

Mutually exclusive splicing regulates the Na_v 1.6 sodium channel function through a combinatorial mechanism that involves three distinct splicing regulatory elements and their ligands

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ABSTRACT

Mutually exclusive splicing is a form of alternative pre-mRNA processing that consists in the use of only one of a set of two or more exons. We have investigated the mechanisms involved in this process for exon 18 of the Na_v 1.6 sodium channel transcript and its significance regarding gene-expression regulation. The 18N exon (neonatal form) has a stop codon in phase and although the mRNA can be detected by amplification methods, the truncated protein has not been observed. The switch from 18N to 18A (adult form) occurs only in a restricted set of neural tissues producing the functional channel while other tissues display the mRNA with the 18N exon also in adulthood. We demonstrate that the mRNA species carrying the stop codon is subjected to Nonsense-Mediated Decay, providing a control mechanism of channel expression. We also map a string of *cis*-elements within the mutually exclusive exons and in the flanking introns responsible for their strict tissue and temporal specificity. These elements bind a series of positive (RbFox-1, SRSF1, SRSF2) and negative (hnRNPA1, PTB, hnRNPA2/B1, hnRNPD-like JKTBP) splicing regulatory proteins. These splicing factors, with the exception of RbFox-1, are ubiquitous but their levels vary during development and differentiation, ensuing unique sets of tissue and temporal levels of splicing factors. The combinatorial nature of these elements is highlighted by the dominance of the elements that bind the ubiquitous factors over the tissue specific RbFox-1.

INTRODUCTION

Mutually exclusive splicing (ME) distinguishes itself from classical cassette exon splicing in that ME exons are never observed together in a given cell/tissue. Therefore, contrary to alternative splicing where a ratio of transcripts containing the alternative exons can arise, ME splicing results in one specific isoform delineating the need for a strict restriction on the isoform(s) present (1). This form of regulation is particularly common in the mammalian nervous system where a large percent (40–60%) of neuronal pre-mRNA undergo alternative splicing in a tissue and developmental regulated manner (2). From a mechanistic perspective the intriguing question regards the mechanisms involved in preventing ME exons from being spliced to each other, considering that the introns between them contain functional 5'- and 3'-splice sites. Several models have been established to be responsible for ME splicing, among these are steric interference between splice sites (3–5) and incompatible splice sites flanking pairs of ME exons (1,6). However, the majority of ME exon pairs do not have a mechanism that absolutely forbids their splicing together and the mechanism(s) that determine the splicing choices are not well known. In these cases, the regulated selection of the individual exons must be sufficiently coordinated to minimize inappropriate splicing without the need for an absolute physical impediment to double-exon inclusion (7). To accomplish this it is evident that the splicing machinery must make use of the specialized regulatory proteins that bind *cis*-elements in the pre-mRNA in either exonic or intronic regions, and alter the splice site recognition functioning as repressors of one ME exon and activators of its partner (8). Elucidating how these *cis*-elements determine which ME exon is chosen is important in understanding the basic mechanism of pre-mRNA splicing and in particular of neural pre-mRNA splicing (4,9–11).

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ME splicing occurs extensively in a tissue and developmentally regulated manner within a family of proteins responsible for the rising phase of action potentials in electrically excitable cells, namely the voltage-gated sodium channels (12–14). This modulation of splicing may result in transcripts that at one extreme have the insertion of an in frame stop codon while on the other alter only a few amino acids, resulting in biochemical and pharmacologically distinct sodium channel isoforms (1,4). It was of interest to see if there could be a common mechanism behind the ME splicing in this family and as a first step of an extensive study we have investigated the ME splicing of exon 18 in the Na_v 1.6 (SCN8A) sodium channel transcript. The voltage gated sodium channel α subunit SCN8A is one of the most abundant sodium channels in neurons. The gene encoding the protein has been shown to undergo ME splicing of exon 18 that encodes transmembrane segments S3 and S4 in domain III of the protein. Prior studies of SCN8A isoforms demonstrated that fetal neurons and non-neuronal cells produce two variant transcripts, one predominant transcript containing the alternative exon 18N (neonatal) and one that skips exon 18. As exon 18N includes a stop codon, unless degraded by NMD, it is predicted that fetal neurons and non-neuronal cells would express a truncated non functional variant of Na_v 1.6 sodium channel (15). 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 (adult), the major transcript in adult brain and spinal cord that results in a functional channel (15). The understanding of the ME mechanism of these exons aside the mechanistic aspect regarding ME choice may also have a therapeutic value as Na_v 1.6 plays an important role in normal axonal conduction and may significantly contribute to the pathophysiology of the injured nervous system (16,17). A greater understanding of how different cell types modulate the ME splicing abolishing channel function may therefore provide clues for possible therapeutic intervention.

Our results support a model in which the ME splicing regulates channel expression through nonsense-mediated mRNA decay of the transcript containing exon 18N. The exclusion of exon 18A in non-neuronal tissue is regulated primarily by the interaction of hnRNP proteins with an exonic splicing silencer (ESS) that we have identified in this exon, while exon 18N inclusion is due to SR proteins that function through an exonic splicing enhancer (ESE) mapped within this sequence. In neuronal cells the ratio of these protein levels differs and in addition a neuron specific factor (RbFox-1) is present. This combination results in exon 18A inclusion in the final transcript and exon 18N exclusion.

MATERIALS AND METHODS

Construction of minigene and expression plasmids

cDNA for muscle-specific RbFox-1 (NM_145891.2), brain-specific RbFox-1 (NM_001142334.1), RbFox-2

(NP_001026865.1) and RbFox-3 (NP_001076044.1) were synthesized by Genescript and subsequently cloned into pFlag CMV-4 Expression vector (Sigma-Aldrich). Hybrid minigenes E18A and E18N were made as previously described (18), using a DNA fragments extending 150-bp upstream and 100-bp downstream of the exon 18A or 18N. SCN8A WT was made by via amplifying three fragments spanning exon 17 to 161 bp of downstream intronic sequence, –141-bp upstream of exon 18N through to 118-bp downstream of exon 18A and a region composed of –470-bp upstream of exon 19 as well as the exon itself. The fragments were then joined together and subsequently cloned into the pcDNA3 plasmid. Subsequent deletion and/or mutagenesis in these minigenes were made through standard PCR mutagenesis or using a QuikChange site directed methodology (Stratagene).

Cell culture, transfections and RT-PCR analysis

HeLa and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (Invitrogen) in standard conditions. Primary cultures of rat trigeminal ganglion (TG) sensory neurons were prepared and maintained in culture as previously described (19). Cycloheximide treatment was performed via the addition of 50 μ g/ μ l to the medium for a period of 3 h. Plasmids used for transfection were purified using Promega Miniprep DNA purification system and transfected using Effectene transfection reagents (Qiagen). In cotransfection experiments, cells were transfected with 0.5 μ g of minigene and 0.5 μ g of muscle-specific RbFox-1, brain-specific RbFox-1, RbFox-2 and RbFox-3 or SRSF1 and SRSF2 expression plasmids (provided by Javier F. Caceres). In the controls, an equivalent amount of the empty expression plasmid was utilized. Total RNA was extracted using TRI reagent solution (Ambion) 24 h after transfection. One microgram of total RNA was used in the retrotranscription reaction with random primers and Moloney murine leukemia virus enzyme (Invitrogen). Spliced products from the transfected minigene were obtained using primers ALFA 5'-caactcaactcctaagcactgc-3' and BRA 5'-gtaccaggaagtgtgtaaatca-3' in the case of the hybrid minigene system and T7 5'-aatacactcactatag-3' and SP6 5'-atataaggtgacatatagaata-3' using a pcDNA3 expression vector. Amplification products were routinely fractionated in 2% (wt/vol) agarose gels.

si-RNA transfections were performed in cells using Oligofectamine Reagent (Invitrogen) according to the manufactures instructions and in all cases two rounds of si-RNA transfections, distanced 24 h, were performed. Cells were harvested for protein and RNA extractions after 36 h. When minigenes were transfected into the cells this was performed 6 h after the second si-RNA transfection using effectene reagent (Qiagen) The si-RNA sequences used were: Rent-1 (Upf1) aaguuucgagagaagauugau (Sigma), Human/Rat Fox-1 5'-ccguuuuguccauucaaau-3' (Sigma), Human hnRNP A1 5'-cagcugaggaagcucuca-3' (Sigma), hnRNP A2/B1 5'-ggaacaguuccgaaagcuc-3' (Sigma), Human hnRNP D-like JKTBP

5'-guuguagacugcacaauua-3' (Sigma), Human PTB 5'gcc uuuuuuucucucucg 3' (Dharmacon), Human nPTB 5'-gaggauucugacgaacua-3' (Dharmacon), SRSF1 5'-gaagauu gaccuauugcaguu-3' (Thermo Scientific), SRSF2 5'-ucgcuucguucgcuucacgacaa-3' (Sigma-Aldrich) and luciferase gene control 5'-gccauucuaucuccuagaggaug-3' (Dharmacon). On the third day, cells were harvested for protein and RNA extractions.

Bioinformatics analysis

Candidate ESS were searched for using <http://genes.mit.edu/fas-ess/>, <http://cubweb.biology.columbia.edu/pesx/> (20). Candidate ESE were searched for using <http://rulai.cshl.edu/tools/ESE> (21).

Pull-down analysis

RNA templates were obtained by amplifying the respective exon/intron sequences using a forward primer carrying a T7 polymerase target sequence (5'-TACgTAATACgAC TCACTATAg-3') with 20nt complementary to the specific exon and a reverse primer carrying 35nt of the target sequence. The amplified products were then purified and ~2µg of DNA was transcribed using T7 RNA Polymerase (Stratagene). Subsequent affinity purification of RNA-binding proteins and western blot analysis, if performed, were carried out as previously described (22,23).

Spliceosomal assembly analysis

Spliceosome complexes were assembled as previously described earlier (24). Briefly, 10 nm of (32)P-labeled RNA was incubated with 40% (v/v) of HeLa nuclear extract under splicing condition. Aliquots were collected at 5 and 30 min. The splicing complexes were separated through electrophoresis using a 4% polyacrylamide native gel run at 300 V for 3 h. Finally, gels were dried and visualized by phosphor imager (Molecular Dynamics). Where specified U2 or U5snRNA was removed from HeLa nuclear extract as previously described (25).

Immunoblotting cell extracts

Cells extracts were prepared by sonication in lysis buffer [15mM HEPES (pH 7.5), 0.25 M NaCl, 0.5% NP-40, 10% glycerol, 1× protease inhibitor (Roche 1873580), 25mM NaF, 10mM β-glycerolphosphate, 0.2mM Na₃VO₄ and 1mM phenylmethylsulfonyl fluoride]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose (0.45µM, Amersham Biosciences), and protein detection was carried out with standard western blotting techniques.

Antibodies

Rent-1 (P-14) Santa Cruz (SC-18260), hnRNP A1, hnRNP A2/B1 (23), hnRNP D-like protein JKTBP (AV40585; Sigma-Aldrich), Anti-SRSF1, SRSF2 and 1H4 from Zymed Laboratories Inc (23). PTB/nPTB and RbFox-1 antibodies were kind gifts from C.W.J Smith and D.L. Black respectively. Anti-flag, anti-tubulin and anti-actin from Sigma Aldrich.

qPCR

qPCR was performed with iQ SYBR Green Supermix on a CFX96 Real-Time PCR Detection system (BIO-RAD). Primers for quantification SCN8A 5'-CGGGATCCCGA TGCCTTCGAGGACATCTACATT-3' (sense) and 5'-G GCGAGCTCGCCCTATACTTGAAGAAGGGCCAG GT-3' (antisense), DCSR1 5'-GCACCAGCTCAAGAAG GAACC-3' (sense) 5'-GGGACTCAAATTTGGCCC TGG -3' (antisense), GAPDH 5'-AAGGTGAAGGTCG GAGTCAA-3' (sense) AATGAAGGGGTCATTGAT GG (antisense). PCRs were performed for 15" at 95°C and 1' at 60°C for 45 cycles followed by the thermal denaturation protocol. Expression levels of the target gene relative to GAPDH RNA were determined using the $2^{-\Delta\Delta CT}$ method. Significant differences were determined by Student's *t*-test wherein *P* < 0.05 was considered to be statistically significant.

RESULTS

Alternative splicing and nonsense-mediated mRNA decay contribute to the regulation of Na_v 1.6 sodium channel expression

Inclusion of exon 18N over 18A in the SCN8A mRNA leads to a transcript with a premature stop codon that has previously been suggested to encode for a non functional sodium channel protein (15). However such protein has never been reported and all the previous work has been carried out using amplification based methodologies. Hence due consideration should be given to the fact that mRNAs seen by amplification based methods may not be present in significant amount in the cells. In fact, mRNAs carrying stop codons can be eliminated by a process known as nonsense-mediated decay (NMD). The coupling of alternative splicing with NMD as a means to regulate the expression of a protein has been previously described (26–31). To investigate if this could also be the case for Na_v 1.6 sodium channel expression NMD was blocked by siRNA-mediated knockdown of Rent-1, a core component of NMD machinery, in HEK 293 cells in which we had observed endogenous SCN8A transcript to be present (Figure 1). Semi-quantitative RT-PCR analysis using primers in flanking exons 17 and 19 of the endogenous SCN8A gene resulted in a substantial increase in the abundance of mRNA including exon 18N (Figure 1B). The levels of exon 18N were quantified by qRT-PCR with primers that specifically amplified the isoform containing exon 18N and found to increase >3-fold upon treatment with siRNA against Rent-1 (Figure 1C). This was also performed in cells in which NMD was blocked pharmacologically with cycloheximide (Figure 1D). These results demonstrate that the mRNA carrying the exon 18N, containing the premature stop codon, is degraded by NMD. As a positive control for NMD we have used the Down syndrome critical region 1 (DSCR1) gene, a well known NMD target (32).

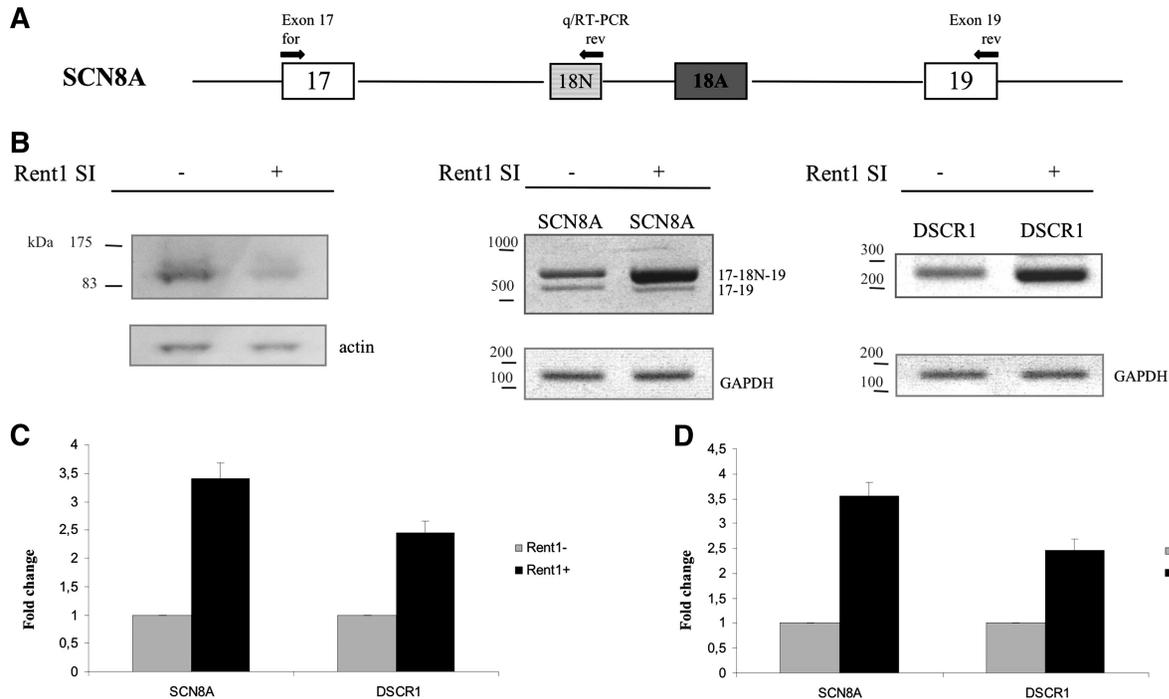


Figure 1. mRNA transcript containing SCN8A exon 18N is subjected to NMD. (A) Schematic representation of region being amplified with the oligos used for semi-quantitative PCR (Exon 17 for, Exon 19 rev) and quantitative PCR (Exon 17 for, q/RT-PCR rev). (B) From left to right: Immunoblot using anti Rent-1 and anti actin as a loading control showing endogenous Rent-1 depletion in the HEK 293 cells. Semi-quantitative mRNA expression levels of endogenous SCN8A treated with an siRNA against Luciferase (–) or an siRNA against Rent-1 (+). Semi-quantitative mRNA expression levels of endogenous DSCR1 treated with an siRNA against Luciferase (–) or an siRNA against Rent-1 (+). (C) Real-time qPCR of SCN8A transcripts containing exon 18N and DSCR1 normalized to GAPDH treated with an siRNA against Luciferase (Rent1–) or an siRNA against Rent-1 (Rent1+). (D) Real-time qPCR of SCN8A transcripts containing exon 18N and DSCR1 normalized to GAPDH before and after treatment with cycloheximide (CHX). The mRNA levels were quantified separately in triplicates and SD values obtained are shown on the bars.

SCN8A exon 18A contains an ESS that inhibits the assembly of the spliceosome in non-neuronal cells

To identify the regulatory *cis*-acting elements that may modulate the tissue and developmentally regulated ME splicing observed for exon 18A we created a hybrid minigene containing exon 18A together with a short amount of flanking intronic sequence (Figure 2A). The mRNA processing of this minigene, following expression of the construct in HeLa cells resulted in a PCR product corresponding to the skipping of the exon 18A from the mature transcript (Figure 2B, Lane 1), reflecting the *in vivo* specificity of non-neuronal tissue for this exon (15).

Exon 18A exclusion may be due to silencing *cis*-acting elements present in the region cloned into the minigene. A bioinformatics screen for candidate exon splicing silencer sequences (ESS) highlighted several hypothetical regions (Figure 2A). As these predictions have a high rate of false positives, in order to test their functional relevance a series of sequential deletions of these areas using the E18A minigene as a backbone were performed (Figure 2A). Transfection of the minigenes E18A Δ 6–27, Δ 27–43 and Δ 43–69 showed that the segment between nucleotides 6 and 27 of the exon define an ESS. In fact deletion of these nucleotides results in almost complete inclusion of exon 18A (Figure 2B, Lane 2), whereas deletions of nucleotides 27–43 and 43–69 behave as the E18A minigene, with exon 18A being totally excluded from the transcript (Figure 2B, Lanes 3 and 4).

The ability of an ESS to exert repression on its upstream 3' splice site is a common feature for the ESS's identified in mammals (33). We therefore investigated if this was also the case for the ESS element mapped in exon 18A through analysis of the spliceosomal complex formation on single exon RNA substrates composed of Exon 18A WT and Exon 18A Δ ESS (Figure 2C). Spliceosome complexes assembled on these RNAs were assembled in HeLa nuclear extract and subsequently separated on a native poly-acrylamide gel, under specific splicing conditions. Complexes H and E, formed on both the substrates, as did an A-like complex after 5 min of incubation in the presence of ATP (Figure 2D), whose identity was confirmed by its absence when using nuclear extract in which U2snRNA had been previously degraded (Supplementary Figure S1). Interestingly, following 30 min of incubation the A-like complex on the Exon 18A WT substrate that contains the ESS element, dissociates pointing towards an unproductive nature of this complex. On the other hand the A-like complex formed on the substrate lacking the ESS was still present even after 30 min (Figure 2D).

SCN8A exon 18N definition is governed by an ESE within this exon

Exon 18N is normally included in the SCN8A transcript in non-neuronal cells (15). Consistent with this, transfection into HeLa cells of a hybrid minigene containing this exon

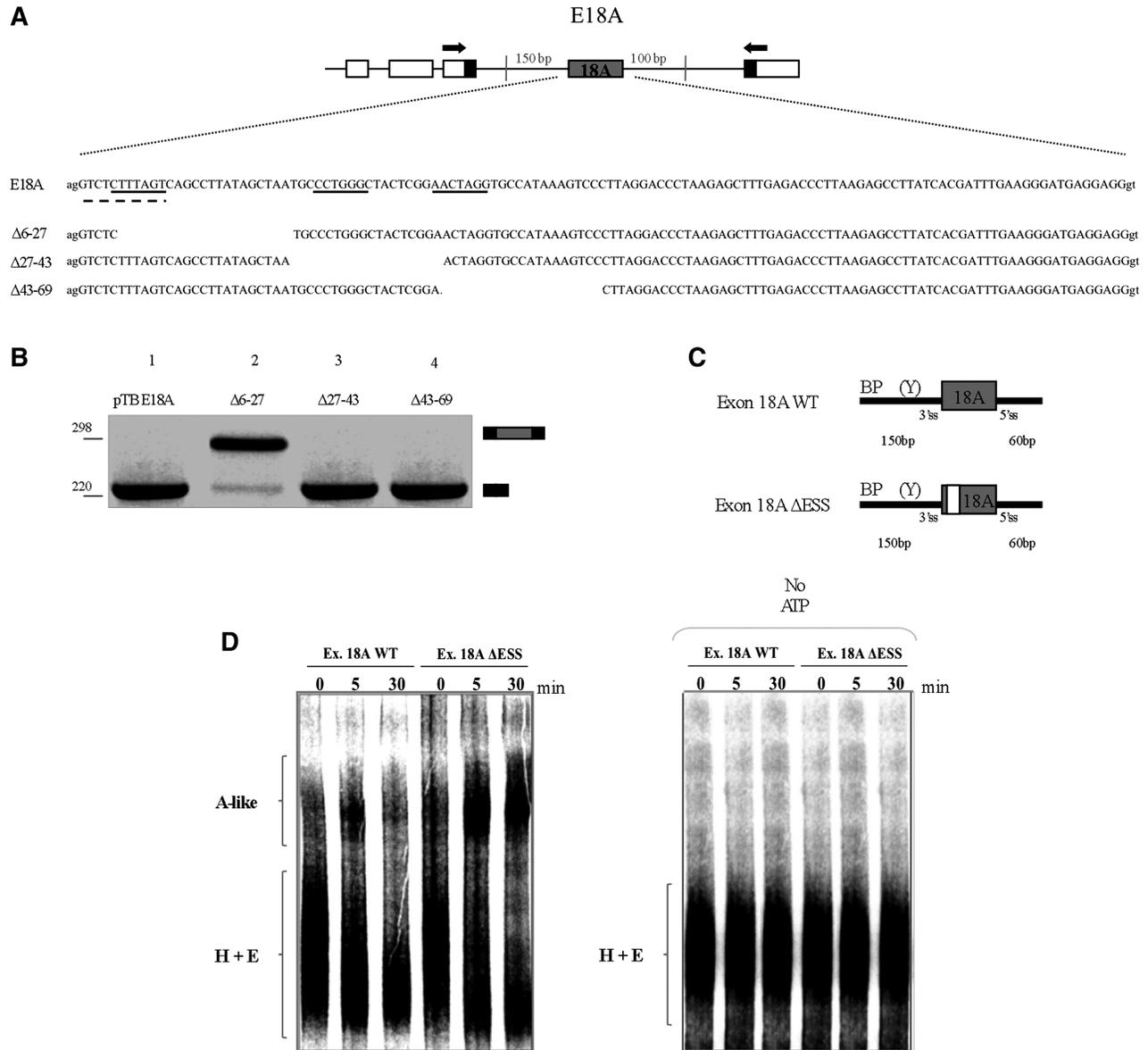


Figure 2. Identification of an ESS element in SCN8A exon 18A. (A) Schematic representation of the minigenes used in transfection experiments. White and black boxes represent the α -globin and fibronectin exons respectively. Sequence corresponding to SCN8A DNA is delimited by vertical lines. Underlined bold and underlined dash nucleotides indicate hypothetical ESS identified bioinformatically with FAS-hex3 (<http://genes.mit.edu/fas-ess/>), and PESS ESS (<http://cubweb.biology.columbia.edu/pesx>) databases respectively. Numbers indicate nucleotide position in exon (upper case). Lower case represents intronic nucleotides (B) RT-PCR analysis of minigene RNA processing after transfection in HeLa cells. Right hand side of gel schematic representation of amplified products. (C) Scheme of 32 P-labeled single exon substrates used for the assembly of splicesomal complexes. (Y) indicates polypyrimidine tract, BP indicates predicted branch point sequence, clear box deletion of ESS. (D) Autoradiography of acrylamide gel electrophoresis of the spliceosomal complexes formed at 0, 5 and 30 min with (left) and without (right) ATP. Regions corresponding to spliceosomal complexes H, E and A-like are indicated on the left hand side of the autoradiograph.

was observed to include exon 18N in the resultant transcript (Figure 3B, Lane 1). In order to determine if exon 18N inclusion may be regulated by exonic enhancer elements we initially used ESE finder ver3.0 that identified multiple putative SR protein responsive elements in exon 18N, in particular corresponding to SRSF1 (ASF/SF2) and SRSF2 (SC35) (Figure 3A) (34–36). To validate functionally these putative ESE's a series of minigene constructs were created with sequential deletion, in the

E18N minigene: E18N Δ 7–26, Δ 27–41 (removes predicted SRSF2 site), Δ 42–61 (removes predicted SRSF2 and SRSF1-binding site) (Figure 3A). Transfection and RT-PCR analysis of E18N Δ 7–26 and Δ 27–41 minigenes showed no changes in the splicing pattern of exon 18N (Figure 3B, Lanes 2 and 3). On the other hand the expression of the E18N Δ 42–61 minigene resulted in complete skipping of exon 18N indicating that this region delimits an ESE (Figure 3B, Lane 4).

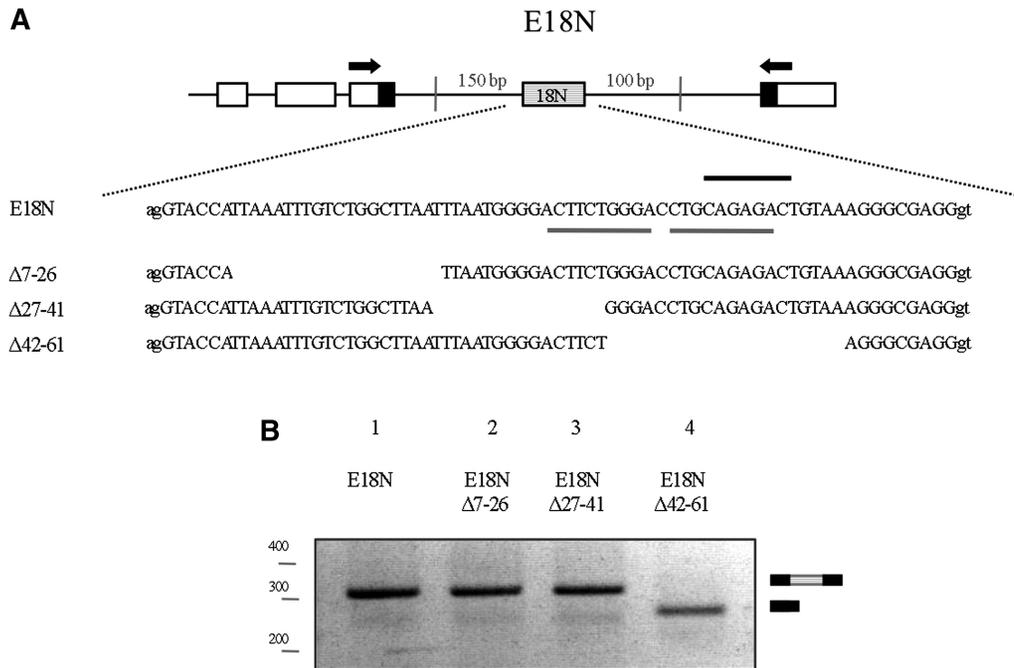


Figure 3. Identification of an ESE element in SCN8A exon 18N. (A) Schematic representation of the minigenes used in transfection experiments. White and black boxes represent the α -globin and fibronectin exons respectively. Sequence corresponding to SCN8A DNA is delimited by vertical lines. Numbers indicate nucleotide position in exon (upper case). Lower case represents intronic nucleotides. The putative SR protein responsive elements identified by ESE finder ver3.0 are indicated by grey lines under the nucleotide sequence in the case of SRSF2 and a black line on top of the nucleotide sequence in the case of SRSF1. (B) RT-PCR analysis of minigene RNA processing after transfection in HeLa cells. Right hand side of gel: indication of exons included in amplicon.

Identification of the *trans*-acting factors binding to the ESS in exon 18A and the ESE in 18N

To determine which *trans*-acting factors may be involved in the repression of exon 18A in HeLa cells affinity purification experiments using RNA extending from -2 bp to $+96$ bp of exon 18A with (RNA WT) and without nucleotides $+6$ to $+27$ (RNA Δ ESS) was performed (Figure 4A). Comparison of the proteins binding the two RNA substrates highlighted several bands specific for the RNA with the ESS, identified through mass spectrometric analysis as hnRNP D-like JKTBP, hnRNP A2/B1 and hnRNP A1 (Figure 4B, bands 1–3). In accordance with this result, select ‘winner’ or known binding sites for hnRNP A1 and hnRNP A2/B1 (37) and JKTBP (38) are observed to be present in the ESS. Interestingly, inspection of the ESS sequence also evidenced a binding site for the proteins PTB/nPTB (39,40) (Figure 4A). Due to the established differential neuronal expression for nPTB with respect to PTB this protein would be an extremely interesting candidate for the binding to the ESS identified in the exon 18A that is included only in the mRNA in neuronal tissue. Although Coomassie differential binding of this protein between the two *in vitro* transcribed RNAs was not observed in the affinity purification it could be the case that this was masked by other proteins running in the gel in the same area as the molecular weight of PTB. In fact, a western blot analysis of the pull-down protein gel fraction using an antibody against PTB showed that the ESS element was associated either directly or indirectly

through protein protein interactions to PTB (Figure 4C). Furthermore, as confirmation of the mass spectrometry results, the differential binding of hnRNP A1, hnRNP A2/B1 and hnRNP D-like JKTBP were tested using specific antibodies against these proteins. As expected, a strong signal for these proteins was observed on the RNA substrate with the ESS element as compared to that without the ESS (Figure 4C). DAZAP1 (41) protein was used in western blot analysis as a loading control (Figure 4C).

To further investigate the functional role of the hnRNPs that bind specifically the ESS element in exon 18A, hnRNP A1, PTB/nPTB, hnRNP D-like JKTBP and hnRNP A2/B1 were depleted via si-RNA. 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 a triple deletion of PTB/nPTB, hnRNP A1 and hnRNP A2/B1 was performed as well as obviously when all five hnRNP proteins were eliminated in HeLa cells prior to the transfection of the E18A minigene (Figure 4E). These results would indicate that hnRNP D-like JKTBP is not playing an important role in the ESS.

To identify the *trans*-acting factors binding the ESE element identified in the exon 18N, an identical approach, as just described, was taken using RNA substrates that were transcribed *in vitro* with and without the ESE element (Figure 4F). This time however, simple inspection of the Coomassie staining of pull downs did not

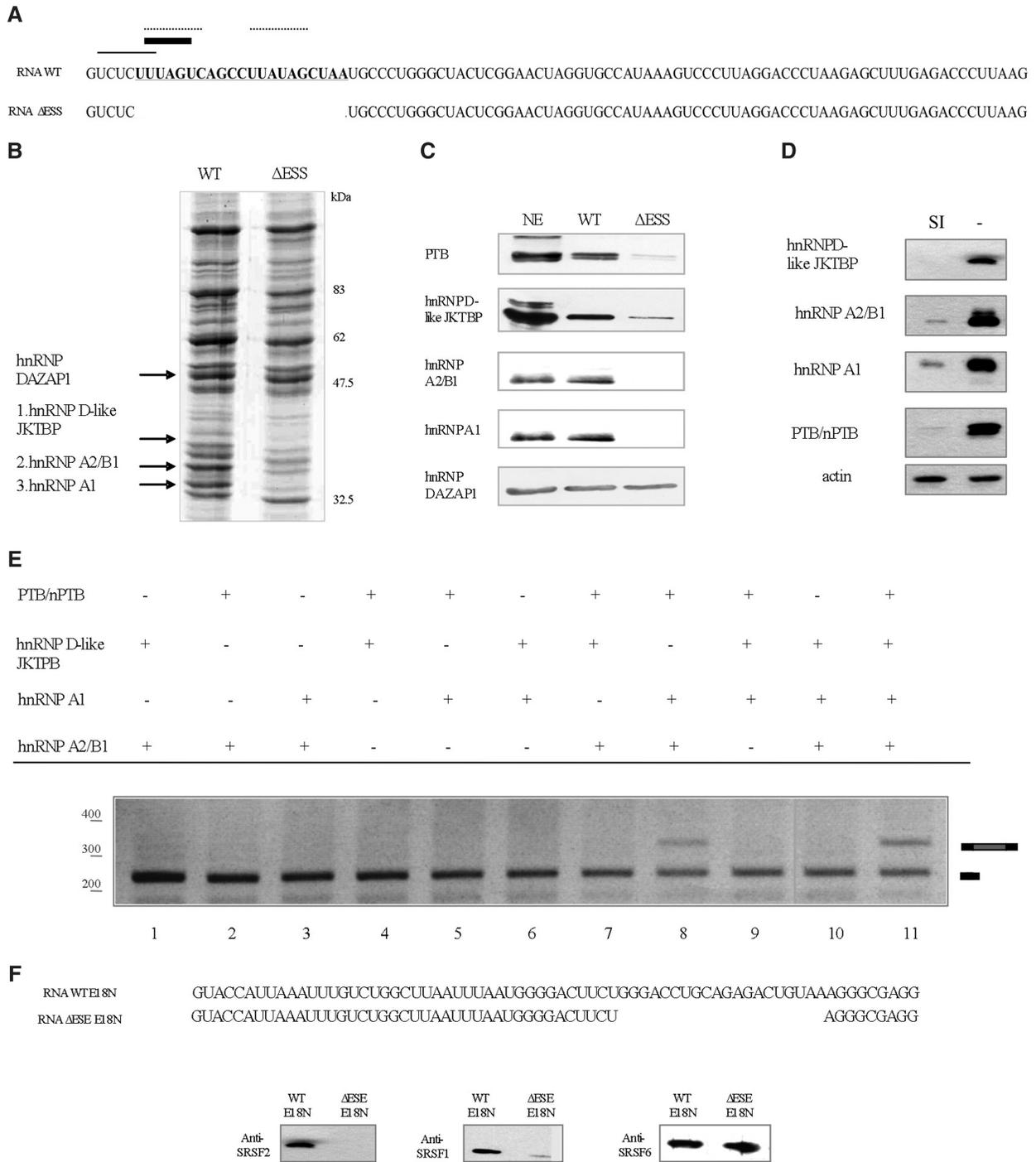


Figure 4. Identification of *trans*-acting factors binding to splicing regulatory elements identified in exon 18A and 18N. (A) RNA's used in pull-down analysis composed of SCN8A exon 18A with and without the ESS element identified between nucleotides 6–27 of the exon. Underlined and bold nucleotides highlight the ESS element identified between nucleotides 6–27 of the exon. On top of the nucleotide sequence-binding sites for hnRNP A1, hnRNPA2/B1 (bold line), hnRNP D-like JKTBP (dotted lines) and PTB (plain line) are indicated. (B) Coomassie blue staining of a pull-down assay using WT RNA and ΔESS RNA. Labels 1–3 indicate the proteins sequenced and identified through mass spectrophotometry analysis specifically observed only with the RNA containing the ESS element (C) Western blot analysis on pull-down assay for PTB, hnRNP D-like JKTBP, hnRNP A2/B1, hnRNP A1 observed to bind specifically the ESS. A western against hnRNP DAZAP1 was performed as a quantitative control. (D) Immunoblot showing typical depletions obtained for the four hnRNPs found to bind or associate with the ESS. Anti actin was used as a loading control. (E) Agarose gel electrophoresis analysis of the RT-PCRs analyzing the effect of the knockdown experiments on the inclusion of exon 18A in the mRNA using the minigene SCN8A E18A. On the right hand side of the gel scheme indicating what each amplicon corresponds to. An antibody against actin was used as a loading control. (F) RNA used in pull-down analysis for identification of *trans*-acting factors binding to the ESE identified in SCN8A exon 18N. Below immunoblots performed on the pull downs that resulted in positive signals against the panel of SR proteins tested.

show any significant variation. As often occurs the SR proteins are masked by other bands, thus an additional step was taken, after pull down and gel electrophoresis fractionation a western blot analyses with specific antibodies against some of the more common *trans*-acting factors binding to the ESE was performed (Figure 4F). As the bioinformatics analyses indicated binding of SRSF1 and SRSF2 would be disrupted through the deletion of the region to the nucleotides 42–61 in the exon we probed the pull-down assays with antibodies for SRSF1 and SRSF2 as well as with the 1H4 antibody that detects SRSF4 (SRp75), SRSF6 (SRp55), SRSF5 (SRp40). Signals for SRSF1 and SRSF2 were observed exclusively in the presence of the RNA with the ESE element. A signal against SRSF6 protein was also observed, however this was seen irrespective of the presence of the ESE element in the RNA sequence (Figure 4F).

In order to test the functional significance of SRSF1 and SRSF2 binding to the ESE, si-RNA of these proteins prior to transfection of the E18A minigene, was performed. However, we could not detect any change in the levels of exon 18N inclusion upon depletion of these proteins (data not shown). This seems to suggest that additional splicing factors may also interact functionally with the ESE as has been previously reported for other systems (42,43).

A wider genomic context plays a role in SCN8A exon 18A exclusion in non-neuronal cells

The analysis of the splicing regulatory elements was up to now performed in a reductive minigene context. It was then of interest to investigate any effect the ESE in exon 18N and the ESS in exon 18A may have on the ME-splicing outcome. Transfections were therefore performed with a SCN8A WT minigene that spans exons 17 to 19, encompassing both the 18N and 18A exons. In addition a series of related constructs were studied, in particular the SCN8A/ Δ ESE E18N minigene in which the ESE was deleted from exon 18N and the minigene SCN8A/ Δ ESS E18A in which the ESS in the exon 18A was deleted. Furthermore, in order to analyze the interplay between the two elements we also created the minigene SCN8A/ Δ ESE E18N/ Δ ESS E18A where both regulatory elements were deleted and the minigene SCN8A/+ ESS E18N in which the ESS from the exon 18A was created in exon 18N in an identical position to that found in exon 18A with minimal nucleotide changes and leaving the ESE intact (Figure 5A).

Transfection of these constructs into HeLa cells, followed by RT-PCR analysis showed that the SCN8A WT minigene displays the same pattern observed for SCN8A exon 18 splicing in non-neuronal tissues (15) with the resulting mRNA species consisting principally of exon 18N inclusion and a minor extent of exon 18 skipping (Figure 5B, Lane 1). Deletion of the ESE in exon 18N resulted in a cDNA in which skipping of both 18N and 18A exons occurs, indicating that lack of exon 18N definition is not enough to aid recognition of 18A (Figure 5B, Lane 2). The SCN8A/ Δ ESS E18A minigene

resulted in partial inclusion of exon 18A (Figure 5B, Lane 3). However, the mRNA including exon 18A also contained the ME exon 18N, indicating that the deletion of the ESS element was insufficient to cause the switch in ME processing of the two exons but represents only one of the combination of *cis*-acting elements necessary for this process. Deletion of both the ESE element in exon 18N and the ESS in exon 18A (The SCN8A/ Δ ESE E18N/ Δ ESS E18A minigene) resulted in a major band corresponding to skipping of exon 18N and 18A and a minor band corresponding to the sole inclusion of exon 18A (Figure 5B, Lane 4). This result highlight that the ME switch between the exons is an interplay between both elements although for a full neuronal switch additional elements may be required.

The fact that the inclusion of exon 18A occurred to a much lesser extent than that previously observed when the same region was deleted from the exon in the minigene context points to the possibility that other *cis*-acting elements may be acting in the larger context repressing exon 18A. Notwithstanding, these would appear to be acting in a lower pecking order of repressors of the exon, if we consider the result observed when we created the ESS element within exon 18N, that is normally included in HeLa cells. In this scenario exon 18N was completely skipped showing that the ESS sequence, identified in the exon 18A, can act in a different context repressing splicing of exons that are normally included in the mRNA (Figure 5B, Lane 5).

Overexpression of RbFox-1 proteins promotes SCN8A exon 18A inclusion only in the absence of the ESS in a (T)GCATG dependent manner

Trans-acting factors expressed specifically in brain have been shown to play vital roles in alternative splicing within this tissue (44–46). The fact that the exon 18A is normally included in the pre-mRNA in neurons together with the observations that deletion of the ESS present in exon 18A did not result in a strong inclusion of this exon in HeLa cells could possibly mean that such a factor(s) were missing from the latter's proteome.

The position and sequence context of a (T)GCATG elements downstream of exon 18A (Figure 6A) has been strongly conserved from Zebrafish to Humans supporting the hypothesis that this element may be a critical component of the splicing switch mechanism that mediates tissue-specific splicing events (47). Furthermore, the (T)GCATG motif is a hallmark of many systems of neuronal splicing regulation (48). The proteins that recognize the hexamers in Humans were found to be homologue of the *Caenorhabditis elegans* RNA-binding protein feminizing on X known in humans as RbFox-1 family of proteins (49). In mammals there are three RbFox Paralogs: RbFox-1 (A2BP1), RbFox-2 (RBM9) and RbFox-3 (HRNBP3). RbFox-1 is expressed in neurons and muscle cells, RbFox-2 has a broader expression pattern, being observed in stem cells, hematopoietic cells, neurons and muscle. RbFox-3 has only been observed in neurons (43). All paralogs contain a single RNA recognition motif that specifically binds the (U)G

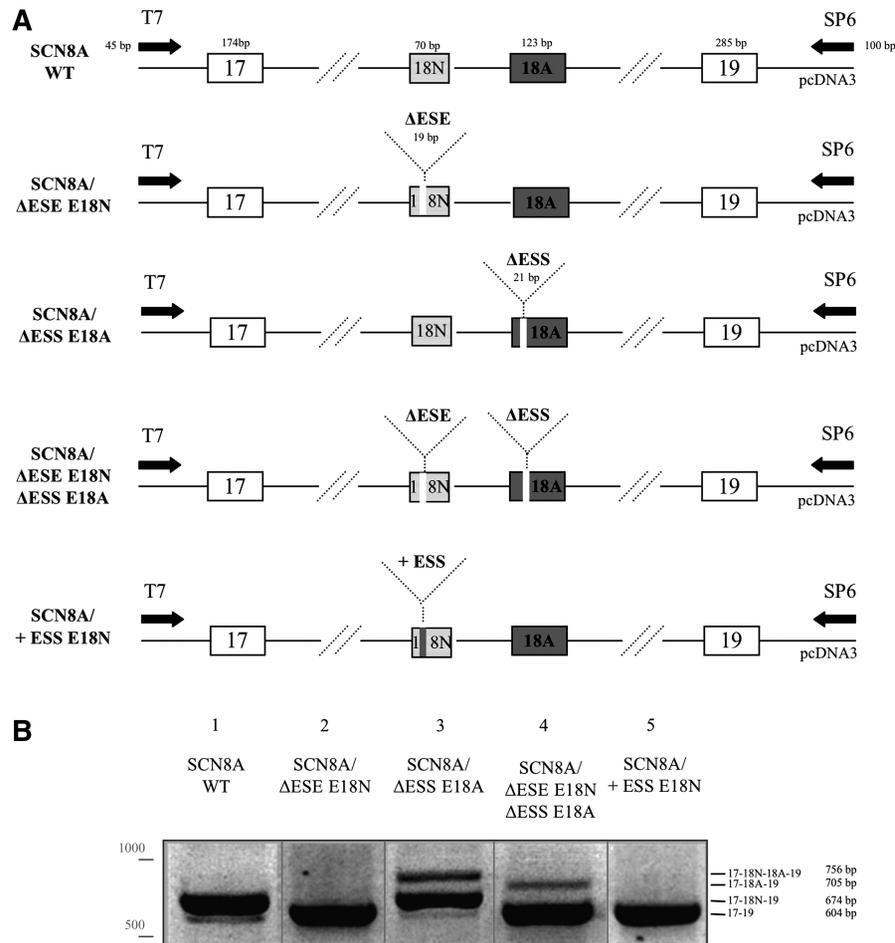


Figure 5. Analysis of effect of the ESE and the ESS on the alternative splicing of SCN8A exon 18N and 18A. **(A)** Schematic representation of the minigenes based on the backbone of the SCN8A WT minigene that spans SCN8A exons 17 through to 19. Two slanted parallel lines indicate SCN8A break in endogenous intronic region. T7 and SP6 indicate primers, specifically to the plasmid, used to amplify specifically the transfected mRNA **(B)** RT-PCR analysis of the minigenes after transfection in HeLa cells. Right hand side of gel: indication of which exons are included in each amplicon.

CAUG sequence. In addition, the Fox paralogs can be expressed in different isoforms that arise through the use of both alternative promoters and alternative exons (40).

The system that we have been using in this study was based on non-neuronal tissue culture cells and we were aware that there was the possibility that many specific factors were missing. In particular the lack of RbFox-1 was a strong candidate behind the reason of exon 18A exclusion, with this protein being absent in HeLa and HEK 293 cells (Supplementary Figure S2). However cotransfection of the plasmid carrying the brain specific isoform of RbFox-1 tagged with the Flag epitope together with the SCN8A WT minigene left the splicing pattern unchanged (Figure 6B, compare Lanes 1 and 2). It was possible that in HeLa cells exon 18A inclusion may be strongly repressed by the hnRNPs previously shown to interact with the ESS element and brain specific RbFox-1 is unable to override their effect. To examine the enhancer activity of brain specific RbFox-1 protein in the absence of ESS repression element we cotransfected the SCN8A/ΔESS E18A minigene with the brain RbFox-1 expression plasmid. In this scenario a strong

increase in exon 18A inclusion was observed (Figure 6B, compare Lanes 3 and 4).

The RbFox-1 protein enhancer activity was previously reported to be dependent on the presence of (T)GCATG (9,48). To confirm that this was the case with exon 18A, mutations were introduced in the minigene SCN8A/ΔESS E18A in each individual repeat or simultaneously in both. These constructs were cotransfected with brain specific RbFox-1 protein (Figure 6C). A single mutation, in the first TGCATG repeat (TGCATG > TGCCTG), had a strong effect on exon 18A resulting in a significant decrease in the inclusion of exon 18A (Figure 6CII, compare Lanes 1 and 2). A mutation in the second GCATG (GCATG > GCGTG) repeat showed the same effect on the reduction of exon 18A inclusion, as was also the case when mutations were introduced in both repeats and the RbFox-1 was expressed (Figure 6C, compare Lanes 1 and 3 and Lanes 1 and 4). The fact that the effect of disruption of more than one repeat is non additive has been previously observed (50).

Considering that the expression of the different RbFox isoforms and paralogs occurs in tissues where exon 18A is

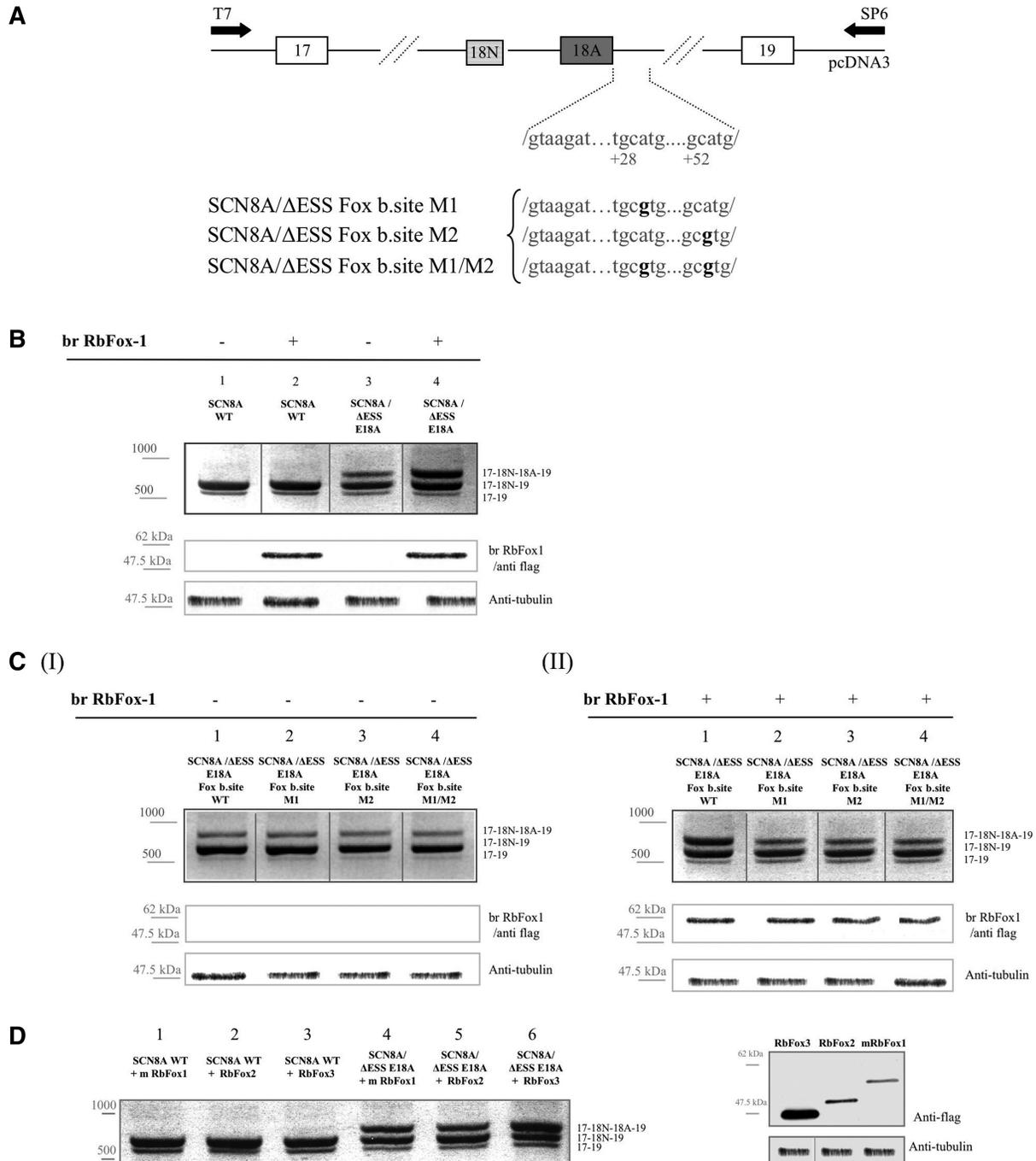


Figure 6. RbFox-1 promotes inclusion of SCN8A exon 18A only in the absence of the ESS in a (T)GCATG dependent manner. **(A)** Scheme of SCN8A wild-type minigenes highlighting the region of the (T)GCATG motifs and subsequent minigenes with RbFox-1-binding sites mutated **(B)** Agarose gel electrophoresis of cotransfections of the SCN8A WT and SCN8A/ Δ ESS E18A minigenes without and with the brain specific RbFox-1 expression plasmid. **(C)** Agarose gel electrophoresis of cotransfections of the SCN8A/ Δ ESS E18A minigene with mutations in RbFox-1-binding sites, without **(CI)** and with **(CII)** brain specific RbFox-1 expression plasmid. Forms of mRNA observed are indicated on the right hand side of the gel. Below analysis of the expression of the RbFox-1 proteins monitored via immunoblotting for the Flag epitope tag present in RbFox-1 expression plasmid and amount of protein loaded on the western blot via immunoblotting against the tubulin. **(D)** The effect of individual isoforms muscle (m) RbFox-1, RbFox-2 and RbFox-3 proteins in promoting SCN8A exon 18A inclusion analyzed by RT-PCR after cotransfection of overexpression plasmids for these proteins with SCN8A WT and SCN8A/ Δ ESS E18A minigene constructs. The mRNA species produced are indicated on the right hand side of the gel. On the right side, immunoblot analysis of protein overexpression via antibodies against the flag epitope. Anti tubulin was used as loading control for western blot.

included it was of interest to compare the relative activity of muscle specific (m) RbFox-1 together with those of RbFox-2 and RbFox-3 proteins in promoting exon 18A inclusion. This was performed using the SCN8A WT and SCN8A/ Δ ESS E18A minigene constructs and over expression plasmids for the specific Fox proteins. After transfection in HeLa cells and RT-PCR analysis, we observed that, as before, in the presence of the ESS in exon 18A (SCN8A WT minigene), RbFox protein overexpression, irrespective of type or isoform, had no significant effect on the RNA splicing (Figure 6D, Lanes 1–3). On the other hand in the absence of the ESS region in exon 18A (SCN8A/ Δ ESS E18A minigene) all Fox isoforms and proteins tested were able to promote the inclusion of exon 18A (Figure 6D, Lanes 4–6).

Endogenous RbFox-1 protein in the primary culture of the TG neurons regulates ME-splicing pattern of endogenous SCN8A exon 18N and 18A

To further ascertain the role played by the RbFox-1 protein in exon 18A ME splicing mechanism it was of interest to test the effect of knock down of the brain RbFox-1 (brRbFox-1) in a neuronal cell system. In the primary culture of rat TG neurons it was observed a very specific ME pattern where 18A was predominantly included in the mRNA rather than exon 18N (Figure 7, Lane 1). Knockdown of brRbFox-1 had a strong effect on the endogenous splicing pattern, from an almost exclusive use of exon 18A, with a minor component of skipping of exon 18, to that where a mRNA containing 18N rather than 18A is now the major transcript (Figure 7, compare Lanes 2 and 3).

DISCUSSION

Eukaryotic genes employ alternative splicing as a mean 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 particular requirements. The mechanisms that determine which splice sites are used and how this process is regulated have still not been precisely defined.

The work performed in this manuscript has begun to unravel the mechanisms behind the ME splicing of SCN8A exons 18N and 18A. Exon 18N inclusion results in a transcript carrying a premature stop codon that we show to be subject to NMD. The alternative splicing outcome of these two exons therefore provides an additional level of control of Na_v 1.6 protein expression in different cell types. It is curious however that the cell has evolved this approach to modulate tissue-specific channel expression that involves full transcription of the gene and degradation of this transcript. The energetic balance and current understanding of regulation processes would indicate that promoter repression might be more economical. A possible explanation behind this type of control may lie in the need to regulate protein function until it is required by the cell in postnatal neurons functioning as a post transcriptional on/off. Another possibility is that the exon 18N isoform may be needed in particular circumstances that we currently ignore. However, a closer look at this issue in the future may shed light on the advantages and disadvantages for such selection that is particularly abundant in specific tissues of the neuronal system. The choice behind selection of exon 18N or 18A appears to be dependent on at least three distinct *cis*-acting sequences present in a 620-nt stretch of DNA. First, an ESS in exon 18A present between nucleotides +6 and +27 represses the inclusion of this exon in HeLa and other non neuronal cells. Four proteins, not counting isoforms and paralogs of these, emerged from affinity purification experiments to bind this element. Three of these represent well characterized splicing repressors: hnRNP A1, hnRNPA2/B1 and PTB (51–59). Depletion of these factors individually does not have any significant effect on exon 18A inclusion but depletion of all three partially relieves exon 18A repression. To date there is no evidence of the involvement of hnRNP-like JKTBP protein in alternative splicing regulation but the fact that the binding consensus sequence of this protein contains an UAG, also present in hnRNPA1-binding consensus sequences, has led suggest a possible role in alternative splice site switching (60). However, analysis of the various combinations of depletions tested would suggest that hnRNP-like JKTBP does not play a significant role as a silencer in this case. These results

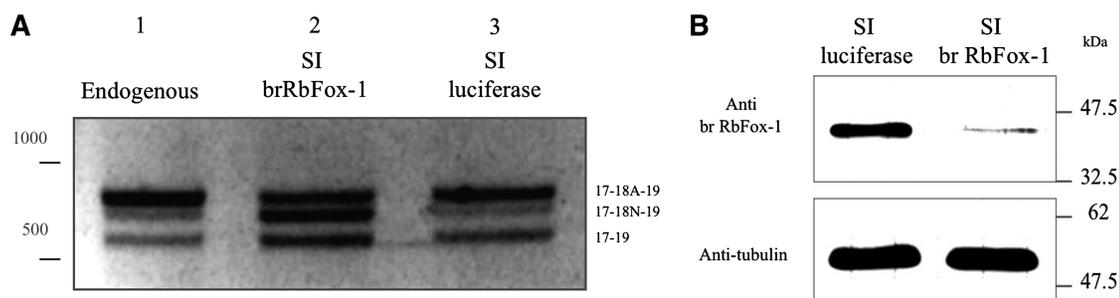


Figure 7. Effect of depletion of br RbFox-1 on exon 18A inclusion in the endogenous SCN8A transcript of rat TG neurons. (A) Agarose gel showing RT-PCR analysis of the effect of brRbFox-1 depletion in rat TG neurons. On the right hand side of the gel the species of mRNA produced are indicated. (B) Immunoblot using anti RbFox-1 and anti tubulin as a loading control showing endogenous brRbFox-1 protein depletion in the TG neurons.

indicate a considerable functional redundancy of the hnRNPs that interact with the ESS in order to maintain the exclusion of exon 18A in HeLa cells. As previously described different hnRNPs can substitute one another in their ability to silence an exon from being included in the pre-mRNA (41).

Two (T)GCATG motifs downstream of the exon 18A were the second *cis*-acting regulatory elements involved in the regulation of the ME splicing of the two exons. These motifs are known to bind RbFox proteins. We demonstrated that brain specific RbFox-1 promoted exon 18A inclusion in HeLa cells only in the absence of the ESS element located within this exon. This effect was observed to be dependent on the presence of both (T)GCATG motifs located downstream of the exon 18A. The fact that exon 18A inclusion was not observed upon overexpression of brain specific RbFox-1 protein, with the WT exon 18A exon in which the ESS silencer is intact, indicated that in the hierarchy of splicing regulation of exon 18A, in HeLa

cells, the hnRNPs binding to the ESS were dominant over the presence of RbFox-1 protein. Analogous results were observed with muscle specific RbFox-1, RbFox-2 and RbFox-3, all of which were capable of increasing the inclusion of the exon 18A in the minigene only with the ESS deleted.

Finally, an ESE responsible for the inclusion of exon 18N in HeLa cells was mapped between nucleotides +41 and +61 of this exon. Deletion of this region in the SCN8A WT minigene results in skipping of exon 18N. This ESE was observed to bind the SR proteins SRSF1 and SRSF2. Down regulation of these proteins however did not result in skipping of the exon 18N. The reason for this however may be similar to the one discussed for the ESS and hnRNPs. In fact SR proteins have often been 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 SRSF1 and SRSF2 observed to bind to it (61–65).

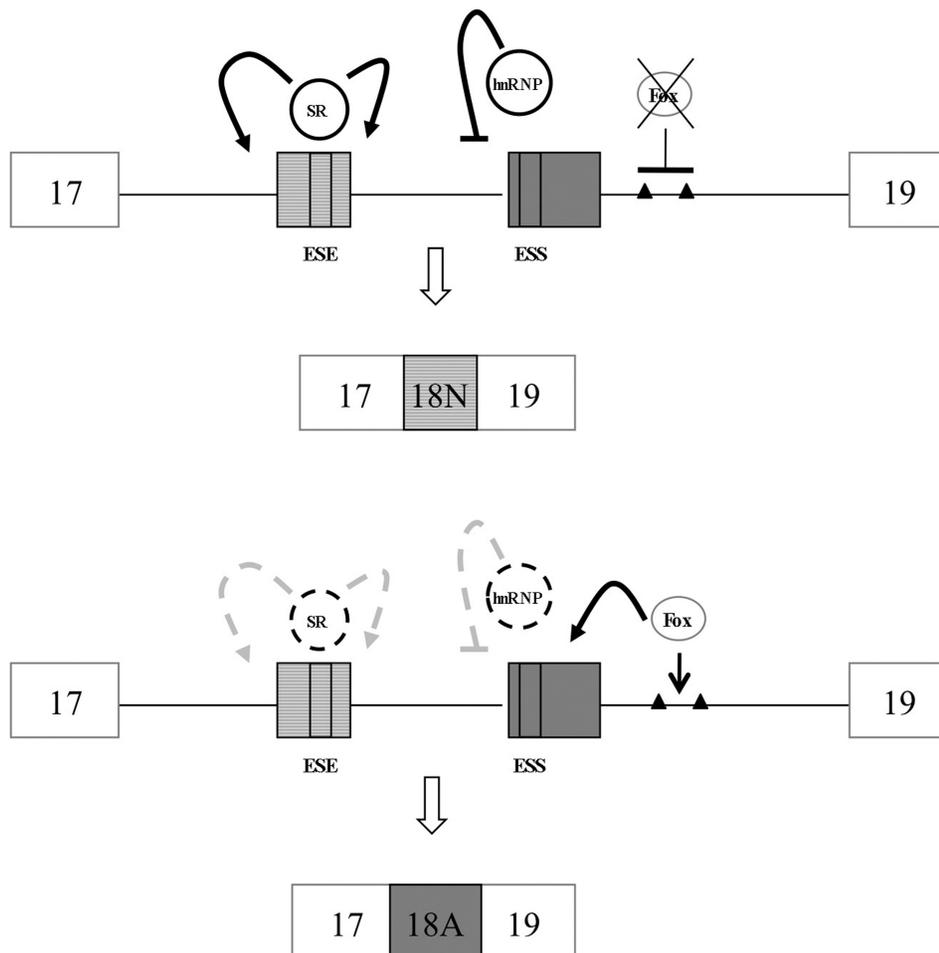


Figure 8. Schematic models for the mechanism of SCN8A ME exon 18N and 18A cell-type specific splicing. Upper section: cartoon of the model for exon 18N inclusion and 18A exclusion in non neuronal cells, that results in the absence of RbFox-1 and hnRNPs binding to the ESS present in the exon 18A. Lower section; cartoon of model for exon 18A inclusion and exon 18N exclusion in neuronal cells that occurs due to variations of stereochemistry ratio of splicing factors together with presence of RbFox-1 protein(s). Bold circles and arrows indicate presence of splicing factors and the mode of action. Dashed circles represents variations in ratios between splicing factors and consequently a decrease in the effect they have on exon definition be it positively or negatively.

An interesting feature of many of the *trans*-acting factors that we found to be associated with the splicing regulatory elements identified is that the expression patterns for these proteins tie in well with the observed tissue specificity of the exons. hnRNP A1 and hnRNP A2/B1 levels have been found to be higher in non-neuronal tissues, as compared to neuronal tissues, such as brain (51), nPTB is a tissue restricted paralog of PTB, expressed only in neuronal cells (39,66–68). Data from *in vitro* analysis showed that nPTB can act either neutrally or as a repressor albeit weaker than PTB in preventing exon inclusion (44–46). The hnRNPD-like protein JKTBP, identified in our study, is a ubiquitously expressed 38-kDa protein, while in neuronal tissue the major form of JKTBP is a 54-kDa protein isoform which may have different function or less repressive effect than 38-kDa JKTBP isoform (60). Thus, the fact that the level of expression of the inhibitory factors in neuronal tissue may be lower or completely absent than in non-neuronal tissues would mean that this ESS would be less functional in this scenario. The expression of certain SRs, such as SRSF1 have been also observed to vary between tissues (64). Furthermore a decrease in SRSF1 levels during neuronal differentiation of P19 cells has been observed (69). The expression pattern of the family of RbFox proteins, all of which we showed were able to result in the inclusion of exon 18A, in the absence of the ESS, varies with each type and isoform, however these are principally expressed in neurons and muscle (70). The variations in the intracellular levels of splicing factors may explain the ME splicing of exon 18N and exon 18A and some abnormal transcripts we have observed with our minigenes during the splicing regulatory element mapping process, such as the simultaneous inclusion of the ME exons observed with the minigene in which the exon 18A ESS element was deleted and brain specific RbFox-1 overexpressed (Figure 5). In tissues where SR proteins are expressed at lower levels or less active isoforms/paralog the ESE in exon 18N would no longer be functional resulting in only inclusion of exon 18A.

Taking into consideration the *cis*- and *trans*-acting elements that we identified and the expression patterns of the latter we are able to propose a model for the molecular mechanisms of the ME splicing of exons 18N and 18A (Figure 8). Exon 18A exclusion in non-neuronal tissue is regulated primarily by the presence in the cell of several hnRNPs proteins including hnRNP A1, hnRNPD-like JKTBP, hnRNPA2/B1 and PTB that interact with an ESS mapped in this exon. We have observed that this *cis*-acting element in HeLa cells is predominant and determines the exclusion of exon 18A even upon the over expression of RbFox-1. Exon 18N is included in non-neuronal cells due to SR proteins that function through an ESE mapped within this exon. In neuronal cells the levels of SR and hnRNP proteins and/or the ratio between them, in conjunction with the presence of RbFox-1, forces the recognition of exon 18A. The end result being that exon 18A is included and exon 18N skipped.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–2.

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