skipping of the cryptic 84 bp exon, also promoted skipping of the essential exon 9, indicating that the level of full-length CFTR transcripts was insufficient for CFTR activation (Nissim-Rafinia et al. 2004). A recent study in mice identified a putative splicing factor (SCNM1) that modifies disease severity in mice homozygous for a splicing mutation in the Scn8a gene. Mice carrying normal alleles of SCMN1 show chronic movement disorder, however, mice carrying a stop mutation in this gene show reduced levels of correctly spliced Scn8a transcripts and a lethal neurological disease (Buchner et al. 2003).

Taken together, the results from humans and mice support the hypothesis that the splicing machinery is a modifier of disease severity in patients carrying splicing mutations.

5
Splicing Modulation by Small Molecules

The effect of small molecules on splicing factors was recently initiated. The effect of the histone deacetylase inhibitor, sodium butyrate (NaBu), on the CFTR function was analyzed, on the basis of previous studies showing that NaBu up-regulated the expression of Htra2-β1 and SR proteins (Brichta

et al. 2003; Chang et al., 2001). In CFP15a cells, NaBu affected the splicing pattern in a concentration-dependent manner. NaBu in the range 5 μM–250 μM promoted skipping of the 84 bp and had no effect on other CFTR exons, nor did lower and higher concentrations have such effect. Activation of the CFTR channel was also achieved by 5 μM–250 μM NaBu, which significantly increased the level of normal CFTR transcripts. Other concentrations had no effect on the CFTR Cl^- efflux, in agreement with the RNA results. These results indicate that the NaBu restoration in CFP15a cells was obtained through modulation of the CFTR splicing pattern.

Since NaBu has the potential to up-regulate gene expression (Brichta et al. 2003; Chang et al. 2001), the possibility that NaBu, in addition to its effect on the splicing pattern, up-regulated the expression of the CFTR gene in CFP15a cells was evaluated. The results revealed no change in the level of CFTR RNA after treatment with NaBu, indicating no effect on the expression of the CFTR gene. Thus, the restoration of the CFTR function by NaBu resulted from splicing modulation only. This splicing modulation is likely to occur through up-regulation of expression of splicing factors.

6
Splicing Modulation by Antisense Oligonucleotides

Correction of the splicing pattern of the 3849+10 kb C→T mutation was also investigated using antisense oligonucleotides (Friedman et al. 1999). Antisense oligonucleotides toward the exon-recognition motifs of the cryptic 84 bp exon, including the aberrant donor and acceptor splice sites or to the 84 bp exon, were shown to disfavor aberrant splicing and enhance an increase in the level of the correctly spliced transcripts. This antisense-mediated correction of splicing was dose and sequence dependent and was accompanied by increased production of CFTR protein that was appropriately glycosylated. However, the effect of this increase on the protein function was not studied.

The various studies discussed in this chapter show that variability in the regulation of the splicing machinery might lead to variations in the level of correctly spliced CFTR mRNA, transcribed from CFTR alleles carrying splicing mutations. This might directly affect the amount of functional proteins and the CF disease severity. Lower levels of correctly spliced transcripts might generate lower levels of functional proteins, leading to a more severe disease. The results from splicing modulation experiments support the role of splicing regulation as a genetic modifier of disease severity. Furthermore, restoration of the CFTR function by splicing modulation provides direct evidence that increasing the level of normal transcripts can restore the protein function. These results explain the correlation between the level of correctly spliced CFTR transcripts and disease severity and

provide the required basis for the development of therapeutic approaches for patients carrying splicing mutations in CF as well as in other human diseases.

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