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## Thesis

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**Characterization of the mechanisms behind the alternative splicing of the mutually exclusive exons 18N and 18A in the sodium channel gene SCN8A and mutually exclusive exons 5N and 5A in the sodium channel gene SCN9A**

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A Thesis Submitted in Fulfillment of the Requirements of the Open University, (UK)  
for the Degree of Doctor of Philosophy

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Trieste, Italy

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June 2011

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# Abbreviations

The standard abbreviations used in this dissertation follow IUPAC rules. All the abbreviations are defined also in the text when they are introduced for the first time. The abbreviations mentioned only once are not included in this list.

bp	Base pairs
nt	Nucleotides
aa	Amino acid
kb	Kilobase
kDa	Kilodalton
dNTPs	Deoxynucleoside triphosphate (A, C, G and T)
N	Nucleotide (A or C or G or T)
DNA	Deoxyribonucleic acid
cDNA	Copy DNA
ESE	Exonic Splicing Enhancer
ISE	Intronic Splicing Enhancer
ESS	Exonic Splicing Silencer
ISS	Intronic Splicing Silencer
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein particles
hnRNP	Heterogenous ribonuclear protein
SR	Arginine-serine rich protein
ss	Splice site
NMD	Nonsense-mediated decay
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
IPTG	Isopropyl- $\beta$ -d-thiogalactopyranoside
TBE	Tris-borate-EDTA (buffer)
PBS	Phosphate buffer saline
ddH <sub>2</sub> O	Double-distilled water
Pu	Purine

## Abstract

Voltage-gated sodium channels are the primary molecules responsible for the rising phase of action potentials in electrically excitable cells. There are 10 distinct sodium channel isoforms  $Na_v$  1.1-1.8 (SCN1A-SCN5A and SCN8A-SCN11A) and the majority of these undergo tissue and developmentally regulated alternative splicing. Two such examples are those of the SCN8A (Nav 1.6) and SCN9A (Nav 1.7) genes. SCN8A gene contains two mutually exclusive exons, 18N and 18A. Transcripts with exon 18N have a conserved in-frame stop codon that predicts the synthesis of a truncated, non functional sodium channel. This protein is expressed in fetal brain and non-neuronal tissues. Once the exon 18A is included, the resulted protein will be a functional channel, that is expressed in adult neurons of CNS and PNS. The SCN9A exon 5N is preferentially expressed in the PNS and CNS of adult tissues and significant usage of exon 5A was found only in DRG. These two isoforms differ in one amino acid in the S3 domain I (exons 5A and 5N). This change of one amino acid induced a small shift of activation to more hyperpolarized potentials for exon 5A compared with exon 5N. Analysis of SCN8A pre-mRNA splicing supports a model in which exon 18A exclusion in non-neuronal tissue is regulated primarily by the presence in the cell types of several hnRNPs proteins that function through an exonic splicing silencer (ESS) found in this exon together with the absence of neuron specific Fox-1 protein. In neuronal cells the absence of these hnRNPs together with the presence of neuron specific Fox-1 cause the exon to be included. The SCN8A exon 18N is included in non neuronal cells due to the SR proteins that function through an exonic splicing enhancer (ESE) found in this exon. In neuronal cells the lower levels of these SR proteins cause the exon 18N to be skipped. This type of control of mutually exclusive splicing through the proteome make-up of a cell type would appear to be influential in the temporal and tissue specific splicing of SCN8A, another member of the voltage gated sodium channels and may indeed represent a more general mechanism.

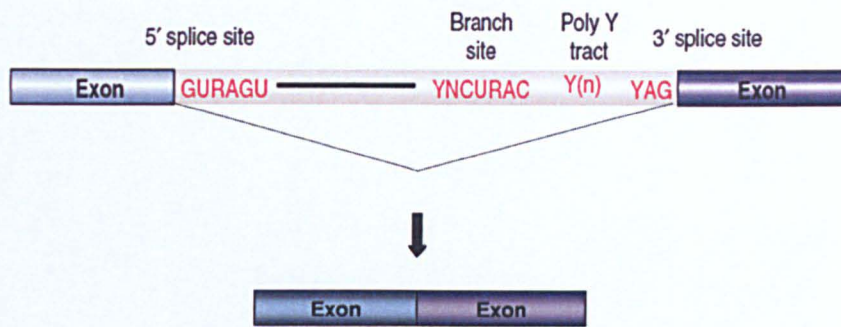
# 1. Introduction

## 1.1 An overview of Pre-mRNA splicing.

The initial primary transcript, precursor RNA (pre-mRNA) is synthesized by RNA polymerase II (Pol II) and is extensively processed before functional mRNA exits the nucleus to be translated into protein in the cytoplasm. Before this final step can occur the pre-mRNA undergoes several modifications that can occur both co and post transcriptionally. Among these, are steps such as the addition of a 7-methyl guanosine cap at the 5' end, splicing of introns, and 3' end formation by cleavage and polyadenylation (de Almeida and Carmo-Fonseca 2008). Splicing and other RNA processing events occur while RNA polymerase II actively transcribes pre-mRNA (Maniatis and Reed 2002).

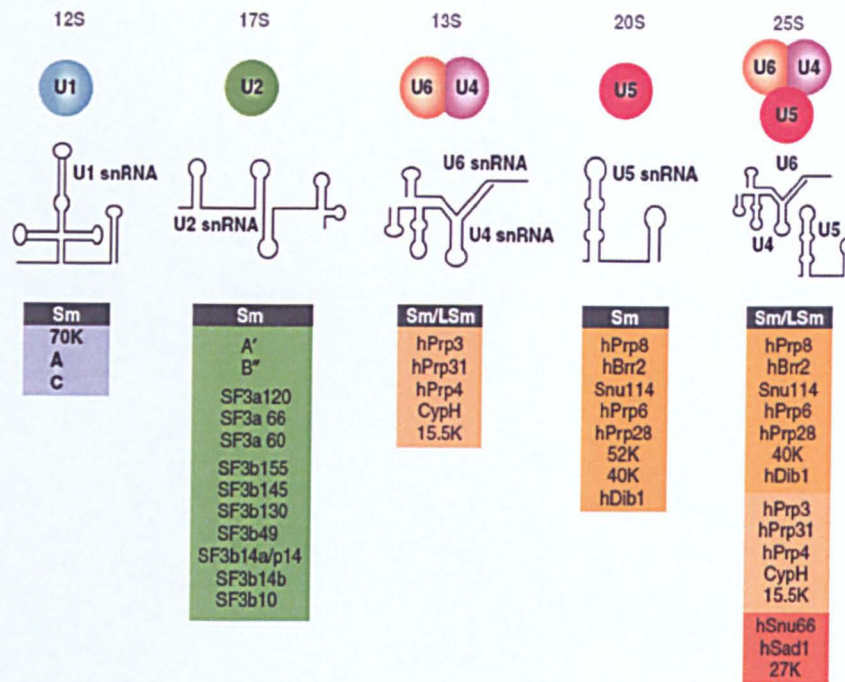
Pre-mRNA splicing is the reaction where introns (non coding intervening sequences present in pre-mRNA) are removed and exons (protein coding regions that will be included in the mature mRNA) are joined together (Gilbert 1978).

To dictate splicing, each intron is marked by a GU dinucleotide at the 5' end and a AG dinucleotide at the 3' end, although exceptions exist (Kreivi and Lamond 1996). These nearly 100% conserved dinucleotides at either end of the intron, in reality, form part of more loosely defined sequences known respectively as the 5' and 3' splice sites. The 5' splice site is a degenerate 8-nucleotide motif with a consensus motif MAG/GTRAGT (where M is A or C and R is A or G) and the holo-3' splice site which is even more loosely defined being composed of three elements: the 3' splice site that has a consensus CAG among which again only dinucleotides AG are universally conserved, a branch point (BP) consensus in which an A nucleotide is universally conserved, and a polypyrimidine tract which are usually spread across the last 40 nucleotides of the intron but they can take up considerably more space (Fig. 1) (Gooding, Clark et al. 2006; Venables 2007).



**Figure 1.** Schematic representation of exon-intron boundaries and consensus sequences for 5' and 3' splice sites and branch point. The two exons and the intron are indicated. Poly (Y) tract means region rich in pyrimidines. The universally conserved nucleotides are the dinucleotide cores of the 5' and 3' splice sites GU and AG respectively together with the branch point (A).

Intron removal is carried out by the spliceosome, a dynamic multi subunit particle containing five small ribonuclear proteins snRNPs, U1, U2, U4, U5 and U6 that function in conjunction with over 200 distinct non-snRNP auxiliary proteins (Hartmuth, Urlaub et al. 2002; Jurica, Licklider et al. 2002; Nilsen 2003; de Almeida and Carmo-Fonseca 2008). Each U snRNP consists of an snRNA (or two in the case of U4/U6), a common set of seven Sm proteins (B/B', D3, D2, D1, E, F, and G) and a variable number of particle-specific proteins (Fig. 2). The most probable secondary structures of the human spliceosomal U1, U2, U4/U6, and U5 snRNAs are shown in Figure 2.

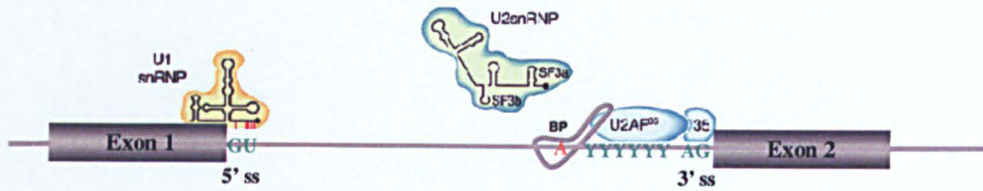


**Figure 2.** Protein composition and snRNA secondary structures of the human spliceosomal snRNPs. All seven Sm proteins (B/B', D3, D2, D1, E, F, and G) or LSm proteins (LSm2-8) are indicated by "Sm" or "LSm" at the top of the boxes showing the proteins associated with each snRNP. The U4/U6.U5 tri-snRNP contains two sets of Sm proteins and one set of LSm proteins (Figure from (Will and Luhrmann)).

Initial recognition of exon/intron junction is based on direct interactions between U1 snRNP with the 5' splice site, the U2 auxiliary factor (U2AF65/35) with the polypyrimidine tract, and U2 snRNP with the branch point sequence (Fig. 3). The interactions between the pre-mRNA and snRNPs are based on their sequence specificity, thus the splice sites are classified by their complementarity to U1 snRNA 5' splice site and the extent of the polypyrimidine tract. Greater complementarity with U1 snRNA and longer polypyrimidine tracts translate into higher affinity binding sites for these spliceosome components and consequently more efficient exon recognition (Hertel 2008). The possibility of ascribing a strength to the 5' and 3' splice site has been the focus of



many studies principally based on position weight matrices that are calculated from collections of splice sites (Shapiro and Senapathy 1987; Senapathy, Shapiro et al. 1990). To date we are still unable given a stretch of DNA if a 8 nucleotide stretch will function as a 5' splice sites or not, nor indeed if a 3' splice site conforming to consensus will do so.

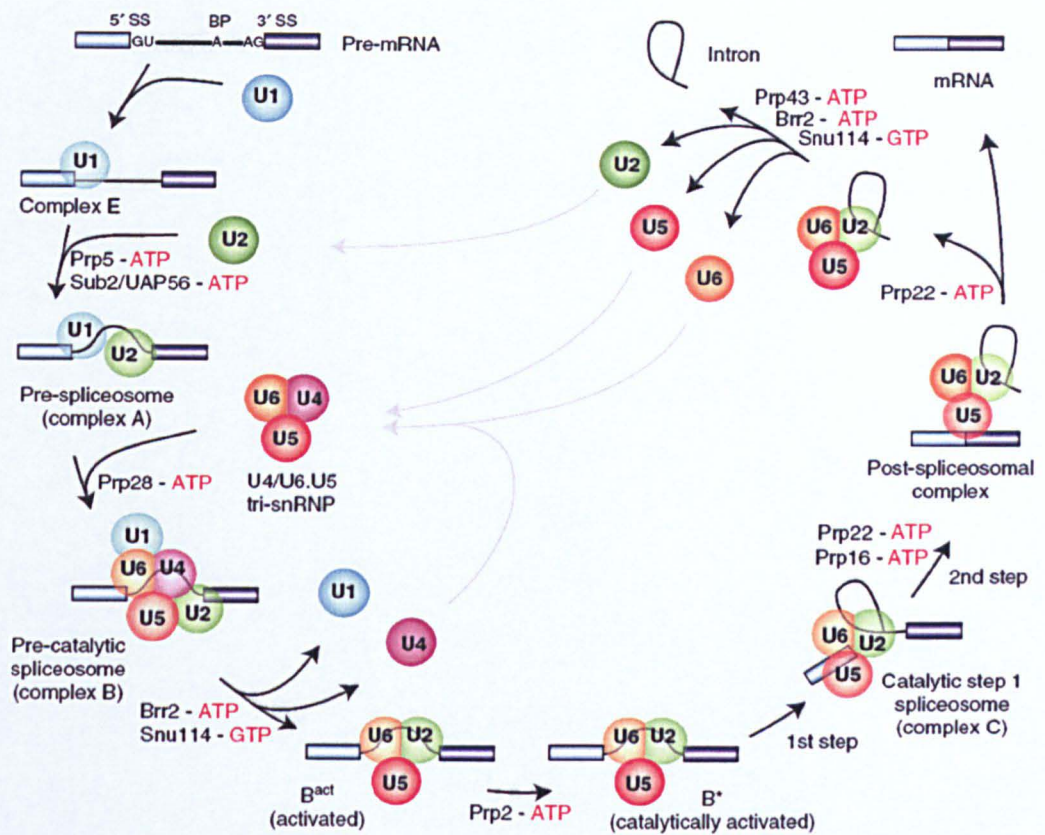


**Figure 3.** Schematic representation of the direct interactions between U1 snRNP with the 5' splice site, the U2 auxiliary factor, U2AF65 and U2AF35, with the polypyrimidine tract and the 3' splice site respectively, and U2 snRNP with the branch point sequence.

### 1.1.1 Spliceosome complex formation.

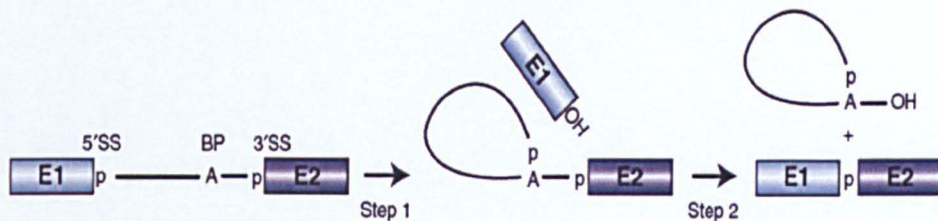
The assembly of the spliceosome has been characterized principally using *in vitro* systems where several distinct intermediates in an assembly pathway can be observed (Query, Strobel et al. 1995). The first complex to be formed is a nonspecific, H complex, and consists of heterogeneous nuclear ribonucleoproteins, hnRNPs, binding with pre-mRNA (Konarska, Grabowski et al. 1985). H complex formation does not require splice sites or ATP and occurs *in vitro* even at 4°C. As mentioned previously, the 5' splice site of an intron is recognized by the snRNP U1, through complementary pairing of the RNA bases. The 3' splice site, with its upstream polypyrimidine tract, is bound by the protein U2

auxiliary factor U2AF, which is a heterodimer that includes U2AF65, a protein containing tandem RNA recognition motifs that binds tightly to the polypyrimidine tract, and U2AF35 which binds the real 3' splice site (Brow 2002). These factors, together with additional proteins, form a first discrete functional spliceosome complex called the E or commitment complex, which bridges the intron and brings the splice sites that are to be cleaved together. E complex formation requires incubation *in vitro* at 30°C and assembles more efficiently on pre-mRNAs containing both a 5' splice site and the polypyrimidine tract at the 3' splice site. However, there are also complexes related to the E complex, so called E5' and E3' complexes, that assemble in half-substrate RNAs containing only a 5' or a 3' splice site, respectively (Michaud and Reed 1993). U2AF recruits the U2 snRNP, and an ATP dependent step allows the RNA portion of the U2 snRNP to base pair with a branchpoint, upstream of the 3' splice site. This base pairing of the U2 with the branch point completes the A complex. Recently, Kotlajich et al, carried out a series of kinetic trap experiments using pre-mRNAs that undergo alternative 5' splice site selection or alternative exon inclusion and, in both cases, they showed that commitment to splice site pairing occurs in the A complex (Kotlajich, Crabb et al. 2009). Subsequent, ATP-dependent steps lead to the binding of the U4-U5-U6 tri-snRNP and the formation of the B complex. Different rearrangements that follow, with the subsequent formation of the catalytic spliceosome C complex, in which two transesterification reactions on the splice sites occurs (Fig. 4).



**Figure 4.** Spliceosome assembly. In the earliest E spliceosomal complex, the U1 snRNP is recruited to the 5'ss. In a subsequent step, the U2 snRNP stably associates with the BP, forming the A complex. The U4/U6.U5 tri-snRNP is then recruited, generating the pre-catalytic B complex. Major rearrangements in RNA–RNA and RNA–protein interactions, leading to the destabilization of the U1 and U4 snRNPs, give rise to the activated spliceosome (the B<sup>act</sup> complex). Subsequent catalytic activation by the DEAH-box RNA helicase Prp2, generates the B catalytically activated complex, which catalyzes the first of the two steps of splicing. This yields the C complex, which in turn catalyzes the second step. The spliceosome then dissociates and, after additional remodeling, the released snRNPs take part in additional rounds of splicing (Figure from (Will and Luhrmann)).

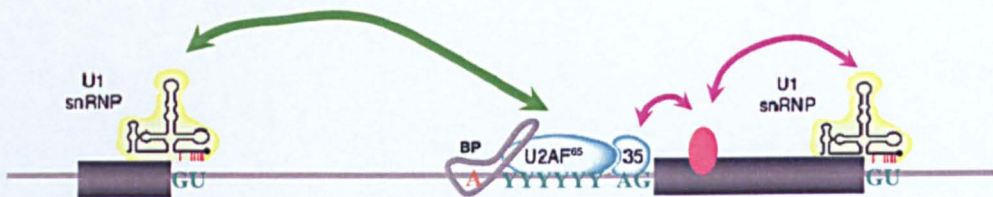
During the first step of transesterification, an adenosine residue generally located within 100 nucleotides of the 3' end of the intron, in a sequence element known as the branch point sequence (BP), carries out a nucleophilic attack on the 5' splice site. This reaction generates the splicing intermediates (free exon 1 and lariat-exon 2). During step 2, exon 1 attacks at the 3' splice site to generate splicing products (spliced exon and lariat intron) (Fig. 5).



**Figure 5.** Schematic representation of sequential transesterification during splicing. In the first reaction, an adenosine residue of the 3' end of the intron, in a sequence element known as the branch point sequence (BP), carries out a nucleophilic attack on the 5' splice site. This reaction generates the splicing intermediates (free exon 1 and lariat-exon 2). During step 2, exon 1 attacks at the 3' splice site of exon 2 to generate splicing products (spliced exon and lariat intron) (Figure from (Will and Luhrmann)).

Spliceosome assembly in cells however is likely to differ from what is happening on simple single-intron RNAs *in vitro*. Before the spliceosome complex forms across the intron, the splice sites are usually defined through interactions between splice-site-bound components across the exons. The assembly of these “exon definition” complexes (EDCs) is stimulated by proteins that bind to special sequences in the exon itself. In particular, E,

A and B complexes have primarily been analyzed and defined in studies using simple two exon/single intron constructs, such that the spliceosome components can only assemble across the intron (Fig. 6). However, a number of latter findings have shown that at least U1 and U2AF subunits of the spliceosome E complex can interact across the single exon as part of an EDC that is essential for high fidelity splice site recognition. Therefore, it is possible for the U1 and U2 snRNPs to first interact across a single exon before pairing with spliceosome components on a flanking exon, indicating that U1 and U2 containing (that is, A-like) EDC may be able to form on natural substrates as part of the spliceosome assembly pathway (Berget 1995; Reed 2000); (House and Lynch 2006).



**Figure 6.** A network of protein-protein interactions across the exon and intron. Before the spliceosome complex forms across the intron (double-headed green arrow), the splice sites are usually defined through interactions between splice-site-bound components across the exons (double-headed pink arrow). The assembly of these “exon definition” complexes (EDCs) is stimulated by proteins that bind to special sequences in the exon itself.

## **1.2 Alternative splicing: where a common precursor pre-mRNA molecule can generate multiple mRNAs.**

As previously explained, the splicing reaction occurs in two transesterification steps within a large spliceosome complex containing more than 100 proteins assembled stepwise onto pre-mRNA introns (Zhou, Licklider et al. 2002). During alternative splicing, the spliceosome assembly is altered so that a splice site is optionally used depending on the cell type, developmental stage or sex, resulting in the inclusion or exclusion of alternative exon sequences in the mature mRNA. Alternative splicing is therefore a process by which the exons of the RNA produced by transcription are reconnected in multiple different ways increasing greatly protein diversity.

Estimates of how commonly alternative splicing occurs in human protein coding genes have increased over the years, from an initial 5 % to more than 95 % (Calarco, Zhen et al.; Sharp 1994). Recent studies have suggested that alternative splicing is nearly ubiquitous in human transcripts and is frequently controlled in a tissue specific manner (Pan, Shai et al. 2004; Wang, Sandberg et al. 2008). In particular, extensive tissue specific alternative splicing has been observed in the mammalian nervous system and is thought to contribute to both its molecular and cellular complexity (Lipscombe 2005). In some cases a single pre-mRNA can generate a large number of protein isoforms, for example, transcripts of the *Dscam*, *Neurexin* and *Slo* genes (Saito, Nelson et al. 1997; Graveley 2001; Xie 2008). The consequences of alternative splicing include altered mRNA stability or subcellular localization and the addition or deletion of specific protein coding sequences. Functional differences among protein isoforms range from subtle modulations to on/off switches or even antagonistic effects (Shipston 2001; Revil, Shkreta et al. 2006)). These mechanisms of alternative splicing are so tightly controlled that even subtle defects in alternative splicing factors or aberrant inclusion of alternative exon can result in genetic diseases

(Hammes, Guo et al. 2001; Faustino and Cooper 2003; Kashima and Manley 2003; Licatalosi and Darnell 2006).

Studies with tissue or sex-specific exons identified both *cis*-acting pre-mRNA elements and trans acting factors that control alternative splicing (Black 2003). Furthermore, pre-mRNA structures are also important for alternative splicing regulation (Grover, Houlden et al. 1999; Xing and Lee 2006). In addition to cell type, developmental stage and sex-specific regulation, alternative splicing can also be dynamically regulated in response to extracellular stimuli such as cytokines, hormones or neurotransmitters, adding a further dimension to the control of the flow of genetic information (Xie and McCobb 1998; Stamm 2002; Lee, Yu et al. 2007). However, it is not clear in most cases how alternative splicing is controlled by external stimuli and intracellular signaling pathways (Rafuse and Landmesser 1996; Hepp, Dupont et al. 2001; Rozic-Kotliroff and Zisapel 2007).

### **1.2.1 Types of alternative splicing.**

Transcripts from a gene can undergo many different patterns of alternative splicing. Typical types of alternative splicing are inclusion or skipping of one or more exons (cassette exons), shortening or lengthening of an exon by alternative 5' and 3' splice site usages, mutual exclusion of two or more exons, and retained introns. Different promoters and different polyadenylation sites may specify alternative 5' and 3' terminal exons, respectively. More complex alternative splicing patterns can be formed by combinations of different basic types (Fig. 7).

Many alternatively spliced genes contain entire exons that are individually included or excluded from the mature mRNA. When such an exon is retained, the splicing pattern resembles that for a constitutive gene in which all potential coding sequences are incorporated into the mature mRNA. When it is removed, it is presumably carried on a

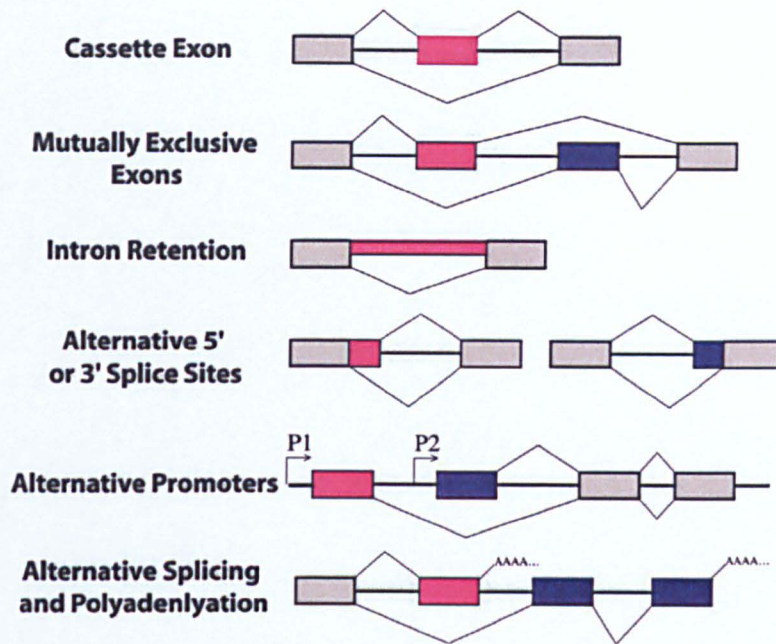
long intron that also contains its flanking noncoding sequences. Such alternatively spliced exons represent discrete cassettes of genetic information encoding peptide subsegments that are differentially incorporated into the mature gene product.

Cassette exons are delimited by splice sites that lie at the boundaries between mRNA-coding and noncoding sequence. While the exon itself may or may not be incorporated, its immediate flanking introns are invariably excluded in the splicing process. There are numbers of genes with internal alternative splice sites which, in contrast, actually lie entirely within potential coding sequence. Splicing at such a site results in the exclusion of some fraction of an otherwise intact exon. Through the use of alternative 5' or 3' splice sites, exons can be extended or shortened in length.

Several other genes incorporate intron sequence into mRNA by failing to splice both members of a donor/acceptor pair altogether. The retained intron necessarily maintains an intact translational reading frame and, in effect, creates a longer fusion exon.

A very special case of paired cassette exons show mutually exclusive splicing. Here, one exon or the other of the mutually exclusive exon pair is invariably spliced into a given mRNA, but the exclusion or inclusion of both simultaneously does not occur.





**Figure 7.** Modes of alternative splicing. Typical types of alternative splicing are inclusion or skipping of one or more exons (cassette exons), mutual exclusion of two or more exons, retained introns and shortening or lengthening of an exon by alternative 5' and 3' splice site. Different promoters (P) and different polyadenylation sites may specify alternative 5' and 3' terminal exons, respectively. In these graphics, exons are represented by boxes and introns by lines. Exon regions included in the messages by alternative splicing are colored while constitutive exons are shown in gray. Promoters are indicated with arrows and polyadenylation sites with AAAA.

### 1.2.2 Mutually exclusive splicing ; “when two is already a crowd”.

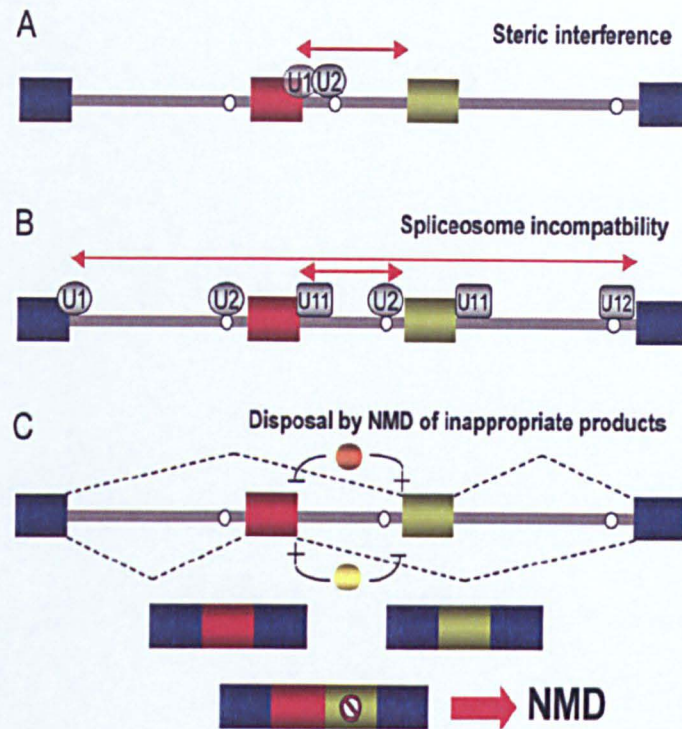
Mutually exclusive splicing (ME) is a process in which only one of a set of two or more exons in a pre-mRNA is included in the final transcript. This form of splicing plays an important role in gene evolution, since it provides the potential to modulate protein functions simply by swapping the mutually exclusive exons without disrupting protein size

or structure (Letunic, Copley et al. 2002). Furthermore, ME splicing can provide a multitude of distinct protein isoforms. An impressive example of ME alternative splicing has been reported for the *Drosophila* homolog of the human Down-syndrome adhesion molecule (*Dscam*) gene. The *Dscam* gene has 115 exons, 95 of which are alternatively spliced. Alternative exons are organized in four clusters with 12 alternative versions of exon 4, 48 versions of exon 6, 33 versions of exon 9, and 2 versions of exon 17. Only one version of a particular exon is chosen, with the exclusion of others, so that by the combinatorial use of alternative exons, the *Dscam* pre-mRNA can potentially encode 38,016 different isoforms. It has been demonstrated that conserved sequence elements in introns that forms RNA secondary structures play a functional role in the mutually exclusive splicing in *Dscam* gene (May, Olson et al.; Graveley 2005).

For genes with mutually exclusive exon pairs, no cellular mRNAs have yet been detected that incorporate both exons in vivo, despite the fact that the introns between them contain functional 5' and 3' splice sites (Mullen, Smith et al. 1991). Therefore the intriguing question is what are the mechanisms involved that prevents ME exons from being spliced to each other? To date different mechanisms have been investigated (Fig. 8). The first is steric interference between splice sites (Smith and Nadal-Ginard 1989). Minimal spacing requirements, around 50 nucleotides for mammalian introns, are necessary between the 5' splice site and the branch point. Below this threshold the distance between, U1 and U2 snRNPs is such that they cannot bind productively to the 5' splice site and the branch point respectively. Thus, the two exons remain free to become spliced to the flanking constitutive exons but not to their mutually exclusive partner. Examples include the  $\alpha$ -tropomyosin and  $\alpha$ -actinin genes (Smith and Nadal-Ginard 1989; Southby, Gooding et al. 1999). Another mechanism is that pairs of ME exons are flanked by splice sites of incompatible types. In addition to the major spliceosome, cells have a minor spliceosome in which the small nuclear ribonucleoproteins (snRNPs) U1 and U2 are replaced by U11 and U12. "U1/U2" and "U11/U12" splice sites have distinct consensus sequences and are

incompatible, so an intron with a U1 5' splice site and a U12 3' splice site cannot be spliced. An example of this type of control is the mutually exclusive splicing of exons 6 and 7 of the human stress-activated protein kinase (*JNK1*) gene that have U1/U2 introns on the upstream side and U11/U12 introns on the downstream side (Kreivi and Lamond 1996; Letunic, Copley et al. 2002). Spliceosomal incompatibility prevents not only splicing together of the ME exons but also exon skipping of both.

However, the majority of ME exon pairs do not have a mechanism that absolutely prohibits their splicing together and in these cases, regulated selection of the individual exons may be sufficiently coordinated to minimize inappropriate splicing without the need for an absolute physical impediment to double-exon inclusion (Grabowski and Black 2001). In these cases trans acting factors, acting through splicing regulatory *cis*-acting elements, functions as repressors of one ME exon and activators of its partner, allow the functional versatility to promote the required coordinated regulation. However, the appearance of strict ME splicing may occur even when the splicing machinery does not achieve the required degree of fidelity. If the two exons are not both a multiple of 3 nucleotides, the alteration in reading frame and consequent introduction of stop codon can lead to disposal by nonsense mediated decay (NMD) of products containing both ME exons and the degradation of a transcript. Close to 60% of predicted ME exons would produce frameshifts upon double inclusion so NMD play an important role in alternative ME splicing regulation (Jones, Carstens et al. 2001).



**Figure 8.** Mechanisms of Mutually Exclusive Splicing.

(A) Steric interference occurs when the branch point (white circle) of the downstream ME exon is too close to the upstream 5' splice site. Mutually exclusive exons are shown in red and green, constitutive exons in blue. (B) A model of spliceosomal incompatibility. The splice sites used by the “U1/U2” and “U11/U12” snRNP containing spliceosomes have distinct consensus sequences and are incompatible. For example, an intron with a U1 5' splice site and a U12 3' splice site cannot be spliced. In panels (A) and (B), impossible splice pathways are shown by the double-headed red arrows. (C) Mutually exclusive splicing can have tightly coordinated regulation by trans acting factors, indicated by the orange and yellow ellipses, some of which can act as both repressors and activators of ME exon pairs. In some cases, nonsense mediated decay (NMD) can dispose of mRNAs containing both ME exons if a premature termination codon is introduced (indicated by stop sign). Figure from (Smith 2005).

### **1.3 Splicing regulatory elements and associated factors.**

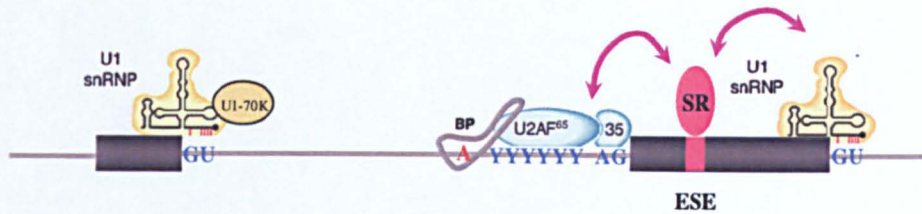
As I previously mentioned the *cis*-acting elements which the spliceosome recognizes (5' and 3' splice site) are highly degenerate resulting in the paradox that such an important and precise process as splicing with the added complexity of alternative splicing is dictated by redundant sequences. Today it is clear that although necessary, these consensus elements are by no means sufficient to define exon/intron borders. A myriad of auxiliary splicing *cis*-acting regulatory elements exist that can either enhance splicing and are termed exon splicing enhancers or intronic splicing enhancers depending on their location or repress exon inclusion and are called exon or intron splicing silencers, again depending on where they are found.

#### **1.3.1 Exonic splicing enhancers and the SR protein family.**

Studies on alternative splicing have identified exonic *cis*-acting elements, referred to as exonic splicing enhancers (ESEs), that facilitate the process of exon definition, thus helping exon inclusion in the mRNA (Mardon, Sebastio et al. 1987; Lam and Hertel 2002). Although originally discovered in regulated exons, ESEs are today also known to be components of constitutively spliced exons (Mayeda, Sreaton et al. 1999; Schaal and Maniatis 1999).

ESEs assist early spliceosomal complex formation by interacting with components of the splicing machinery that make up the previously described E complex (Reed 1996). This is done generally through the serine/arginine-rich proteins, SRs, and SR related proteins that

generally assemble on ESEs elements to promote both regulated and constitutive splicing by forming networks of interaction with each other (Fig. 9) (Blencowe 2000).



**Figure 9.** Mechanistic model connecting ESE and SRs. Splicing enhancer sequences within the exon (ESE) recruit proteins of the SR protein family, which establish a network of protein-protein interactions across the exon that stabilize the exon defined complex.

The SR proteins are nuclear phosphoproteins that are concentrated, together with most other splicing factors, in nuclear subregions termed speckles, which are believed to be sites of storage or assembly of splicing factors. It has been proposed that SR proteins and other splicing factors are recruited from the speckles to the sites of active transcription. Thus, certain SR proteins shuttle continuously between the nucleus and cytoplasm (Caceres, Sreaton et al. 1998). The SR proteins have a common domain structure of one or two RNA binding domains followed by an RS domain containing repeated arginine/serine dipeptides, which can be highly phosphorylated. This phosphorylation modulates protein-protein interaction, that serves as a bridge between the 5' and 3' splice sites across the introns and across the exons and/or between enhancers and adjacent splice site, within the spliceosome (Caceres, Sreaton et al. 1998; Black 2003; Ram and Ast 2007). Much of initial understanding of ESEs derives from the regulation of inclusion of exon 4 in the *doublesex* (*dsx*) gene in *Drosophila*. The doublesex repeat element (*dsxRE*), in this exon,

one of the first best characterized ESE element consisting of six repeats of a 13-nucleotide consensus sequence and a purine-rich element located between repeat 5 and 6. A multisubunit complex, containing SR-related proteins, alternative splicing factors Transformer (Tra), Transformer 2 (Tra2) and additional SR proteins assembles on *dsx* ESE element and once fully assembled, the dsxRE complex activates the recognition of a weak, sex specific 3' splice site, thereby promoting exon 4 inclusion and the female differentiation pathway (Ryner and Baker 1991; Tian and Maniatis 1992). Similarly, mammalian ESEs were identified initially as a purine rich sequences that associate with SR family proteins and promotes the utilization of adjacent splice site (Mardon, Sebastio et al. 1987; Lavigne, La Branche et al. 1993). Some of the mammalian SR family includes the proteins: SRp20, SRp30c, 9G8, SRp40, SRp55, SRp70, SC35 and ASF/SF2. SR-related family of proteins have RS domain but serve different role. These include, for example, U2AF, U1-70K, SRm160/300, Tra2 and numerous others (Table 1).

**Table 1.** SR and SR-related proteins with their respective binding sites.

<b>Name*</b>	<b>Domains</b>	<b>Binding sequence</b>
<b>Canonical SR proteins</b>		
SRp20 (SFRS3)	RRM and RS	GCUCCUCUUC
SC35 (SFRS2)	RRM and RS	UGCUGUU
ASF/SF2 (SFRS1)	RRM, RRMH and RS	RGAAGAAC
SRp40 (SFRS5)	RRM, RRMH and RS	AGGAGAAGGGA
SRp55 (SFRS6)	RRM, RRMH and RS	GGCAGCACCCUG
SRp75 (SFRS4)	RRM, RRMH and RS	GAAGGA
9G8 (SFRS7)	RRM, zinc finger and RS	(GAC) <sub>n</sub>
SRp30c (SFRS9)	RRM, RRMH and RS	CUGGAUU
SRp38 (FUSIP1)	RRM and RS	AAAGACAAA
<b>Other SR proteins</b>		
SRp54	RRM and RS	ND
SRp46 (SFRS2B)	RRM and RS	ND
RNPS1	RRM and Ser-rich	ND
SRrp35	RRM and RS	ND
SRrp86 (SRrp508 and SFRS12)	RRM and RS	ND
TRA2 $\alpha$	RRM and two Arg-rich	GAAARGARR
TRA2 $\beta$	RRM and two RS	(GAA) <sub>n</sub>
RBM5	RRM and RS	ND
CAPER (RBM39)	RRM and RS	ND



Different members of SR proteins are generally interchangeable in their ability to fulfill a wide range of activities in the splicing reaction. SR proteins seem to be also important in splicing regulation being able to modulate selection of alternative splice sites in a concentration dependent manner (Tacke and Manley 1999).

Protein-protein interaction screens have indicated, that through their RS domains, SR-family proteins, such as SC35 and ASF/SF2 can interact with each other, with U1-70K SR related protein, and with small U2AF35 subunit of the heterodimeric U2AF35/65 auxiliary splicing factor, which contains a short RS domain (Fu 1995). These interaction studies led to the proposal that SR family and SR related proteins facilitate splicing by forming interactions across the exons and introns. However, it was proposed that, in naturally occurring enhancers, SR proteins apparently may bind as a component of a multiprotein complex, whose components are not yet identified but may include known splicing factors as the large RS domain proteins SRm160/300, Tra2, U1 snRNP and heterogeneous ribonucleoproteins, such as hnRNPs A1 and hnRNP H. The SRm160/300 subunits are new SR-related proteins that lack RNA binding domain. SRm160/300 in particular is thought to be required for a typical purine rich mammalian ESE (consisting of six GAA repeats) to promote splicing of a pre-mRNA. The stable association of SRm160/300 with this pre-mRNA required the ESE, the U1 snRNP binding at the 5' splice site and the hTra2 $\beta$  binding to (GAA) $n$  sequence element. Thus, SRm160/300 promotes critical interaction between SR proteins, SR related proteins and hTra2 $\beta$  bound to ESE, and snRNP components of the spliceosome complex (Blencowe, Bauren et al. 2000). The binding sites for a SR protein members can be fairly degenerate (Black 2003).

Due to the importance of these ESEs in pre mRNA splicing a lot of work has gone into the possibility of predicting the presence of these enhancers. Several ESE prediction programs are presently available (Fairbrother, Yeh et al. 2002; Zhang and Chasin 2004). The majority of these are developed on either functional consensus motifs for SR proteins identified through a functional SELEX (systemic evolution of ligands by exponential

enrichment) method, on the statistical analysis of differences in hexamer frequencies between exons and introns and between exons with weak and strong splice sites based on the presumption that the ESE will be more common in exons rather than introns and again more common in exons with weak splices sites or on consensus sequences derived from known ESE and corresponding trans acting factors. Although extremely useful as a starting point in investigation of splicing mechanism, the presence of a high score motif in a sequence does not necessarily identify that sequence as an exonic splicing enhancer in its native context (Goren, Ram et al. 2006).

### **1.3.2 Exonic splicing silencer and hnRNP proteins.**

While the positive regulation of splicing is thought to occur as a result of protein-protein interaction strengthening the recognition of the splice site, negative regulation of splice site choice often result from the prevention of the splice site to be recognized. These negative regulatory sequences, within an exon, are called exonic splicing silencer, ESSs, and in the majority of cases are bound by particular hnRNP family proteins.

The hnRNP proteins are large group of molecules identified by their association with pre-mRNA or hnRNAs (heterogeneous nuclear RNAs). At least 20 hnRNPs have been identified, and are designated from hnRNP A1 to hnRNP U (Table 2) (Dreyfuss, Kim et al. 2002). hnRNP proteins contain one or more of RNA binding motifs. The most common is the RNP motif (RBD, also called RNA recognition motif, RRM), KH domains and RGG (Arg-Gly-Gly) boxes (Burd and Dreyfuss 1994).

**Table 2.** hnRNPs involved in pre-mRNA splicing with respective binding sequences.

Name	Other names	Domains*	Binding sequences
hnRNP A1	NA	RRM, RGG and G	UAGGGAU
hnRNP A2	NA	RRM, RGG and G	(UUAGGG) <sub>n</sub>
hnRNP B1			
hnRNP C1	AUF1	RRM	U rich
hnRNP C2			
hnRNP F	NA	RRM, RGG and GY	GGGA and G rich
hnRNP G	NA	RRM and SRGY	CC(A/C) and AAGU
hnRNP H	DSEF1	RRM, RGG, GYR and GY	GGGA and G rich
hnRNP H'			
hnRNP I	PTB	RRM	UCUU and CUCUCU
hnRNPL	NA	RRM	C and A rich
hnRNPLL	SRRF	RRM	C and A rich
hnRNPM	NA	RRM and GY	ND
hnRNPO	NA	RRM and RGG	ND

The structure of hnRNPs is modular, apart from the RNA binding domain, each hnRNP protein also contains the protein-protein interaction domain that mediates the localization of the protein. hnRNP proteins participate in various nuclear events, such as transcriptional regulation (Tomonaga and Levens 1995; Michelotti, Michelotti et al. 1996; Miao, Chang et al. 1998), telomere length maintenance (Eversole and Maizels 2000; Fiset and Chabot 2001), immunoglobulin gene recombination (Dempsey, Sun et al. 1999), splicing (Ashiya and Grabowski 1997; Chan and Black 1997; Chou, Rooke et al. 1999; Del Gatto-Konczak, Olive et al. 1999; Tange, Damgaard et al. 2001; Chen and Manley 2009), pre-ribosomal-RNA (Russell and Tollervey 1992), and 3' end processing (Kessler, Henry et al. 1997). These proteins are also important in nucleo-cytoplasmic transport of mRNA (Lee, Henry et al. 1996), in mRNA localization (Hoek, Kidd et al. 1998; Mouland, Xu et al. 2001), translation (Habelhah, Shah et al. 2001) and stability (Xu, Chen et al. 2001). Like SRs proteins also hnRNPs continuously shuttle between nucleus and cytoplasm.

Although a role as a negative regulator for splicing can be found in the literature for the majority of hnRNPs, some major players involved in pre-mRNA splicing regulation emerge. The most abundant hnRNPs belong to the A/B type. This family consists of a number of isoforms (A1, A2/B1, B2 and A3) showing extensive post transcriptional and translational modifications and sharing common RNA binding motif (Pozzoli and Sironi 2005). hnRNP A1, the most studied, contains two RNA binding domains and a glycine rich auxiliary domain. Using model substrates containing competing 5' splice sites, hnRNP A1 was shown to induce a shift toward the use of distal 5' splice site (Mayeda, Munroe et al. 1994). hnRNP A1 has been shown to also affect the use of the 3' splice site, as in the case of K-SAM exon in FGFR2 receptor mRNA, resulting in skipping of this exon (Del Gatto, Gesnel et al. 1996). The application of the SELEX approach has identified UAGGGA/U as the highest "winner" sequence for hnRNP A1 (Burd and Dreyfuss 1994). hnRNP A1 can interact with itself and with other hnRNPs, as well as with U2 and U4 snRNPs (Buvoli, Cobianchi et al. 1992; Cartegni, Maconi et al. 1996).

hnRNP proteins belonging to the H group (hnRNPs F, H, H' and 2H9) are encoded by different genes but share common binding site (GGGA) and similar structure. hnRNP H has been shown to act both as a activator and repressor of splicing. For example, hnRNP H acts as a powerful repressor in the rat  $\beta$ -tropomyosin gene by binding to the UGUGGG motif and causing exon 7 skipping in non muscle cells (Chen, Kobayashi et al. 1999; Chou, Rooke et al. 1999). It was also demonstrated that insertion of a UGUGGG motif in CFTR exon 9 results in lower inclusion of this exon (Pagani, Stuani et al. 2003; Buratti, Baralle et al. 2004).

hnRNP G proteins, in humans, are encoded by two genes RBMY and RBMX located on chromosome Y and X respectively. The product of RBMX has been implicated in splicing regulation of two mutually exclusive exons in the  $\alpha$ -tropomyosin gene, in particular hnRNP G was shown to promote skipping of the skeletal muscle specific exon (SK) and to enhance the inclusion of the non muscle specific exon (NM) (Elliott, Oghene et al. 1998).

HnRNP DL (hnRNPD-like) JKTBP protein it is closely related to hnRNPD/AUF family. There are at least two isoforms of JKTBP1 and 2 in HeLa cells. The major isoform JKTBP1, although it is primarily nuclear, shuttles between the nucleus and cytoplasm in HeLa cells (Akagi, Kamei et al. 2000). Little is known about the RNA binding properties of JKTBP. The consensus sequence for JKTBP2 is the 7 nt sequence ACUAGC/T (Kamei and Yamada 2002). A UAG present in these consensus splice sites is also found in the JKTBP high-affinity binding sites, thus JKTBP may play a role in alternative splice site switching (Akagi, Kamei et al. 2000).

Other hnRNP proteins, such as hnRNP L, K, E1 and E2, have traditionally been thought to regulate cytoplasmic export, stability and translation of specific mRNA (Liu and Mertz 1995; Makeyev, Eastmond et al. 2002; Perrotti and Calabretta 2002). However, each of these proteins was found also in the nucleus and may be involved in splicing reactions (Pinol-Roma 1999). Rothrock et al. showed that hnRNP L and hnRNP E1 are the primary ESS binding proteins found in nuclear extracts from a T-derived cell line, and that a hnRNP L is the key regulatory protein responsible for ESS dependent exon 4 silencing of the CD45 gene (Rothrock, House et al. 2005).

### 1.3.3 Intronic splicing silencers and enhancers.

*Cis*-acting regulatory elements in the intronic sequences have been studied to a lesser extent than those present in exons. Like exonic regulation, there are both positive and negative *cis*-acting sequences in the intron, so called intronic splicing enhancer (ISE) and intronic splicing silencer (ISS).

Some of the best characterized ISSs include binding sites for previously described hnRNP A1 protein (Hui and Bindereif 2005; Matlin, Clark et al. 2005; Wagner, Baraniak et al. 2005; Kashima, Rao et al. 2007). hnRNP A1 has been shown to interact with a novel intronic silencer element (ISS), important for the splicing of the second HIV-1 *tat* intron. The removal of the second *tat* intron is regulated by a combination of a suboptimal 3' splice site and *cis*-acting splicing enhancers and silencers. The positional overlap between the ISS element and the branch point -26, as well as the anchoring sites for U2 snRNP at the branch point -26, suggested that hnRNP A1 binding physically blocks the association of branch point binding factors, such as SF1 and U2 snRNP. Consistent with this view, Tange et al., observed that hnRNP A1 blocked U2 snRNP binding to the branch point, in the ISS dependent manner, of the second *tat* intron (Tange, Damgaard et al. 2001).

hnRNP I, also called PTB and PTBP1 is another major player in splicing control. PTB has strong RNA binding activity, and its preferred binding site is UCUU, often flanked by pyrimidines (Perez, Lin et al. 1997). Initially it was hypothesized that PTB is a positive splicing regulator, but later studies revealed that PTB is rather a splicing repressor (Garcia-Blanco, Jamison et al. 1989; Valcarcel and Gebauer 1997). Early models of PTB repression suggested that it may compete with U2AF65 for polypyrimidine tract, however regulation by PTB often requires additional PTB binding elements. In the case of exon N1 of the *c-src* gene, for example, cooperative binding sites on both exon sides are required for its repression in non neuronal cells (Chou, Underwood et al. 2000). The presence of a tissue restricted counterpart of the ubiquitously expressed PTB was initially detected in rat

brain extracts based on its different electrophoretic mobility but similar RNA binding activity to PTB. Two independent studies later identified and cloned nPTB (also known as PTBP2 and brPTB), confirming that is tissue restricted paralog of PTB. Subsequently, nPTB has been identified as a key regulator of exon N1 splicing of the *c-src* gene and as a protein that interacts with Nova-1/2, a neuron specific protein. Data from in vitro analysis showed that nPTB can act either neutrally or as a weaker repressor than PTB in preventing exon N1 inclusion of *c-src* gene (Ashiya and Grabowski 1997; Markovtsov, Nikolic et al. 2000; Polydorides, Okano et al. 2000). Additional focused and genome-wide analyses of PTB and nPTB have shed light on the mechanism by which these factors regulate alternative splicing. Both proteins display mutually exclusive expression patterns in the brain, with PTB being expressed in glial and non-neuronal cells, and nPTB in neurons. When PTB is present at sufficient levels, it represses splicing of exon 10 in nPTB transcripts, producing an isoform containing a stop codon that is degraded by NMD pathway. PTB also appears to further inhibit nPTB expression or turnover at the protein level independent of its effect on splicing of exon 10. Thus, down-regulation of PTB should lead to the expression of functional nPTB. In neurons, the silencing of PTB expression is achieved by the neuron-specific micro-RNA miR-124, which effectively derepresses nPTB exon 10 splicing, although efficient inclusion of this exon in neurons also requires positive-acting splicing regulators, such as neural specific SR-related protein of 100 kDa, nSR100 protein (also known as SRRM4), which promotes exon 10 inclusion of nPTB. This mutually exclusive expression pattern appears to be established after cells have committed to a neuronal fate, because neural progenitor cells appear to express both PTB and nPTB (Boutz, Chawla et al. 2007; Makeyev, Zhang et al. 2007; Spellman, Llorian et al. 2007; Calarco, Superina et al. 2009).

There are several elements known to act as intronic splicing enhancers, ISE's, but the proteins that mediate their effects are less well characterized than for ESE's. One well characterized ISE element is the triplet (GGG) which often occurs in clusters and can

enhance recognition of adjacent 5' or 3' splice sites (McCullough and Berget 1997). Intronic (CA) repeats in several cases can enhance splicing of upstream exons, probably through binding of hnRNP L protein (Hui and Bindereif 2005; Hung, Heiner et al. 2008). TIA-1 is an important ISE protein that binds pyrimidine-rich sequences that binds downstream of exon 9, of the CFTR (cystic fibrosis transmembrane conductance regulator) gene (Zuccato, Buratti et al. 2004; Venables 2007). Another example in which TIA-1 regulates splicing via ISE elements is Fas exon 6 that can be included or skipped to generate mRNA encoding a membrane bound form of the receptor that promotes apoptosis or a soluble isoform that prevents programmed cell death respectively. TIA-1 protein promotes U1 snRNP binding to the 5' splice site of intron 6, by binding to the ISE element in the +6 to +20 position downstream of Fas exon 6, which in turn facilitate exon inclusion (Izquierdo, Majos et al. 2005).

Two RNA-binding proteins with family members expressed specifically in nervous system and/or muscle tissues are the muscleblind-like (MBNL) and CUGBP/ETR-like (CELF; also known as Bruno-like) factors (Barreau, Paillard et al. 2006; Pascual, Vicente et al. 2006). These factors have been implicated in neurological disorders and in CUG trinucleotide expansion diseases such as myotonic dystrophy (Gallo and Spickett; Cooper 2009). These factors are also involved in several aspects of mRNA metabolism, including alternative splicing (Ladd, Charlet et al. 2001; Ho, Charlet et al. 2004). Several studies using model pre-mRNA substrates have demonstrated that MBNL and CELF bind intronic CUG-repeat and UG-rich elements respectively. At least some members of this family activate splicing through intronic enhancer elements (Charlet, Logan et al. 2002; Ho, Charlet et al. 2004).

Another common ISE element is the hexanucleotide (U)GCAUG motif that represents highly specific recognition element for the Fox family proteins. This element strongly enhances the splicing and since is an important subject of this thesis it will be discussed in more detail below.



### 1.3.4 (U)GCAUG element as a cell type specific splicing signal.

(U)GCAUG element has the peculiarity of functioning both as an enhancer or silencer (Kuroyanagi 2009). As previously mentioned computational target prediction for the binding of specific splicing factors is often very difficult, largely due to the small size and degeneracy of splicing factor binding motif. An exception to this degeneracy is the hexanucleotide (U)GCAUG motif, which is an important intronic element regulating the splicing of several exons (Huh and Hynes 1994; Modafferi and Black 1997; Lim and Sharp 1998). Computational analysis further showed that the (U)GCAUG elements were preferentially localized to the proximal downstream intron with greatest enrichment of sites in the first 100 nucleotides downstream, however it has been shown to function at a distance of up to 1 kb from the regulated exon. Further evidence for the importance of this regulatory element comes from the fact that it is evolutionarily conserved near the orthologous alternative exons from fish to humans (Minovitsky, Gee et al. 2005).

The hexamer (U)GCAUG is the functional motif shared among many brain enriched exons, but also an enhancer of alternative exons with different tissue specific regulation. Thus, (U)GCAUG is a marker specifically of regulated alternative splicing, but not a general marker for all alternative exons (Jin, Suzuki et al. 2003; Nakahata and Kawamoto 2005; Underwood, Boutz et al. 2005). The majority of brain-enriched exons possess at least one copy of (U)GCAUG in the flanking intron, and the remaining few generally have closely related GCAUG pentameric motif(s) in the proximal intron (Brudno, Gelfand et al. 2001). As these hexamer elements can act from within different sequence contexts, specific RNA secondary structures are probably not critical for hexamer function. Therefore, it is likely that the (U)GCAUG motif comprises the essential *cis*-active element. Repeated (U)GCAUG sequences are capable of functioning in a cell type dependent fashion in a heterologous context, a finding that has not been established for many of the other potential vertebrate elements (Huh and Hynes 1994).

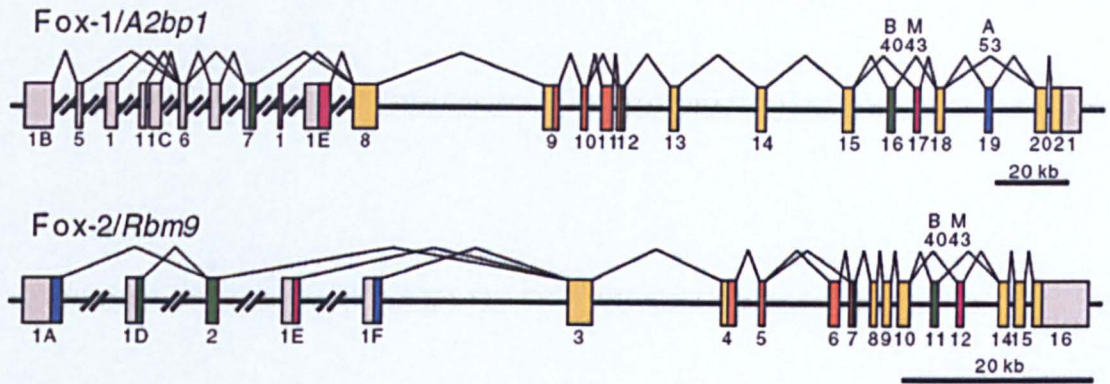
The patterns of alternative polyadenylation were strongly correlated with those of alternative splicing across tissues, and (U)GCAUG(U) heptamer was highly enriched in the extension region of tandem 3' untranslated regions (UTRs) (Wang, Sandberg et al. 2008).

### **1.3.5 The Fox protein family: evolutionarily conserved regulators of tissue-specific alternative splicing in metazoans.**

(U)GCAUG represents a highly specific recognition element for the Fox family of proteins. The Fox-1 (feminizing locus on X) gene was originally identified in *C. elegans*, where it acts as a numerator element in counting the number of X chromosomes relative to ploidy, and determining male or hermaphrodite development (Auweter, Fasan et al. 2006). There are several homologues of *C.elegans* Fox-1 in mouse and human genomes. In addition to human Fox-1 (Ataxin-2 binding protein, A2BP1 gene), there are other paralogous genes in the mammalian genomes. One of them is Fox-2 (RNA binding motif 9, RBM9 gene) and the other is Fox-3 (hexaribonucleotide binding protein 3, HRNbp3, NeuN gene) (Shibata, Huynh et al. 2000; Lieberman, Friedlich et al. 2001; McKee, Minet et al. 2005; Kim, Adelstein et al. 2009).

Fox-1 expression has been observed to be high in brain, heart and skeletal muscle, while Fox-2 protein shows much broader tissue expression as well as higher expression levels in the embryo relative to the adult than Fox-1. Fox-3 protein expression has only been observed in neurons (Kuroyanagi 2009). Recently, it has been reported that Fox-3 gene product is NeuN, neuron specific protein, whose expression has been used widely as a reliable tool to detect most postmitotic neuronal cell type in neuroscience, developmental biology and stem cell research fields as well as diagnostic histopathology (Kim, Adelstein

et al. 2009). Alignments of the cDNA, EST and genomic databases indicate that both the A2BP1 (Fox-1) and RBM9 (Fox-2) genes have multiple promoters and multiple internal cassette exons that are variably included in the mRNA (Fig. 10).



**Figure 10.** Schematic structure and major splicing patterns of the mouse Fox-1/A2bp1 and Fox-2/Rbm9 genes. Boxes indicate exons and horizontal lines indicate introns. Use of these exons is supported by cDNA and/or EST sequences in GenBank/EMBL/DDBJ databases. Coding regions are coloured; RRM domains in orange, brain-specific region in green, muscle-specific region in magenta, other isoform-specific regions in blue, and common regions in yellow. The size of the exons is not proportional to that of the introns (Figure from (Kuroyanagi 2009)).

Both gene transcripts are found to undergo tissue specific alternative splicing. In both cases, brain and striated muscles express unique splice variants generated by mutually exclusive splicing of exons B40 and M43, respectively, which provide different coding sequences in the middle of the C-terminal region. A2BP1 gene (Fox-1) contains an additional cassette type alternative exon A53, consisting of 53 nucleotides. Inclusion or exclusion of exon A53 results in two different amino acid sequences at the C-terminal

region (Nakahata and Kawamoto 2005; Baraniak, Chen et al. 2006; Fukumura, Kato et al. 2007).

In the case of the A2BP1 gene, moreover, it appears that brain and striated muscle utilize alternative promoters, resulting in different amino acid sequences at the N terminus. The 5' RACE of skeletal muscle mRNAs yielded essentially a single species of sequence with 29 unique N-terminal amino acids. The 5' RACE, using brain mRNAs, however, yielded multiple products with multiple deduced amino acid sequences, all of which differ from the muscle amino acid sequence at the very N-terminus (Nakahata and Kawamoto 2005). Exon/intron organization of RBM9 and A2BP1 genes shows remarkable similarities at the RNA binding domain (RBD), encoded in four exons. Of note is that the significant amounts of RBM9 and A2BP1 mRNAs from skeletal muscles are missing a part of the RBD by exon skipping. Specifically, they lack the 93 nt exon that encodes RNP1, one of the two most critical motifs of the RBD (Burd and Dreyfuss 1994). This isoform is missing crucial residues for RNA binding, and thus, will not mediate splicing enhancement or repression through the (U)GCAUG element. Instead, the intact N- and C-terminal domains of this isoform can counteract the effect of full length Fox proteins in enhancing a Fox dependent exon. Thus, rather than the autoregulated splicing reducing the overall level of the protein, the new isoform directly antagonizes Fox activity (Damianov and Black).

### **1.3.6 Fox family proteins targets and implications.**

The Fox-1 family of proteins for the first time was identified in *C.elegans*, while the Fox-1 protein in humans was originally identified as an interactor with ataxin-2 and both Fox-1 and Fox-2 interact with ataxin-1, which are mutated in spinal cerebellar ataxia (Shibata, Huynh et al. 2000; Lam, Bowman et al. 2006). Indeed, genes with Fox dependent

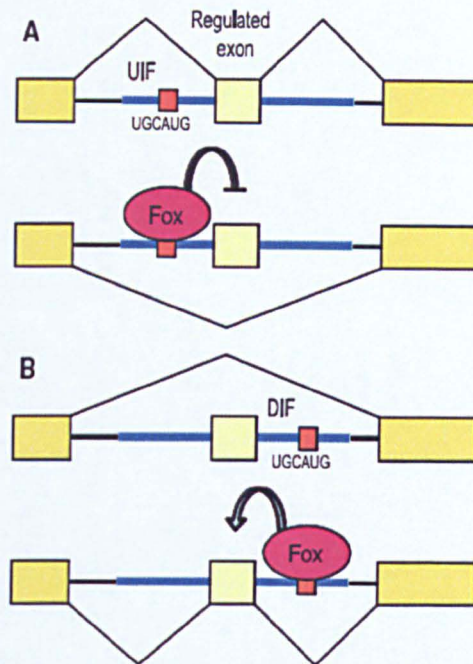
alternative splicing events have been frequently associated with diseases, including several neurological, heart, and muscular disorders. Furthermore, mutations in the Fox-1 gene itself or alteration of its expression are associated with neurological disorders and heart disease (Kaynak, von Heydebreck et al. 2003; Bhalla, Phillips et al. 2004; Szatmari, Paterson et al. 2007). Two recent alternative splicing profiling studies have also found a connection between the Fox family of splicing regulators and breast and ovarian cancer. These studies implicate the altered regulation of Fox-dependent splicing networks in various cancers, and analysis of these networks should uncover how these isoforms contribute to the development and progression of the cancer disease (Lapuk, Marr et al.; Venables, Klinck et al. 2009).

Fox-2 CLIP (ultraviolet cross-linking and immunoprecipitation assay) tag clusters in human embryonic stem cell (hESCs) revealed an enrichment of RNA-binding proteins, nuclear mRNA splicing factors, and serine/threonine kinases. Among these Fox-2 target genes were heterogeneous ribonucleoproteins (hnRNPs) such as A2/B1, H1, H2, and PTB, alternative splicing regulators including Fox-1, nPTB, SRp20, SRp40, SRp55, and Tra2a, and other RNA-binding proteins important for stem cell biology (Yeo, Coufal et al. 2009).

### **1.3.7 Mechanisms of alternative splicing regulation by the Fox family.**

Experimental validation of many predicted target genes for the Fox family confirmed a previously demonstrated trend with respect to exon inclusion or skipping, depending on the location of the (U)GCAUG element. The alternative exon is included when the Fox-1 family binds to the (U)GCAUG element in the downstream intronic region, while the exon is skipped when the Fox family binds to the (U)GCAUG element in the upstream intronic

region (Fig. 11) (Llorian, Schwartz et al.; Racca, Gardiol et al.; Licatalosi, Mele et al. 2008; Zhang, Zhang et al. 2008; Yeo, Coufal et al. 2009).



**Figure 11.** Schematic illustration of alternative splicing regulation by the Fox-1 family. (A) The Fox-1 family represses exon inclusion by binding to (U)GCAUG element(s) in the upstream intronic flanking (UIF) region. (B) The Fox-1 family enhances exon inclusion by binding to the (U)GCAUG element(s) in the downstream intronic flanking (DIF) region. Boxes indicate exons and horizontal lines indicate introns. Blue horizontal lines indicate the UIF and DIF regions. Orange boxes indicate (U)GCAUG elements. Figure from (Kuroyanagi 2009).

However, to date very little is known about the molecular mechanisms through which Fox-1 and Fox-2 proteins regulate alternative splicing. It is of interest to note that the solution

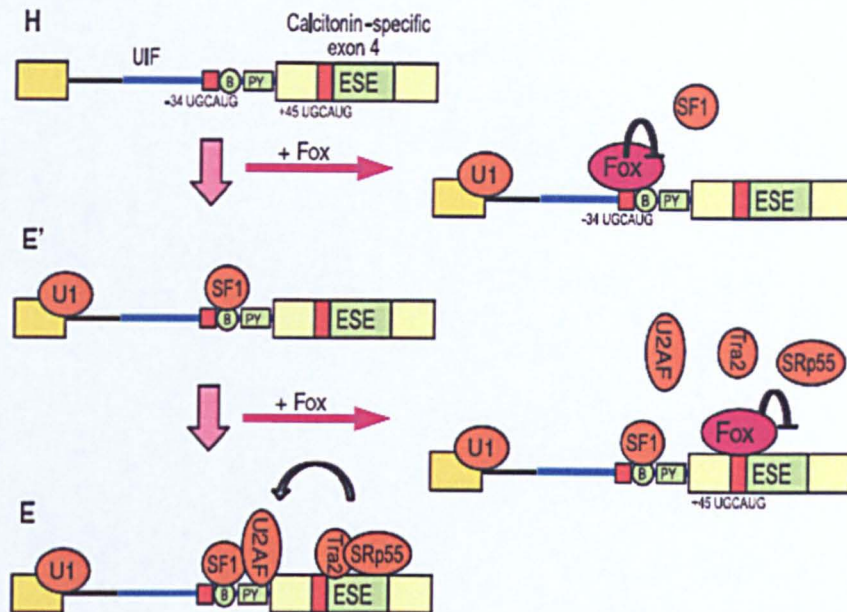
structure of the Fox-1 RNA binding domain (RBD) in complex with (U)GCAUG(U) heptamer showed that the last three nucleotides, UG(U), are recognized in a canonical way by the four stranded  $\beta$  sheet of the RBD. In contrast, the first four nucleotides, (U)GCA, are bound by two loops of the protein in an unprecedented manner. Nucleotides U1, G2 and C3 are wrapped around a single phenylalanine in the Fox protein, while G2 and A4 form a base pair. This novel RNA binding site is independent from the  $\beta$  sheet binding interface. These results demonstrate the unusual molecular mechanism of sequence specific RNA recognition by Fox-1, which is exceptional in its high affinity for a defined but short sequence element. The structure of Fox-1 in complex with (U)GCAUG(U) is particularly interesting for understanding the function of the protein. Fox-1 induces curvature in the RNA upon binding. Therefore, the binding of Fox-1 to its RNA targets might lead to a conformational changes in the RNA that in turn many influence splicing regulation (Auweter, Fasan et al. 2006).

Studies performed on specific genes have focused on mechanism of Fox-1/Fox-2 mediating repression while little is known about how the Fox-1 proteins promote inclusion of an exon via binding to (U)GCAUG element(s) in the downstream intronic region. For example, exon 9 of the human mitochondrial ATP synthase  $\gamma$  subunit (F1 $\gamma$ ) gene is one of the model systems of Fox-1 mediated exon skipping. Fox-1 repressed F1 $\gamma$  exon 9 by inhibiting splicing of the downstream intron 9 via GCAUG elements in the upstream intronic region without affecting efficiency of splicing of intron 8 where the GCAUG elements reside (Fukumura, Kato et al. 2007). Unexpectedly, U1 snRNP components were specifically absent from prespliceosomal E complex on the intron 9 formed, and Fox-1 prevented formation of the E complex in the *in vitro* splicing reaction with HeLa nuclear extract. Mutations in the 5' splice site of intron 9 conferred U1 dependency and concomitantly impaired Fox-1 mediated regulation, indicating that U1 independent splicing of hF1 $\gamma$  intron 9 is indispensable for induction of exon 9 skipping by Fox-1 (Fukumura and Inoue 2009). Still, it is not known whether Fox-1 mediated repression of

alternative exons in other target genes is generally accompanied by U1 independent splicing (Fukumura and Inoue 2009).

Calcitonin-specific exon 4 is regulated by a balance between competing effects of the Fox-1 family proteins binding to (U)GCAUG elements at positions -34 in the UIF region and +45 in exon 4, and of Tra2b and SRp55 protein binding to exonic splicing enhancers (ESEs) (Tran, Coleman et al. 2003; Zhou, Baraniak et al. 2007). Furthermore, Zhou et al. demonstrated that the Fox-1 family proteins bound to the -34 (U)GCAUG silencer element to prevent SF1 from binding to the branch point without affecting U1 snRNP binding to the pre-mRNA, and that the -34 (U)GCAUG element repressed formation of prespliceosome E' complex, a pre-spliceosome complex formed in U2AF-depleted HeLa nuclear extracts prior to early (E) complex formation. They also demonstrated that the Fox-1 family of proteins interfered with binding of Tra2 $\beta$  and SRp55 to the ESEs via the +45 (U)GCAUG element and that the +45 (U)GCAUG element blocked recruitment of U2AF65 and formation of the pre-spliceosome E complex. These results raised a fail-safe two step model of repression of calcitonin-specific exon 4 by the Fox-1 family and two (U)GCAUG elements (Fig. 12) (Zhou, Baraniak et al. 2007).





**Figure 12.** A model for repression of pre-spliceosome complex formation by the Fox-1 family. The repression of calcitonin specific exon 4 of calcitonin/CGRP pre-mRNA in neuronal cells by the Fox-1 family involves two distinct regulatory events. First, the -34 element in the UIF region prevents E' complex formation through repressing SF1 binding to the branch point (BP). Second, the + 45 exonic element blocks transition to E complex via inhibiting U2AF65 binding to polypyrimidine tract (PY). Figure from (Kuroyanagi 2009).

Expression of Fox-1 promoted inclusion of EIIIB exon in the rat fibronectin gene via highly repeated and evolutionarily conserved UGCAUG motifs in the downstream intronic region. Expression of Fox-1 or Fox-2 enhanced inclusion of a short neuron-specific exon, N1, of the *c-src* gene through UGCAUG element in the DIF region in non-neuronal HeLa cells. Fox-2 positively regulated inclusion of exon 16 of protein 4.1R pre-mRNA via conserved three copies of UGCAUG motifs in the downstream intronic region in late

erythroleukemia cell differentiation as well as in heterologous HeLa cells (Kuroyanagi 2009).

A clue to elucidating the promotion mechanism, may come from the interaction between Fox protein and a U1 snRNP specific protein U1-C in a yeast two-hybrid system, which is also the case of TIA-1 recruiting U1 snRNP to the 5' splice site (Forch, Puig et al. 2002; Ohkura, Takahashi et al. 2005). Furthermore, Fox-1 activity on model substrates indicate that the protein can activate splicing from a multimerized (U)GCAUG element. This activation is independent of other binding elements. Thus, Fox-1 can apparently activate splicing and not just release an exon from repression by other proteins (Auweter, Fasan et al. 2006).

## **1.4 Alternative splicing in sodium channel genes.**

Sodium channels are large integral membrane proteins, encoded by at least ten genes in mammals. Selective permeation of sodium ions through voltage dependent sodium channels is fundamental to the generation of action potentials in excitable cells. The different sodium channels have remarkably similar functional properties, but small albeit relevant changes in sodium channel function and/or different sodium channel isoforms, generated by alternative splicing, are biologically important as underscored by mutations that cause several sodium channel human diseases, or channelopathies.

### **1.4.1 Sodium channel isoforms.**

Voltage gated sodium channels are responsible for the generation of action potential and thereby play an important role in propagation of electrical impulses in muscle, nerve and

heart tissues (Hodgkin and Huxley 1952). Sodium channels are integral membrane proteins that form ion channel, conducting sodium ions through a plasma membrane. Influx of sodium ions depolarizes the membrane further and initiates the rising phase of the action potential. Sodium channels are classified according to the trigger that opens the channel for such ions, either a voltage-change (voltage-gated sodium channels) or binding of a substance (a ligand) to the channel (ligand-gated sodium channels). The channel is composed of pore forming  $\alpha$  subunit of ~ 260 kDa associated with one or two  $\beta$  subunits,  $\beta$ 1 (SCN1B, 36 kDa), localized in brain, skeletal and cardiac muscle, and  $\beta$ 2 (SCN2B, 33 kDa), localized in central nervous system and cardiac muscle (Catterall and Epstein 1992). Additionally, two other members of the  $\beta$  subunit have been described,  $\beta$ 1A, a splice variant of the SCN1B gene, and  $\beta$ 3 (SCN3B) localized primarily in neuronal tissue (Kazen-Gillespie, Ragsdale et al. 2000). The  $\alpha$  subunit gene family consist of nine genes that are highly conserved across species. The gene names are referred to as SCN1A through SCN11A (the SCN6/7A gene is part of the  $\text{Na}_x$  sub-family and has uncertain function). The proteins of these channels are named  $\text{Na}_v1.1$  through  $\text{Na}_v1.9$  (Table 3). The channels are characterized by differential sensitivities to the sodium channel blocker tetrodotoxin and inactivation kinetics. Highly tetrodotoxin sensitive channels are encoded by SCN1A, SCN2A, SCN3A, SCN4A, SCN8A and SCN9A genes, and all of them have faster inactivation kinetics compared to the sodium channel genes SCN5A, SCN10A and SCN11A, that are also less sensitive to tetrodotoxin. SCN9A, SCN10A and SCN11A are expressed in peripheral nervous system (PNS). SCN1A, SCN2A, SCN3A and SCN8A are also expressed in PNS, but are more abundant in the central nervous system (CNS). SCN4A and SCN5A are highly expressed in skeletal muscle and heart, respectively. SCN6A/SCN7A is expressed in different tissues, but still is debated whether this gene encodes a functional sodium channel (Table 3) (Isom, De Jongh et al. 1992; Yoshida 1994; Goldin, Barchi et al. 2000; Goldin 2001).

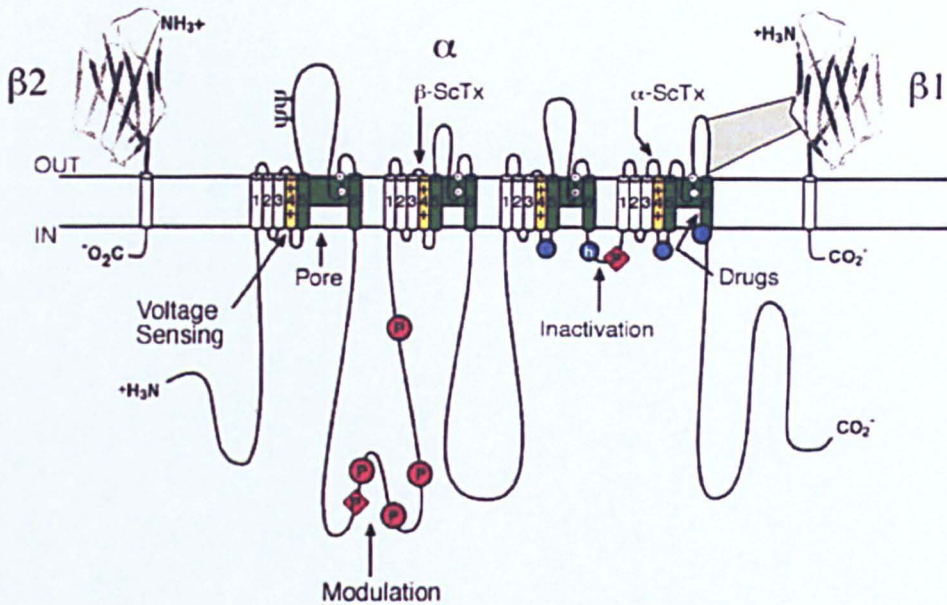
**Table 3.** The voltage gated sodium channel gene family and tissue specificity, modified from (Koopmann, Bezzina et al. 2006).

Channel	Gene	Location	Primary tissues where channels are expressed
Na <sub>v</sub> 1.1	<i>SCN1A</i>	2q24	CNS; brain
Na <sub>v</sub> 1.2	<i>SCN2A</i>	2q23-q24.3	CNS; brain
Na <sub>v</sub> 1.3	<i>SCN3A</i>	2q24	CNS; brain
Na <sub>v</sub> 1.4	<i>SCN4A</i>	17q23.1-q25.3	adult skeletal muscle
Na <sub>v</sub> 1.5	<i>SCN5A</i>	3p21	heart muscle; skeletal muscle
Na <sub>x</sub>	<i>SCN6A/SCN7A</i>	2q21-q23	PNS; heart muscle; skeletal muscle; uterus
Na <sub>v</sub> 1.6	<i>SCN8A</i>	12q13	CNS; brain
Na <sub>v</sub> 1.7	<i>SCN9A</i>	2q24	PNS;
Na <sub>v</sub> 1.8	<i>SCN10A</i>	3p24.2-p22	PNS
Na <sub>v</sub> 1.9	<i>SCN11A</i>	3p24-p21	PNS
Na <sub>v</sub> β.1	<i>SCN1B</i>	19q13.1	brain; heart muscle; skeletal muscle
Na <sub>v</sub> β.4	<i>SCN4B</i>	11q23	brain; PNS; heart muscle; skeletal muscle

### 1.4.2 Structure of voltage gated sodium channel family.

Sodium channels can often be isolated from cells as a complex of two types of protein subunits,  $\alpha$  and  $\beta$ . An  $\alpha$  subunit forms the core of the channel. The  $\alpha$ -subunit has four repeat domains, labeled I through IV, each containing six membrane-spanning regions, labeled S1 through S6 (Fig. 13). The highly conserved S4 region acts as the channel's voltage sensor, which is due to positive amino acids located at every third position. When stimulated by a change in transmembrane voltage, this region moves toward the extracellular side of the cell membrane, allowing the channel to become permeable to ions

(Yang and Horn 1995). The ions are conducted through a pore, which can be broken into two regions. The more external portion of the pore is formed by the "P-loops" (the region between S5 and S6) of the four domains. This region is the most narrow part of the pore and is responsible for its ion selectivity. The inner portion (more cytoplasmic) of the pore is formed by the combined S5 and S6 regions of the four domains. The region linking domains III and IV plugs the channel after prolonged activation, inactivating it, thus this region is important for channel function (Kellenberger, Scheuer et al. 1996). The  $\beta$  subunit consist of one transmembrane segment, an intracellular domain and a glycosylated extracellular domain. The function of the  $\beta$  subunit is to control channel gating, regulate the level of expression of the  $\alpha$  subunit at the plasma membrane, and are most likely involved in cell adhesion through interaction with the cytoskeleton, extracellular matrix, and some other adhesion molecules, important for cell migration and aggregation (Goldin 1993; Isom and Catterall 1996; Isom 2001). When the  $\alpha$  subunit protein is expressed by a cell, it is able to form channels which conduct sodium ions, even if  $\beta$  subunits are not expressed, but when  $\beta$  subunits assemble with  $\alpha$  subunits the resulting complex can display altered voltage dependence and cellular localization (Isom, De Jongh et al. 1992).



**Figure 13.** The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane folding diagrams. Cylinders represent  $\alpha$ -helical segments (S1-S6). Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the  $\beta 1$  and  $\beta 2$  subunits are shown as immunoglobulin-like folds.  $\Psi$ , sites of probable N-linked glycosylation; P in red circles, sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds); green, pore-lining segments; white circles, the amino residues that form the ion selectivity filter and the tetrodotoxin binding site; yellow, S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor. Sites of binding of  $\alpha$  and  $\beta$  scorpion toxins ( $\alpha$ -ScTx and  $\beta$ -ScTx) and a site of interaction between  $\alpha$  and  $\beta 1$  subunits are also shown (modified from (Yu and Catterall 2003).

### **1.4.3 Expression profiles of sodium channels during development and subcellular localizations.**

Mammalian sodium channels, in addition to the differences in cellular and tissue expression, also have different expression profiles during development, and different subcellular localizations, consistent with a distinct role of each of these in mammalian physiology. As mentioned previously,  $Na_v$  1.3 is highly expressed in fetal nervous system, whereas  $Na_v$  1.1,  $Na_v$  1.2 and  $Na_v$  1.6 are abundant in the adult CNS.  $Na_v$  1.1 and  $Na_v$  1.2 are localized to the soma of the neuron, where they can control neuronal excitability through interaction of synaptic impulses to set the threshold for action potential initiation and propagation to the dendritic and axonal parts of the neuron.  $Na_v$  1.6, during development has been shown to replace  $Na_v$  1.2 in maturing nodes of Ranvier, the gaps in myelin sheaths of myelinated axons where action potential conduction takes place (Boiko, Rasband et al. 2001; Kaplan, Cho et al. 2001; Craner, Hains et al. 2004). The most abundant sodium channels, expressed in PNS are  $Na_v$  1.7,  $Na_v$  1.8 and  $Na_v$  1.9.  $Na_v$  1.7 appears to be localized to the axons, where it may function in initiating and conducting the action potential in PNS neurons. More restricted expression patterns are observed for  $Na_v$  1.8 and  $Na_v$  1.9 sodium channel, expressed in small sensory neurons of dorsal root ganglion and trigeminal ganglia, where they, together with  $Na_v$  1.7 have an important role in perception of pain (Akopian, Souslova et al. 1999; Black, Fjell et al. 1999).

#### **1.4.4 Developmentally regulated alternative splicing of sodium channel genes.**

For each particular sodium channel gene, subtle differences in channel properties can be attributed to alternative splicing, post-translational modifications, changes in the expression of ancillary  $\beta$ -subunits, and mutations. Importantly, alternative splicing of transcripts derived from a common gene has been shown to generate biochemical and pharmacologically distinct sodium channel isoforms (Goldin 2001). Minor changes in the properties of specific isoforms result in human diseases of muscle, heart, and the nervous system (Onkal and Djamgoz 2008).

Alternative RNA splicing has been described for several sodium channel genes (Table 4), for example SCN1A with extended exon 11, SCN3A has three different variants of exon 12, SCN5A can alternative splice resulting in a difference in one amino acid in exon 18, SCN9A undergoes alternative splicing resulting in an extended exon 11, and SCN11A can result in an alternative spliced isoform lacking exon 16 (Schaller, Krzemien et al. 1992; Makielski, Ye et al. 2003; Raymond, Castle et al. 2004; Thimmapaya, Neelands et al. 2005). Additionally, developmentally regulated and tissue specific splice variants of SCN2A, SCN3A, SCN8A and SCN9A, exon 5N and 5A, are expressed predominantly neonatally (N) or in adults (A), have been described (Lu and Brown 1998; Heron, Crossland et al. 2002). Two such alternative splicing events, in particular ME splicing are of central importance to this PhD thesis and concern the SCN8A and SCN9A sodium channel genes.

Previous research has shown developmentally regulated alternative splicing of SCN8A coding exon 18. Exons, 18N (neonatal) and 18A (adult), encodes transmembrane segments S3 and S4 in domain III. Prior studies of SCN8A variants demonstrated that fetal neurons and non-neuronal cells produce two variant transcripts, one containing the alternative exon



18N and one that skips exon 18. Because SCN8A exon 18N includes a stop codon, the prediction was that fetal neurons and non-neuronal cells would express a truncated, non-functional variant of Na<sub>v</sub>1.6 sodium channel (Plummer, McBurney et al. 1997). The proportion of transcripts containing exon 18N is highest in mouse fetal brain between E12.5 and P1.5, while at later stages the predominant transcripts contain exon 18A (Plummer, McBurney et al. 1997). In a recent paper it was suggested that macrophages can express a full-length variant of Na<sub>v</sub>1.6 through deletion of exon 18N in SCN8A transcripts. From a functional standpoint, these Na<sub>v</sub>1.6 channels are required for macrophage podosome formation, melanoma invadopodia formation and cellular invasion (Carrithers, Chatterjee et al. 2009). In adult brain and spinal cord, the major transcript contains exon 18A. In addition, four alternatively spliced noncoding exons in the 5' UTR region of SCN8A gene have been reported (Drews, Lieberman et al. 2005). Regarding SCN9A sodium channel gene, previous research has identified exons 5N and 5A of the gene that undergo mutually exclusive alternative RNA splicing to produce mRNAs coding either for an isoform predominant in dorsal root ganglion (5A) or a different isoform (5N) preferentially expressed in the PNS and CNS neurons of adult tissues. These exons are 92 bp in length and encode amino acids which correspond to part of the S3 and most of S4 transmembrane segments within domain I and the extracellular loop between them. Despite 22 nucleotide differences between the exons, only a single amino acid is altered, specifying either aspartic acid (in exon 5A) or serine (in exon 5N).

It is still debated what the functional consequences of this amino acid change might be, but the outcome may be significant. First, because the alternative splicing to generate (5N) and (5A) isoforms is developmentally regulated, thus representing an investment by the organism in maintaining this differential expression (Sarao, Gupta et al. 1991). Thus, a channel with a neutral charged residue may be advantageous in the neonate when cells are migrating and differentiating whereas channels with a negatively charged residue are preferable in the mature cells of the adult (Auld, Goldin et al. 1990). The finding that

virtually the same alternative splicing module, (exons 5N and 5A), has been preserved in distinct sodium channel genes provides further support to the idea that the single amino acid difference has substantial functional significance (Onkal and Djamgoz 2008). The electrophysiological differences have been observed between the 'adult' and 'neonatal' isoforms, that could have a number of physiological (developmental) and pathophysiological consequences. Compared to the 'adult', for example the 'neonatal' channel was associated with a larger charge of sodium ions entry into the cell during each opening of the channel. The additional sodium ions influx may be important for intracellular pH regulation (Levi, Dalton et al. 1997; Cooper, Schell et al. 1998; Blaustein and Lederer 1999; Karmazyn, Gan et al. 1999; Bers, Barry et al. 2003). It has been shown, in particular, that alternatively spliced variants of the human Nav 1.7 channel (5N and 5A) can have different functional properties. The change of one amino acid in the S3 domain I (exons 5A and 5N) induced a small shift of activation to more hyperpolarized potentials for exon 5A compared with exon 5N. Thus, channels with exon 5A inactivate more slowly than channels with exon 5N, causing a delay in inactivation for 5A variants compared with 5N during a slow depolarization ramp protocol. As a consequence, fewer channels are inactivated at the potential range where the inward current is present. This increase in channel availability will therefore induce larger inward current amplitudes that might increase cell excitability (Chatelier, Dahllund et al. 2008).

Alternative splicing have been described also for the  $\beta$  subunits; SCN1Ba and SCN1Bb differ beyond the immunoglobulin region (Ig)-loop region, resulting in a distinct C-terminal domain and longer SCNB1b transcript (Kazen-Gillespie, Ragsdale et al. 2000).

**Table 4.** Different splice variants of voltage-gated sodium channels modified from (Koopmann, Bezzina et al. 2006).

Gene name	Splice variants	GenBank accession number	Comments
<i>SCN1A</i>	variant 1	AB093548	variant 1 contains extended exon 11
	variant 2	NM_006920	
<i>SCN2A</i>	neonatal isoform		exon 5N
	adult isoform		exon 5A
<i>SCN3A</i>	neonatal isoform	AF035685	exon 5N
	adult isoform	AF035686	exon 5A
	splice variant	NM_006922	this variant contains extended exon 12
<i>SCN5A</i>	variant 1	NM_198056	variant 1 contains 1 extra amino acid in exon 18
	variant 2	NM_000335	
<i>SCN8A</i>	neonatal isoform	AY682082	exon 5N
	adult isoform	AY682081	exon 5A
	neonatal isoform	AF050730	exon 18N
	adult isoform	AF050730	exon 18A
<i>SCN9A</i>	splice variant	AY682083	this variant contains extended exon 12
	neonatal isoform	AY682085	exon 5N
	adult isoform	AY682084	exon 5A
<i>SCN11A</i>	splice variant	AY682086	this variant contains extended exon 11
	variant a	NM_001037	variant b encodes longer transcript
	variant b	NM_199037	

N=neonataly; A=adult; ND=not determined.

## 1.4.5 Chanelopathies: the role of sodium channels in disease.

Mutations in *SCN1A* and *SCN2A* cause different epilepsy syndromes (Escayg, MacDonald et al. 2000; Sugawara, Mazaki-Miyazaki et al. 2001)), whereas mutations in *SCN4A* have been linked to myotonia conditions (Mitrovic, George et al. 1994). Mutations in *SCN5A* are linked to Brugada syndrome and long QT syndrome (Bennett, Yazawa et al. 1995; Veldkamp, Wilders et al. 2003; Antzelevitch 2005). Mutations in the mouse ortholog of *SCN8A* gene cause ataxia and other movement disorders (Meisler, Plummer et al. 2004). So far, only one protein truncation mutation in *SCN8A* sodium channel gene has been described in humans, associated with a case of cerebellar atrophy, ataxia, and mental retardation (Trudeau, Dalton et al. 2006). A single nucleotide polymorphism in *SCN8A*

gene may contribute to the risk for suicide attempts, possibly through alterations in neuronal conduction which hypothetically could lead to disturbed analysis of incoming information in periods of emotional and physical stress (Wasserman, Geijer et al. 2005). Dysfunction of the  $Na_v1.6$  sodium channel alters sleep architecture, reduces diurnal corticosterone levels, and enhances spatial memory (Papale, Paul et al.). There is strong evidence indicating that  $Na_v1.6$  is the predominant sodium channel located in the nodes of Ranvier in both PNS and CNS myelinated neurons, and transgenic mice in which  $Na_v1.6$  has been made non-functional typically die around postnatal day 15, a time at which myelination is rapidly progressing (Garcia, Sprunger et al. 1998; Caldwell, Schaller et al. 2000). Recent studies have identified changes in the expression pattern of  $Na_v1.6$  sodium channel, as an important contributor to remission and progression in multiple sclerosis (MS). The disease process in MS attacks myelinated axons, denuding them of myelin, or causing them to degenerate. Following the loss of the myelin, remyelination does not always occur. Recovery of clinical function in cases such as this requires the restoration of secure action potential conduction along demyelinated axons. After demyelination, some of the axon membrane can develop higher than normal densities of  $Na_v1.6$  sodium channel, restoring in this way proper action potential conduction. The available evidence suggests that  $Na_v1.6$  sodium channels are important participant in axonal degeneration in MS, caused by sustained influx of sodium ions, which drives reverse operation of the sodium-calcium exchanger, an antiport molecule that can import damaging level of calcium into axon. Thus,  $Na_v1.6$  are being investigated as a potential therapeutical target in MS. Although, subtype specific sodium channel blocker are not yet available, some of the nonspecific blockers, such as phenytoin and flecainide have been shown to be protective in mouse and rat model of MS, EAE (experimental allergic encephalomyelitis). However, in a recent publications it has been observed a clinical worsening following withdrawal of phenytoin in mouse model of MS, after prolonged exposure to this sodium

channel inhibitor (Caldwell, Schaller et al. 2000; Craner, Newcombe et al. 2004; Stys 2004; Waxman 2006; Smith 2007; Waxman 2008).

SCN9A (Na<sub>v</sub> 1.7), SCN10A (Na<sub>v</sub> 1.8) and SCN11A (Na<sub>v</sub> 1.9) sodium channels have been implicated in different pain conditions, but only SCN9A (Na<sub>v</sub> 1.7) channelopathies have been described so far. Na<sub>v</sub>1.7 plays a crucial role in our ability to perceive pain sensations. Cox et al. studied several families from Northern Pakistan that contained members with an inability to experience pain ((Cox, Reimann et al. 2006). Neurological examinations indicated that while these individuals apparently had never experienced any pain sensations, otherwise they appeared normal. The only noted exceptions to this, were deficits in olfaction in some patients (Weiss, Pyrski et al.; Goldberg, MacFarlane et al. 2007). It was determined that the pain insensitivity was congenital and could be mapped as an autosomal recessive trait linked to a region of chromosome 2. Goldberg et al. identified Na<sub>v</sub>1.7 truncating mutations in patients with congenital indifference to pain, demonstrating that a loss-of-function of Na<sub>v</sub>1.7 can result in an insensitivity to pain (Goldberg, MacFarlane et al. 2007). As individuals that lack functional Na<sub>v</sub>1.7 channels can appear normal except for their complete insensitivity to pain, it was proposed that drugs that selectively target Na<sub>v</sub>1.7 might be ideal analgesics (Cox, Reimann et al. 2006; Ahmad, Dahllund et al. 2007). It is important to note that while humans lacking functional Na<sub>v</sub>1.7 were generally deemed to be healthy except for the apparently complete lack of ability to experience pain, mice that have a global deficit in Na<sub>v</sub>1.7 die just after birth (Nassar, Stirling et al. 2004), suggesting the role(s) of Na<sub>v</sub>1.7 could be different in mice and humans.

At least nine distinct point mutations in SCN9A sodium channel gene that alter the amino acid sequence of this channel have been identified in patients with erythromelalgia, another SCN9A-linked disease (Yang, Wang et al. 2004; Dib-Hajj, Rush et al. 2005). These mutations, which are thought to underlie the severe chronic burning pain sensations in the hands and feet that is the primary symptom associated with erythromelalgia, all cause

significant hyperpolarizing shifts in the voltage-dependence of activation (Cummins, Dib-Hajj et al. 2004; Dib-Hajj, Rush et al. 2005; Choi, Dib-Hajj et al. 2006) Indeed, several studies on Na<sub>v</sub>1.7 mutations in erythralgia have correlated a larger ramp current amplitude with an increase in dorsal root ganglion excitability (Lee, Yu et al. 2007). There is also an evidence that Na<sub>v</sub> 1.7 channels containing exon 5A displayed a larger ramp current than those containing exon 5N, as mentioned previously. Mutations involved in erythralgia that increase the ramp current might thus be more harmful in the presence of exon 5A. Interestingly, symptoms of primary erythralgia arise in childhood or adolescence and may progress and become constant with age. The increased symptoms of erythralgia with age might be related to an increase in the proportion of exon 5A. Similarly, changes in the proportions of exons 5N and 5A with age might also be related to differences in pain sensitivity (Chatelier, Dahllund et al. 2008).

Other Na<sub>v</sub>1.7 point mutations underlie a second autosomal dominant chronic pain disorder, paroxysmal extreme pain disorder (PEPD), characterized by severe burning rectal, ocular, and submandibular pain sensations (Fertleman, Baker et al. 2006).

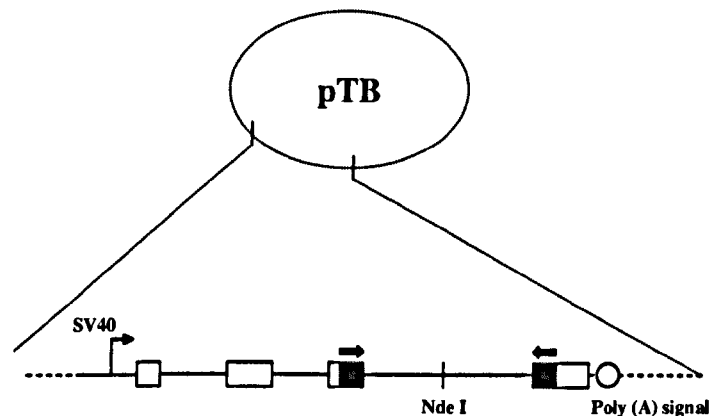
## 2. Aim of the project

Our knowledge of the various *cis*- and trans-acting factors playing a role on both normal and aberrant splicing pathways has been greatly enhanced in the last years. However, the resulting information explosion has also uncovered the fact that many splicing systems are not easy to model. In fact due to the unexpectedly complex network of interactions involved in pre-mRNA splicing we are still in part ignorant of the exact molecular mechanism involved and this is more so the case in mutually exclusive alternative splicing. The Aim of this thesis is to gain insights into the molecular mechanisms involved behind the control of ME splicing by studying this process in selective genes of the voltage gated sodium channel family whose many members undergo tissue and developmentally regulated ME splicing. In particular I have chosen to start with two ME splicing events in two of the family members SCN8A exons 18N and 18A and exons 5N and 5A in the SCN9A gene. The choice behind these two specific ME splicing events stems from the possible implications as therapeutic targets these ME events represents.

### 3. Results

#### 3.1 Analysis of SCN8A exon 18N and 18A mutually exclusive splicing regulation in a heterologous minigene context.

In order to create a tool with which to start to investigate the control mechanisms behind the tissue and developmentally regulated mutually exclusive splicing observed for SCN8A exon 18N and exon 18A I initially cloned these exons individually, together with a limited amount of respective flanking intronic sequence in the pTB minigene system, which is a modified version of the  $\alpha$ -globin-fibronectin-EDB minigene (Muro, Iaconcig et al. 1998) (Fig. 14).



**Figure 14.** Schematic representation of pTB minigene. White and black boxes represents the  $\alpha$ -globin and fibronectin exons respectively. The minigene contains a functional polyadenylation site at the 3' end and at the 5' end an  $\alpha$ -globin gene promoter and SV40 enhancer sequences to allow polymerase II transcription in the transfected cell lines. An Nde I restriction site in the intron between the two  $\alpha$ -globin/fibronectin hybrid exons is present, allowing for quick insertion of the exon and flanking intronic sequence one would wish to study. Black arrows in the two  $\alpha$ -globin/fibronectin hybrid exons indicate the



position of primers, used in RT-PCR reaction when analyzing the mRNA produced by the minigene.

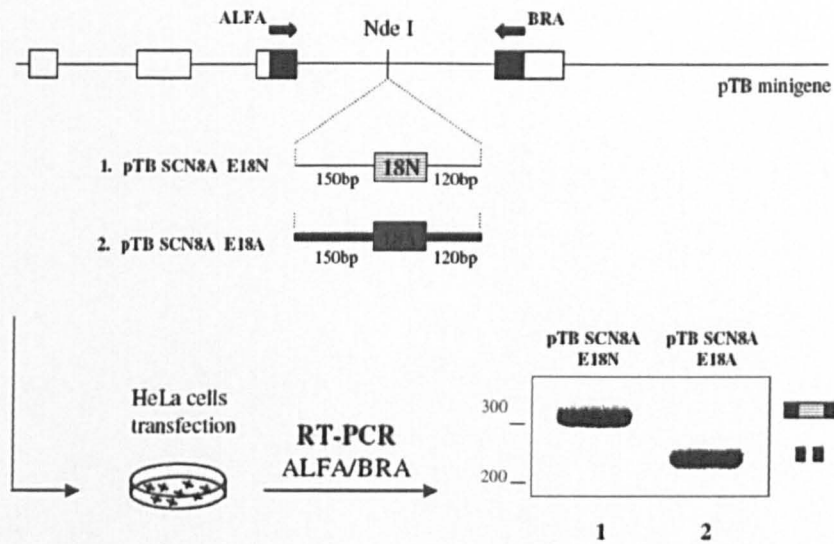
The rationale behind the use of the pTB minigene as a backbone was to isolate a relatively small DNA fragment containing the SCN8A exons under study, that would however allow this exon to behave as observed *in vivo*. This region of DNA could then be more easily manipulated and used as a starting point with which to investigate the *cis*-acting elements controlling the inclusion or exclusion of SCN8A exon 18N or 18A.

### **3.1.1 Splicing of the pTB minigene carrying either SCN8A exon 18N or 18A follow the observed splicing pattern observed for non-neuronal tissue.**

The pTB SCN8A exon 18N and pTB SCN8A exon 18A were made by standard methods described in material and methods section 6.17.1. Briefly, I initially subcloned the individual exons, SCN8A E18N and SCN8A E18A, together with 150 bp of upstream and 120 bp of downstream intronic sequence in the case of the former and 150 bp of upstream and 120 bp of downstream intronic sequence in the case of the later in the pUC19 vector. This vector was used primarily for the purpose of sequencing of the minigene, and after the exact sequence was confirmed, both DNA fragments were sub-cloned into the pTB minigene, using Nde I restriction enzyme site present between the two  $\alpha$ -globin/fibronectin hybrid exons (Fig. 14).

The mRNA processing of these minigenes was analyzed following expression of the constructs in HeLa cells, through RT-PCR using oligonucleotides specific for the minigene

ALFA and BRA. As can be seen in Fig. 15, in the case of pTB minigene carrying SCN8A exon 18N with its flanking intronic sequences, a unique PCR product of 311 bp is obtained, identified as a normal inclusion of exon 18N in the mature transcript (Fig. 15, Lane 1). On the other hand with the minigene carrying SCN8A exon 18A, together with its flanking intronic sequence, a PCR product of 239 bp is observed, corresponding to skipping of the exon 18A from the mature transcript (Fig. 15, Lane 2). These experiments showed that the recognition of the SCN8A exons 18N and 18A have different requirements even in an heterologous context and that the HeLa cells reflected the *in vivo* specificity of the expression.



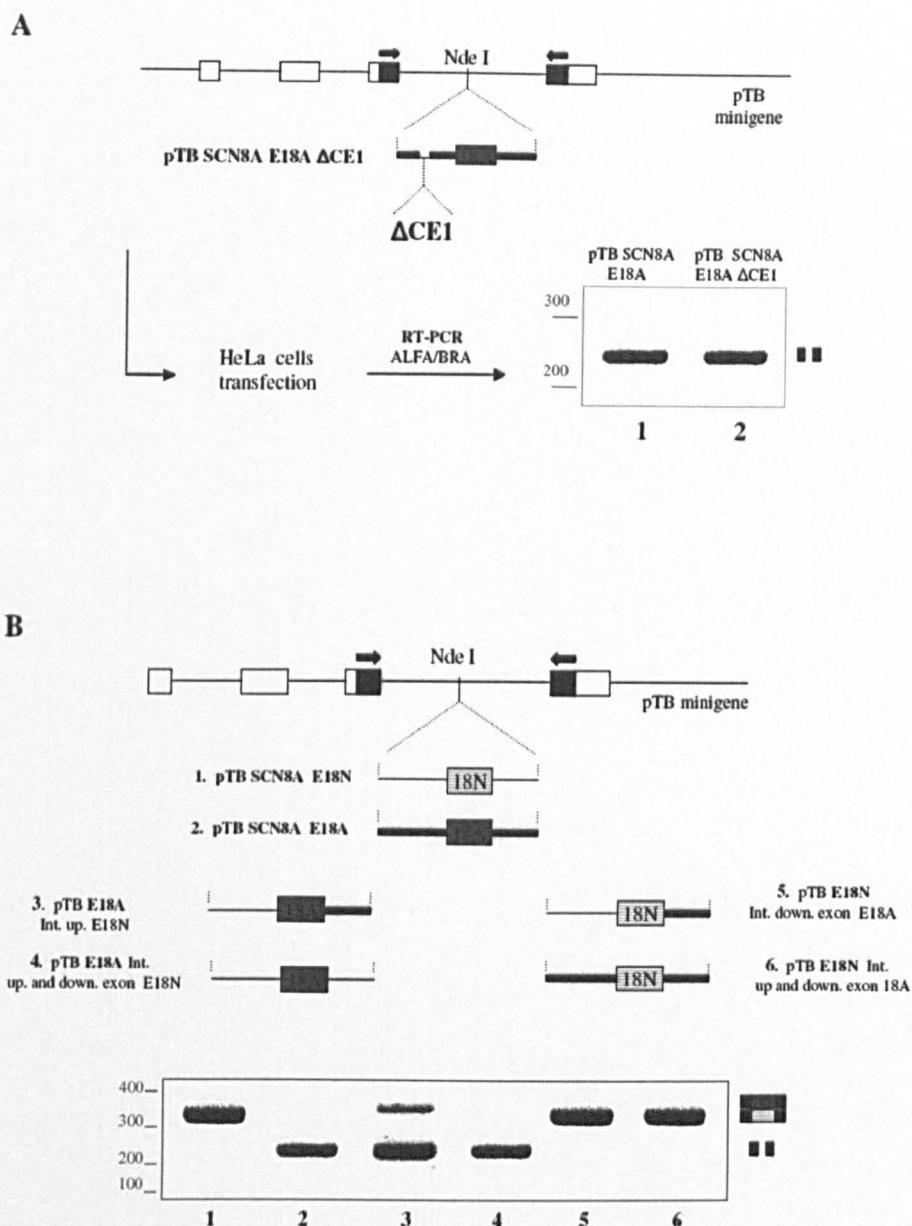
**Figure 15.** Analysis of the minigene splicing assay performed with pTB SCN8A exon 18N and pTB SCN8A exon 18A minigenes. In the upper panel a schematic representation of the pTB SCN8A E18N and pTB SCN8A E18A minigenes. Lower panel: Analysis of expression of the constructs in HeLa cells. The mRNA produced by the minigenes was analyzed through RT-PCR using oligonucleotides specific for the minigene ALFA and BRA (black arrows) and agarose gel electrophoresis. In the case of pTB minigene carrying exon 18N with its flanking intronic sequences (pTB SCN8A E18N) a unique PCR product is observed corresponding to the inclusion of exon 18N in the mature transcript (Lane 1). On the other hand with the minigene carrying exon 18A together with its flanking intronic sequence (pTB SCN8A E18A) PCR product corresponding to the skipping of the exon 18A from the mature transcript is observed (Lane 2).

### **3.1.2 The respective Inclusion and Exclusion of the SCN8A exon 18N and 18A in the mRNA produced from the pTB minigene in HeLa cells appears to be primarily dependent on exonic *cis*-acting elements.**

A genomic analysis of SCN8A from human, mouse and fish had previously highlighted an unusually high sequence identity of 70% in the human and mouse intron between SCN8A ME exons 18N and 18A (counting insertions and deletions as single changes) (Plummer, McBurney et al. 1997). Three-way sequence comparison identified one highly conserved element (CE1) adjacent to a eukaryotic branchpoint consensus site located upstream of the 3' splice site of exon 18A. The observed evolutionary conservation strongly suggested that this element may have a functional role that has been maintained by selection. I therefore decided to start the investigation of splicing regulatory elements that may be involved in the SCN8A mutually exclusive splicing by eliminating this 24 bp long CE1 element through site directed deletion, in the pTB SCN8A E18A minigene, creating the pTB SCN8A E18A  $\Delta$ CE1 minigene. After transfection in HeLa cells and RT-PCR analysis, using pTB SCN8A E18A and pTB SCN8A E18A  $\Delta$ CE1 minigenes, a unique PCR product of 239 bp is observed, corresponding to a skipping of exon 18A (Fig. 16A, Lanes 1 and 2). Thus, at least in this context, this element does not appear to play a role in the exclusion of the SCN8A exon 18A in HeLa cells.

The *cis*-acting elements, aside the classical core elements that define an exon, controlling the inclusion of the SCN8A exon 18N and exclusion of the SCN8A exon 18A in the pTB minigene, when transfected in HeLa cells, may be present either in the exonic or intronic portion of the DNA used to construct the minigenes, or indeed both, as outlined in the introduction section 1.3. To obtain an indication whether *cis*-acting elements controlling exon inclusion or exclusion, of the two SCN8A ME exon 18N and 18A, lie in the intronic

or exonic segment of the gene, I made a series of different constructs composed of SCN8A exons 18N or 18A, where I replaced the intronic sequence upstream or downstream of either SCN8A exon with those of the other as well as both sides of flanking intronic sequence (Fig. 16B, minigenes 3-6). The substitution of the upstream intron of exon 18A with the upstream intron of exon 18N, resulted in two products, as can be seen on agarose gel. A major band of 239 bp band representing a PCR product lacking exon 18A and the 362 bp band representing a PCR product including exon 18A (Fig. 16B, Lane 3). Curiously, skipping of SCN8A exon 18A was restored after transfection with minigene containing the intronic sequence flanking the SCN8A exon 18N upstream and downstream of SCN8A exon 18A (Fig. 16B, Lane 4). On the other hand the SCN8A exon 18N, independently of the intron context, always a unique PCR product of 311 bp corresponding to inclusion of exon 18N was observed (Fig. 16B, Lane 5 and 6). These results indicate that the intronic region immediately upstream of exon 18N may have some enhancing capacity on the inclusion of exon 18A, but the principal elements responsible for the inclusion or exclusion of exons 18N and 18A principally lie in the exonic portions of the DNA placed into the minigenes.



**Figure 16.** The respective inclusion and exclusion of the SCN8A exon 18N and 18A in the mRNA produced from the pTB minigene in HeLa cells appear to be primarily dependent on the exonic *cis*-acting elements. (A) In the upper panel a schematic representation of the pTB SCN8A exon 18A  $\Delta$ CE1 minigene. Lower panel: analysis of expression of the constructs pTB SCN8A E18A and pTB SCN8A E18A  $\Delta$ CE1 in HeLa cells. The mRNA produced by the minigenes was analyzed through RT-PCR using oligonucleotides specific for the minigene ALFA and BRA (black arrows) and agarose gel electrophoresis. A unique PCR product of 239 bp is observed, corresponding to a skipping of exon 18A in both cases

and is depicted on the right hand side of the gel (Lanes 1 and 2). (B) In the upper panel schematic representations of a series of minigene constructs composed of SCN8A exons 18N or 18A, with different combinations of intronic sequence upstream and downstream of the two ME exon. Lower panel: Results of the RT-PCR analysis of these minigenes in agarose gel electrophoresis. On the right hand side of the gel is a illustration indicating what each amplification corresponds to.

### **3.2 Identification of an exonic splicing silencer element (ESS) in SCN8A exon 18A.**

I initially decided to set out to identify the regulatory *cis*-acting elements that may modulate the mutually exclusive splicing of SCN8A exon 18A by trying to identify the reason as to why this exon was excluded from the pTB mRNA when transfected in Hela cells. The previous experiments carried out in section 3.1.2 indicated that it was highly likely that these lie in the exonic portion of the SCN8A sequence used in the minigene pTB SCN8A E18A.

An initial approach to identify possible exon silencer elements was performed through scans of SCN8A exon 18A for sequence elements with similarity to known exon silencer elements, predicted by bioinformatic programs, that matches the functional silencers found in the FAS-hex3 or PESS ESS databases; <http://genes.mit.edu/fas-ess/>, <http://cubweb.biology.columbia.edu/pesx> respectively (Wang, Rolish et al. 2004; Zhang and Chasin 2004). These analysis highlighted several important regions that may be involved (Fig. 17A). In particular putative silencer elements were predicted in the 5' region of SCN8A exon 18A. In order to test the functional consequence of these predictions, using the pTB SCN8A E18A minigene as a backbone, I made a series of

sequential deletions of this area. In particular derivatives of pTB SCN8A E18A minigene (6-27 $\Delta$ , 27-43 $\Delta$  and 43-69 $\Delta$ ) were made (Fig. 17B). These minigenes were transfected in HeLa cells, RNA was extracted and subjected to RT-PCR analysis. As can be seen after electrophoresis the 6-27 $\Delta$  minigene gave almost complete inclusion of exon 18A while 27-43 $\Delta$  and 43-69 $\Delta$  minigenes had no consequences on the inclusion of exon 18A (Fig. 17B). It therefore appears that the segment between nucleotides 6-27 of the exon, that I will refer to as the ESS element, of the SCN8A exon 18A, contains an ESS element, responsible for repressing 18A exon splicing in HeLa cells.



**A**

FAS-ESS web server results

<http://genes.mit.edu/fas-ess/>

Set: FAS-hex3

Name: seq

Length: 123 **SCN8A Exon 18A**

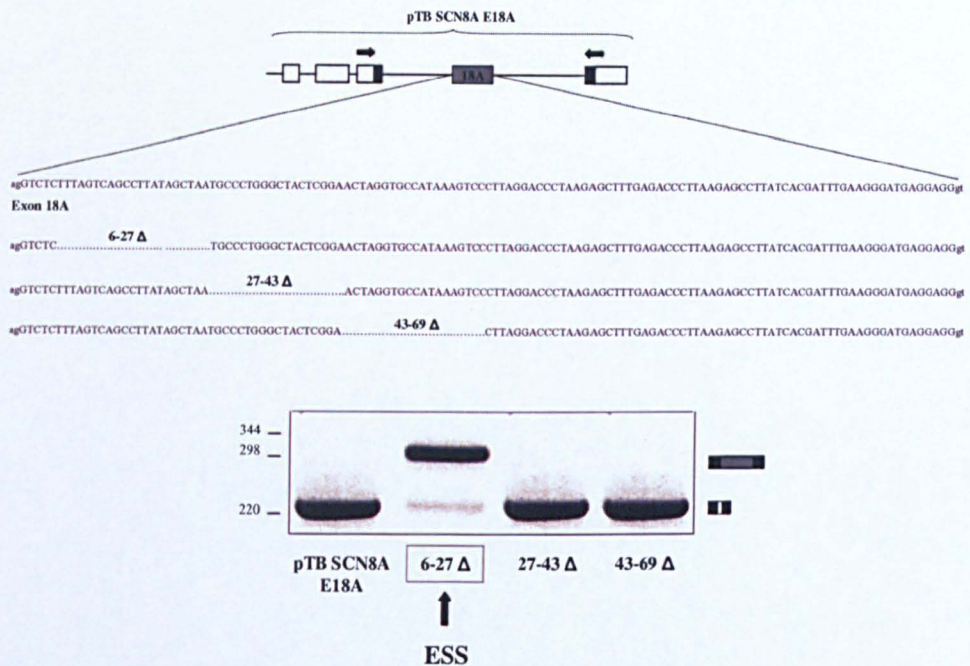
GTCTCTTTAGTCAGCCTTATAGCTAATGCCCTGGGCTACTCGGAACTAGGTGCCATAAAGTCCCTTAGGACCCTAAGAGCTTTGAGACCCTTAAGAGCCTTATCACGATTTGAAGGGATGAGGAGG

PESXs in your sequence:

<http://cubweb.biology.columbia.edu/pesx>

GTCTCTTTAGTCAGCCTTATAGCTAATGCCCTGGGCTACTCGGAACTAGGTGCCATAAAGTCCCTTAGGACCCTAAGAGCTTTGAGACCCTTAAGAGCCTTATCACGATTTGAAGGGATGAGGAGG

**B**



**Figure 17.** Identification of an exon splicing silencer element (ESS) in SCN8A exon 18A. **(A)** Analysis of SCN8A exon 18A for sequence elements with similarity to known exon silencer elements, using a bioinformatic programs, FAS-hex3 and PESS ESS databases; <http://genes.mit.edu/fas-ess/>, <http://cubweb.biology.columbia.edu/pesx>. Underlined and bold nucleotides indicate areas identified by the programs as possible ESS. **(B)** Upper panel: Schematic representation of the wt pTB SCN8A exon 18A minigene carrying deletion of the nucleotides 6-27 $\Delta$ , 27-43 $\Delta$  and 43-69 $\Delta$ . Lower panel. Splicing

analysis of the exon 18A showed that only the 6-27 $\Delta$  minigene gave complete inclusion of exon 18A, while 27-43 $\Delta$  and 43-69 $\Delta$  minigene had no consequences on exon 18A inclusion as illustrated on the right hand side of the gel.

### **3.2.1 The ESS identified in the SCN8A exon 18A inhibits the assembly of multiple proteins during spliceosome formation.**

The ability of an ESS to exert repression on its upstream 3' splice site is a common feature for the ESSs identified in mammals (Carstens, Wagner et al. 2000). If the SCN8A exon 18A regulatory element has a dominant ESS, spliceosome assembly should be inhibited at an early step. The spliceosome assembly occurs stepwise and is characterized by the interaction of multiple proteins and ribonuclear proteins with the nascent transcript (Sharp 1994). Although already discussed in the introduction (section 1.1.1), it is important to remember that these RNA and protein complexes are in order of formation as follows: H, E, A, B and C. H is a nonspecific complex formed by interaction of the pre-mRNA with multiple hnRNPs (Jamison, Crow et al. 1992). The composition of this complex can vary greatly between different RNAs but will include splicing regulatory proteins involved in the formation of the catalytically component spliceosome. Its formation occurs even at 0°C and does not require ATP. Incubation of the pre-mRNA at 30°C leads to formation of complex E (House and Lynch 2006). In addition to splicing regulatory elements, such as SR proteins and hnRNPs, complex E contains U1 snRNP and the U2AF splicing factors at the 5' and 3' splice sites of the intron, respectively. Complex A forms following the addition of ATP and involves binding of the splicing factor U2 snRNP to the branch point in the intron. Incorporation of the U4/U5/U6 tri-snRNP leads to formation of B complex.

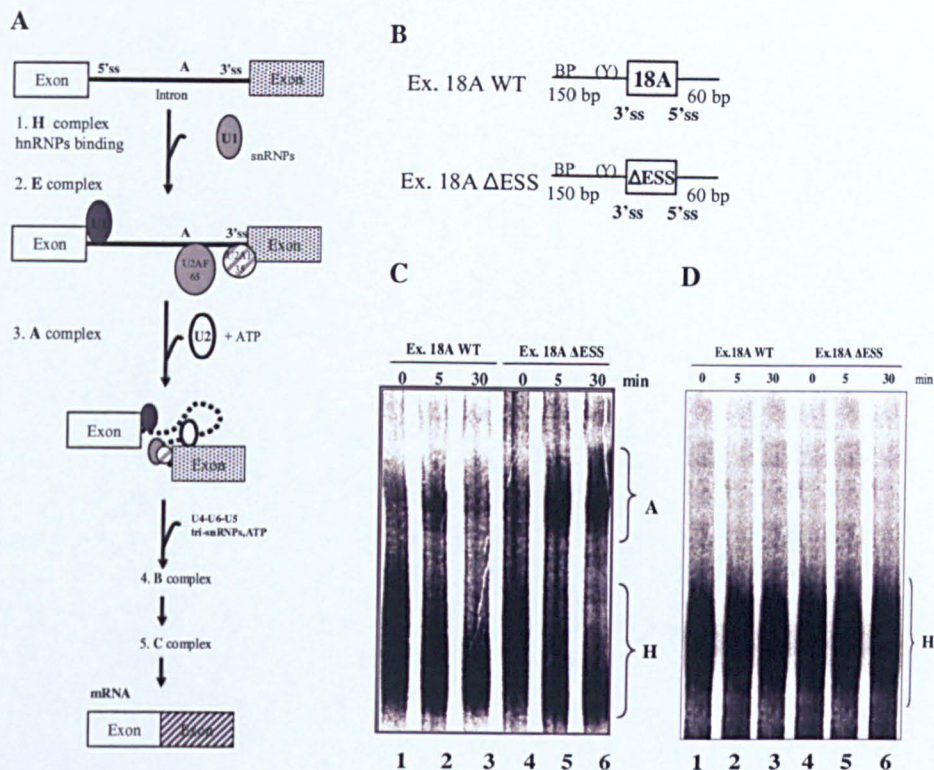
Subsequently, after massive structural rearrangements, the catalytically activated spliceosome, complex C forms, and the first transesterification step of splicing occurs (Fig. 18A) (Fukumura and Inoue 2009).

To further investigate the mechanism of SCN8A exon 18A repression by the ESS identified, I wanted to determine if this indeed was causing a block in the spliceosome formation and if so at what step in spliceosome assembly this was occurring. The most commonly used method for resolving the H, A, B and C complexes is electrophoresis on a vertical, 4% nondenaturing polyacrilamide gel (Konarska, Grabowski et al. 1985). The buffer containing Tris-glycine and heparin is usually added to the splicing reaction to dissociate nonspecific interactions. However, under these conditions, the E complex coimmigrates with the H complex (Michaud and Reed 1993).

I analyzed the assembly of spliceosome complexes formation on single exon substrates composed of Ex.18A WT and Ex.18A  $\Delta$ ESS RNAs, carrying the upstream 3' splice site, the polypyrimidine/predicted branch point (BP) sequences and the downstream 5' splice site of which schematic representations can be seen in Figure 18B.

At time point 0, only complexes H and E, that under these experimental conditions co-migrates, formed on both the substrates (Fig. 18C, lanes 1 and 4). After 5 minutes of incubation of the splicing substrates with HeLa nuclear extract at 30 °C with ATP, complex A was detected in both substrates but was much less abundant in the Ex.18A WT substrate compared with the Ex.18A  $\Delta$ ESS substrate where the ESS was missing (Fig. 18C, lanes 2 and 5). After 30 minutes of incubation at 30 °C, complex A was observed to be much more abundant and stable during time on the substrate without the ESS element (Ex.18A  $\Delta$ ESS) as can be seen by comparison with the Ex.18A WT substrate (Fig. 18C, lanes 3 and 6). Gel analysis further showed that the complex A was not detected in both substrates in the absence of ATP (Fig. 18D, Lane 1-6). These results further confirm the presence of the ESS element in SCN8A exon 18A identified in section 3.2 and suggest that the ESS inhibits the assembly of multiple proteins during spliceosome formation. The

reduction in complex A formation may be caused by a repressive effect on the exon 18A ESS on the strength of binding of U2 or other splicing factors during the formation of complex A.



**Figure 18.** The ESS identified in the SCN8A exon 18A inhibits the assembly of multiple proteins during spliceosome formation. (A) Schematic representation of spliceosome assembly that occurs stepwise and is characterized by the interaction of multiple proteins and ribonuclear proteins with the nascent transcript. These RNA and protein complexes are formed in a stepwise manner with names letters designated certain stages: H - E - A - B - C. (B) Cartoon illustrating the two different  $^{32}\text{P}$ -labeled *in vitro* splicing substrates were used. (Y) indicates polypyrimidine tract, BP indicates predicted branch point sequences. (C) Autoradiography of acrylamide gel electrophoresis of the spliceosome complexes of the two substrates at 0, 5 and 30 minutes. The regions corresponding to complexes H and A are indicated on the right hand side of the autoradiograph. (D) Autoradiography of acrylamide gel electrophoresis of the spliceosome complexes of the two substrates at 0, 5

and 30 minutes without ATP. The region corresponding to complex H is indicated on the right hand side of the autoradiograph.

### **3.3 Identification of trans-acting factors binding to the ESS identified in the SCN8A exon 18A.**

The vast majority of splicing enhancer and silencing sequences characterized to date function as binding sites for splicing regulatory proteins that in turn alter the mRNA processing by mechanisms previously described in the introduction (section 1.3). To identify the trans-acting factors binding to the ESS identified in SCN8A exon 18A, I performed a pull down analysis as previously described (Buratti, Baralle et al. 2004). The effect observed when the 6-27 $\Delta$  region was eliminated in the pTB SCN8A E18 minigene and the inhibition of spliceosome assembly in the presence of this region indicate that the 6-27 $\Delta$  region indeed contains an ESS element. It was therefore of interest to try and identify the trans-acting factors through which this splicing *cis*-acting regulatory element acts.

Briefly, pTB SCN8A E18A (with the ESS identified present) and pTB SCN8A E18A  $\Delta$ ESS minigenes (without the ESS element) were used as a template for the amplification with the primer that added a T7 promoter at the start of the sequences (Fig. 19A).

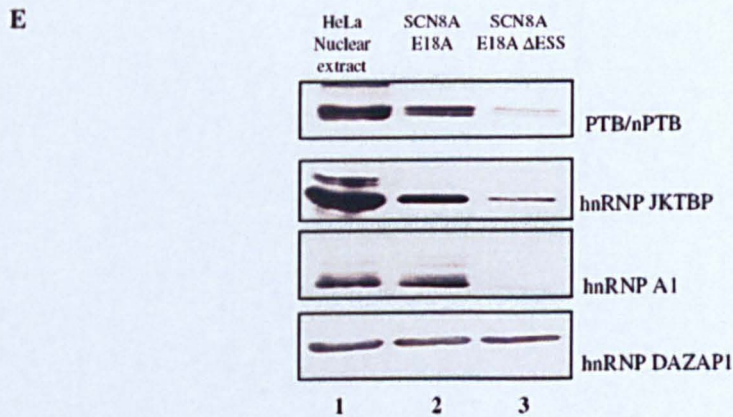
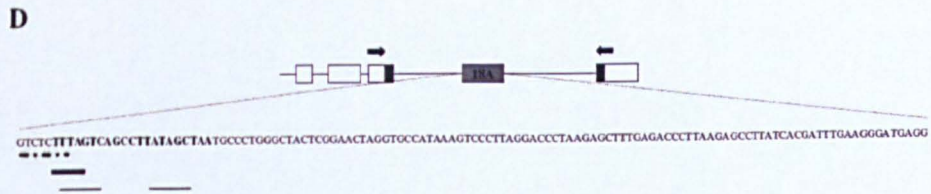
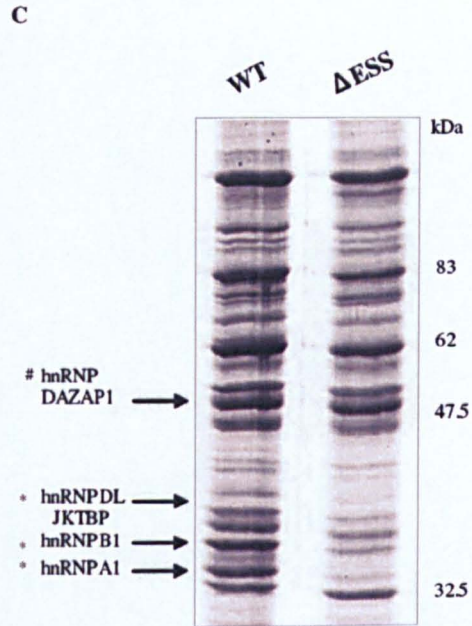
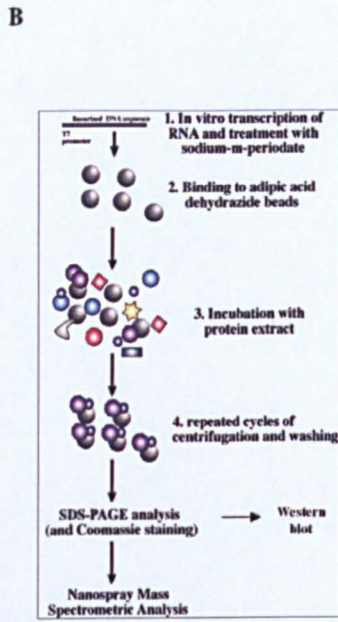
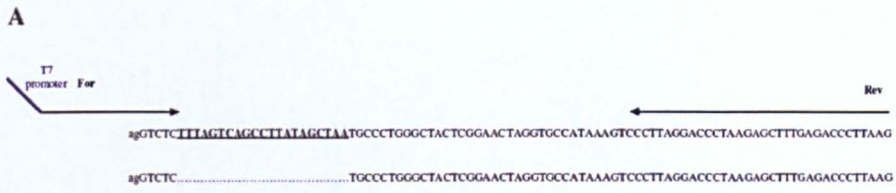
These RNA's were then covalently linked to adipic acidic beads, incubated with nuclear extract prepared from HeLa cells, precipitated and washed. RNA-associated proteins were then eluted with SDS sample buffer, separated by SDS-PAGE and visualized by Coomassie blue staining (Fig. 19B) and eventually proteins were identified by mass spectrometry and western blot. As can be seen from the coomassie gel (Fig. 19C) both RNAs (with and without ESS element) were observed to bind multiple proteins. However, comparison of the proteins bound to RNA with ESS versus RNA without ESS, revealed

several bands that associate specifically with RNA that contained ESS, indicating possible candidate proteins specific for the ESS element (Fig. 19C \*). Each of the ESS-specific bands was excised from the gel and analyzed by mass spectrometry by the Protein Networks Group at ICGEB (Dr. Michael P. Meyers ICGEB), as well as a band that was common to both the RNA with and without ESS element (Fig. 19C #). The mass spectrometry analysis identified protein band common to both RNA substrates as hnRNP DAZAP1 and the three protein bands unique to the RNA substrate with the ESS element; as hnRNP JKTBP, hnRNP B1 and hnRNP A1 protein. With the exception of hnRNP B1, for which I did not find in literature strong evidence regarding the potential involvement in the repression of the splicing, the other two proteins have been strongly implicated in splicing regulation.

Consistent with the identification of the ligands binding to the ESS are the presence of binding sites for these in the ESS. For example hnRNPA1, a “winner” sequence identified by SELEX, UAGGGA/U is present in the ESS region, as is the 7 nt sequence ACUAGC/T for hnRNP JKTBP (Fig. 19D). While searching for the binding site of these proteins in the region of the ESS we also noted that there was a binding site for the PTB/nPTB protein which is UCUU (Perez, Lin et al. 1997). As PTB/nPTB would be an extremely interesting candidate for the binding to ESS identified in the SCN8A exon 18A, I decided to test if this protein was also differentially being pulled down with the two *in vitro* transcribed RNAs. Even if the differential binding of these proteins with the two *in vitro* transcribed RNAs was not observed in the coomassie gel (Fig.19C) it could be the case that this would be masked by other proteins running in the gel in the same area as the MW of PTB/nPTB. This was performed by using a specific antibody against this protein in western blots performed on the pulled down proteins (Fig. 19E). As further confirmation of the mass spectrometry results, hnRNP A1 and hnRNP JKTBP were also tested using specific antibodies against these proteins in western blot analysis. The antibody against the hnRNP DAZAP1 protein was used in western blot analysis as a loading control. As expected, I

observed a very strong signal on the RNA substrate with the ESS element for hnRNP JKTBP and hnRNP A1. Interestingly, this was also the case for PTB/nPTB protein (Fig. 19E, Lane 2).

Thus, although I cannot fully rule out the presence or importance of other proteins which may be involved, RNA-affinity purification clearly identifies the hnRNP family members, hnRNP A1, PTB/nPTB and hnRNP JKTBP as the primary proteins associated with the ESS regulated exonic silencer.





**Figure 19.** Identification of trans-acting factors binding to the exonic splicing silencer identified in SCN8A exon 18A. (A) DNA sequence used for the *in vitro* transcription of WT RNA and mutant  $\Delta$ ESS RNA. (B) Schematic representation of the pull down procedure analysis. The steps are indicated on the right hand side of the figure. Following separation of the proteins in SDS PAGE acrylamide gel the protein bands can be visualized by commassie staining or recognized by western blot analysis through incubation with specific antibodies. (C) Coomassie blue staining of a pull down assay using WT RNA and mutant  $\Delta$ ESS RNA. Arrows indicate the proteins sequenced and identified through mass spectrophotometry analysis. (D) Schematic representation of the binding sites identified in the region of the ESS for the proteins (PTB/nPTB (dotted line), hnRNP A1 (bold line) and JKTBP (plain lines)). (E) Western blot analysis for PTB/nPTB, JKTBP hnRNP A1 and hnRNP DAZAP1 performed on a pull down of the WT RNA and mutant  $\Delta$ ESS RNA.

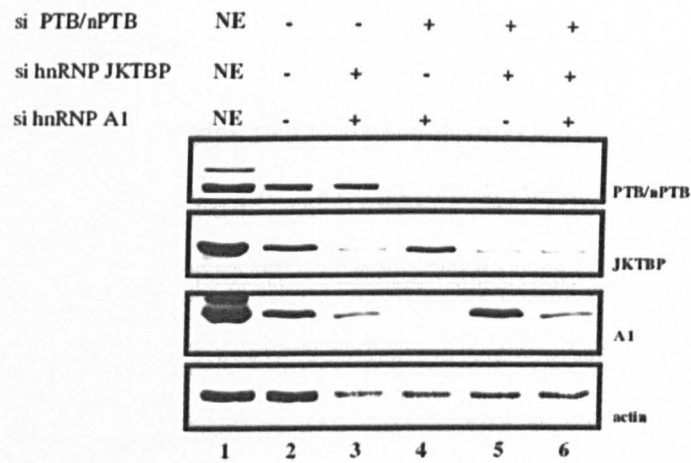
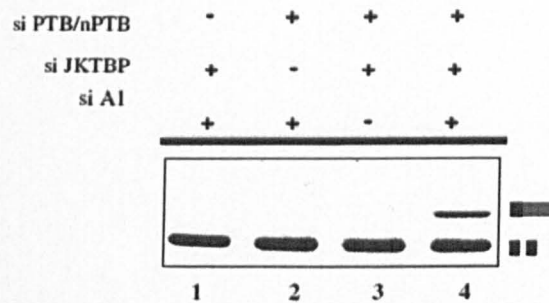
### **3.3.1. Knockdown of hnRNPs A1, PTB/nPTB and JKTBP protein decreases the effect of ESS identified in the exon 18A.**

To establish if hnRNPs identified to bind specifically the ESS element in SCN8A exon 18A (section 3.2.2.) have a functional role in the repression of SCN8A exon 18A I performed a si-RNA experiment (SI), in which I depleted hnRNPA1, PTB/nPTB and hnRNP JKTBP individually as well as in different combinations from HeLa cells. As no strong evidence was found in the literature regarding the potential involvement of hnRNP B1 in the repression of the splicing I decided for the time being not to test this candidate as a potential splicing regulator of SCN8A exon 18A inclusion.

All si-RNA treatments were effective at reducing the protein levels of the target protein as can be observed from their reduction in western blot analysis on protein cells extracts of

the treated cells (Fig. 20A, Lanes 3-6). The effect the knock down of these proteins had on the mRNA processing of SCN8A exon 18A was analysed by transfecting the pTB SCN8A E18A minigene in a background where these proteins had being knocked down singularly or in different combinations. Whereas the individual knock down of the proteins did not have any effect as the exon 18A was still skipped, exon 18A inclusion was observed to some extent when all four hnRNP proteins were eliminated (Fig. 20B, Lane 4). These results indicate that there is a considerable redundancy of the hnRNPs in the interactions with the ESS in order to maintain the exclusion of SCN8A exon 18A in HeLa cells and presumably “neonatal tissues”. This type of redundancy is similarly to what has being documented for SR proteins (Tacke and Manley 1999).

How these hnRNPs participates in the regulation of tissue and developmental specific alternative splicing regulation of these ME exons remains to be understood. Tissue specific changes in the expression level of hnRNPs may be involved, or another possibility is that additional tissue specific factors are involved that function together with these hnRNPs in regulation of this alternative splicing. Furthermore, neuronal cells may possess different isoforms and amounts of hnRNPs, or another possibility is tissue-specific phosphorylation. Indeed, phosphorylation has been shown to affect binding of hnRNP C to pre-mRNA (Auboeuf, Batsche et al. 2007).

**A****B**

**Figure 20.** Analysis by si-RNA of the functional role played by PTB/nPTB, JKTBP and hnRNP A1 in exon 18A exclusion from mRNA. **(A)** The western blot analysis of si-RNA experiments, depleting the hnRNP's PTB/nPTB, JKTBP and hnRNP A1 individually and in different combinations. All si-RNA treatments were effective at reducing the protein levels of the target protein as can be observed comparing the western blot signals and those of actin used as a control. **(B)** Agarose gel electrophoresis analysis of the RT-PCRs analyzing the effect of the knockdown experiments on the inclusion of SCN8A exon 18A in the mRNA using the minigene pTB SCN8A E18A. On the right hand side of the gel is a illustration indicating what each amplification corresponds to.

### **3.4 Deletion of the ESS, identified in the SCN8A exon 18A, in a minigene composed of a larger homologous context, is insufficient to cause inclusion of exon 18A in the mRNA.**

Over the last few years it has emerged that pre-mRNA is embedded with splicing regulatory elements. The practical consequence of this is that the correct control of splicing often depends on multiple signals whose cumulative effect results to give the splicing outcome desired (Buratti, Baralle et al. 2004). Till this moment the analysis of the ESS silencer element, on the inclusion of SCN8A exon 18A, was performed in a reductive minigene in respect to the homologous sequence context that could not provide any information on what effect the deletion of the ESS would have in a more homologous environment, or any subsequent effect may have on SCN8A exon 18N.

I therefore constructed a SCN8A WT minigene, spanning SCN8A exons 17 to 19, encompassing both the 18N and 18A exons (Fig. 21A). Transfection into HeLa cells, followed by RT-PCR analysis and agarose gel electrophoresis allowed me to analyze the RNA processing of the two SCN8A ME exon 18N and 18A in this minigene. A major amplicon of 620 bp, and a very minor amplicon of 549 bp were obtained (Fig. 21A). Sequencing of the gel-purified cDNA fragments showed that the 620 bp product contains exon 17, 18N and 19. This transcript, as outlined in the introduction (section 1.4.4) contains an in-frame stop codon that would generate a truncated protein of 1034 amino acids, 60% of the full-length channel protein (Plummer, McBurney et al. 1997). The 549 bp fragment is derived from an mRNA product spliced directly from exon 17 to exon 19, excluding both SCN8A ME exons 18N and 18A from the mRNA. This transcript maintains an open reading frame but lacks sequences encoding S3 and S4 segments of domain III as explained in the introduction (section 1.4.4). These results indicate that the

SCN8A WT minigene splicing behaves in a manner observed of SCN8A splicing in non-neuronal tissues (Plummer, McBurney et al. 1997).

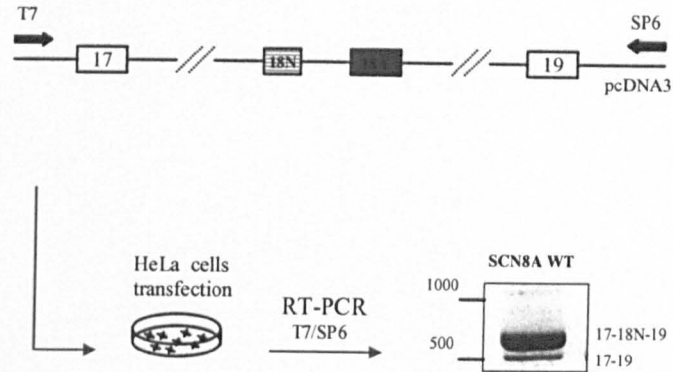
To test if ESS, identified in the SCN8A exon 18A, had the same effect in the homologous minigene context as it did in the pTB minigene, I deleted this silencer element in the SCN8A WT minigene, creating the minigene SCN8A/ $\Delta$ ESS E18A. Furthermore as a number of exon sequences which repress splicing have been described, capable of repressing splicing of heterologous exon, suggesting that they can function independently in a relatively simple way by recruitment of a protein, I decided to test whether the ESS identified could function autonomously and represents a phenomenon of more general interest. This was done by introducing this sequence element into the SCN8A exon 18N, in the context of SCN8A WT minigene, which is normally included in HeLa cells, at an analogous location as to that in which it is found in exon 18A exon (Fig. 21C), creating the minigene SCN8A/+ESS E18N.

RT-PCR analysis of SCN8A/ $\Delta$ ESS E18A (Fig. 21B) resulted in partial inclusion of SCN8A exon 18A. The mRNA including exon 18A also contained ME exon 18N, indicating that the deletion of the ESS element was not sufficient to cause the switch in ME of the two exons. Curiously, the inclusion of SCN8A exon 18A occurred to a much lesser extent than that previously observed when the same region was deleted from the exon in the pTB minigene context (Fig. 17B). This fact could indicate that other *cis*-acting elements may be acting in the larger context, necessary to aid the inclusion of this exon or repress it. However, the former seems to be the likely if we consider the result observed when we created the ESS element in SCN8A exon 18N as in this scenario the exon 18N carrying the ESS element, identified in exon 18A, was skipped showing that this sequence can act in a different context repressing splicing of exon which is normally included (Fig. 21C).

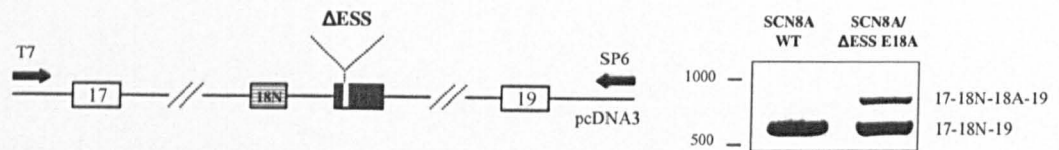
Taken together these results would indicate that the ESS thus far identified is extremely active, as it can function out of context, and more interestingly that there are indeed other

*cis*-acting elements, present in the larger homologous context spanning SCN8A exons 17-19 that also play a role in recognition of the SCN8A exon 18A.

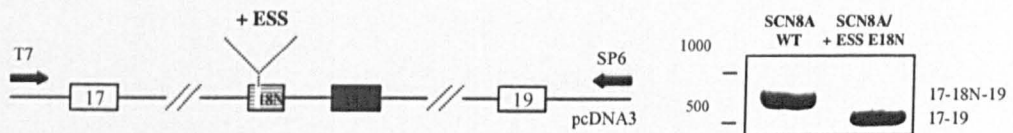
**A**



**B**



**C**



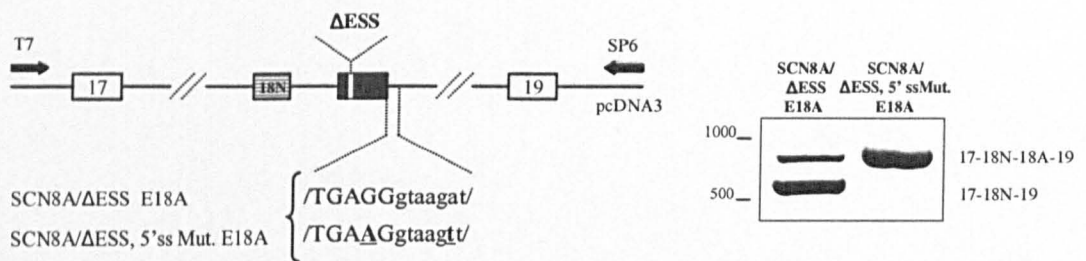
**Figure 21.** Analysis of the alternative splicing of SCN8A exon 18N and 18A in a homologous minigene system. **(A)** Schematic representation of the minigene SCN8A WT that spans SCN8A exons 17 through to 19. // indicates SCN8A endogenous intronic region not included in the minigene construct. Transfection in HeLa cells followed by RT-PCR resulted in two products. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. The major band corresponds to exon 17, 18N and 19 whereas the lower band was composed of exons 17 and 19. **(B)** Left,

schematic representation of the minigene SCN8A/ $\Delta$ ESS E18A. Right, analysis of the mRNA processing of this minigene after transfection in HeLa cells. Two bands can be observed on the agarose gel, the upper band corresponds to exons 17-18N-18A-19 and the lower band to exons 17-18N-19 (C) Left, schematic representation of the minigene SCN8A/+ ESS E18N. Right, analysis of the mRNA processing of this minigene after transfection in HeLa cells where the effect of the introduction of the ESS result in exclusion of the exon 18N.

### **3.5 Changing the 5' splice site of SCN8A exon 18A towards consensus aids in the inclusion of the exon in mRNA.**

The previous results have shown that deletion of the ESS element in the SCN8A homologous minigene context was insufficient to cause complete inclusion of the SCN8A exon 18A in the mRNA when a larger homologous context was included in the minigene as opposed to what was observed when SCN8A exon 18A together with a limited amount of intronic sequence was placed in a heterologous context. This fact could indicate that other *cis*-acting elements may be needed to aid in the inclusion of this exon and whose trans-acting factors are missing from HeLa cells or that the larger context carries elements that repress the SCN8A exon 18A inclusion. These possibility's was further accented by the fact that I observed that the 5' splice site of the SCN8A exon 18A differs from the consensus (canonical sequence of a mammalian 5' ss is MAG/GTRAGT in which / indicates the exon-intron junction at two positions). The differences between the consensus 5' splice site of SCN8A exon 18A suggest that the 5' splice site of exon 18A might be inherently weak and thus more prone to the effects of "weak" *cis*-acting elements. To test this possibility and investigate the influence of SCN8A exon 18A 5' splice site strength on

SCN8A exon 18A splicing, using the minigene composed of SCN8A exons 17-19 with the ESS sequences deleted (SCN8A/ $\Delta$ ESS E18A), I mutated the SCN8A exon 18A 5' splice site in manner that brought it to match exactly the canonical mammalian 5' splice site, creating a new minigene, SCN8A/ $\Delta$ ESS, 5'ss Mut. E18A (Fig. 22). Comparison of the two minigenes, SCN8A/ $\Delta$ ESS E18A and SCN8A/ $\Delta$ ESS, 5'ss Mut. E18A, shows that an improvement of the 5' splice site drastically increases the inclusion of SCN8A exon 18A with the mRNA processing of the minigene resulting in the complete inclusion of both mutually exclusive exon, when compared to the mRNA processing of the SCN8A/ $\Delta$ ESS E18A. Thus, the 5' splice site of SCN8A exon 18A is inherently weak, and this characteristic is important for its incomplete inclusion and susceptibility to regulation.



**Figure 22.** The 5'ss of exon 18A is inherently weak. Left, schematic representation of the minigene SCN8A/ $\Delta$ ESS E18A illustrating the 5'ss nucleotide sequence as well as the nucleotide modification performed in order to bring the 5'ss to match the canonical mammalian 5' splice site creating the minigene SCN8A/ $\Delta$ ESS, 5'ss Mut. E18A. Right, analysis of the mRNA processing of this minigene after transfection in HeLa cells where the effect of the introduction of a canonical 5'ss results in complete inclusion of exon 18A in an mRNA species composed of exons 17-18N-18A-19 as opposed to the partial inclusion of exon 18A in such an mRNA species observed in the presence of the wild type 5'ss. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid.



### **3.6 Identification and characterization of further *cis*-acting regulatory elements in the homologous minigene SCN8A WT context affecting exon 18A inclusion or exclusion in the mRNA.**

The results presented in section 3.5 showed that the 5' splice site of SCN8A exon 18A is intrinsically weak. An exon with a weak 5' splice site would be prone to the influence of even “weak” *cis*-acting splicing regulatory sequences adding a layer of control to its inclusion or exclusion from the final mRNA other than the ESS thus far identified. This further layer of complexity could be in the form of further splicing silencers or enhancers, the latter who's function in HeLa cells may not be active due to lack of the appropriate trans-acting factors in this cell line, as after all SCN8A exon 18A in non-neuronal cells lines is typically excluded from the final mRNA.

#### **3.6.1 A region in the vicinity of the 3' splice site of SCN8A exon 18A mediates the splicing regulation of SCN8A gene.**

The fact that the deletion of the ESS resulted in a drastically reduced inclusion of the SCN8A exon 18A in the mRNA when I used the SCN8A/ $\Delta$ ESS E18A minigene, as opposed to the full inclusion observed when the ESS was deleted in the heterologous minigene (pTB vector), led me to hypothesise that other *cis*-acting elements may be present in the larger homologous context. Indeed as I showed in previous paragraph, the SCN8A exon 18A 5' splice site was inherently weak and this characteristic was one of the important factors contributing to the skipping of exon 18A in non neuronal cells, such as HeLa.

As has been shown previously, in the pTB minigene context (Fig. 16B, Lane 3), the exclusion of SCN8A exons 18A, in part, was also determined by its upstream intronic flanking region. To further analyze the *cis*-acting elements in the intronic portion between the two SCN8A ME exon 18N and 18A that may be critical in determining the ME exon selected in the mRNA I designed six new minigene constructs in which different deletions of the intronic region and/or exon 18N were made, in the presence or absence of the ESS thus far identified in SCN8A exon 18A (Fig. 23A). These constructs were transfected in HeLa cells, and analyzed by RT-PCR.

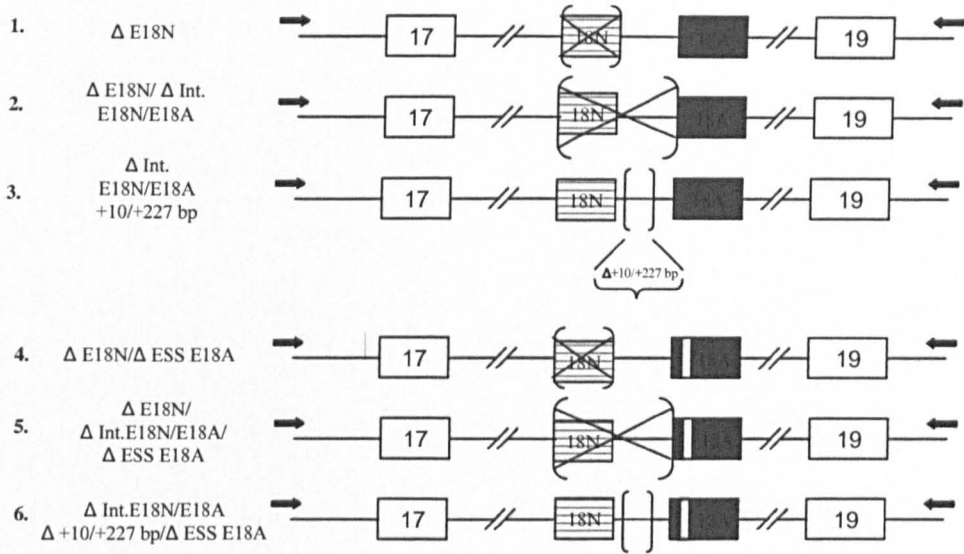
The first construct was made in order to determine whether deletion of only exon 18N from the SCN8A WT minigene would have any effect on the inclusion of exon 18A. To this end a DNA fragment containing exons 17, 18A (with and without the ESS element) and 19, together with the respective intronic regions, but without exon 18N, was introduced in pcDNA3 expression vector (Fig. 23A, constructs 1 and 4). The mRNA produced, after transfection and RT-PCR analysis continued to skip SCN8A exon 18A if the ESS was present resulting in an mRNA species composed of exons 17 and 19 (Fig. 23B, Lane 1) and showed similar levels of exon 18A inclusion, albeit this time in an mRNA species obviously missing exon 18N (Fig. 23B, Lane 4).

Subsequently, I tested the effect on the mRNA processing of the deletion of exon 18N together with the deletion of the intronic region between the two SCN8A ME exon, 18N and 18A, on inclusion of SCN8A exon 18A, again in minigenes with and without the ESS identified in exon 18A (Fig. 23A, constructs 2 and 5). As suspected from the previous results with pTB minigene, inclusion of SCN8A exon 18A was observed (Fig. 23B, Lane 2) even in the presence of the ESS element in SCN8A exon 18A and was more significant in the absence of the ESS (Fig. 23B, Lane 5)

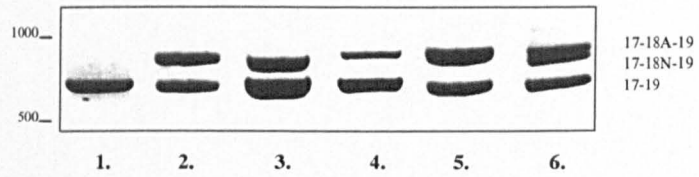
A further construct was made in order to try and isolate the intronic sequence responsible for the observed exon 18A inclusion. To this end I removed a region corresponding to the +10 bp nucleotide downstream of the 5'ss of exon 18N to +227 bp in a minigenes with and

without the ESS in exon 18A (Fig. 23A, constructs 3 and 6). Unexpectedly in this scenario we observed that with the minigene in which the ESS was present (Fig. 23B, Lane 3) exon 18N was drastically affected with the major band corresponding to mRNA composed only of exons 17 and 19 as opposed to the mRNA species resulting from the SCN8A WT minigene (Fig. 23B, Lane 1). It would therefore appear that the intronic region +10 bp to +227 bp between two SCN8A ME exon 18N and 18A, contains *cis*-acting elements that promote the inclusion of exon 18N. The same +10 bp to + 227 bp deletion, in the minigene with the ESS in exon 18A deleted, results in three mRNA products. The principal species was a skipping of both ME exon, 18N and 18A. The two other species of approximately equal intensity were ones of exons 17-18A-19 and another 17-18N-19 (Fig. 23B, Lane 6). This experiments start to paint an extremely complex picture of the splicing control provided by the intronic region between the two ME exons that I have only just began to comprehend and whose effect on the two ME exons are schematically represented in figure 23C. From the data on mRNA splicing patterns observed, with construct number 2, I can hypothesize that the intronic region between two ME exon, 18N and 18A, contains elements that repress the inclusion of exon 18A. Furthermore these elements, based the data from construct number 3, would appear to be outside the region deleted, +10 bp/+227 bp, and therefore present in the intronic region located -150 bp upstream of the 3' ss of exon 18A. The construct number 3, on the other hand, would indicates that this region contains an ISE for the inclusion of SCN8A exon 18N.

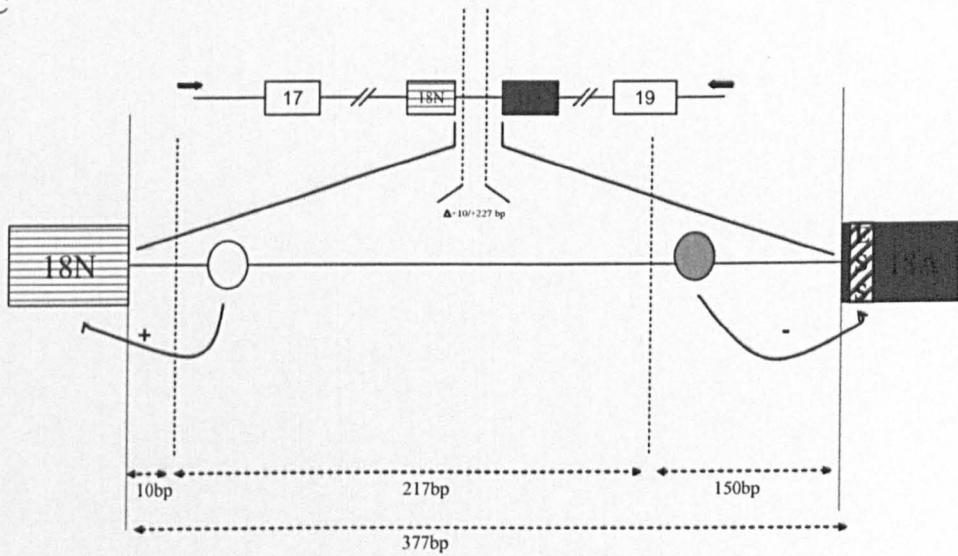
**A**



**B**



**C**



**Figure 23.** Analysis of the effect of the DNA sequence upstream of exon 18A on mRNA processing. (A) Schematic representation of the minigenes created in order to analyze the effect on mRNA splicing of the DNA region upstream of exon 18A. The deletions were performed both in the SCN8A WT minigene (upper section) as well as the minigene SCN8A/ $\Delta$ ESS E18A (lower section). // indicates the region of endogenous DNA deleted from the minigene. (B) Agarose gel electrophoresis of the RT-PCR analysis of the constructs performed after transfection in HeLa cells. Three forms of mRNA are observed as indicated on the right hand side of the gel and are composed of exons 17-19, exons 17-18A-19 and exons 17-18N-19. (C) Schematic representation of the deletions that were made in the homologous minigene context and the position of the possible *cis*-acting elements and their role they may play for the inclusion of SCN8A exon 18N and skipping of exon 18A as inferred from section (B) of this figure 10.

### **3.6.2 A conserved sequence in the downstream intron of exon 18A defines an important region required for its regulation.**

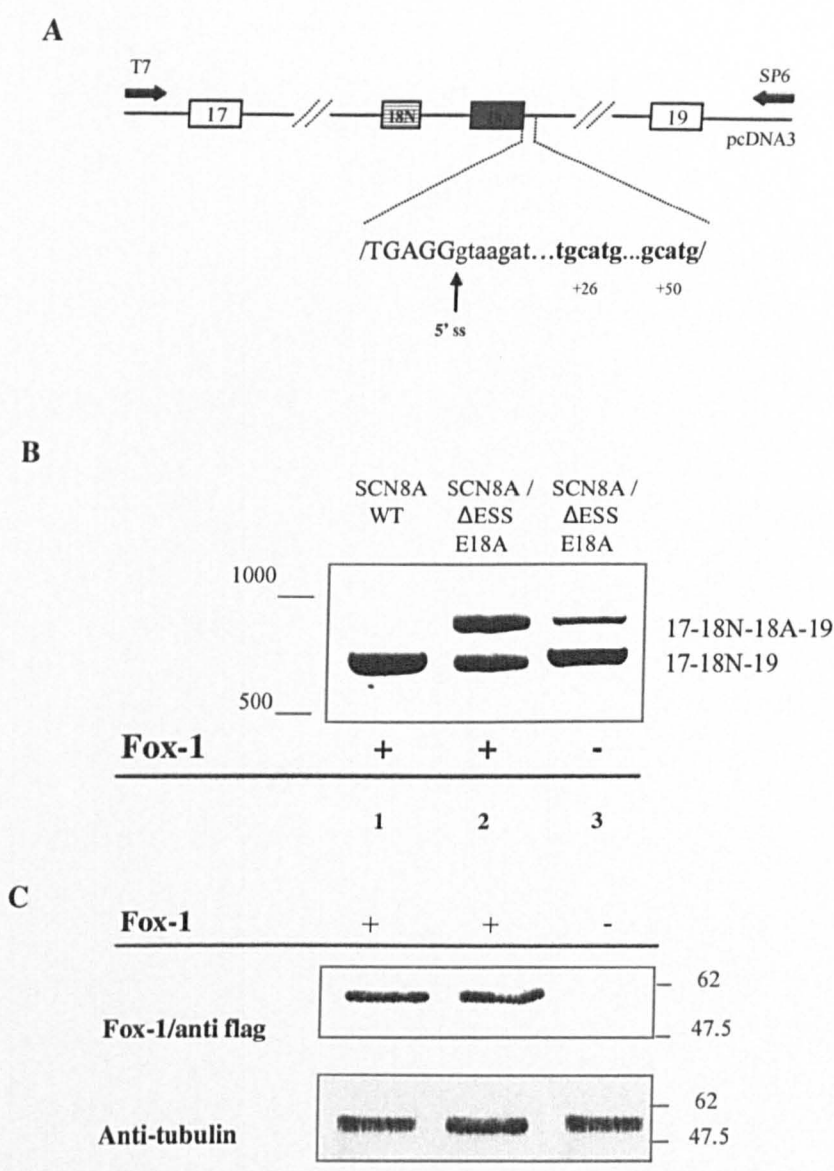
Lately several trans-acting factor expressed specifically in neuronal tissues have been identified (section 1.3.5). The fact that the 5' splice site of SCN8A exon 18A is intrinsically weak means its efficient use may depend on one of these trans-acting factor, present possibly only in neuronal cells, where SCN8A exon 18A is included.

To identify possible intron enhancer elements, I examined the intronic sequence around the SCN8A exon 18A for *cis*-acting elements that may function as an enhancer element. I observed that there are two (T)GCATG elements in the sequence downstream of SCN8A exon 18A, in close proximity to its 5' splice site (Fig. 24A). Interestingly, it has been

shown that the position and sequence context of (T)GCATG elements of SCN8A exon 18A has been strongly conserved, from fish to humans (Minovitsky, Gee et al. 2005), supporting a hypothesis that this element may be a critical component of the splicing switch mechanism that mediates tissue-specific splicing events. The (T)GCATG sequence is a previously characterized splicing enhancer element that has been shown to be important for the proper splicing regulation of a different genes, such as fibronectin, *c-src* and calcitonin/CGRP (Hedjran, Yeakley et al. 1997; Lim and Sharp 1998). In addition, a computational study demonstrated an overrepresentation of (T)GCATG hexamers in the downstream intron of neural and muscle-specific alternatively spliced exons (Underwood, Boutz et al. 2005). Thus, this element is a hallmark of many system of neuronal splicing regulation. The factors responsible for recognizing the hexamers were first recognized by Jin et al., who showed these proteins to be homologous of the *Caenorhabditis elegans* RNA binding protein feminizing on X (Fox-1). Members of Fox protein family regulate the splicing of many neuron and muscle specific genes. There are three mammalian family members, Fox-1 (A2BP1), Fox-2 (RBM9) and Fox-3 (hnrbp3), each containing a nearly identical RNA binding domain that recognize the hexanucleotide element (U)GCAUG. Fox-1 is expressed in neurons and muscle, Fox-3 is expressed only in neurons, while expression of Fox-2 shows broader range of the expression, being found in embryo, neurons and muscle (Kuroyanagi 2009). Brain specific Fox-1 may represent a trans-acting factor, missing from the HeLa cells needed to enhance the recognition of the SCN8A exon 18A in neuronal cells.

To investigate if brain specific Fox-1 protein could indeed play a role in aiding the recognition of SCN8A exon 18A, I created an expression plasmid carrying the sequence for the expression of the brain specific isoform of Fox-1 together with a tag for the Flag epitope. This expression plasmid was transfected into HeLa cells together with the either the SCN8A WT or SCN8A/ $\Delta$ ESS E18A minigene. The splicing of the exon 18A was assayed by RT-PCR analysis (Fig. 24B). The expression of the Fox-1 was monitored by

immunoblotting for the Flag epitope tag (Fig. 24C). As previously shown, the SCN8A WT minigene results in complete inclusion of exon 18N and skipping of exon 18A in HeLa cells (Fig. 21A). Cotransfection of SCN8A WT minigene construct with the Fox-1 expression plasmid did not result in a change in SCN8A exon 18A inclusion, since this exon was still completely skipped (Fig. 24B, Lane 1). In HeLa cells the SCN8A exon 18A inclusion may be strongly repressed by different hnRNPs protein, binding the ESS element previously identified, even in the presence of Fox-1 protein, thus masquerading any effect this latter may have. This was in accordance with the results showed by Minovitsky et al., suggesting that (T)GCATG elements alone are not sufficient to determine precise tissue specificity (Minovitsky, Gee et al. 2005). To examine the enhancer activity of brain specific Fox-1 protein in the absence of ESS repression element, I used a SCN8A/ $\Delta$ ESS E18A minigene, where the ESS element was removed from the SCN8A exon 18A. Cotransfection with the Fox-1 expression plasmid, strongly increased the SCN8A exon 18A inclusion, resulting in an additional band corresponding to a product including both exons, 18N and 18A (Fig. 24B, Lane 2). Identity of this band was confirmed by sequencing of the gel purified product. Cotransfection of the SCN8A/ $\Delta$ ESS E18A minigene with an empty expression vector, CMV-4, did not have an effect on the inclusion of exon 18A (Fig. 24C, Lane 3).



**Figure 24.** Effect of Fox-1 protein expression on the mRNA splicing of the SCN8A WT and SCN8A/ $\Delta$ ESS E18A minigenes. **(A)** Schematic representation of the two (T)GCATG elements in the sequence downstream of exon 18A. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. **(B)** Agarose gel electrophoresis of co-transfection of the minigenes the SCN8A WT and SCN8A/ $\Delta$ ESS E18A with a brain specific Fox-1 expression plasmid. When Fox-1 expression plasmid was not transfected a empty CMV-4 plasmid was used (Lane 3). The species of mRNA produced are indicated on the right hand side of the gel. **(C)** Analysis of the expression of



the Fox-1 proteins was monitored by immunoblotting for the Flag epitope tag and amount of protein loaded on the western blot controlled through western blots against the tubulin.

### **3.6.3 Overexpression of Fox-1 protein aids in SCN8A exon 18A inclusion in the mRNA in a (T)GCATG dependent manner.**

As already mentioned, previous studies have shown that (T)GCATG is an intronic splicing regulatory element bound with unusually high specificity by the Fox-1 protein, and this interaction is required for efficient splicing of neural specific alternative exons (Underwood, Boutz et al. 2005). To test whether this observed Fox protein enhancer activity was dependent on the presence of (T)GCATG in the case of SCN8A exon 18A, further minigenes were constructed and tested in co-transfections with the brain specific Fox-1 expression plasmid. The minigenes were made in such a fashion as to evaluate the relative contribution of each intrinsic repeat individually or in combination, by mutating these in the SCN8A/ $\Delta$ ESS E18A minigene together with co-transfection with brain specific Fox-1 protein (Fig. 25A).

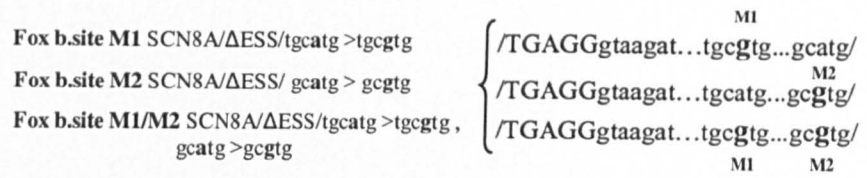
A single mutation in the first (T)GCATG (TGCATG > TGCGTG) repeat had a drastic effect on SCN8A exon 18A inclusion, since the exon was almost completely skipped (Fig. 25B, Lane 2), as can be seen by comparison with the SCN8A/ $\Delta$ ESS E18A minigene in which Fox-1 binding sites were not mutated (Fig. 25B, Lane 1). A mutation in the second GCATG (GCATG > GCGTG) repeat, showed an identical effect on the reduction of exon 18A inclusion, as was also the case when mutations were introduced in both repeats and the Fox-1 was expressed (Fig. 25B, Lanes 3 and 4).

Thus, splicing enhancement is strongly dependent on both (T)GCATG elements, as their mutation results in ineffectiveness of Fox-1 to aid in the inclusion of the SCN8A exon

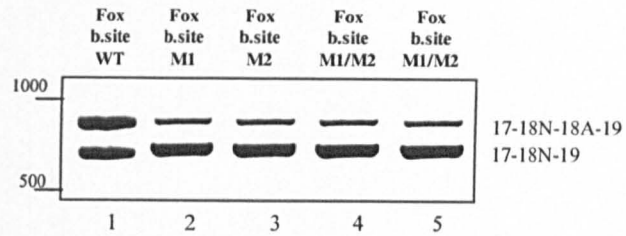
18A. Cotransfection of the minigene where both Fox binding sites were mutated, with an empty expression vector, CMV-4, did not have an effect on the inclusion of exon 18A (Fig. 25B, Lane 5). The expression of the Fox-1 was monitored by immunoblotting for the Flag epitope tag and antibody for the tubulin was used as a loading control (Fig. 25C).

The results obtained allow me to conclude that the deletion of a conserved element (ESS) is not sufficient for regulated SCN8A exon 18A inclusion in the WT minigene construct. The region downstream of exon 18A, containing two (T)GCATG repeats, defines an important region required for its regulation which functions through Fox-1, demonstrating that the Fox-1 protein is an important trans-acting factor for the inclusion of the SCN8A exon 18A.

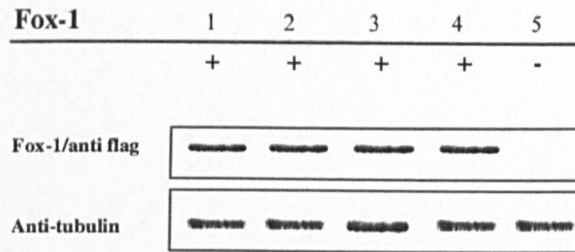
**A**



**B**



**C**



**Figure 25.** Fox-1 enhancer activity is dependent on the presence of the (T)GCATG regulatory elements. (A) Schematic representation of the minigenes created in order to analyze the effect of the mutations in Fox-1 binding sites, singularly or in combination. (B) Agarose gel electrophoresis of co-transfections of the minigenes with and without the mutations in Fox-1 binding sites, with a brain specific Fox-1 expression plasmid. Two forms of mRNA are observed as indicated on the right hand side of the gel. (C) Analysis of the expression of the Fox-1 proteins was monitored by immunoblotting for the Flag epitope tag and amount of protein loaded on the western blot controlled through western blots against the tubulin.

### **3.7 Differential activities of different Fox proteins in promoting SCN8A exon 18A inclusion.**

In mammals, Fox-1, Fox-2 and Fox-3 are members of an extended family of proteins arising from multiple genes and complex alternative splicing. Previously it has been demonstrated that the expression of certain spliced isoform is specific to particular tissues and that their enhancer activities are variable (section 1.3.5). It was therefore of interest to compare the relative activity of individual isoform of muscle specific Fox-1 and brain specific Fox-1, Fox-2 and Fox-3 proteins in promoting SCN8A exon 18A inclusion. This was performed using the SCN8A WT and SCN8A/ $\Delta$ ESS E18A minigene constructs and over-expression plasmids for the specific Fox proteins created in an identical manner to that of the brain specific Fox-1 expression plasmid.

After the transfection in HeLa cells and RT-PCR analysis, I observed that, as before in the presence of the ESS in SCN8A exon 18A (SCN8A WT minigene), Fox protein(s) over expression, irrespective of type or isoform, had no effect on mRNA produced (Fig. 26A, Lanes 1-4). On the other hand in the absence of the ESS region in SCN8A exon 18A the greater effect on the inclusion of SCN8A exon 18A occurred with co-transfection of the brain specific Fox-3 expression plasmid was observed (Fig. 26A, Lane 8). The effect on exon 18A inclusion after the cotransfection with brain specific Fox-2 and muscle specific Fox-1 was the same as with the previously observed effect with the brain specific Fox-1 protein (Fig. 26A, Lanes 7, 6 and 5 respectively). To confirm that muscle specific Fox-1 and the brain specific Fox-1, Fox-2 and Fox-3 proteins are indeed expressed, immunoblot analysis was performed against the flag epitope (Fig. 26B).

A possible explanation as to why a greater inclusion of SCN8A exon 18A is observed, transfecting the cells with the brain specific Fox-3 protein may lie in the efficiency of this protein in entering the nucleus rather than the actual function of the protein per se. Indeed

although all of expressed Fox proteins share an identical RNA binding domain, and are equally capable of binding to the (T)GCATG element, each isoforms shows quantitative differences in splicing activity and nuclear distribution in transfected cells (Kim, Adelstein et al. 2009).

However, more interestingly is the outcome that the presence of Fox proteins, and not in particular of specific isoform, is necessary but not sufficient to get complete inclusion of SCN8A exon 18A in neuronal cells, but rather the amount of other trans-acting factors together with the Fox protein expression is what dictate the inclusion of SCN8A 18A exon.



### **3.8 Different cell lines tested in search for the SCN8A exon 18A inclusion.**

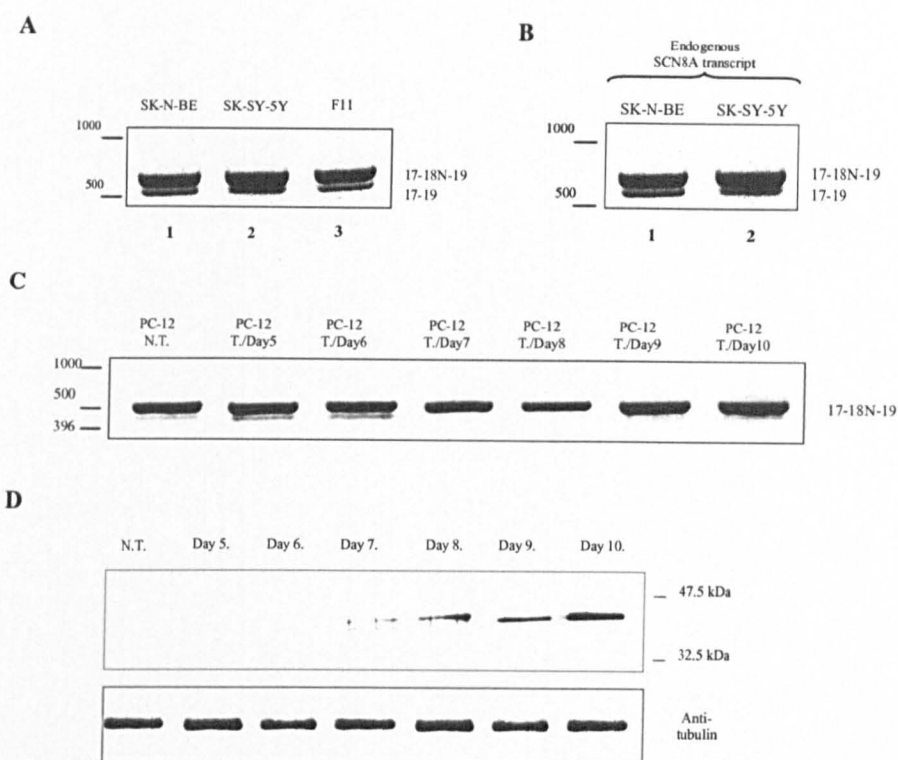
In order to ascertain the role played by Fox protein in SCN8A exon 18A inclusion in the mRNA as well as any possible effect it may have on the ME splicing mechanism it was important to find a cell line in which the ME nature of these exons was respected and SCN8A exon 18A included in the mRNA rather than SCN8A exon 18N.

Neuronal clonal cell lines have often been used to study the differentiated features of authentic neurons. It can often be demonstrated that a particular neuronal property is retained in a neuroblastoma cell line with characteristics that are apparently identical to authentic neuronal tissue. Thus, I decided to test some of the neuroblastoma cell lines. To this end, I used two neuroblastoma SK-N-BE (ATCC Number: CRL-2271™) and SH-SY-5Y (ATCC Number: CRL-2266™) cell lines. Unfortunately, in both cell lines tested, two products after the transfection and the RT-PCR analysis, of the SCN8A WT minigene, were observed, with a major band corresponding to a product including exon 18N and a minor band corresponding to a skipping of both ME exon (Fig. 27A, Lanes 1 and 2). In these two cell lines, SCN8A transcript was also endogenously present with a similar pattern of inclusion than the minigene, that is only SCN8A exon 18N inclusion or skipping of both ME exon (Fig. 27B). I also tested the F11 cell line (provided by Prof. E. Wanke, Università degli studi di Milano-Bicocca) that is hybrid cell line derived from a mouse N18TG2 neuroblastoma and rat DRG sensory neuron, and the rat pheochromocytoma cell line, PC-12 (ATCC Number: CRL-1721™).

F11 cell, morphologically in culture showed characteristics of neuronal cells. As a minigene for a transfection experiment, I used a SCN8A WT minigene, and after the RT-PCR analysis, the same pattern as in neuroblastoma cell lines was observed (Fig. 27A, Lane 3).

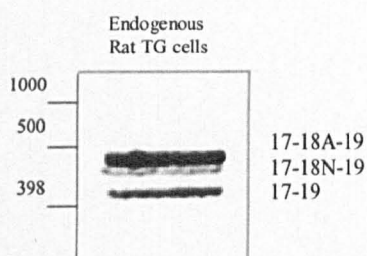
Previously, it has been shown that clonal cell lines may be grown under different culture conditions in which they exhibit altered morphologies (Nelson, Christian et al. 1976; Nirenberg, Wilson et al. 1983). These alterations may be associated with changes in the expression of various functions. Such changes may provide insights into the factors and mechanisms underlying the regulation and expression of neuronal characteristics during normal differentiation. A classic example of this is the use of the PC12 pheochromocytoma cell line for study of the mechanism of action of nerve growth factor (NGF) (Race and Wagner 1985). I therefore decided to investigate if these cells could provide a suitable substrate in which to further ascertain for the role Fox-1 had on SCN8A exon 18A splicing regulation *in vivo*. In order to induce differentiation, PC-12 cells were cultured in DMEM containing 1% serum and 50ng/mL NGF as described in material and methods (section 6.19). After 10 days of treatment with NGF, RNA and protein were extracted from PC-12 cells at several different time points. Expression of Fox-1 protein was observed to be induced in PC-12 cells treated with NGF from day 7, steadily increasing till day 10 (Fig. 27D). Surprisingly however RT-PCR analysis using primers that flanked exons 17 and 19 of the endogenous rat SCN8A gene resulted in mRNA species only including SCN8A exon 18N (Fig. 27C), even if the Fox-1 protein expression increased after the treatment with NGF. Thus, even if in some of these cells, such as PC-12, Fox-1 protein was expressed, the cellular environment was not appropriate for the inclusion of SCN8A exon 18A. This may be due to the presence or the amount of hnRNPs that bind to the silencer element being such that, the repression of SCN8A exon 18A inclusion via the ESS is overpowered, or maybe there are some other factors that somehow aid the inclusion of SCN8A exon 18N, factors that are in direct competition with tissue specific factor, such as Fox-1. What is certain is that, amounts of different, ubiquitously expressed proteins, such as hnRNPs or SRs, and/or the presence of some tissue specific factors and their interactions, all together are the part of the complex cellular environment, that dictate the splicing pattern of the genes, such as SCN8A transcript.





**Figure 27.** Analysis of endogenous and exogenous SCN8A exon 18A inclusion in neuronal cell lines. **(A)** RT-PCR analysis of the transfection of SCN8A wild type minigene in neuroblastoma SK-N-BE, SH-SY-5Y and F11 cell lines. The mRNA species produced are indicated on the right hand side of the gel. **(B)** RT-PCR analysis of the endogenous SCN8A gene in neuroblastoma SK-N-BE and SH-SY-5Y cells using primers in exons 17 and 19. The mRNAs species produced are indicated on the right hand side of the gel. **(C)** RT-PCR analysis using primers that flanked exons 17 and 19 of the endogenous rat SCN8A gene in PC-12 cells cultured in DMEM containing 1% serum and treated for 10 days with 50ng/mL of NGF. Inclusion of SCN8A exon 18N was consistently detected irrespective of the time point. **(D)** Fox-1 protein expression analysis at different time points of differentiation, of the PC-12 cells. The treatment with NGF as can be observed in the western blot analysis causes Fox-1 to appear and steadily increase from day 7. The antibody against the tubulin was used as a loading control (the lower panel).

Finally I decided to examine the splicing pattern of endogenous SCN8A transcript in the primary culture of rat trigeminal ganglion neurons. To this end, the primary culture of a rat TG neurons was prepared as described in material and methods (section 6.20). To demonstrate that the SCN8A channel transcript is indeed expressed in these cells, and that the ME exon included is the exon 18A, I performed a simple RT-PCR analysis, using primers in rat SCN8A exons 17 and 19 on RNA isolated from these neurons, and after the agarose gel electrophoresis I observed three amplicons, one major and two very minor (Fig. 28). Sequencing of the gel-purified cDNA fragments showed that the major amplicon product consisted of SCN8A exon 17-18A-19. The band in the middle corresponded to the inclusion of exon 17-18N-19, while the lowest band corresponded to the skipping of both SCN8A ME exons 18N and 18A.



**Figure 28.** Endogenous SCN8A transcript in the primary culture of the rat trigeminal ganglion (TG) neurons. RT-PCR analysis, using primers in rat SCN8A exons 17 and 19 and agarose gel electrophoresis. On the right hand side of the gel the species of mRNA produced are indicated.

### **3.9 Endogenous Fox-1 protein in the primary culture of the trigeminal ganglion (TG) neurons regulates ME splicing pattern of endogenous SCN8A exon 18N and 18A.**

The primary culture of rat trigeminal ganglion neurons fulfilled the criteria of the cell type I was searching for in order to further analyse the role played by Fox-1 in the ME control of SCN8A exons 18N and 18A, namely the inclusion of SCN8A exon 18A rather than 18N in the mRNA.

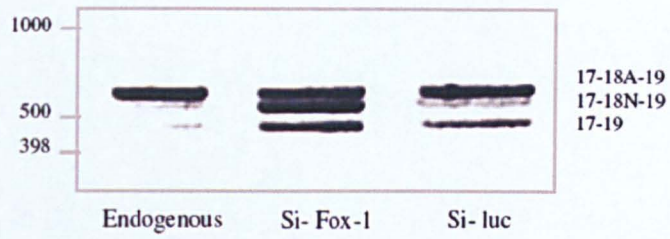
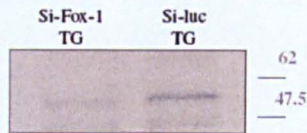
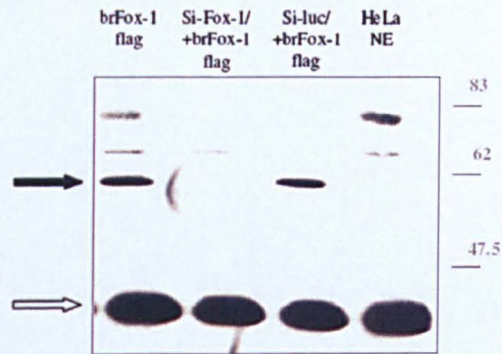
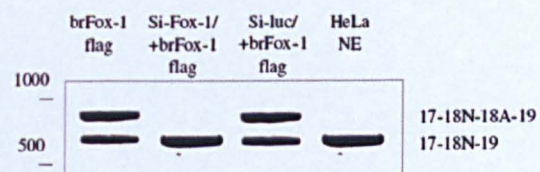
I therefore sought to determine the effect knockdown of Fox-1 protein would have in these cells. To be sure that the Fox-1 protein would be silenced and uncertain which of the six protein isoforms of Fox-1 may or may not be present in this cell type I designed an si-RNA to target all six isoforms of Fox-1 protein. As a control, for si-experiment I used a si-RNA against a luciferase gene. The knockdown of Fox-1 was performed as described in materials and methods section 6.25. Briefly two subsequent si-RNA transfections were carried out distanced 24 hours apart and after a further 24 hours the cells were harvested and total RNA was isolated for RT-PCR analysis using primers that correspond to sequences in the rat SCN8A exons 17 and 19. Analysis of alternative splicing of endogenous SCN8A ME 18A and 18N, in these cells demonstrated that Fox-1 is indeed a key player in the mechanism of ME splicing as from an almost exclusive cDNA consisting of SCN8A exon 17-18A-19, I now observe three cDNA species. The principal band is composed of SCN8A 17-18N-19 and two minor bands of approximately the same intensity corresponding to SCN8A 17-18A-19 and SCN8A 17-19 revealed changes in splicing pattern, with an increase of exon 18N and decrease of exon 18A inclusion (Fig. 29A).

Unfortunately, the amount of trigeminal ganglion neurons in culture after 2 days of treatments with SI for the Fox-1 protein, was not sufficient to get enough of material for

the Western blot analysis, as can be seen in Fig. 29B. Indeed an antibody against the tubulin resulted in a signal that was barely detectable (Fig. 29B). Notwithstanding the fact that the si-RNA experiments were repeated three times and resulted in the same result in regards to the splicing patterns of the two ME exons, I was never able to obtain enough material for a good western blot signal. The animals utilized were a generous gift from Prof. Giacca's laboratory. In order to have enough cells in a p35 to perform the si-RNA experiment, one rat needs to be sacrificed per well. To be able to increase this number, with the aim of been able to extract enough material for RT-PCR experiments as well as western blots, I have now set up my own breeding program and hope to be able to complete the experiments in the near future.

However, as a partially indication that the SI against Fox-1 can indeed knockdown this protein, comes from previous experiments where I initially tested the SI efficiency for the Fox-1 protein in HeLa cell, transfected with brain specific Fox-1 overexpression plasmid with the flag epitope.

Overexpressed Fox-1 protein was observed to be greatly decreased, after the SI experiments as can be seen in the western blot analysis using an antibody against Fox-1 (Fig. 29C).

**A****B**Antibody  
anti tubulin**C**Antibody  
anti Fox-1Antibody  
anti tubulin**D**RT-PCR  
SCN8A/ $\Delta$ ESS E18A

**Figure 29.** Analysis of the endogenous Fox-1 protein on the regulation of the ME splicing of SCN8A exon 18N and 18A endogenously present in rat TG neurons. **(A)** RT-PCR analysis of the endogenous SCN8A gene after SI experiment against Fox-1 and luciferase proteins. The mRNA species produced are indicated on the right hand side of the gel. **(B)** Immunoblot analysis of tubulin protein in TG neurons after the knockdown of Fox-1. **(C)** In the upper panel, immunoblot analysis of Fox-1 protein overexpression before and after the SI of Fox-1 and luciferase protein in HeLa cells (black arrow), and unspecific bands (white arrow). NE; nuclear extract. Lower panel: the antibody against the tubulin was used as a loading control. **(D)** The knock down was also correlated to the functional effect in the RT-PCR analysis using SCN8A/ $\Delta$ ESS E18A minigene construct.

#### **4. Investigation into the *cis*-acting elements controlling the inclusion of exon 18N in non neuronal cell line.**

Till now I have been investigating into the molecular mechanisms principally responsible for SCN8A exon 18A exclusion in the mRNA. However, it is obvious that the ME splicing will also depend on strict regulation of SCN8A 18N via auxiliary *cis*-acting splicing elements. We have observed in section 3.6.1 that, in part, there appears to exist some ME control for SCN8A exon 18N through elements in the intronic sequence between the two ME exons.

Furthermore, we have already observed that in trigeminal ganglion when we constrain the inclusion of SCN8A exon 18A in the endogenous transcript via si-RNA of Fox-1 protein the mRNA species goes from nearly a 100% pure inclusion of 17-18A-19 to a dominant mRNA species containing 18N as well as two other mRNA species, one carrying the 18A exon and also complete skipping of the two ME exons (Fig. 29A). This data would indicate

that the trans-acting factors responsible for 18A inclusion are still to some extent present in the trigeminal ganglion

#### **4.1 An exonic enhancer element (ESE) necessary for SCN8A exon 18N inclusion in non-neuronal cells is present within this exon.**

Alternatively spliced exons often contain ESEs for binding of splicing regulators that determine their inclusion. Different amounts of the trans-acting factors in different cell types can then subsequently determine their inclusion or exclusion in specific cells or under specific circumstances. Previous studies have indeed shown that SR proteins, the family of proteins principally shown to exert their effect through ESE's, are differentially expressed in different tissues including neuronal (Hanamura, Caceres et al. 1998).

As SCN8A exon 18N was always observed to be included in the mRNA when the minigenes were transfected in HeLa cells, I decided to initiate the investigation of the splicing control mechanisms underlying SCN8A exon 18N by determining if any ESE could be identified within this exon necessary for its inclusion in mRNA in HeLa cells.

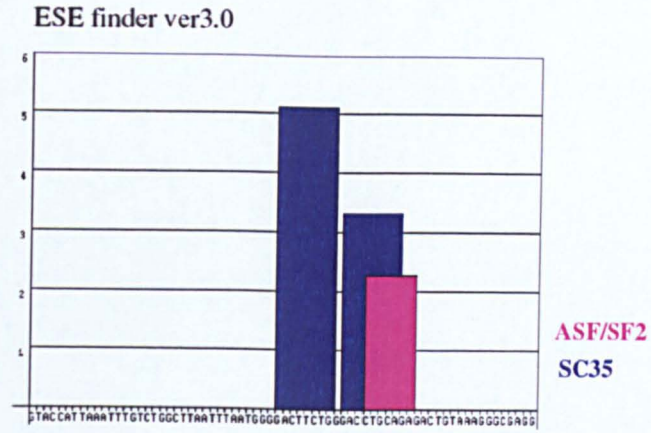
A first approach using ESE finder ver3.0, a bioinformatic program that searches for some of the more common ESE sequences was undertaken. The ESE finder program identified multiple putative SR protein responsive elements in SCN8A exon 18N (Fig. 30A). In order to see if these putative ESE's had a functional role in the inclusion of SCN8A exon 18N I created a series of constructs with sequential and overlapping deletions in the minigene SCN8A WT: minigenes SCN8A/E18N  $\Delta 1$ ,  $\Delta 2$ ,  $\Delta 3$ ,  $\Delta 4$ ,  $\Delta 5$  and  $\Delta 6$  (Fig. 30B).

After RT-PCR analysis I observed that the SCN8A/E18N  $\Delta 2$ ,  $\Delta 3$ ,  $\Delta 4$  and  $\Delta 5$  minigenes resulted in complete skipping of SCN8A exon 18N (Fig. 30C, Lanes 3-6), while SCN8A E18N  $\Delta 1$  and  $\Delta 6$  minigenes had no consequences on the inclusion of SCN8A exon 18N in

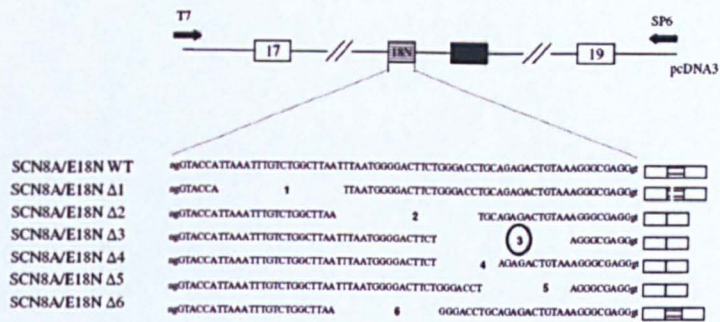
the mRNA (Fig. 30C, Lanes 2 and 7). The SCN8A E18N  $\Delta$ 2 minigene construct overlaps with SCN8A E18N  $\Delta$ 3 which also overlaps with SCN8A E18N  $\Delta$ 4 and  $\Delta$ 5, all of these deletions cause exon 18N to be skipped. As SCN8A E18N  $\Delta$ 6 minigene construct, that does not have a negative effect on the inclusion of exon 18N, partially overlaps with  $\Delta$ 2, I therefore concluded that the segment  $\Delta$ 3, that I shall now refer to as the ESE element, is likely to cover the region containing most of the ESE activity and decided to use this minigene to further investigate the ESE element responsible for inclusion of SCN8A exon 18N.



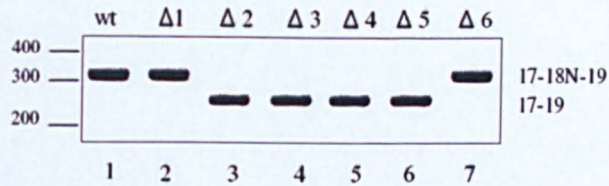
**A**



**B**



**C**



**Figure 30.** Identification of an exonic splicing enhancer element (ESE) in the SCN8A exon 18N. **(A)** The score matrix of SC35 and ASF/SF2 SR proteins predicted to bind SCN8A exon 18N, as predicted by ESE finder ver3.0. **(B)** Schematic representation of the SCN8A/E18N minigene carrying deletions Δ1, Δ2, Δ3, Δ4, Δ5 and Δ6. **(C)** Results of the RT-PCR analysis of these minigenes in agarose gel electrophoresis. On the right hand side of the gel are the forms of mRNA observed.

## **4.2 Identification of the trans-acting factors binding to the ESE element identified in SCN8A exon 18N.**

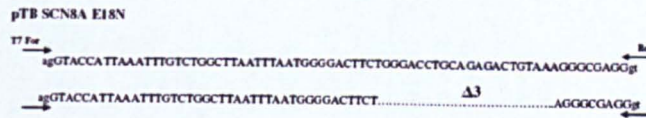
In order to identify the trans-acting factors binding the ESE element identified in the SCN8A exon 18N, I performed a pull down experiment, in a identical manner as to that described in section 3.3. Therefore, pTB E18N (with the ESE identified present) and pTB E18N  $\Delta$ 3 minigenes (without the ESE element) were used as a template for the synthesis *in vitro* of the corresponding RNAs (Fig. 31A). These RNA's were then covalently linked to adipic acid beads, incubated with nuclear extract prepared from HeLa cells, precipitated and washed. RNA-associated proteins were then eluted with SDS sample buffer and separated by SDS-PAGE. The proteins from the gel were immediately transferred to the nitrocellulose membrane, and after the transfer of the proteins, specific antibodies against some of the more common trans-acting factors binding to ESE were used for the detection of the proteins.

As a first approach we probed the pulldowns with the an IH4 antibody which in theory is recommended for detection of SR RNA processing factors, including SRp75, SRp55, SRp40. I was only able to observe a signal against SRp55 protein that was however visible irrespective of the presence or not of the RNA sequence containing the ESE element (Fig 31B). As the binding SC35 and ASF/SF2 highlighted in the bioinformatics analysis would be disrupted by deletion of region 3, I probed the pull down assay with antibodies for SC35 and ASF/SF2. From the western blot shown in figure 31B, I can see that the signal for these proteins was observed exclusively in the pull down experiment using the RNA with the ESE element (Fig. 31B).

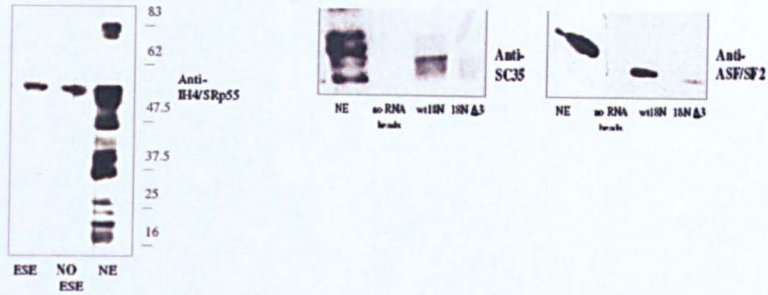
In order to test the functional significance of this differential binding to the RNA with or without the presence of the region ESE, I decided to perform an si-RNA experiment, depleting these two proteins, singularly or in combination, in HeLa cells. Both si-RNA treatments were effective at reducing the protein levels of the target protein as can be

observed from their reduction in Western blot analysis (Fig. 31C). The effect the knock down of these proteins had on the mRNA processing of SCN8A exon 18N was analysed by transfecting the WT SCN8A minigene in these backgrounds, where both these proteins had being knocked down. Unfortunately, we could not detect any change in the levels of SCN8A exon 18N inclusion, depleting these proteins (Fig. 31C). These results may mean that these proteins do not have a functional role in the inclusion of SCN8A exon 18N. However it is much more likely, as has being shown in previous studies, where it was observed that different members of SR proteins are generally interchangeable in their ability to fulfill a wide range of activities in the splicing reaction (Tacke and Manley 1999) since the binding sites for a SR protein members can be fairly degenerate (Black 2003), that a redundancy in the occupation of the ESE by the SR proteins is occurring upon depletion of SC35 and ASF/SF2 proteins.

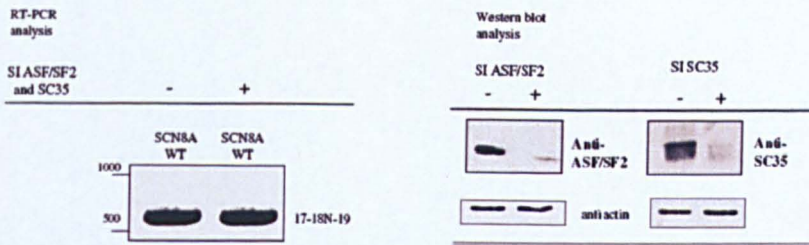
A



B



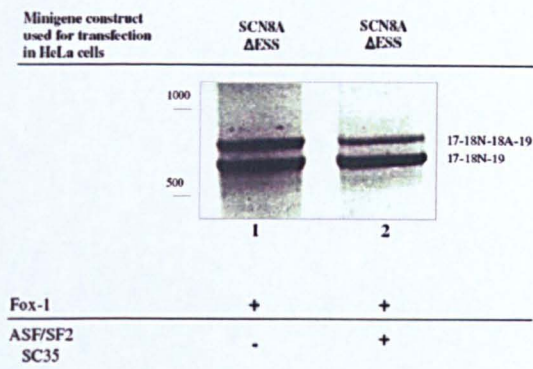
C



**Figure 31.** Identification of trans-acting factors binding to the exonic splicing enhancer identified in the SCN8A exon 18N. (A) DNA sequences used in T7 *in vitro* transcription for the production of WT RNA and mutant  $\Delta$ 3 RNA. (B) Western blot analysis for SRp55 (left panel), SC35 and ASF/SF2 proteins (right panel), performed on a pull down of the WT RNA (with the ESE) and  $\Delta$ 3 RNA (without the ESE). NE; HeLa nuclear extract (C) Left panel: agarose gel electrophoresis analysis of the RT-PCRs analyzing the effect of the knockdown experiments on the skipping of SCN8A exon 18N in the mRNA using the SCN8A WT minigene. Right panel: the western blot analysis of si-RNA experiments, depleting the SC35 and ASF/SF2. An antibody against actin protein was used as a loading control.

### **4.3 Overexpression of SC35, ASF/SF2 and Fox-1 proteins in HeLa cells.**

To further analyse the possible role played by SC35 and ASF/SF2 in the ME splicing of SCN8A exon 18N through overexpression of these two trans-acting factors ideally we would want a minigene system in which this exon is not completely recognized. In HeLa cells this is partially provided by the minigene SCN8A/ $\Delta$ ESS E18A with over expression of Fox-1 protein. In these case, exon 18N inclusion is not excluded, as two mRNA species composed of SCN8A exons 17-18N-18A-19 and 17-18N-19 (Fig. 24B, Lane 2) can be observed, however it is incorrectly proceeded in that the mRNA species 17-18N-18A-19 containing the two mutually exons does not occur in vivo. I therefore decided to determine the effects of elevated levels of the SR proteins, ASF/SF2 and SC35, on the splicing of SCN8A/ $\Delta$ ESS E18A minigene in HeLa cells co-transfected with Fox-1 expression plasmid. The RT-PCR analyses showed that overexpression of ASF/SF2, SC35 and Fox-1 decreased the relative intensity of the band corresponding to the mRNA product including both the SCN8A 18N and 18A exons, relative to that containing SCN8A exon 18N (Fig. 32). Thus, it would appear that elevated levels of both ASF/SF2 and SC35 enhance correct processing of exon 18N inclusion of the SCN8A transcript in HeLa cells via binding to enhancer elements within the exon, indicating that these proteins aid the correct ME processing of the mRNA overpowering the effects exerted by the deletion of the ESS element in SCN8A exon 18A and Fox-1 over expression and pushing the splicing processing towards exclusive inclusion of SCN8A exon 18N.



**Figure 32.** Effect of SC35, ASF/SF2 and Fox-1 over expression on the mRNA splicing of the SCN8A/ΔESS E18A minigene in HeLa cells. Agarose gel electrophoresis analysis of the RT-PCR analyzing the effect of SC35, ASF/SF2 and Fox-1 over expression experiments. On the right hand side of the gel are the forms of mRNA observed.

## **5. Mutually exclusive splicing regulation of SCN9A exons 5N and 5A sodium channel gene.**

In order to see if the control mechanism that I have begun to elucidate for the SCN8A exons 18A and 18N ME splicing could represent a more general mechanism, I also investigated the ME splicing regulation of SCN9A exons 5N and 5A.

Exons 5N and 5A of the SCN9A sodium channel gene are mutually exclusive, and their selection is regulated during development in a tissue specific manner (Raymond, Castle et al. 2004). The SCN9A exon 5N is preferentially expressed in the peripheral (PNS) and central (CNS) nervous system of adult tissues and significant usage of SCN9A exon 5A was found only in dorsal root ganglion (DRG). The alternative SCN9A exons 5N and 5A encode part of segment S3 and all of S4 of domain I in the  $\alpha$  subunits of SCN9A sodium channel gene. The two splice variants differ by one amino acid substitution, residue 7 is aspartic acid (Asp) in 5A and serine (Ser) in 5N. The functional consequences of this change is thought to be significant, for two principal reasons. Firstly, the alternative splicing to generate N and A isoforms is developmentally regulated, thus representing a substantial investment by the organism in maintaining this differential expression (Sarao, Gupta et al. 1991). This suggests that channels with a neutral charged residue (SCN9A with exon 5N) are advantageous in the neonate when cells are migrating and differentiating whereas channels with a negatively charged residue (SCN9A with exon 5A) are preferable in the mature cells of the adult (Onkal and Djamgoz 2008). Secondly, the same alternative splicing module, (exons 5N and 5A), has been preserved in distinct sodium channel genes (SCN2A, SCN3A, SCN5A and SCN8A) provides further support to the idea that the single amino acid difference has substantial functional significance that may be related to the different perception of pain believe existing between adults and neonates.

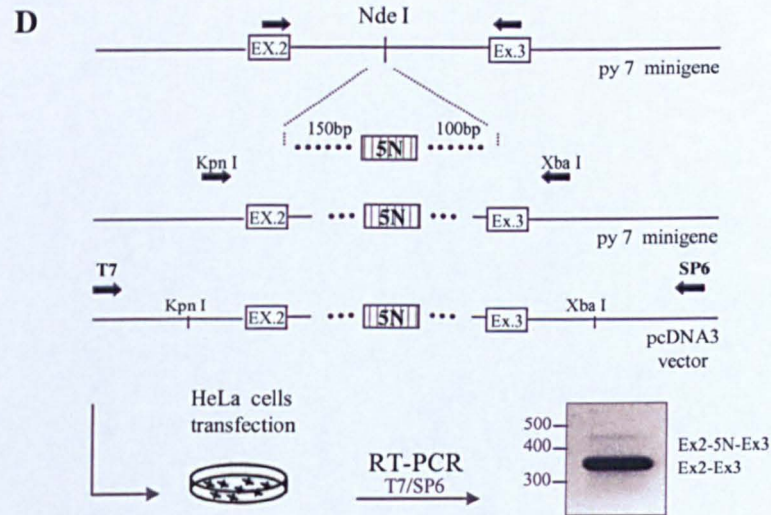
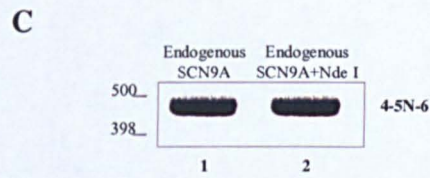
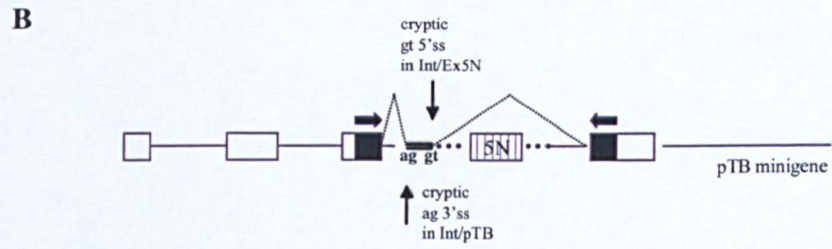
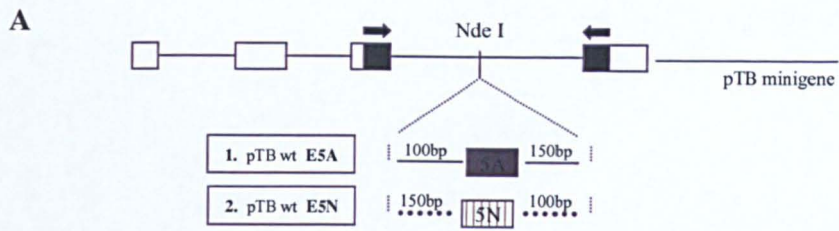
## 5.1 Splicing regulation of SCN9A ME exons 5N and 5A in heterologous minigene systems.

As with SCN8A gene, in order to be able to better map the individual elements that control exon inclusion/exclusion of SCN9A exon 5N and 5A I decided to clone the individual exons together with flanking intronic upstream and downstream sequence in the pTB minigene system. Creating pTB wt 5N and pTB wt 5A minigene, the splicing assay was performed by transfecting each minigene plasmid into HeLa cells. Using the pTB wt 5A minigene I observed a unique PCR product identified as inclusion of exon 5A in the mature transcript (Fig. 33, Lane 1). This result was unexpected as SCN9A exon 5A inclusion, from the literature is only documented in DRG cells. This result therefore demonstrate that the *cis*-acting regulatory elements that dictate the exclusion of SCN9A exon 5A are missing from the DNA fragment inserted in the pTB minigene, or HeLa cells may be expressing important trans-acting factors positively regulating SCN9A exon 5A inclusion. To determine if this latter option was the case I attempted to amplify the endogenous transcript in this cells line. In order to ascertain the identify of the amplification, as SCN9A exons 5N and 5A are of identical size, the RT-PCR product was digested with restriction enzyme, Nde I, specific for only one alternatively spliced isoform, specifically 4-5A-6. In figure 33C we can observe that HeLa cells express the SCN9A gene, with inclusion of SCN9A exon 5N making the former assumption as to why the SCN9A exon 5A was present in the cDNA the more likely explanation.

The pTB wt E5N minigene, resulted in a cDNA product corresponding to the use of cryptic exon with the exclusion of the SCN9A exon 5N (Fig. 33A, Lane 2), using as a 3' splice site an ag in the intron of pTB minigene and as the 5'splice site the gt in the intronic region upstream of exon 5N (Fig. 33B). The use of the cryptic splice site could just be a fortuitous occurrence. To establish if the context of pTB minigene system was responsible



for aberrant splicing of exon 5N we cloned this exon together with the flanking intronic sequence in another minigene system, called pY7, described in materials and methods. After transfection and RT-PCR skipping of exon 5N was observed (Fig. 33D). However it is also important to note that the pY7 alpha-tropomyosin exons 2 and 3 splice sites are very well defined therefore that this pair is preferred over those we inserted could be expected.

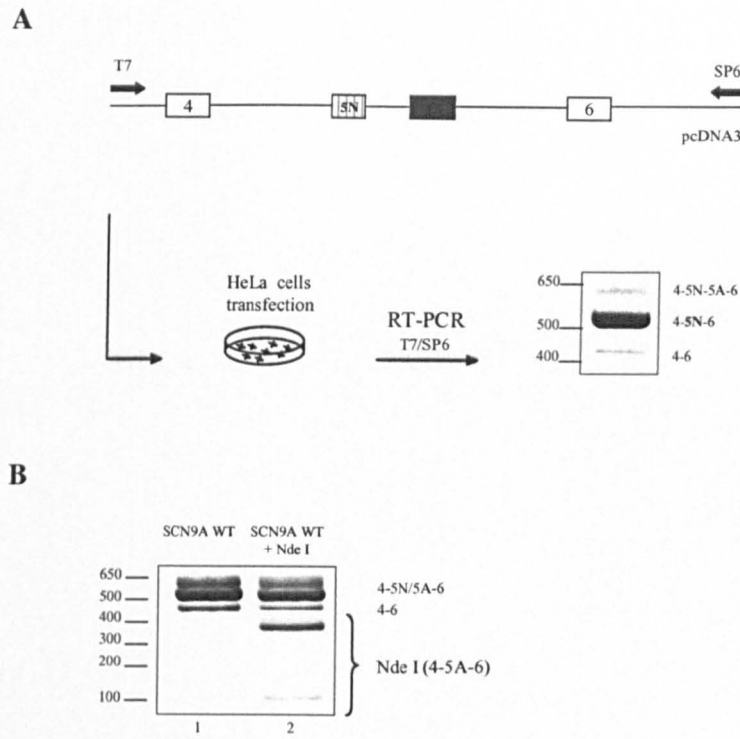


**Figure 33.** Analysis of minigene splicing assay performed with pTB wt E5A and pTB wt E5N minigenes. (A) In the upper panel a schematic representation of the pTB wt E5A and pTB wt E5N minigenes. Lower panel: analysis of expression of the constructs in HeLa cells. The mRNA produced was analyzed through RT-PCR using oligonucleotides ALFA and BRA (black arrows) and agarose gel electrophoresis. On the right hand side of the gel is a illustration indicating what each amplification corresponds to. (B) Schematic representation of the pTB wt E5N minigene together with the position of the cryptic splice sites used. (C) RT-PCR analysis of the endogenous SCN9A gene in HeLa cells using primers in exons 4 and 6. (D) In the upper panel a schematic representation of the pY7 E5N minigene. Lower panel: The mRNA produced was analyzed through RT-PCR using oligonucleotides T7 and SP6 (black arrows) and agarose gel electrophoresis. On the right hand side of the gel is a illustration indicating what each amplification corresponds to.

## **5.2 SCN9A ME exons 5N and 5A splicing in homologous minigene system.**

The results obtained for both SCN9A exons 5N and 5A in heterologous context highlighted the fact that the splicing control of these ME exons would appear to be dependent on a lot more than the “core” *cis* elements that are generally used to define an exon and depend on splicing *cis*-acting regulatory elements that I failed to include in the minigenes constructs thus far made. Therefore to verify this possibility I subcloned a genomic DNA fragment from exon 4 through to exon 6, encompassing both mutually exclusive SCN9A exon 5N and 5A (Fig. 34A). Transient transfection experiments with WT SCN9A minigene in HeLa cells and subsequent RT-PCR analysis showed that the processing of RNA of the minigene in this case was very similar to the endogenous processing of SCN9A previously observed

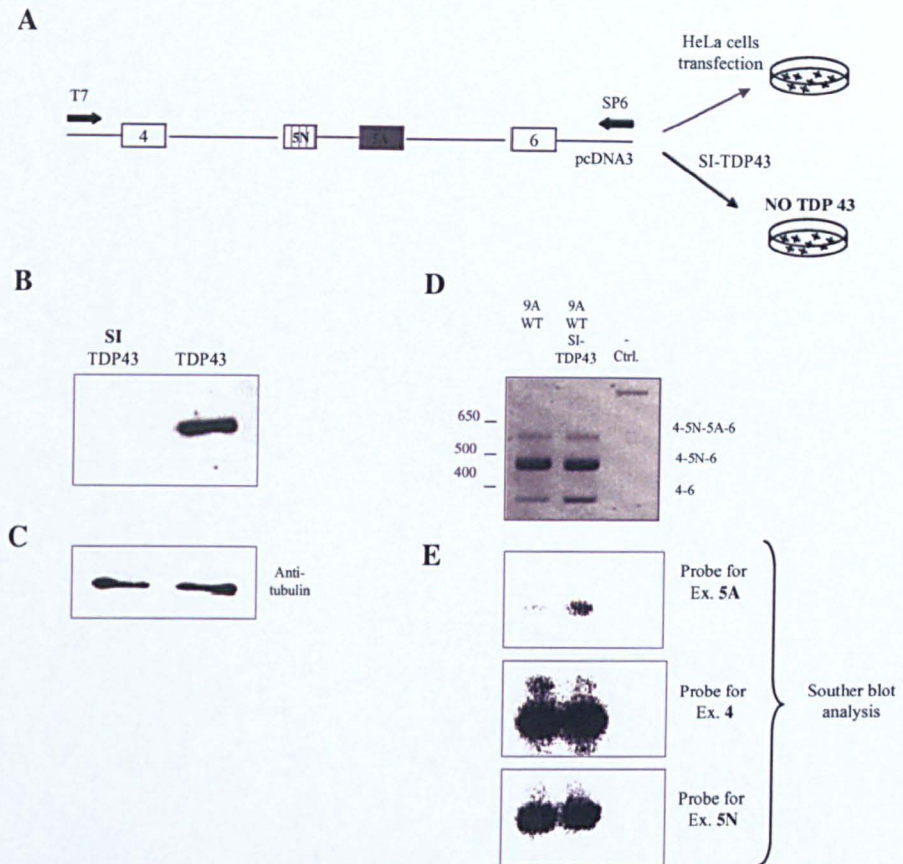
(Fig. 33C). In fact three products are seen on agarose gel electrophoresis, with the lowest band representing a cDNA product lacking exons 5N and 5A, the middle and major band by far representing a cDNA product including either exon 5N or 5A and the upper band representing a cDNA product including exon 5N and 5A. The identity of the major band was analysed as before through digestion with Nde I enzyme, showing that although this corresponding principally to cDNA product composed of SCN9A exons 4-5N-6 a small amount of cDNA composed of SCN9A exons 4-5A-6 was also present (Fig. 34B, Lane 2). Notwithstanding this fact and that normally for genes with mutually exclusive exon pairs, no cellular mRNAs have yet been detected that incorporate both exons, despite the fact that the introns between them contain functional 5' and 3' splice sites, I deemed this type of splicing outcome acceptable to begin to study the mechanisms behind the ME splicing of the two exons.



**Figure 34.** Analysis of the alternative splicing of SCN9A exon 5N and 5A in a homologous minigene system. **(A)** Schematic representation of the minigene SCN9A WT that spans SCN9A exons 4 through to 6. Transfection in HeLa cells followed by RT-PCR analysis and agarose gel electrophoresis. On the right hand side of the gel are indicated the species of mRNA produced. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. **(B)** Agarose gel electrophoresis analysis of the RT-PCRs, analyzing the effect of the digestion with Nde I enzyme with the species of mRNA produced that are indicated on the right hand side of the gel.

### **5.3 TDP43 protein, as a potential trans-acting factor that binds the (TG)<sub>n</sub> repeats in the intron between SCN9A ME exons 5N and 5A.**

Previous research in our laboratory have been identified a TDP43 protein as a trans-acting factor binding to the (TG)<sub>n</sub> repeat region near the 3' splice site of human CFTR exon 9 and inducing exon skipping (Buratti, Brindisi et al. 2004). The intronic sequence between the SCN9A ME exons 5N and 5A was observed to be rich in (TG) repeats. It was therefore of interest to test if TDP43 is also acting here in an analogous fashion. In order to investigate if TDP43 has a role in SCN9A exons 5N and 5A ME splicing, I tested the RNA processing of WT SCN9A minigene in the absence of TDP43 (Fig. 35A). Specifically a double transfection distanced 48h from each other of the si-RNA were performed for an effective reduction of the endogenous protein as can be observed in HeLa cell lysates collected and analysed for TDP43 endogenous protein by western blot assay after the double transfection (Fig. 35B and C). Following successful knock down of TDP43 in HeLa cells through si-RNA, I transfected WT SCN9A minigene construct. The processing of the minigenes was then analyzed through RT-PCR analysis (Fig. 35D) were I observed that knockdown of TDP43 had no effect on the previously observed splicing patterns. To differentiate weather the principle amplification was composed of the cDNA SCN9A exons 4-5N-6 or 4-5A-6 this time I performed radioactive labeled hybridisation analysis (Fig. 35E). Briefly three probes were used that were specific for exon 4 (common to both cDNA species), exon 5A and 5N with the probe being in a region where the nucleotide sequence is different from each other (5'-CTCAGCATTGAGAACA-3' on exon 5N, and 5'-TTCAGCTCTTCGAACT-3' on exon 5A).

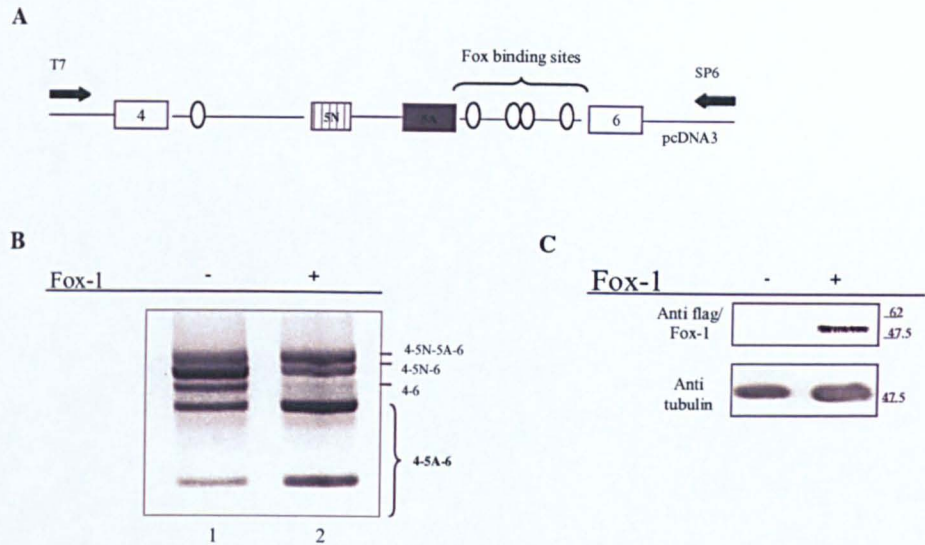


**Figure 35.** Analysis by si-RNA of the functional role played by TDP-43 protein in a ME splicing regulation of SCN9A gene. **(A)** Schematic representation of the minigene SCN9A WT that spans SCN9A exons 4 through to 6 together with the transfections in HeLa cells, before and after the SI experiment. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. **(B)** The western blot analysis of si-RNA experiment, depleting the TDP-43 protein. **(C)** The SI treatment was effective at reducing the protein level of the target protein as can be observed comparing the western blot signal and that of tubulin used as a control. **(D)** Agarose gel electrophoresis analysis of the RT-PCR analyzing the effect of the knock down experiment using the SCN9A WT minigene. On the right hand side of the gel are indicated the species of mRNA produced. **(E)** Southern blot analysis performed on PCR amplicons using radioactive labeled probes specific for exon 5A, 4 and 5N.

## **5.4 The Fox-1 protein plays a critical role in mediating splicing switches in SCN9A sodium channel gene.**

Another obvious starting point considering the results obtained with SCN8A exons 18N and 18A was to investigate the possible role Fox-1 may play in the control of the ME splicing of SCN9A exons 5N and 5A. Inspection of the intron's sequence downstream of exon 5A, revealed four (T)GCATG sequences (Fig. 36A), all of them conserved during the evolution, further emphasizing potential regulation by Fox-1 protein. Furthermore, one (T)GCATG motif is also present upstream of SCN9A exon 5N. Several studies have indicated that the position of a Fox protein binding site relative to a target exon will determine whether the protein will enhance or repress splicing. Upstream binding sites are repressive, but downstream Fox sites generally act as enhancers (Llorian, Schwartz et al.; Licatalosi, Mele et al. 2008; Zhang, Zhang et al. 2008; Yeo, Coufal et al. 2009). To investigate if Fox-1 is also playing a critical role in the ME splicing regulation of the SCN9A sodium channel, I co-transfected the WT SCN9A minigene with the brain specific Fox-1 overexpression plasmid previously described (paragraph 3.6.2). The RT-PCR products were then digested with Nde I restriction enzyme, specific only for exon 4-5A-6 transcript. From the restriction enzyme digestions expression of brain specific Fox-1 protein in HeLa cells resulted in almost completely inclusion of SCN9A exon 5A in the mature transcript (Fig. 36B, compare lines 1 and 2). Simultaneously, the inclusion of exon 5N was strongly inhibited. Curiously over expression of Fox -1 also reduces the cDNA species in which both ME are skipped, SCN9A 4-6 (Fig. 36).



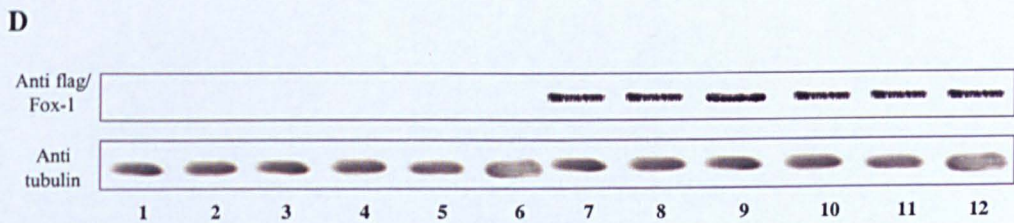
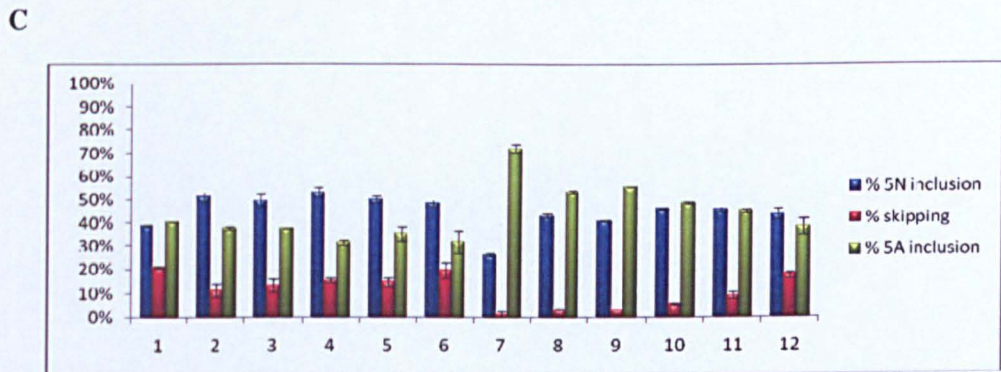
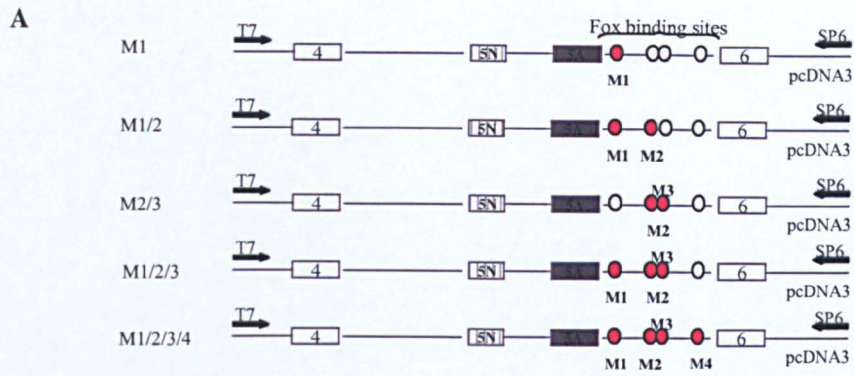


**Figure 36.** Effect of Fox-1 protein expression on the mRNA splicing of the SCN9A WT minigene. (A) Schematic representation indicating the positions of the (T)GCATG motifs in the minigene. The circles are for Fox-1 binding sites. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. (B) Agarose gel electrophoresis analysis of the RT-PCRs digested with Nde I enzyme of the cotransfections experiments with SCN9A WT and Fox-1 expression plasmid. The species of mRNA produced are indicated on the right hand side of the gel. (C) Analysis of the expression of Fox-1 protein was monitored by immunoblotting for the Flag epitope tag and amount of the protein loaded on the western blot controlled through western blot against tubulin.

## **5.5 Mutational analysis of individual (T)GCATG repeats in the downstream intronic region of exon 5A.**

To determine which Fox binding sites are most important for the effect observed on the splicing of the SCN9A WT minigene, each of the putative Fox binding elements downstream of exon 5A was mutated either singly or in combination with the other elements by changing one nucleotide in the motifs, in particular TGCATG to TGCGAG. These mutant minigenes were cotransfected with the Fox-1 expression plasmid or with an empty vector (CMV-4) as a control. The splicing pattern was assayed by RT-PCR analysis, and Fox-1 protein expression was assayed by western blot analysis. A complete panel of mutant constructs is shown in figure 37A (constructs 1-5).

The resultant RT-PCRs of the transient transfection experiments using the WT SCN9A and four mutant SCN9A minigenes, together with Fox-1 expression plasmid were digested using Nde I restriction enzyme in order to be able to distinguish between the cDNA species exon 4-5N-6 and 4-5N-6. As can be observed in figure 37B the increased inclusion of exon 5A in the WT SCN9A minigene was reduced significantly irrespective of which motif was mutated notwithstanding the fact that overall some increased inclusion of SCN9A exon 5A was still observed and in order to eliminate any effect of Fox-1 on splicing all four motifs had to be disrupted. Interestingly it was only when all four motifs had being disrupted did an equivalent amount of the cDNA species 4-5 return to the amounts observed when the wildtype construct was transfected on its own without the Fox expression plasmid.



**Figure 37.** Fox-1 enhancer activity is dependent on four (T)GCATG repeats present in the intronic region downstream of exon 5A. **(A)** Schematic representation of a complete panel of Fox-1 binding sites mutants in the SCN9A WT minigene. The circles are for Fox-1 binding sites. T7 and SP6 primers are present in the 5' and 3' region, respectively, of the translated region in the plasmid. **(B)** RT-PCRs analysis of the transfections of mutant constructs (M1, M1/2, M2/3, M1,2,3, M1,2,3,4) and the SCN9A WT minigenes in HeLa cells, without and with overexpression of the brain Fox-1 protein, digested with Nde I enzyme. The species of mRNA produced are indicated on the right hand side of the gel. **(C)** Quantification of the percentage of exon 5N or 5A inclusion and skipping of both was performed using ImageJ 1.38 software. Standard deviation values from three independent experiments are shown. **(D)** Analysis of the expression of Fox-1 protein was monitored by immunoblotting for the Flag epitope tag and amount of the protein loaded on the western blot controlled through western blot against tubulin.

## 4. Discussion

Many eucaryotic genes employ alternative splicing as a means of generating protein diversity. The differential incorporation of the exons into the mature RNA is often under developmental and/or tissue specific control and enables the cell to tailor the protein to suit its own particular requirements. The mechanisms that determine which splice sites are used and how this process is regulated in different cell types or developmental stages have still not be precisely defined. However, much progress has been made in identifying the *cis*-acting elements involved in alternative splicing some of which are responsible for setting the default competition between splicing events, while others are necessary for the cell specific switch to a regulated splicing pattern. Less is known about the trans-acting factors involved in regulation of tissue specific splicing. Such factors may be tissue specific regulators of splicing or alternatively, tissue specific splicing may be regulated by alterations in the concentrations or activities of general splicing factors.

The work performed in this thesis has begun to unravel the mechanisms behind the ME splicing of SCN8A exons 18A and 18N and SCN9A exons 5A and 5N. The choice by the splicing machinery of ME exons 18A and 18N of the SCN8A gene appears to be dependent on at least four distinct *cis*-acting sequences present in a 620 nucleotide stretch of DNA and the trans-acting factors through which these exert their role on the splicing outcome. In section 3.2 through deletion mapping I identified an ESS in SCN8A exon 18A present between nucleotides +5/+26 of the exon that represses the inclusion of this exon in mRNA in Hela cells by making the spliceosome A complex less stable. In fact deleting this sequence from this exon in a minigene causes the SCN8A exon 18A to be efficiently included in the mRNA. Further proof of this elements ability to act as a ESS comes from the fact that when placed in a heterologous exon it was also observed to repress this exon inclusion in the final mRNA transcript (Fig. 8C).

Through pull down analysis I identified the trans-acting factors that bind to this ESS element. Four proteins emerged from this study and they were formally identified by mass spectrometry and western blot analyses as hnRNP A1, hnRNP JKTBP and PTB/nPTB. I also demonstrated that down regulation of these splicing repressors hnRNP A1, PTB/nPTB and JKTBP in HeLa cells partially relieves repression of SCN8A exon 18A inclusion in a heterologous minigene context. hnRNP A1 is a splicing repressor protein that, typically, but not always recognize splicing silencer elements, and once bound to these elements it can interfere with the binding of spliceosomal components or activator proteins. PTB is also an abundant RNA binding protein that may binds to the ESS element and it can regulate alternative splicing by creating zone of silencing. Till now, there is no evidence of the involvement of hnRNP JKTBP protein in splicing, but an UAG present in these consensus binding site that was found in the JKTBP high-affinity binding sites, suggest a possible role in alternative splice site switching (Akagi, Kamei et al. 2000). It is of interest to note that the expression patterns for these proteins tie in well with the observed inclusion of SCN8A exon 18A neuronal tissue. It is known for example that levels of hnRNP A1 are high in non neuronal tissues, while there is less of this protein in neuronal tissues, such as brain (Clower, Chatterjee et al.). The presence of a tissue restricted counterpart of the ubiquitously expressed PTB was initially detected in rat brain extracts. Two independent studies latter identified and cloned nPTB confirming that is a tissue restricted paralog of PTB, expressed only in neuronal cells. Data from in vitro analysis showed that nPTB can act either neutrally or as a repressor weaker than PTB in preventing exon inclusion. The mRNA levels of PTB were also found to decrease rapidly during differentiation (Shinozaki, Arahata et al. 1999). There is also an evidence that Fox-1/2 strongly activate inclusion of the *nPTB* exon, presumably by interacting with the two downstream hexamer sequences (T)GCATG, in contrast, the effect of Fox-1/2 on the paralogous *PTB* exon is more subtle (Zhang, Zhang et al. 2008). The JKTBP protein that we identified in a pull down experiments is a 38kDa protein ubiquitously expressed, while

in neuronal tissue the major form of JKTBP is a 54kDa protein isoform which may have different function or less repressive effect than 38kDa JKTBP isoform (Akagi, Kamei et al. 2000). Thus the fact that the level of expression of the inhibitor factors in neuronal tissue is lower than in other tissues or completely absent would mean that this ESS would be less functional in this scenario.

Deletion of the ESS element from SCN8A exon 18A in a homologous minigene context containing both ME exon 18N and 18A together with the constitutive exons 17 and 19, however resulted in only a minor rescue of SCN8A exon 18A inclusion and furthermore the inclusion occurred in concert with that of SCN8A exon 18N. This indicated a much more complex ME control mechanism behind the switch of SCN8A exons 18A and 18N observed between neuronal and non neuronal cells respectively and clearly of a much more intricate control behind the inclusion of SCN8A exon 18A in the mRNA transcript. This difference between the heterologous and homologous minigenes was likely due to the fact that in the latter multiple *cis*-acting splicing regulatory signals exist whose cumulative effect result in the desired splicing outcome. Indeed, I showed that the 5'ss of SCN8A exon 18A is intrinsically weak and therefore easily subjective to the influence of weak *cis*-acting splicing elements. Furthermore, the trans-acting factors binding these elements may or may not be present in HeLa cells. In this regard it had been shown that a (T)GCATG motif association with regulated exon 18A has been highly conserved in evolution particularly in the downstream proximal intron of vertebrates from fish to humans (Underwood, Boutz et al. 2005). This observation supports the hypothesis that this element may be a critical component of the splicing switch mechanism that mediates tissue-specific splicing events. The (T)GCATG motif has been identified as a binding site for the family of Fox proteins (Minovitsky, Gee et al. 2005). There are three mammalian family members, Fox-1, Fox-2 and Fox-3, together with different isoforms of each of them, expressed predominantly in neuronal and striated muscle tissues and was therefore feasible that these protein played a role in the inclusion of SCN8A exon 18A in neuronal tissues.

This motif revealed itself to be the second *cis*-acting regulatory element involved in the regulation of SCN8A exon 18A. Over expression of different members of the Fox family as well as different isoforms (sections 3.6.2 and 3.7 and figures 11 and 13) were all capable of increasing the inclusion of the SCN8A exon 18A in the minigene with the ESS deleted (SCN8A/ $\Delta$ ESS E18A). The increased inclusion due to the brain specific Fox-1 over expressed was linked to the presence of both (T)GCATG motifs downstream of SCN8A exon 18A (section 3.6.3 figure 12). The fact that no SCN8A exon 18A inclusion was observed upon over expression of these proteins in the presence of the ESS enhancer (SCN8A WT minigene section 3.6.2 and figure 11) indicated that in the hierarchy of splicing regulation of SCN8A exon 18A the hnRNPs binding to the ESS were dominant over the presence of Fox protein. It is interesting to note that the over expression of Fox-3 had a more pronounced effect on SCN8A exon 18A inclusion, but this may be because the more efficient entry of this protein into the HeLa cell nucleus. In a recent paper it has been shown that all Fox-1 and Fox-2 isoforms were diffusely distributed in both the nuclei and cytoplasm or predominantly localized to the cytoplasm when they were exogenously expressed in cultured cells. In contrast, exogenously expressed Fox-3 isoforms localize almost exclusively to nuclei in cultured cells (Kim, Adelstein et al. 2009). I have been unable as yet to ascertain the identity of the member of the Fox-1 family of proteins responsible for the inclusion of exon 18A in its endogenous context as this family of proteins share an identical RNA binding domain, all of them binds to the (T)GCATG hexamer in an identical manner and transfecting each of them singularly with the SCN8A/ $\Delta$ ESS E18A minigene, I observed that all three Fox proteins are capable of activating exon 18A inclusion when they are over-expressed in cultured cells (Section 3.7, figure 13). However, some hypothesis as to which is the more likely candidate can be made based on the literature. SCN8A exon 18A is included in neurons of CNS and PNS, where all three Fox proteins are expressed, but this exon seems to be also included in Purkinje cells, in which expression of Fox-3 was not observed (Kim, Kim et al.), thus we



can exclude this protein as a specific trans-acting factor necessary for the inclusion of exon 18A in its endogenous context. This leaves the two other candidates, Fox-1 and Fox-2. Fox-2 however, is known to have a broader tissue expression, being mainly found in neurons and striated muscles, but also in embryo (Kuroyanagi 2009). If we consider the fact that SCN8A exon 18N, instead of exon 18A, is included in embryo (Plummer, McBurney et al. 1997), this leaves us with Fox-1 as the potential candidate behind the increased inclusion of SCN8A exon 18A in neuronal cells. I reiterate that this is a hypothesis as there may be other or different players in different neuronal tissues.

A third *cis*-acting regulatory element, this time responsible for the inclusion of SCN8A exon 18N in Hela cells, was mapped between nucleotides +41 and +61 of this exon. Deletion of this region in the SCN8A WT minigene gave rise to the minigene SCN8A/E18N  $\Delta$ 3 (section 4.1 and figure 17) whose transfection in cells produced an mRNA species that skipped both SCN8A exons 18A and 18N. This ESE was observed to bind the SR proteins SC35 and ASF/SF2. Notwithstanding that the removal of these proteins through siRNA knock down differed from the deletion of this region with the SCN8A exon 18A still being included in the mRNA the fact that we are in the presence of an ESE is indisputable. ESE's are known to act in the vast majority of cases through members of the SR protein family as discussed in sections 1.3.1. These proteins furthermore have being often shown to be able to substitute one another, providing an explanation as to the discrepancies between the effect on the splicing outcome between deletion of the ESE and the knockdown of the trans acting factors observed to bind to it (Caceres, Sreaton et al. 1998; Hanamura, Caceres et al. 1998; Tacke and Manley 1999; Rooke, Markovtsov et al. 2003; Long and Caceres 2009).

Finally, a fourth element or rather a fourth region containing two *cis*-acting regulatory elements is present in the intronic region between the two ME exons. In particular, an ISE for SCN8A exon 18N is present within the intronic region +10/+227 downstream of the SCN8A exon 18N and an ISS for SCN8A exon 18A would appear to be present within the

first 150 bp of intronic sequence upstream of the SCN8A exon 18A (section 3.6.1 figure 10). However these assumptions are based on preliminary data and further experimentation is needed in order to refine the area of these *cis*-acting elements as well as the trans acting factors involved.

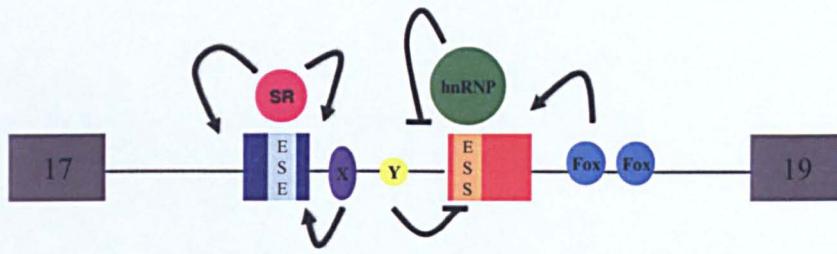
The over expression experiments of Fox proteins in HeLa cells with the minigene the SCN8A/ $\Delta$ ESS E18A resulted in a mRNA processing that does not occur in vivo, namely the inclusion of both SCN8A exons 18N and 18A in the same transcript. This as mentioned previously is most likely due to other *cis*-acting splicing regulatory signals or indeed lack of trans acting factors in HeLa cells that play an important role in the determination of the splicing event. In order to obtain a clear idea of the role of the Fox protein in the ME of SCN8A exons 18A and 18N I turned to a cell line (primary rat trigeminal neurons) in which SCN8A exon 18A was included in the transcript rather than 18N. The effect of the depletion of neuron specific Fox-1 protein on the inclusion of SCN8A exon 18A was impressive, as we observed a decrease in the exon 18A inclusion, together with the appearance of the transcripts containing exon 18N and skipping of both ME exons (section 3.9 and figure 16). Unfortunately a formal proof of these results needs further experiments mainly to complete the controls, see section 3.9. Although all the direct and circumstantial evidence are extremely clear and support the tentative model for the regulation of ME splicing of SCN8A exon 18A and 18N presented in figure 25A that takes into account all the experimental evidence collected in this thesis.

The expression of certain SRs, such as ASF/SF2, is highly regulated during development, such that, for example, in neurons, at an early stage the amount of ASF/SF2 protein is high, but progression during development versus adult organism, shows a decrease in its level of expression (Shinozaki, Arahata et al. 1999). From the previous research it appears that ASF/SF2 and other members of the SR protein family regulate alternative splicing, usually by promoting the use of the proximal 5' splice sites (Caceres and Kornblihtt 2002). This activity of SR proteins is often counteracted by hnRNP A1 and related proteins,

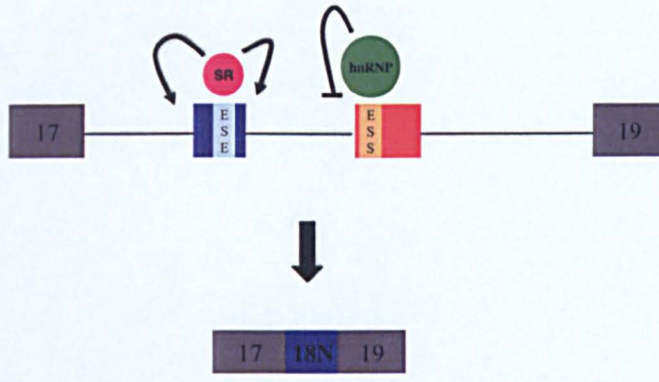
which generally favor the use of the distal 5' splice sites. Therefore, the antagonistic activities of SR proteins and hnRNP A1-like proteins are important determinants of alternative 5' splice site selection *in vitro* and *in vivo*. The hypothetical mechanism of this alternative splicing regulation requires that the relative abundances or activities of these antagonistic factors, SRs and hnRNPs, vary under conditions in which the transcript undergo differential splicing in different cell types and during development. The expression of specific trans-acting factor and the relative levels of SRs and hnRNPs, rather than their absolute amounts, together with the myriad of *cis*-acting elements present in and around the SCN8A exons 18A and 18N determine the selection of alternative SCN8A ME exons 18A and 18N.

This type of control in part appears to be what I am beginning to observe behind the regulation of SCN9A ME splicing of ME exons 5A and 5N. Although still at the initial stages I have been able to determine that one of the principal factors in the switch between the two ME exons is the Fox protein. Indeed over expression of Fox-1 protein in HeLa cells, together with the SCN9A WT minigene resulted in inclusion of SCN9A exon 5A in the mature transcript and again was associated with the presence of the (T)GCATG motifs downstream of the exon (section 5.4, figure 23). In this case however the inclusion of SCN9A exon 5N was strongly inhibited. The fact that over expression of the Fox protein even in Hela cells results in the ME switch unlike the scenario observed with SCN8A ME of exons 18A and 18N is indicates that the hierarchy of *cis*-acting elements in this case may be dependent on Fox family of proteins.

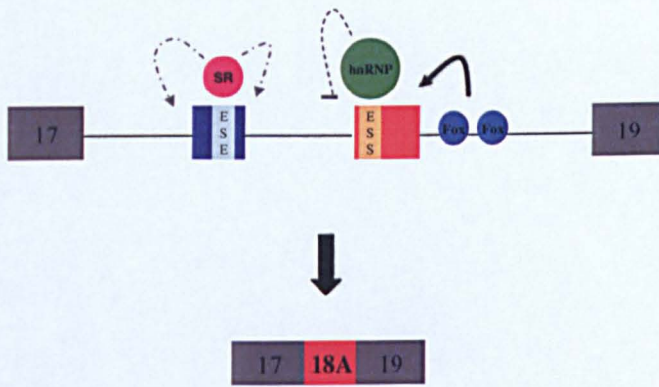
A



B



C



**Figure 38.** Model for the Mechanism of SCN8A ME exon 18N and 18A Cell-Type-Specific Splicing. (A) Schematic representation of all *cis* and *trans* acting factors identified controlling the inclusion or exclusion of SCN8A ME exons 18N and 18A. An ISE for SCN8A exon 18N and an ISS for SCN8A exon 18A exist in the intronic region between the two ME exons however as the *trans* acting factors through which these function have yet to be identified they are denoted X and Y respectively. (B) In tissues where SCN8A exon 18N is included such as non neuronal cells, hnRNPs, among which hnRNPA1, JKTBP and PTB, bind to the ESS in SCN8A exon 18A repressing its inclusion in the mRNA. SR proteins, among which ASF/SF2 and SC35, bind to the ESE in SCN8A exon 18N stimulating its inclusion in the mRNA. (C) In tissues where SCN8A exon 18A is included, such as neuronal cells, a decrease in the quantity of SR proteins binding the ESE in SCN8A exon 18N in combination with a decrease or absence of hnRNPs binding to the ESS in SCN8A exon 18A and the increased definition of the SCN8A exon 18A provided by the presence of Fox-1 protein occurs. Thickness and fullness of arrows indicate relative amount of the *trans* acting factors.

## 5. Conclusion

The molecular mechanisms behind the ME splicing of SCN8A exons 18A and 18N have been investigated and my analysis is consistent with the model depicted in figure 25, B and C in which exon 18A exclusion in non-neuronal tissue is regulated primarily by the presence in the cell types of several hnRNPs proteins (hnRNP A1, hnRNP JKTBP and PTB) that function through an exonic splicing silencer (ESS) mapped in this exon together with the absence of neuron specific Fox-1 protein. In neuronal cells the reduction of these hnRNPs together with the presence of Fox-1 allow the exon to be included. The SCN8A exon 18N is included in non neuronal cells due to SR proteins that function through an exonic splicing enhancer (ESE) mapped in this exon. In neuronal cells the lower levels of these SR proteins together with the enhanced recognition of SCN8A exon 18A, due to the presence of Fox-1 and lower levels of the hnRNPs that bind to the ESS element in SCN8A exon 18A, causes the exon 18N to be skipped and the SCN8A exon 18A to be included.

It is tempting to hypothesize that considering the results obtained thus far from the study of SCN9A ME splicing of exons 5A and 5N this type of control of mutually exclusive splicing through the proteome make-up of a cell type could be influential in the temporal and tissue specific splicing of other members of voltage gated sodium channels representing a more general mechanism.

## 6. Materials and Methods

### 6.1 Chemical reagents

General chemicals were purchased from Sigma Chemical Co., Merck, Gibco BRL, Boehringer Mannheim, Carlo Erba and Serva.

#### 6.1.1 Standard solutions

All the solutions are identified in the text when used apart from the following:

- a) TE: 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 7.4)
- b) PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4
- c) 10X TBE: 108 g/l Tris, 55 g/l Boric acid, 9.5 g/l EDTA
- d) 6X DNA sample buffer: 0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol FF, 30 % v/v glycerol in H<sub>2</sub>O.
- e) 10X protein sample buffer: 20 % w/v SDS, 1 M DTT, 0.63 M Tris-HCl (pH 7), 0.2% w/v bromophenol blue, 20 % v/v glycerol, 10 mM EDTA (pH 7).

### 6.2 Enzymes

Restriction enzymes were from New England Biolabs, Inc. DNA modifying enzymes such as Taq Polymerase, DNaseI RNase free, and T4 DNA ligase were obtained from Roche Diagnostic. Klenow fragment of *E. coli* DNA polymerase I and T4 polynucleotide Kinase were from New England Biolabs, Inc. RNase A was purchased from Sigma Chemicals Ltd. A 10 mg/ml solution of RNase A was prepared in sterile water and boiled for 10 minutes to destroy trace amounts of DNase activity. All enzymes were used following manufacturer's instructions.

### 6.3 Synthetic oligonucleotides

Synthetic DNA oligonucleotides were purchased from Sigma-Genosy and IDT and Integrated DNA Biotechnologies.

### 6.4 Radioactive isotopes

Radioactive [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\alpha$ -<sup>32</sup>P]UTP were supplied by Amersham U.K. Ltd.

### 6.5 Bacterial culture

The *E. Coli* K12 strain DH5 $\alpha$  was transformed with the plasmids described in this study and used for their amplification. Plasmids were maintained in the short term as single colonies on agar plates at 4 °C but for long term storage they were kept on glycerol stocks made by adding sterile glycerol to a final 30% v/v concentration to liquid bacterial cultures. Glycerol stocks were stored at -80°C. When necessary, from the glycerol stocks an overnight culture of bacteria was grown in Luria-Bertani medium [LB medium: per litre: 10 g Difco Bactotryptone, 5 g Oxoid yeast extract, 10 g NaCl, (pH 7.5)]. Bacterial growth media were sterilized before use by autoclaving. When appropriate, ampicillin was added to the media at a final concentration of 200  $\mu$ g/ml.

### 6.6 Cell culture

The cell line used for transfection and cotransfection experiments were:

- a) HeLa cells, an immortal cell line derived from cervical cancer cells.
- b) SK-N-BE (ATCC Number: CRL-2271™), neuroblastoma cell line.
- c) SH-SY-5Y (ATCC Number: CRL-2266™), neuroblastoma cell line.



- d) F11 (provided by Prof. E. Wanke, Università degli studi di Milano-Bicocca), a hybrid cell line derived from a mouse N18TG2 neuroblastoma and rat DRG sensory neuron.
- e) PC-12 (ATCC Number: CRL-1721™), the rat pheochromocytoma cell line.
- f) Primary rat trigeminal neuronal culture.

## **6.7 DNA preparation**

### **6.7.1 Small scale preparation of plasmid DNA from bacterial cultures**

The alkaline lysis of recombinant bacteria was performed by resuspending the bacterial pellet in 200 µl of ddH<sub>2</sub>O; 150 µl of solution II (0.2 M NaOH, 1 % w/v SDS) were then added and the contents mixed by inversion. 250µl of solution III (3 M potassium acetate pH 5.2) were then added and the contents mixed by inversion. The bacterial lysate was then centrifuged in an Eppendorf microcentrifuge at maximum speed and the supernatant transferred to a new tube. An equal volume of 1:1 v/v phenol:chloroform solution was added to the supernatant. The tube was then vortexed and centrifuged as above. The aqueous phase containing the DNA was transferred to a new tube. An equal volume of chloroform was added to the supernatant. The tube was then vortexed and centrifuged as above. The aqueous phase containing the DNA was then recovered and the DNA pelleted by ethanol precipitation. The final pellet was resuspended in 50 µl of ddH<sub>2</sub>O and 5 µl of such preparation were routinely taken for analysis by restriction enzyme digests.

### **6.7.2 Large scale preparations of plasmid DNA from bacterial cultures**

For large-scale preparations of plasmid DNA that was necessary for the transfection experiments, JetStar purification kit (Genomed) was used according to the manufacturer's

instructions. In order to get a good amount of plasmid, an inoculation in 50 ml of TB medium is grown overnight at 37°C.

## **6.8 RNA preparation from cultured cells**

Cultured cells were washed with PBS and RNA extracted using RNA Trizol, (Invitrogen inc) according to the manufactures instructions. Briefly, 750ul of Trizol was added/p6 well and allowed to incubate for 5 minutes. The solution was subsequently moved to a 1.5ml eppendoff tube and 200ul of ,chloroform was added. After centrifugation at 10000rpm the supernatant was collected and the RNA precipitated with 0.75 vol. of isopropanol. The pellet was resuspended in 100 µl of ddH<sub>2</sub>O and digested with 1U of DNase RNase free by incubateion at 37 °C for 30 minutes, and then the RNA was purified by acid phenol extraction. The final pellet was resuspended in 35 µl of ddH<sub>2</sub>O and frozen at -80 °C. The RNA quality was checked by electrophoresis on 1% agarose gels.

## **6.9 Estimation of nucleic acid concentration**

An optical density of 1.0 at 260 nm is usually taken to be equivalent to a concentration of 50 µg/ml for double stranded DNA, 40 µg/ml for single stranded DNA and RNA, and approximately 20 µg/ml for single-stranded oligonucleotides samples. The ratio of values for optical densities measured at 260 nm and 280 nm is considered as 1.8 for pure sample of DNA and 2 for RNA and these are reduced by protein contaminants (Sambrook et al., 1989). Therefore, these values were used to determinate not only the concentration but also the purity of the samples.

## **6.10 Enzymatic modification of DNA**

### **6.10.1 Restriction enzymes**

Restriction endonucleases were used in the construction and analysis of recombinant plasmids. Each restriction enzyme functions optimally in a buffer of specific ionic strength. All buffers were supplied by the same company that supplied the enzymes and were used according to the manufacturer's instructions.

For analytical digests 100-500 ng of DNA were digested in a volume of 20  $\mu$ l containing 5 U of the appropriate restriction enzyme. The reaction was incubated for 2-3 hours at 37 °C. Preparative digestion was made of 5-10  $\mu$ g DNA using the above conditions and 5 U of restriction enzyme for  $\mu$ g of DNA in 200  $\mu$ l reaction volume.

### **6.10.2 Large fragment of *E. coli* Polymerase I and T4 Polynucleotide Kinase**

These enzymes were used to treat PCR products for blunt-end ligation during construction of recombinant plasmids. The large fragment of DNA Polymerase I (Klenow) is a proteolytic product of *E. coli* DNA Polymerase I. It retains polymerization and 3'->5' exonuclease activity, but has lost 5'->3' exonuclease activity. This was useful for digesting specific residues added by Taq DNA polymerase at the 3' terminus to create compatible ends for ligation. T4 Polynucleotide Kinase catalyses the transfer of phosphate from ATP to the 5' hydroxyl terminus of DNA. It was used for example in the addition of 5'-phosphate to PCR products to allow subsequent ligation. Klenow fragment (2.5 U) was added to 23  $\mu$ l of PCR product in 5 mM MgCl<sub>2</sub> buffer. The mixture was incubated at room temperature for 10 minutes. EDTA to a final concentration of 0.2 mM, ATP to a final concentration of 1 mM, 10 U of T4 Polynucleotide Kinase and the proper quantity of

Kinase buffer were added to the above mixture and incubated at 37 °C for 30 min. The enzymes were inactivated by incubation at 80 °C for 20 min.

### **6.10.3 T4 DNA ligase**

T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3' hydroxyl and 5' phosphoryl termini in DNA, requiring ATP as a cofactor in this reaction. This enzyme was used to join double stranded DNA fragments with compatible sticky or blunt ends, during generation of recombinant plasmid DNAs.

20 ng of linearized vector were ligated with a 5-10 fold molar excess of insert in a total volume of 20 µl containing 1X ligase buffer and 1U of T4 DNA ligase. Reaction was carried out at room temperature for 6-12 hours.

In some reactions synthetic oligonucleotide were included in the reaction. In these cases, the amounts added to each reaction to obtain inclusion of oligonucleotides in the resulting plasmid were about 100 fold molar excess over the DNA vector.

### **6.11 Agarose gel electrophoresis of DNA**

DNA samples were size fractionated by electrophoresis in agarose gels ranging in concentrations from 0.8 % w/v (large fragments) to 2 % w/v (small fragments). The gels contained ethidium bromide (0.5 µg /ml) and 1X TBE. Horizontal gels were routinely used for fast analysis of DNA restriction enzyme digests, estimation of DNA concentration, or DNA fragment separation prior to elution from the gel. Samples of 20 µl containing 1X DNA loading buffer were loaded into submerged wells. The gels were electrophoresed at 50-80 mA in 1X TBE running buffer for a time depending on the fragment length expected and gel concentration. DNA was visualized by UV trans illumination and the result recorded by digital photography.

## **6.12 Elution and purification of DNA fragments from agarose gels**

This protocol was used to purify small amounts (less than 1 µg) of DNA for sub-cloning. The DNA samples were electrophoresed onto an agarose gel as described previously. The DNA was visualized with UV light and the required DNA fragment band was excised from the gel. This slab was cut into pieces, and the JETquick Spin Column Technique (Genomed) was used according to the manufacturer's instructions. Briefly, 600 µl of gel solubilisation solution L1 (NaClO<sub>4</sub>, Na acetate and TBE) were added for each 100 mg of the gel slice pieces and incubated at 55 °C for 15 min vortexing every 5 min. The mixture was loaded into a prepared JETquick column and it was centrifuged at maximum speed for 1 min. The flowthrough was discarded. 700 µl of washing and reconstituted solution L2 (ethanol, NaCl, EDTA and Tris-HCl) were added into the spin column and after 5 min, the column was centrifuged in the same conditions twice. The flowthrough was again discarded both times. To elute the bound DNA, 30-50 µl of pre-warmed sterile water were added onto the centre of the silica matrix of the spin column and the system was centrifuged for 2 min. The amount of DNA recovered was approximately calculated by UV fluorescence of intercalated ethidium bromide in an agarose gel electrophoresis.

## **6.13 Preparation of bacterial competent cells**

Bacterial competent cells, *E. Coli* strains, were grown overnight in 3 ml of LB at 37°C. The following day, 300 ml of fresh LB were added and the cells were grown at room temperature for 4-5 h until the OD<sub>600</sub> was 0.3-0.4. The cells were then put in ice and centrifuged at 4 °C and 1000g for 15 min. The pellet was resuspended in 30 ml of cold TSS solution (10% w/v PEG, 5% v/v DMSO, 35mM Mg Cl<sub>2</sub>, pH 6.5 in LB medium). The cells were aliquoted, rapidly frozen in liquid nitrogen and stored at -80°C. Competence was determined by transformation with 0.1 ng of pUC19 and was deemed satisfactory if this procedure resulted in more than 100 colonies.

## **6.14 Transformation of bacteria**

Transformations of ligation reactions were performed using 1/2 of the reaction volume. Transformation of clones was carried out using 20 ng of the plasmid DNA. The DNA was incubated with 60  $\mu$ l of competent cells for 20 min on ice and at 42°C for 1.5 minutes. At this point 60  $\mu$ l of LB were added and the bacteria allowed to recover for 10 min at 37 °C. The cells were then spread onto agarose plates containing the appropriate antibiotic. The plates were then incubated for 12-15 hours. When DNA inserts were cloned into  $\beta$ -galactosidase-based virgin plasmids, 25  $\mu$ l of IPTG 100 mM and 25  $\mu$ l of X-Gal (4 % w/v in dimethylformamide) were spread onto the surface of the agarose before plating to facilitate screening of positive clones (white colonies) through identification of  $\beta$ -galactosidase activity (blue colonies).

## **6.15 Amplification of selected DNA fragments**

The polymerase chain reaction was performed on genomic or plasmid DNA following the basic protocols of the Roche Diagnostic Taq DNA Polymerases. The volume of the reaction was 50  $\mu$ l. The reaction buffer was: 1X Taq buffer, dNTP mix 200  $\mu$ M each, oligonucleotide primers 1 nM each, Taq DNA Polymerase 2.5 U. As DNA template, 0.1 ng of plasmid or 100-500 ng of genomic DNA were used for amplification. When a DNA fragment longer than 2000bp was amplified, DMSO 3% was also added to the mixture. The amplification conditions are described for each particular PCR. The amplifications were performed on a Cetus DNA Thermal Cycler (Perkin Elmer) or on a Gene Amp PCR System (Applied Biosystems).

## **6.16 Sequence analysis for cloning purpose**

Sequence analysis of plasmid DNA was performed using the CEQ 2000 sequencer (Beckman Coulter) or sequencing service (BMR Genomics). The plasmid DNA of interest

(approximately 100 ng) was purified through a MicroSpin S-400 HR Column (Amersham Pharmacia Biotech). The DNA was then amplified using fluorescent labeled dideoxy nucleotide terminators according to the manufacturer's instructions. The samples were analyzed by loading them into the automatic sequencer.

## **6.17 Minigene systems**

In this study, the splicing pattern of several exons has been investigated through the use of two minigene backbones.

The PTB minigene is a hybrid construct containing exons from  $\alpha$ -globin and fibronectin, under the control of the  $\alpha$ -globin promoter. The intronic region between the two fibronectin exons contains a unique Nde I site in which the exon along with its flanking regions with and without the nucleotide substitution under study can be inserted. In fact this system allows us to insert a single exon with its intronic flanking regions, which could contain splicing regulatory elements, and analyze its splicing outcome.

In this study, the splicing pattern of one exon has been investigated by using pY7 minigenes/splicing-construct which contains the alpha-tropomyosin exons 2 and 3 separated by a 111-nucleotides synthetic intron sequence with a 5' splice site partially derived from the human beta-globin gene. The SP6 promoter controls the expression of this reporter minigene inserted into the pBMN vector backbone.

### **6.17.1 Generation of minigenes**

In this thesis several minigenes have been created and this has been done through PCR amplification of the exon under study with its intronic flanking regions, treatment with Klenow-Kinase enzymes as described previously (section 6.10.2), purification from agarose gel (section 6.12) and cloning in pUC19 vector (Fermentas) using the enzyme restriction site Sma I and through sequencing using oligonucleotides Universal For and

Rev the absence of any other nucleotide variation in the entire amplified fragments was checked. Following sequencing in order to check the nucleotide sequence to be correct, the subcloning in pTB or pY7 minigenes is performed using the enzyme restriction site Nde I. The orientation of the inserted fragment in the pTB minigene was checked through colony PCR using, respectively, specific oligonucleotides forward and pTB1950 antisense (as) that recognized respectively the inserted fragment and the pTB plasmid; thus the amplification of the product could have been possible only in presence of a fragment with the correct orientation. Subsequent positive clones were further controlled through sequencing using oligonucleotide pTB1698 sense (s).

In addition, in this work one construct was created by inserting the fragment of interest in the pY7 minigene system using an Nde I restriction site present in this minigene, together with the two alpha-tropomyosin exons. The DNA fragment containing the fragment of interest together with flanking alpha-tropomyosin exons was now amplified by PCR reaction using the two external oligos in the pY7 minigene, pY7-KpnI-sense and pY7-XbaI-antisense. Finally, the obtained DNA inserts was digested with the KpnI and XbaI enzymes (New England Biolabs), gel extracted (EuroClone) and ligated with T4 DNA ligase (New England Biolabs) into the pcDNA3 plasmid using the KpnI and XbaI restriction sites, which carries the CMV promoter suitable for *in vitro* coupled transcription-splicing and in cell culture experiments. When it was possible to make a minigene consisting of at least two exons three introns I preferred to make a homologous minigene by cloning of the appropriate fragment in pcDNA3 vector, using specific primers containing BamH I and Xho I restriction sites, via PCR method. The splicing assay was performed by transfecting each minigene into HeLa cells.



Universal forward

5'GTAAAACGACGGCCAGT3'

Universal reverse

5'GAAACAGCTATGACCAT3'

pTB1689s

5'CTAAAACAGTTTTGGAATA3'

pTB1950as

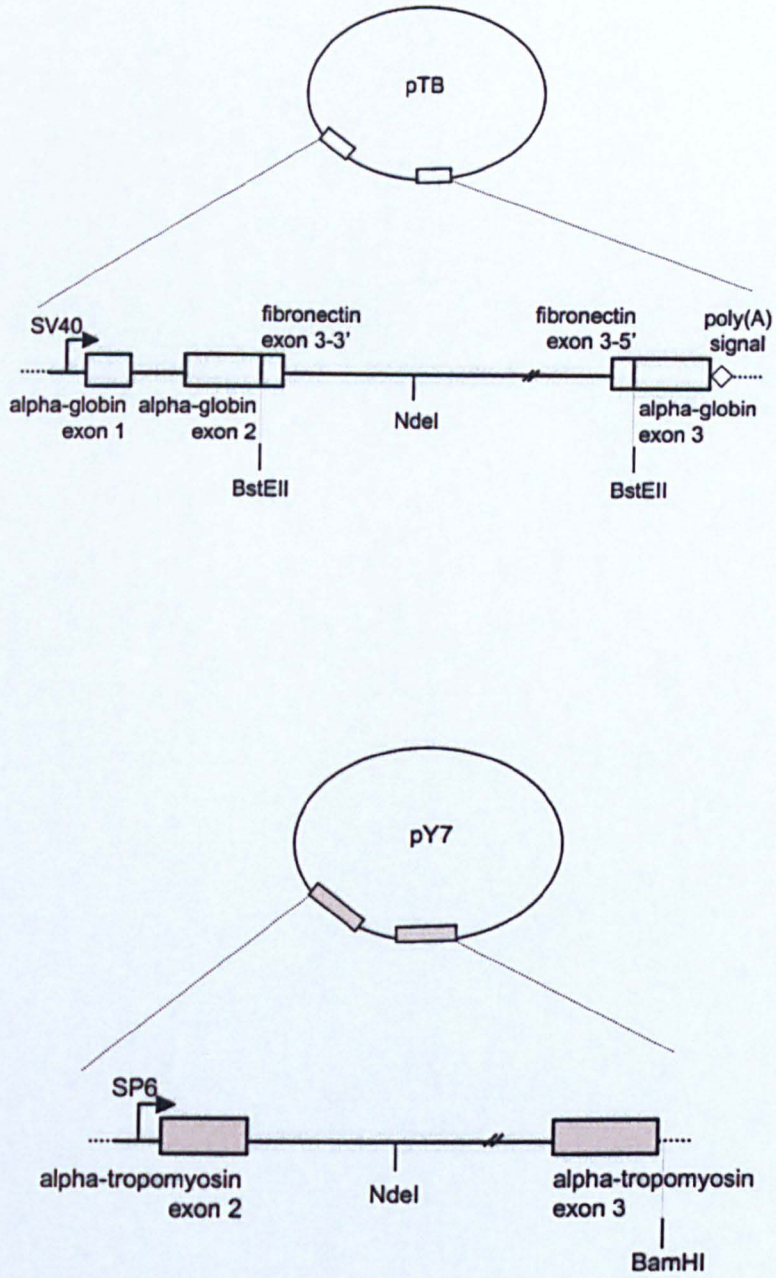
5'CGAAGAGGCTTAAGTTGCCAC3'

pY7-KpnI-s

5' GGGGTACCGAATACAAGCTTGTCGAG3'

pY7-Xba I-as

Rev: 5'GCTCTAGAGTCGGACCTG3'



**Figure 39.** Schematic representation of the reporter minigenes used in this study. Upper panel: pTB minigene. Lower panel: pY7 minigene.

### 6.17.2 PCR amplifications and Quick Change Mutagenesis PCR method

External primers that contained an restriction site were used for the PCR amplifications of the fragments that were subsequently cloned, as described previously, in the pTB minigene system or pcDNA3 vector (for the primers that were used see section 6.17.3).

Two steps PCR technique has been used for generating the hybrid mutants using different minigenes as a templates. A first set of PCR reactions was performed using a combination of primers: forward external primer and reverse internal primer and forward internal and reverse external primer. The DNA fragments were gel extracted and used as templates for a second PCR reaction using both external primers. The DNA insert obtained was then cloned into pTB vector using the NdeI restriction site or pcDNA3 vector using BamH I and Xho I restriction site (for the primers that were used see section 6.17.3).

Mutagenesis and deletions were also at times performed using the QuikChange site-directed methodology. Briefly, oligonucleotide primers, each complementary to opposite strands of the area of interest and carrying the mutant nucleotides are designed and utilized during temperature cycling by *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA containing the desired mutations is then transformed into competent cells. The small amount of starting DNA template required to perform this method, the high fidelity of the *PfuTurbo* DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction (for the primers that were used see section 6.17.3).

Briefly, two separate primer extension reactions were set up (one for each forward and reverse primer) containing:

5 microliters 10X Pfu Buffer (supplied with enzyme)

1 microliter 10 micromolar primer (0.13 microgram 45-mer)

0.1 – 0.2 microgram plasmid template

1 microliter 10 mM dNTP mix

H<sub>2</sub>O to a final volume of 50 microliters

1 microliter Pfu turbo polymerase (Stratagene)

PCR reaction was done using standard conditions:

1. 94 deg, 30 sec
2. 95 deg, 30 sec
3. 55 deg, 1 min
4. 68 deg, 2 min/kb up to 10 KB plasmid

Digestion of amplification product was done by adding into a PCR reaction 10 units of Dpn I enzyme, mixed well and incubated at 37 deg for at least one hour. 1 microliter of the reaction was used to transform DH5 $\alpha$  competent cells. 60 microliters of cells on separate plates were plated.

### **6.17.3 A complete list of the primers used in the section 3 in this thesis.**

The amplifications for the generation of the fragments to clone in the minigene were performed through using the following oligonucleotides.

The results section 3.1.1.

External primers that contained an Nde I restriction site were used for the PCR amplifications of the fragments that were subsequently cloned as described previously in the pTB minigene system using as a template SCN8A WT minigene (see bellow).

Name of the minigene: pTB SCN8A E18N

For: 5'GGAATTCCATATGGAAATAGCACTGAGATCTTCATATATG 3'

Rev: 5'TTCCATATGGAATTCCGGGAAGAGAAAATCGAGAAGGC 3'

Name of the minigene: pTB SCN8A E18A

For: 5'GGAATTCCATATGGAAAATCTGTGTCTGCAGTGCTAG 3'

Rev: 5'TTCCATATGGAATTCCGCTGCATCACAGAATTCGTGTTTG3'

The results section 3.1.2

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant pTB SCN8A E18A  $\Delta$ CE1 using pTB SCN8A E18A minigene as a template using two primers:

Name of the minigene: pTB SCN8A E18A  $\Delta$ CE1

For: 5'CTTTATTCTAACAGAGAGGAATGGCTGTTTTCTTTCTTTT3'

Rev: 5'GAAAGAAAACAGCCATTCCTCTCTGTTAGAATAAAGCCCCTAGCAC3'

Two steps PCR technique has been used for generating the hybrid mutants using both pTB SCN8A E18N and pTB SCN8A E18A as a templates. A first set of PCR reactions was performed using a combination of primers: forward external primer and reverse internal

primer and forward internal and reverse external primer. The DNA fragments were gel extracted and used as templates for a second PCR reaction using both external primers. The DNA insert obtained was then cloned into pTB vector using the NdeI restriction site.

Name of the minigene: pTB E18A Int.up. E18N

For External: 5'GGAATTCCATATGGAAATAGCACTGAGATCTTCATATATG 3'

Rev Internal: 3'ATCTGTATTCTTTTCCATAGGTCTCTTTAGTCAGC5'

For Internal: 5'TCCATAGGTCTCTTTAGTCAGCCTTA3'

Rev External: 5'GGAATTCCATATGGAAATAGCACTGAGATCTTCATATATG 3'

Name of the minigene: pTB E18A Int. up and down. exon E18N

For External: 5'GGAATTCCATATGGAAATAGCACTGAGATCTTCATATATG 3'

Rev Internal: 3'CACGATTTGAAGGGATGAGGGTAAGGCTCT5'

For Internal: 5'GAAGGGATGAGGGTAAGGCTCTTGTCGTCTGTTC3'

Rev External: 5'TTCCATATGGAATTCCGGAAGAGAAAATCGAGAAGGC 3'

Name of the minigene: pTB E18N Int. down. exon E18A

For External: 5'GGAATTCCATATGGAAATAGCACTGAGATCTTCATATATG 3'

Rev Internal: 3'AGACTGTAAAGGGCGAGGGTAAGATACTA5'

For Internal : 5'AAGGGCGAGGGTAAGATACTAAGAGCAG3'

Rev External: 5'TTCCATATGGAATTCCGCTGCATCACAGAATTCGTGTTTG3'

Name of the minigene: pTB E18N Int. up and down. exon E18A

For External: 5'GGAATTCCATATGGAAAATCTGTGTCTGCAGTGCTAG 3'

Rev Internal: 3'CTCTTCTGTTTCTGTGTAGGTACCATTAAT5'

For Internal: 5'CTGTGTAGGTACCATTAATTTGTC3'

Rev External: 5'TTCCATATGGAATTCCGCTGCATCACAGAATTCGTGTTTG3'

## The results section 3.2

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant minigenes using pTB SCN8A E18A minigene as a template.

Name of the minigene: pTB SCN8A E18A

6-27  $\Delta$

For: 5'TCTGTGTAGGTCTCTGCCCTGGGCTACTCGGAACT3'

Rev: 3'TCTCTTCTGTTTCTGTGTAGGTCTCTGCCCTGGGC5'

27-43  $\Delta$

For: 5'TATAGCTAAACTAGGTGCCATAAAGTCCCT3'

Rev: 3'CTTTAGTCAGCCTTATAGCTAAACTAGGTGCCAS'

43-69  $\Delta$

For: 5'CTACTCGGACTTAGGACCCTAAGAGCTTTG3'

Rev: 3'TAGCTAATGCCCTGGGCTACTCGGACTTAGGACCCT5'

## The results section 3.2.1 and 3.3

For the *in vitro* transcription analysis the RNA was transcribed from PCR templates amplified from the pTB SCN8A E18A and pTB SCN8A E18A 6-27  $\Delta$ . A T7 promoter sequence was added towards the 5' end of the templates using forward primer carrying T7 sequence and the primer.

Name of the minigene: Ex. 18A WT and Ex. 18A/ $\Delta$ ESS

For: 5'TACGTAATACGACTCACTATAGGGTGCTAGGGGCTTTATTCTAACAC3'

Rev: 3'GAAACTGTTTAAGCATGCTAGAACTGAT5'

Name of the minigene: pTB SCN8A WT E18A T7 promoter

For: 5'TACGTAATACGACTCACTATAGGAGGTCTCTTTAGTCAGCCTTAT3'

Rev: 3'TCCCTTAGGACCCTAAGAGCTTTGAGACCCTTAAG5'

Name of the minigene: pTB SCN8A/ $\Delta$ ESS E18A T7 promoter

For: 5'TACGTAATACGACTCACTATAGGAGGTCTCTGCCCTGGGCTACTCGG3'

Rev: 3'TCCCTTAGGACCCTAAGAGCTTTGAGACCCTTAAG5'

#### The results section 3.4

Human SCN8A WT minigene was amplified from genomic DNA using forward primer containing BamH I restriction site and reverse with the Xho I restriction site. Amplification of this fragment was performed by PCR analysis.

Name of the minigene: SCN8A WT

For: 5'CGGGATCCCGATGCCTTCGAGGACATCTACATT3'

Rev: 5'GGCGAGCTCGCCCTATACTTGAAGAAGGGCCAGGT3'

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant minigenes using SCN8A WT minigene as a template.

Name of the minigene: SCN8A/ $\Delta$ ESS E18A

For: 5'GTCTCTGCCCTGGGCTACTCGGAACTAGGTG3'

Rev: 5'CTCTTCTGTTTCTGTGTAGCGTCTCTTGCCCTGGG3'

Name of the minigene: SCN8A/+ESS E18N

For: 5'TAGGTACCTTTAGTCAGCCTTATAGCTAATTAATGGGGACTTCTGGGA3'

Rev:3'GTATTCTTTTCCATAGGTACCTTTAGTCAGCCTTATAGCTAATTAATGGG  
G5'

#### The results section 3.5

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant minigenes using SCN8A/ $\Delta$ ESS E18A minigene as a template.

Name of the minigene: SCN8A/ $\Delta$ ESS E18A, 5'ss Mut. E18A

For: 5'GAAGGGATGAAGGTAAGTTACTAAGAGCAGCTGATC3'

Rev: 5'AGCTGCTCTTAGTAACTTACCTTCATCCCTTCAAATCGTGATAAGGC3'



### The results section 3.6.1

Two steps PCR technique has been used for generating the hybrid mutants using both SCN8A WT and SCN8A/ $\Delta$ ESS E18A as a templates. A first set of PCR reactions was performed using a combination of primers: forward external primer and reverse internal primer and forward internal and reverse external primer. The DNA fragments were gel extracted and used as templates for a second PCR reaction using both external primers. The DNA insert obtained was then cloned into pcDNA3 vector using the BamH I and Xho I restriction sites.

Name of the minigene:  $\Delta$  E18N

For External: 5'CGGGATCCCGATGCCTTCGAGGACATCTACATT3'

Rev Internal: 3'ATTTATCTGTATTCTTTTCCATAGGTAAGGC5'

For Internal: 5'TATTCTTTTCCATAGGTAAGGCTCTTGTCGTCTGTTC3'

Rev External: 5'GGCGAGCTCGCCCTATACTTGAAGAAGGGCCAGGT3'

Name of the minigene:  $\Delta$  E18N/ $\Delta$  Int. E18N/E18A

For External: 5'CGGGATCCCGATGCCTTCGAGGACATCTACATT3'

Rev Internal: 3'ATTTATCTGTATTCTTTTCCATAGGTCTCTTTA5'

For Internal: 5'ATTCTTTTCCATAGGTCTCTTTAGTCAGCCTTAT3'

Rev External: 5'GGCGAGCTCGCCCTATACTTGAAGAAGGGCCAGGT3'

Name of the minigene:  $\Delta$  Int. E18N/E18A +10/+227 bp

For External: 5'CGGGATCCCGATGCCTTCGAGGACATCTACATT3'

Rev Internal:

5'TAGCACTGGAAGAGAAAATCGAGAAGGCGGTAGAACATACAC3'

For Internal: 5'GATTTTCTCTTCCAGTGCTAGGGGCTTTATTCTAAC3'

Rev External: 5'GGCGAGCTCGCCCTATACTTGAAGAAGGGCCAGGT3'

## The results section 3.6.3 and 4.1

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant minigenes using SCN8A/ $\Delta$ ESS E18A minigene as a template.

Name of the minigene: Fox b.site M1 SCN8A/ $\Delta$ ESS/tgcatg > tgcgtg

For: 5'CAGCTGATCCTTCTGCGTGCCAGTGGAAACTGTTTAAGC3'

Rev: 3'CTAAGAGCAGCTGATCCTTCTGCGTGCCAGTGGAA5'

Name of the minigene: Fox b.site M2 SCN8A/ $\Delta$ ESS/gcatg > gcgtg

For: 5'CTGTTTAAGCGTGCTAGAACTGATCACATG3'

Rev: 3'CCAGTGGAAACTGTTTAAGCGTGCTAGA5'

Name of the minigene: SCN8A/E18N  $\Delta$ 1

For: 5'TCCATAGGTACCTTAATGGGGACTTCTGGGA3'

Rev: 3'CTGTATTCTTTTCCATAGGTACCTTAATGGGGA5'

Name of the minigene: SCN8A/E18N  $\Delta$ 2

For: 5'TCTGGCTTAATGCAGAGACTGTAAAGGGC3'

Rev: 3'CCATTAAATTTGTCTGGCTTAATGCAGAGACT5'

Name of the minigene: SCN8A/E18N  $\Delta$ 3

For: 5'TGGGGACTTCTAGGGCGAGGGTAAGGCTCTTG3'

Rev: 3'CTTAATTTAATGGGGACTTCTAGGGCGAG5'

Name of the minigene: SCN8A/E18N  $\Delta$ 4

For: 5'TGGGGACTTCTAGAGACTGTAAAGGGCGA3'

Rev: 3'CTTAATTTAATGGGGACTTCTAGAGACTGTA5'

Name of the minigene: SCN8A/E18N  $\Delta$ 5

For: 5'ACTTCTGGGACCTAGGGCGAGGGTAAGGCTCT3'

Rev: 3'TAATTTAATGGGGACTTCTGGGACCTAGGGCGAGG5'

Name of the minigene: SCN8A/E18N  $\Delta$ 6

For: 5'TCTGGCTTAAGGGACCTGCAGAGACTGTAAAG3'

Rev: 3'TAAATTTGTCTGGCTTAAGGGACCTGC5'

#### The results section 4.2

For the *in vitro* transcription analysis the RNA was transcribed from PCR templates amplified from the pTB SCN8A E18N and pTB SCN8A E18N  $\Delta$ 3. A T7 promoter sequence was added towards the 5' end of the templates using forward primer carrying T7 sequence and the primer.

Name of the minigene: pTB SCN8A E18N and pTB SCN8A E18N  $\Delta$ 3

For:

5'TACGTAATACGACTCACTATAGGAAGGTACCATTAAATTTGTCTGGCTTA3'

Rev: 3'AGGGCGAGGTGAAGGCTCTTGTCGTC5'

#### The results section 5.2

Human SCN9A WT minigene was amplified from genomic DNA using forward primer containing BamH I restriction site and reverse with the Xho I restriction site. Amplification of this fragment was performed by PCR analysis.

Name of the minigene: SCN9A WT (For BamH I/Rev Xho I)

For: 5'CGGGATCCCGATTACACTTTTACTGGAATATATAC3'

Rev: 3'GAGTGAAGAAGAAGACTTTAGAATAGGGCGAGCTCGCC5'

## The results section 5.1

External primers that contained an Nde I restriction site were used for the PCR amplifications of the fragments that were subsequently cloned as described previously in the pTB minigene system using as a template SCN9A WT minigene (see below).

Name of the minigene: pTB wt E5A

For: 5'GGAATTCCATATGGAATATTTGGGAGTTGTTCTTTGTTTCG3'

Rev: 5'TTCCATATGGAATTCCAGCCAAGTAGTCATCCTCATC3'

Name of the minigene: pTB wt E5N

For: 5'GGAATTCCATATGGAAGTGCAGAACTATAACATTTAAAAT3'

Rev: 5'TTCCATATGGAATTCCAAACGAACAAAGAACAACCTCCCAA3'

## The results section 5.5.

Quickchange site directed mutagenesis PCR technique has been used for generating the hybrid mutant minigenes using SCN9A WT minigene as a template.

Name of the minigene: SCN9A Fox b.site M1

For: 5'GTTTAATCAATTGCGTGGGTCTTTAGGATGAGGATGACT3'

Rev: 3'CTTTGAGTTTAATCAATTGCGTGGGTCTTTAG5'

Name of the minigene: SCN9A Fox b.site M2/M3

For: 5'TAAATATCAGCGTGGGGATATTGCGTGACATTGTTTTTTATTTTTTC3'

Rev: 3'AGAGATCATAAATATCAGCGTGGGGATATTGCGTGACATTG3'

Name of the minigene: SCN9A Fox b.site M4

For: 5'TGATATAATGCGTGACTTTCTAGGAAAGCTTG3'

Rev: 3'TCATTGATTTGATATAATGCGTGACTTTCTAG5'

## **6.18 Maintenance and analysis of cells in culture**

HeLa, SK-N-BE, SH-SY-5Y, F11 and cell lines was grown in Dulbecco's Mem with Glutamax I (Gibco) (Dulbecco's modified Eagle's medium with glutamine, sodium pyruvate, pyridoxine and 4.5 g/l glucose) supplemented with 10% fetal calf serum (Euro Clone) and Antibiotic Antimycotic (Sigma) according to the manufacturer's instructions. Plates containing a confluent monolayer of cells were treated with 0.1% w/v trypsin as follows. Cells washed with PBS solution, were incubated at 37°C with 1-2 ml of PBS/EDTA/trypsin solution (PBS containing 0.04% w/v EDTA and 0.1% w/v trypsin) for 2 minutes or until cells were dislodged. After adding 10 ml of media, cells were pelleted by centrifugation and resuspended in 5 ml pre-warmed medium. 1-2 ml of this cell suspension was added to 10 ml medium in a fresh plate and was gently mixed before incubation.

## **6.19 Maintenance and differentiation of the PC-12 cell line in culture**

Rat pheochromocytoma (PC-12) cells were grown in Dulbecco's Minimal Eagle Media (DMEM; CellGro, Manassas, VA) supplemented with 10% heat-inactivated horse serum (HS, Sigma-Aldrich), 5% fetal bovine serum (FBS, Hyclone) and 1% antibiotic/antimycotic mixture (Gibco). Cells were plated in 35-mm collagen-coated dishes (10 µg/ml, Sigma). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> until reaching the desired confluence (~ 50% or ~90%). To differentiate PC-12 cells (dPC-12), DMEM containing 1% FBS and 1% antibiotic/antimycotic mixture, was supplemented with 50 ng/ml of Nerve Growth Factor 7S (Sigma) for 10 days.

## **6.20 Trigeminal neuron cells preparation**

P9-P12 rats were anesthetized by diethyl ether and decapitated. Trigeminal ganglion (TG) were rapidly excised and placed in F12 medium (Sigma) without serum and cleaned from meningeal residues. For each animal (2TGs) enzymatic dissociation was carried in 500

microliters of F12 medium containing 0.25mg/ml trypsin, 1 mg/ml collagenase and 0.2 mg/ml DNase at 37 °C in agitation for 25 min. The digestion was stopped by adding 50 microliters of FBS. The cells were dissociated mechanically through pipetting. After centrifugation of the suspension for 3 min at 1000 rpm, the supernatant was removed and cells were resuspended in F12 +10% FBS (500 microliters). Cells were plated on collagene-coated (10 µg/mL) 12-well treated Petri dishes with 500 microliters of the F12 medium with 10% fetal calf serum and left at 37 °C 5% CO<sub>2</sub>.

Enzimatic Solution used for 50ml F12 + P/S (without serum)

ENZYME	CONCENTRATION	UNITS	COMPANY COD	+
trypsin	0.5 mg/ml	6000BAEE units/mg	Sigma T-1005	
collagenase	1 mg/ml	300 units/mg	Sigma C-0130	
DNase	0.2 mg/ml	2,580 kunits/mg solid	Sigma D-5025	

## 6.21 Transfections

The DNA used for transfections was prepared with JetStar purification kit (Genomed) as previously described. Liposome-mediated transfections of 3x10<sup>5</sup> HeLa cells were performed using Effectene reagent (Qiagen). 0.5 µg of construct DNA was mixed with 4 µl of Enhancer for each transfection and the mixture was incubated at room temperature for 5 minutes to allow the condensation of the DNA. Then, 5 µl of Effectene were added to the mixture and an incubation of 10 minutes has been performed. After the addition of 500 µl of complete culture medium the mixture was added to the cells in 3 ml of the same medium and incubated at 37°C. After 6 h the medium was replaced with fresh medium and 12 h later, the cells were harvested. RNA isolation followed as described.

## **6.22 Construction of the expression plasmids.**

Muscle specific Fox-1 and brain specific Fox-1, Fox-2 and Fox-3 were synthetically made, the cDNA sequence of whoms NCBI Reference Sequence is: NM\_145891.2, NM\_001142334.1, NP\_001026865.1 and NP\_001076044.1 respectively. The synthetic cDNA was delivered in GenScript's standard vector pUC57 (GenScript). Defined cDNA fragments encoding various Fox proteins were generated by digestion with appropriate sets of enzymes, Hind III and Kpn I for muscle specific Fox-1, brain specific Fox-1 and Fox-3, Hind III and Xba I for brain specific Fox-2, and cloned in the CMV-4 vector, using the same restriction sites that were used for digestion, which contained a flag epitope tag.

## **6.23 Cotransfections**

In cotransfection experiments, cells were transfected with 0.5 µg of minigene and 0.5 µg of muscle specific Fox-1, brain specific Fox-1, Fox-2 and Fox-3 separately or ASF/SF2 and SC35 expression plasmids provided by Javier F. Caceres.

## **6.24 mRNA analysis by Polymerase Chain Reaction**

### **6.24.1 cDNA synthesis**

In order to synthesize cDNA, the 3 µg of total RNA extracted from cells were mixed with random primers (Pharmacia) in a final volume of 20 µl. After denaturation at 70°C the RNA and the primer were incubated for 1 hour at 37 °C in the following solution: 1X First Strand Buffer (Gibco), 10 mM DTT, 1 mM dNTPs, RNase inhibitor 20 U (Ambion) and Moloney murine leukemia virus reverse transcriptase 100 U (Gibco). 1µl of the cDNA reaction mix was used for the PCR analysis.

### 6.24.2 cDNA analysis

PCR analysis of cDNA was carried out for 35 cycles (94 °C 30 sec, 58 °C 30 sec, 72 °C 1 min) in 50 µl reaction volumes using the following oligonucleotides which recognize specific regions of the pTB minigene or pcDNA3 expression vector.

Oligonucleotide name pTB minigene	Sequence of the oligonucleotide (5'-3')
ALFA	CAACTTCAACTCCTAAGCCACTGC
BRA	GTCACCAGGAAGTTGGTTAAATCA
Oligonucleotide name pcDNA3 vector	Sequence of the oligonucleotide (5'-3')
T7	AATACGACTCACTATAG
SP6	ATTAGGTGACACTATAGAATA

### 6.25 Small interfering RNA (si-RNA) transfections

Si-RNA transfections were performed in cells using Oligofectamine Reagent (Invitrogen) according to the manufactures instructions. Briefly, 40-50% confluent 35 mm well plates were prepared. 25 µM of si-RNA were mixed with 175µl of Opti-MEM medium and, separately, 5 µl Oligofectamine with 15 µl of Opti-MEM medium.

The si-RNA sequence used for depletion were:

Human/Rat Fox-1 5'-CCGUUUUGCUCCAUACUAAUU-3' (Sigma);

Human hnRNP A1 5'-CAGCUGAGGAAGCUCUUCA-3' (Sigma);

Human JKTBP 5'- GUUGUAGACUGCACAAUUA-3' (Sigma);

Human PTB 5'-GCCUCUUUAUUCUCUUCGG-3'; (provided by C.W.J. Smith)

Human nPTB 5'-GAGAGGAUCUGACGAACUA-3' (provided by C.W.J. Smith)



Luciferase 5'-GCCAUUCUAUCCUCUAGAGGAUG-3' (Dharmacon).

Small interfering RNA oligonucleotide specific for TDP-43 is a Y. M. Ayala personal communication. The samples were incubated for 7 min at room temperature. Then the Oligofectamine mix was added to precomplexed siRNA mix and incubated for 20 min at room temperature. Finally, the mix was dropped onto the cells maintained in 800µl of Opti-MEM without antibiotics and FBS. After 24h, a second siRNA transfection was performed as described above, followed by the transfection with the different minigenes. The following day, cells were harvested for RNA extractions, RT-PCR analyses and Western blots. Particularly, western blot was performed in order to check the depletion of each protein.

## **6.26 Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)**

Whole-protein extracts were obtained by cell sonication in lysis buffer (1X PBS and 1X Protease inhibitor cocktail). Upon quantification with Bradford assay, proteins from each sample were mixed with 10X protein loading buffer (20% w/v SDS, 1M DTT, 0.63M Tris-HCl pH 7, 0.2% w/v bromophenol blue, 20% v/v glycerol, 10mM EDTA pH 7). Conventional slab gel SDS PAGE (Laemmli, 1970) was performed in vertical gels with the required percentage of polyacrylamide (37,5:1 acrylamide:bis-acrylamide, ProtoGel, National Diagnostics), depending on each case. The gels were run at 40 mA in 1X SDS-PAGE running buffer (50 mM Tris, 0.38 M glycine, 0.1 % w/v SDS). After running, gels were either stained with coomassie Blue R250 in methanol-water-acetic acid 45:45:10 (v/v/v) or Western blot analysis was performed.

## **6.27 Western blots and antibodies**

SDS-PAGE gels were blotted on standard nitrocellulose membrane and incubated in blocking solution (PBS1X, 0.2% Tween, 2% milk) over night. Primary antibodies were

incubated with the membrane in the same buffer for 2 hours. Afterwards, the membrane was then washed three times in washing solution (PBS1X, 2%Tween), incubated with the proper HRP-secondary antibodies (anti-mouse/rabbit, Dako) for 1 hour, washed twice and stained with ECL reagent (Thermo Scientific). Finally, an autoradiography was taken on Kodak Biomax XAR films.

The following primary antibodies were used in this study:

Fox-1: N-14 (sc-135476, Lot#1709, Santa Cruz Biotechnology)

hnRNP A1: donated by Emanuele Buratti

PTB/nPTB: donated by C.W.J Smith

hnRNP JKTBP: (Product number: AV40585, Sigma-Aldrich)

DAZAP1: donated by Natasa Skoko

ASF/SF2, SC35 and 1H4 (against SRp 75, 55, 40): Zymed Laboratories Inc.

Tubulin: (1:5000, Sigma T5168)

Actin: (1:1000, Sigma-Aldrich)

Anti-flag epitope: (1:2000) (Sigma F1804)

TDP-43: donated by Emanuele Buratti.

## **6.28 RNA preparation for pull down and band-shift analysis**

RNA templates were obtained by amplifying the respective exon/intron sequences using a forward primer carrying a T7 polymerase target sequence (5'-TACgTAATACgACTCACTATAg-3') with complementary to the specific exon and a reverse primer carrying of the target sequence. The amplified products were then purified and ~2 µg of DNA was transcribed using T7 RNA Polymerase (Stratagene) in the presence of transcription buffer (350 mM HEPES, pH 7.5, 30 mM MgCl<sub>2</sub>, 2 mM spermidine, and 40 mM dithiothreitol), 40 units of RNasin, 7 mM each of the four NTPs, and 60 units of T7 polymerase (1.5 units/µg). Following incubation for 2 h at 37 °C, the RNA was purified

using NICK Columns (Amersham Biosciences), precipitated and resuspended in RNase-free water.

## 6.29 Pull down analysis

One nanomole of cold RNAs was placed in a 400  $\mu$ l reaction mixture containing fresh 0.1 M NaOAc, pH 5.0, and 5 mM sodium *m*-periodate (Sigma). Reaction mixtures were incubated for 1 h in the dark at room temperature. The RNA was ethanol-precipitated and resuspended in 500  $\mu$ l of 0.1 M NaOAc, pH 5.0. Then, 400  $\mu$ l of adipic acid dehydrazide-agarose bead 50% slurry (Sigma) was washed four times in 10 ml of 0.1 M NaOAc, pH 5.0, and pelleted after each wash at 3000 rpm for 3 min in an Eppendorf minifuge. After the final wash, 300  $\mu$ l of 0.1 M NaOAc, pH 5.0, was added to the beads. The slurry was then divided into equal aliquots that were mixed with each periodate-treated RNA sample and incubated for 12 h at 4 °C on a rotator. The beads with the bound RNA were then pelleted and washed three times in 2 ml of 2 M NaCl and three times in 3 ml of RNA washing buffer (52 mM HEPES-KOH, pH 7.5, 10 mM MgCl<sub>2</sub>, 8 mM Mg(acetate), 5.2 mM dithiothreitol, 38% v/v glycerol). They were incubated in 1X RNA binding buffer (*i.e.* RNA washing buffer added with 7.5 mM ATP, 10 mM GTP, and 5 mg/ml heparin) with 0.3 mg of HeLa cell nuclear extract for 20 min at 30 °C in a 500  $\mu$ l final volume, pelleted by centrifugation at 1000 rpm for 3 min, and washed five times with 5 ml of RNA washing buffer. After the final centrifugation 60  $\mu$ l of SDS-PAGE sample buffer were added to the beads and heated for 5 min at 90 °C before loading onto a 10% SDS-PAGE gel. Internal sequence analysis from the Coomassie Blue-stained bands excised from the SDS-PAGE gel was performed using an electrospray ionization mass spectrometer (LCQ DECA XP, ThermoFinnigan). The bands were digested by trypsin, and the resulting peptides were extracted with water and 60% acetonitrile/1% trifluoroacetic acid. The fragments were then analyzed by mass spectrometry by the Protein Networks Group at ICGEB.

Alternatively, a western blot analysis can be performed using the specific antibody against the protein we want to check the presence.

### **6.30 Spliceosomal assembly analysis**

Spliceosome complexes were assembled as described earlier (Behzadnia, Hartmuth et al. 2006). Briefly, 10 Nm of (32)P-labelled RNA was incubated with 40% (v/v) of HeLa nuclear extract under splicing condition. Aliquots were collected at 5 and 30 minutes time points and reaction was stopped by addition of 2.5µl of 5X spliceosome gel loading buffer (1X TBE, 30% v/v glycerol, 0.5g/l Heparin, 0.1% w/v bromophenol blue). The splicing complexes were separated through electrophoresis using a 4% polyacrylamide native gel run at 300V for 3 hours. Finally, gels were dried and visualized by phosphor imager (Molecular Dynamics).

### **6.31 Southern blot analysis**

Briefly, RT-PCR amplicons were checked in an agarose gel. The resulting fragments were separated according to size by electrophoresis through a 0.6% (w/v) agarose gel (120V). The gel was photographed under UV light and washed twice (for 10 minutes each) with 0.2N HCl for depurination of DNA, then twice (10 minutes each) with Denaturation Solution (1.5 M NaCl; 0.5 M NaOH). The DNA was transferred from the gel to the nylon membrane (Z-Probe, BioRad) by upwardcapillary transfer using Denaturation solution (overnight). The day after the blotting was removed. To checked blotting efficiency the membrane was observed under UV light, whereas the agarose gel was stained with a solution containing ethidium bromide. After the membrane was neutralized twice (10 minutes each) with Neutralizing Solution (1 M Tris-Cl; 1.5 M NaCl) and dried at room temperature on a Whatman 3 MM filter paper. The DNA was bound to the membrane by UV cross-linking (2X, 0.25 J). Then, the membrane was prehybridised for at least 1h at 65°C in 20 ml of Pre-hybridization solution (6X SCC, 5X Denhardt's reagent, 0.5% (w/v)

SDS, 100 µg/ml salmon sperm DNA) and hybridized overnight at 65°C to the radiolabelled probe, in 10 ml of a pre-hybridisation solution. The day after the hybridization solution was removed and the membrane was washed with several washing solutions at 65°C: 4X SSC, 0.1% SDS (w/v) to remove the excess of the hybridisation probe; 2X SSC, 0.1% SDS (w/v) and 0.5X SSC, 0.2% SDS (w/v) with more stringent conditions to eliminate non specific hybridisation. After each wash the membrane was checked for radioactive signal using a geiger (Canberra Packard SRL) to evaluate if continue or suspend the washing. Finally, the membrane was immediately covered with plastic wrap mounted on Whitman 3 MM filter paper and exposed overnight using Cyclone screen (Cyclone, Storage Phosphor System, Canberra Packard SAL).

### **6.31.1 Hybridization probes preparation**

The different DNA fragment used as Southern blot hybridisation probes were oligonucleotides for SCN9A exon 4, 5N and 5A; 5'CTTCTGTGTAGGAGAATTC3', 5'CTCAGCATTGAGAACA3' and 5'TTCAGCTCTTCGAACT3' respectively (IDT Integrated DNA Technologies). Radioactive probes were generated from 50 ng of DNA by the Rediprime II Random Prime Labelling System (Amersham Bioscience) using [ $\alpha$ -<sup>32</sup>P] dCTP (Amersham Bioscience) and were then purified from unincorporated nucleotides by Nick columns (Amersham bioscience). Both the labelling and the probe purification were carried out according to the manufacturers' instructions.

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## Acknowledgement

Foremost, I would like to express my sincere gratitude to my Ph.D. supervisor, Prof. Francisco E. Baralle for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study. My sincere thanks also goes to my external supervisor Dr. Geoffrey Woods, for his encouragement, insightful comments, and discussions. A very special thanks goes out to Marco Baralle Ph.D, Staff Scientist, for his expertise, understanding, patience, stimulating discussions and for the sleepless nights we were working before deadlines. He provided me with direction, technical support and helped me in all the time of research and writing of this thesis. I want to thank and give credit to all people in my laboratory, Molecular Pathology Group, who have provided me with invaluable assistance. Whether be it gentle guidance or access to materials or services that helped me a lot in my research and it is greatly appreciated. I would like to thank the friends of my entire extended family at ICGEB for providing a loving environment for me. I wish to thank my best friend Mirjana for helping me get through the difficult times, and for all the emotional support, entertainment, and caring she provided. Lastly, and most importantly, I wish to thank my parents, Katica Zubovic and Ivan Zubovic and my brother Arijan. They supported me, taught me, and loved me. To them I dedicate this thesis.



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