ELSEVIER

Molecular Brain Research 101 (2002) 109–121

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

Modulation of the membrane-binding projection domain of tau protein: splicing regulation of exon 3

Meltem Cevik Arikan[a], John Memmott[a], Jennifer A. Broderick[a], Robert Lafyatis[c], Gavin Screaton[d], Stefan Stamm[e], Athena Andreadis[a,b,*]

[a]Department of Biomedical Sciences, E.K. Shriver Center for Mental Retardation, Waltham, MA 02452, USA
[b]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA 01655, USA
[c]Arthritis Center, Boston University School of Medicine, Boston, MA 02118, USA
[d]Human Immunology Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK
[e]University of Erlangen-Nurenberg, Institute of Biochemistry, 91054 Erlangen, Germany

Accepted 6 March 2002

Abstract

Tau is a microtubule-associated protein whose transcript undergoes complex regulated splicing in the mammalian nervous system. The N-terminal domain of the protein interacts with the axonal membrane, and is modulated by differential inclusion of exons 2 and 3. These two tau exons are alternatively spliced cassettes, in which exon 3 never appears independently of exon 2. Previous work with tau minigene constructs indicated that exon 3 is intrinsically suboptimal and its primary regulator is a weak branch point. In this study, we confirm the role of the weak branch point in the regulation of exon 3 but also show that the exon is additionally regulated by a combination of exonic enhancers and silencers. Furthermore, we demonstrate that known splicing regulators affect the ratio of exon 3 isoforms, Lastly, we tentatively pinpoint the site of action of several splicing factors which regulate tau exon 3. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Cellular and molecular biology

Topic: Gene structure and function: general

Keywords: MAP tau; Regulated isoform; Membrane-interacting domain; Alternative splicing; *cis* and *trans* splicing regulator

1. Introduction

Alternative splicing is a versatile and widespread mechanism for generating multiple mRNAs from a single transcript [47,49,75,105]. Splicing choices are spatially and temporally regulated and the ensuing mRNAs produce functionally diverse proteins, contributing significantly to proteomic complexity [68].

Splicing is effected by the spliceosome, a large and dynamic complex of small nuclear riboproteins (snRNPs) [10,90,98,103]. A major question in splicing, and an obvious point of regulation, is how the spliceosome recognizes authentic splicing sites [10]. This is a critical decision, because mammalian splice site sequences are loosely defined and thus redundant in the genome [112], though the exon definition model has provided a partial mechanism for splice site authentication [7].

Numerous in-depth studies of *cis* determinants of alternative splicing have shown that splice site selection occurs via intrinsic hierarchies defined by complementarity of splice sites, branch points and their associated polypyrimidine (polyY) tracts to their cognate snRNPs [47,75,78]. In an increasing number of systems, exon inclusion is achieved by utilization of exonic enhancers [10,12,114]. Their mutation can result in human disease by causing aberrant splicing [24,114]. However, regulation is sometimes conferred by exonic silencers or distal intronic elements [2,27,29,30,38,57,64,109,110].

On the *trans* side of regulation, mammalian splicing

*Corresponding author. E.K. Shriver Center, 200 Trapelo Road, Waltham, MA 02452, USA. Tel.: +1-781-642-0279; fax: +1-781-894-9968.

E-mail address: athena.andreadis@umassmed.edu (A. Andreadis).

0169-328X/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0169-328X(02)00178-X

regulators mostly belong to two superfamilies, the serine/arginine-rich (SR) proteins and heterogeneous ribonuclear proteins (hnRNPs), neither of which is exclusively involved in alternative splicing [18,67,78,116,118]. The former are also components of the spliceosome, whereas the latter are also involved in pre-mRNA transport, RNA stability and translational regulation. Several mammalian splicing factors are enhanced in or restricted to neurons—Nova1, KH-type splicing regulatory protein (KSRP), suppressor of white apricot (SWAP) and human transformer (htra2β1) [14,60,81,82,96,111]. Nevertheless, it appears that the exquisite calibration of mammalian alternative splicing is primarily achieved by SR and hnRNP proteins which, despite their ubiquitous distribution, show distinct tissue and developmental ratios [28,51,62,123].

Tau is a microtubule-associated protein (MAP) enriched in axons of mature and growing neurons [9,65]. Tau is also found in the cell nucleus [119], in the distal ends of growing neurons [11,35] in oligodendrocytes [46,76] and in muscle [120]. Hyperphosphorylated, microtubule-dissociated tau protein is the major component of neurofibrillary tangles, a hallmark of several neurodegenerative diseases [107].

Tau is encoded by a single copy gene [54,83]. It produces three transcripts of 2, 6 and 9 kilobases (kb) which are differentially expressed in the nervous system, depending upon stage of neuronal maturation and neuron type [25,36,41–43,55,85,119]. The 6 kb codes for the axonal tau; the 2 kb tau mRNA is localized to the nucleus [119] whereas the 9 kb tau transcript is restricted to the retina and peripheral nervous system [25,41,85]. These three transcripts undergo complex alternative splicing: six of the 16 tau exons are regulated cassettes [3,4,25,41–43,54,55,66,69,92].

The N-terminus of the tau protein interacts with the plasma membrane [13]. The C-terminus of the tau protein contains four imperfect repeats (encoded by exons 9 to 12) which act as microtubule binding domains [55,70]. Altered splicing regulation of tau exon 10 can cause inherited frontotemporal dementia with parkinsonism, almost certainly by disturbing the normal tau isoform ratio [22,53,58,108].

The structure and function of the tau N-terminus is modulated by cassette exons 2 and 3 (Fig. 1A). These exons are adult-specific and remain regulated in the central nervous system [43,55,66,120], but become constitutive in

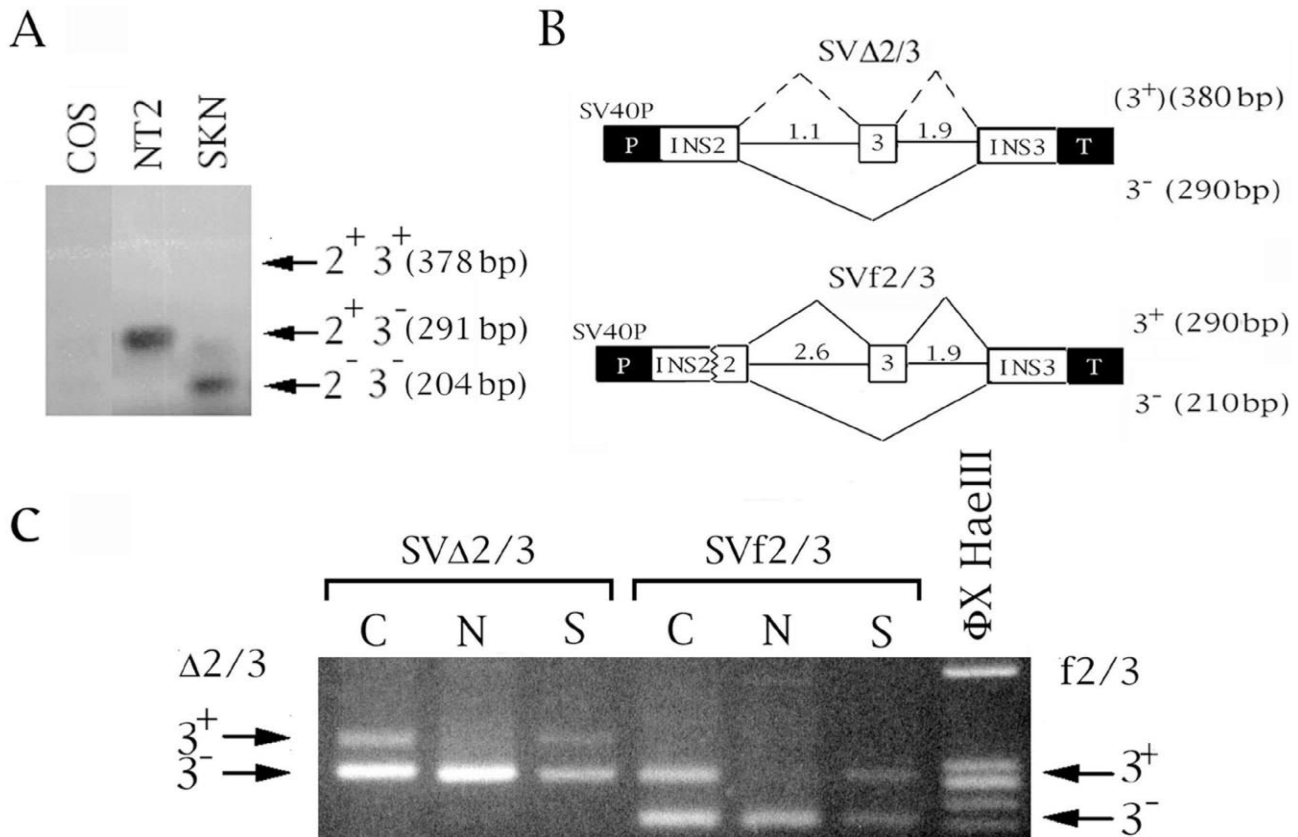

Fig. 1. The behavior of tau exon 3 minigenes reflects their endogenous expression. (A) The expression profile of human tau exon 3 in nonneuronal COS, teratocarcinoma NT2, and neuroblastoma SKN cells. The positions of the expected species produced by RT-PCR are shown. Primer pair: HT1S/HT4PCN; hybridization probe: HT1NL. (B) Schematic representation of expression constructs containing tau exon 3. P represents the SV40 early promoter, T the insulin terminator. The small intron between insulin exons 1 and 2 is not shown for the sake of clarity. The numbers on each side of tau exon 3 show how many kbp of flanking introns are present. Major splicing pathways are shown by solid, minor by dashed lines. (C) RT-PCR analysis of exon 3 expression constructs in COS (C), NT2 (N), and SKN (S) cells. The identities of the spliced species are indicated. Primer pair: INS1/INS3.

the peripheral nervous system [25,41]. In a previous study [3] we showed that exon 3 is incorporated inefficiently, unless either the region around its putative branch point is modified or exon 2 is present in a pre-spliced configuration (that is, if the branch point of exon 2 is no longer present). In this report we expand and refine our knowledge of the splicing regulation of exon 3.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

The human tau genomic fragments originated from cosmid or λ clones [4]. The parental vector used for cloning was pSVIRB [3,5,120]. Constructs SVΔ2/3 and SVf2/3 were previously generated [3] and used in the present study as 'background' controls (Fig. 1B). SVΔ2/3 contains tau exon 3 and its flanking introns [1.1 kilobase pairs (kbp) upstream and 1.4 kbp downstream]. SVf2/3 contains the 3′ two thirds of tau exon 2 fused to insulin exon 2, the entire 2.6 kbp intron between tau exons 2 and 3, tau exon 3 and 1.4 kbp of its downstream intron. The additional intron lengths shown in Fig. 1B come from the insulin gene. Additionally, the following minigene constructs were generated by directed mutagenesis (the primers used for mutageneses are listed in Table 1).

Constructs SVΔ2/3TM, SVΔ2/3TM_{BP} and SVΔ2/3TM_{SS} (Fig. 2A) contain various combinations of branch points and 3′ splice sites. SVΔ2/3TM is SVΔ2/3 in which the region just before tau exon 3 bears a substitution of 300 nucleotides from the expanded intron between exons 2 and 3 of rat α-tropomyosin (α-TM) [102]. The substitution sequence contains a strong branch point with a very long associated polyY tract and the 3′ splice site of α-TM exon 3; the expansion comes from an insert which completely relieves the steric hindrance between the branch point and the 3′ splice site and results in constitutive inclusion of the α-TM exon [102]. SVΔ2/3TM_{BP} is a variant of SVΔ2/3TM in which the 3′ splice site has been altered so that it is identical to that of tau exon 3. SVΔ2/3TM_{SS} is a variant of SVΔ2/3 in which the 3′ splice site has been mutated so that it is identical to that of α-TM exon 3.

Constructs E3-1 to -7 arise from SVf2/3; the alterations to wild-type exon 3 are shown in Fig. 3A. To generate the mutant constructs, whole-plasmid mutagenesis was performed on SVΔ2/3, SVΔ2/3TM or SVf2/3 using the QuikChange Site Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions, except that the polymerase chain reaction (PCR) products were digested with *Dpn*I overnight. The mutations were verified either by sequencing (for the constructs in Fig. 2) or by the appearance of a restriction site embedded in the oligonucleotides used in the procedure (for the constructs in Fig. 3).

Construct intermediates generated by PCR and selected

Table 1
Primers

Primer name or use	Length	Orientation	Location	Sequence
Mouse tau cloning and sequencing				
MT3S	30	Sense	Beginning of mouse tau exon 3	ACGTGACTGCGCCCCTAGTGGATGAGAGAG
MT3N	30	Antisense	End of mouse tau exon 3	CTGTAATTCCTTCTGGGATCTCCGTGTGGG
Human tau wild type and mutant constructs				
E3-1S*	30	Sense	Within human tau exon 3	GGTTTCTAGATGTGACGTCACCCTTAGTGG
E3-2S*	30	Sense	Within human tau exon 3	GTGACAGCACCCATGGTGGATGAGGGAGCT
E3-3S*	30	Sense	Within human tau exon 3	GAGGGAGCTCCCGGCGCGCAGGCTGCCGCG
E3-4S*	30	Sense	Within human tau exon 3	GGCTGCCGCGGATCCCCACACGGAGATCCC
E3-5S*	30	Sense	Within human tau exon 3	CAGCCCCACACGGGGGCCCCAGAAGGAACC
E3-6S*	30	Sense	Within human tau exon 3	CCACACGGAGATCCCGGGAGGAACCACAGG
E3-7S*	30	Sense	Within human tau exon 3	ATCCCAGAAGGAACCCCGGGTGAGGGTAAG
TM_{BP}*	32	Sense	Upstream intron of exon 3	CTGCCAACTCCCATC**TAG**ATGTGACAGCACCC
TM_{SS}*	32	Sense	Upstream intron of exon 3	GGATCTCGGTGGTTAC**CAG**GTGTGACAGCACC
HTI3N	22	Sense	Upstream intron of exon 3	CCTACTTCAGGCTGCTTTCTGG
HT3PCN	20	Antisense	Downstream intron of exon 3	GAATCCACCCATGTTCCTGC
RT-PCR of endogenous and transfected human tau				
INS1	30	Sense	End of insulin exon 1	CAGCTACAGTCGGAAACCATCAGCAAGCAG
INS3	30	Antisense	Within insulin exon 3	CACCTCCAGTGCCAAGGTCTGAAGGTCACC
HT1S	24	Sense	Near 5′ of human tau exon 1	GAACCAGGATGGCTGAGCCCCGCC
HT4PCN	23	Antisense	Near 3′ of human tau exon 4	GGGTCACGTGACCAGCAGCTTCG
Hybridization of endogenous human tau				
HT1NL	24	Antisense	Near 3′ of human tau exon 1	CCCTCTTGGTCTTGGTGCATGGTG

*Mutations in these are shown or diagrammed in Figs. 2 and 3. For site directed mutagenesis, we used pairs of these primers and their reverse complements (not shown). The nucleotides shown in bold type within TM_{BP} and TM_{SS} correspond to the 3′ splice site of tau exon 3 and α-TM, respectively.

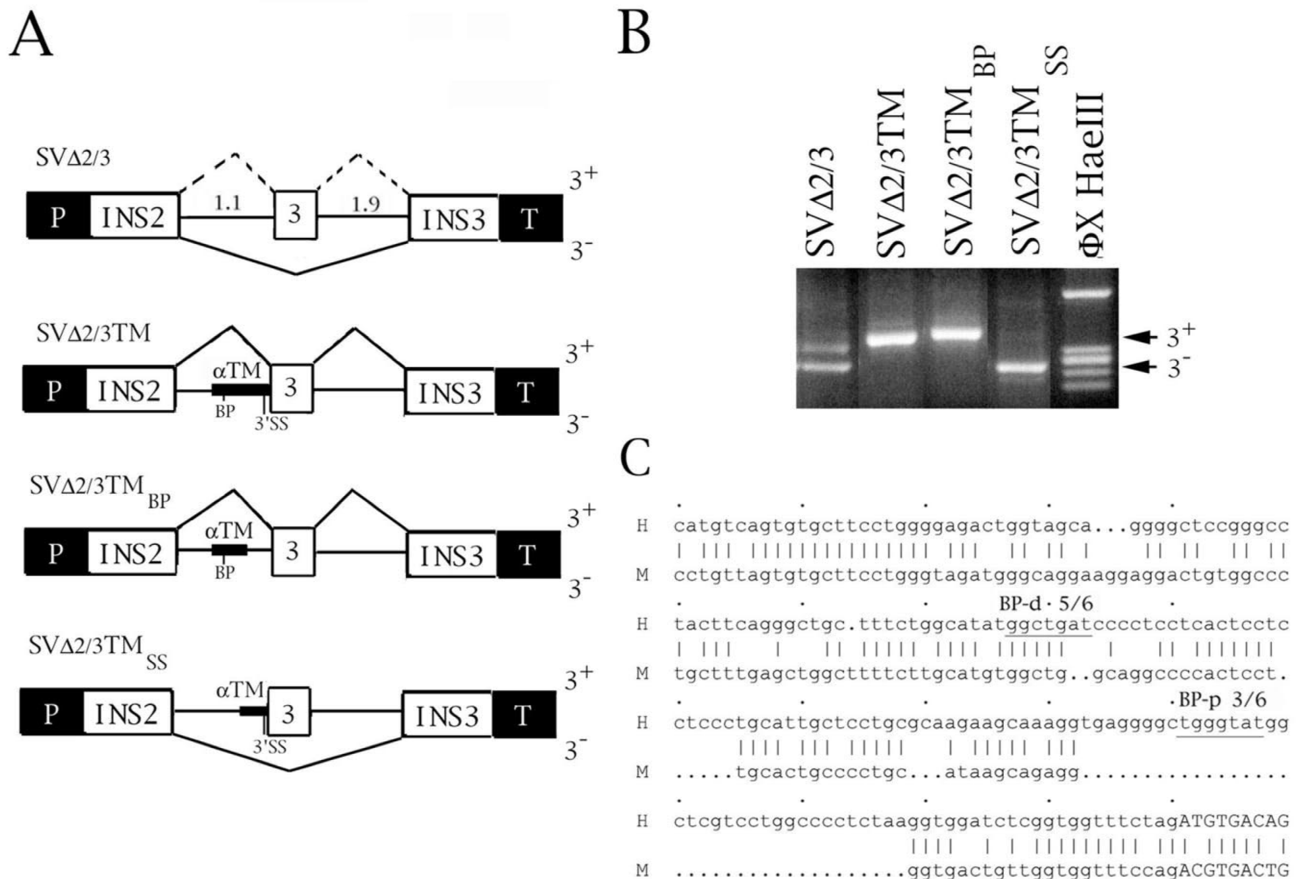

Fig. 2. The primary splicing defect of exon 3 is a weak branch point. (A) Schematic representation of expression constructs which contain branch point and 3′ splice site alterations. The thick line indicates the extent and identity of α-tropomyosin (α-TM) substitutions. Other conventions for the constructs are as in Fig. 1B. (B) RT-PCR analysis of exon 3 branch point mutation constructs in COS cells. The identities of the spliced species are indicated. Primer pair: INS1/INS3. (C) Sequence comparison of the region upstream of tau exon 3 between human (H) and mouse (M). Exonic sequences are in uppercase, intronic in lowercase. The two putative branch points of the human exon 3 [3] are underlined. BP-d=Distal, BP-p=proximal.

reverse transcription (RT)-PCR products were cloned by either of two methods: (1) denatured for 2 min at 90 °C in 'forward heat' buffer [93], phosphorylated in the presence of kinase forward buffer and T4 polynucleotide kinase (New England Biolabs), run on a low melt agarose gel, extracted from the agarose by treatment with β-agarase (New England Biolabs) and ligated into pKS(+) (Stratagene) or pCRII (InVitrogen); or (2) run unphosphorylated on a regular agarose gel, extracted by the GeneClean method (kit II or III, Bio 101) and ligated into pCRII Topo-TA (InVitrogen).

Plasmid DNA was prepared from 5 ml of saturated cultures using Qiagen Tip-20s, then sequenced either in an automatic fluorescent ABI Prism sequencer or by the modified dideoxy method [8,95] with Sequenase 2.0 (US Biochemicals) and [^{35}S]α-dATP (Amersham). After manual sequencing, the sequences were resolved on 8.3 M urea/6% polyacrylamide gels and analyzed with the GCG software [33].

Vectors bearing splicing factor cDNAs expressed them from the following promoters: CMV for hnRNPA1, htra2β1, Nova1, U2snRNP auxiliary factor (U2AF); SR proteins 20, ASF/SF2, 30c, SC35, 40, 55, 75 [15,38,82,97,122]; adenovirus major late for polypyrimidine tract binding (PTB) protein [86]; SV40 early for SWAP [71]. Previous Westerns as well as in vivo co-transfections (described in the references just listed) have shown that these constructs express functional proteins.

2.2. Cloning of the mouse tau gene

Two primers were prepared which corresponded to the beginning and end of mouse tau exon 3 (MT3S and MT3N, shown in Table 1). These were used to obtain three P1 phages containing mouse tau genomic inserts from Genome Systems by PCR. The P1 plasmids were transduced into the Cre^{+} strain NS3516; P1 plasmid DNA was prepared according to the P1 manual of Genome Systems and sequenced using the mouse primers listed in Table 1.

2.3. Cell culture, transfections and RNA preparation

Monkey kidney (COS) and human teratocarcinoma cells

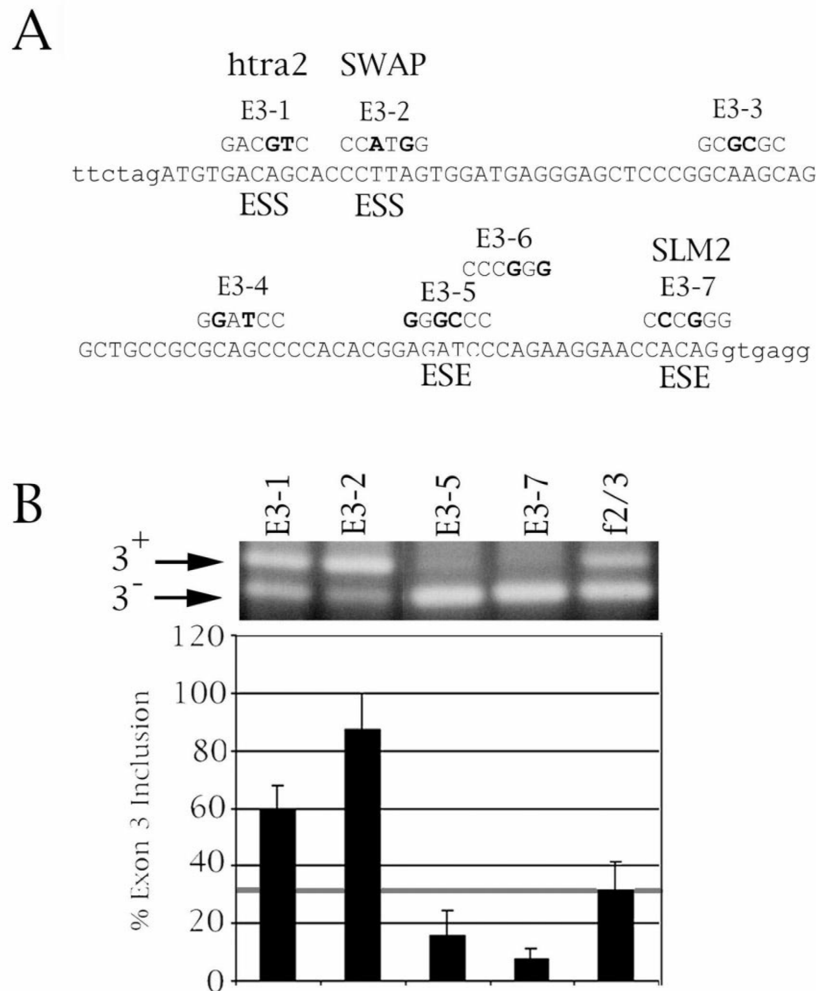

Fig. 3. Exon 3 contains several exonic enhancers and silencers, as discovered by directed mutagenesis. (A) Outline of mutations within exon 3. Exonic sequences are in uppercase, intronic in lowercase. The changes are shown above the wild-type sequence, with the altered nucleotides in bold. Shown below each mutated sequence is its action, if any: ESE=exonic enhancer, ESS=exonic silencer. Shown above each mutated sequence is the tentatively assigned splicing factor, if any. (B) Splicing behavior of mutated constructs of exon 3 in COS cells, as seen by RT-PCR analysis. Only the mutants which affect exon 3 behavior are shown. The gray bar across the graph shows the level of the wild type. The identities of the spliced species are indicated. Primer pair: INS1/INS3. % Exon inclusion was calculated by scanning the bands from three independent transfections and measuring their areas using the OneDscan analysis program.

(NT2) and SK-N-SH human neuroblastoma cells (SKN) were maintained as previously described [3,38]. Plasmid DNA was prepared by cesium chloride banding [93] and introduced into cells by the lipofection method (LT1, Panvera). Total RNA was isolated by the TRIzol method (Life Technologies).

2.4. Reverse transcription and PCR reactions

For PCR analysis of RNA, 5 μg of total RNA was treated with RNAase-free DNAase I (Promega). The DNAase I was subsequently heat-inactivated and cDNA generated using RNAase H^- Superscript (Life Technologies), in 20 μl for 1 h at 42 °C. A 3-μl volume of this reaction mix was then added to two Ready-to-Go PCR beads (Pharmacia) in a total volume of 50 μl. The PCR conditions were: for the endogenous gene, denaturation 94 °C/1 min, annealing 58 °C/1 min, extension 72 °C/1 min, 25 cycles; for the constructs, denaturation 94 °C/1 min, annealing 65 °C/1 min, extension 72 °C/1 min, 25 cycles (for COS) or 30 cycles (for NT2 or SKN). In the case of transfected cells, the primers (INS1 and INS3, Table 1) were chosen to only amplify products arising from the constructs.

The RT-PCR experiments were done with RNAs from three independent transfections, to ensure reproducibility. The isoform ratio was calculated by scanning the bands from three independent transfections using the One-Dscan program and the IP Lab analysis software from Scanalytics. For the expression constructs, the predicted sizes of the $3^-/3^+$ RT-PCR products are: 290/380 for SVΔ2/3 and 210/290 for SVf2/3.

2.5. Blotting and hybridization

RT-PCR products from endogenous RNAs were run on 2% agarose/1× TAE. The gel was denatured in 1.5 M NaCl/0.5 M NaOH for 30 min, renatured in 1.5 M NaCl/0.5 M Tris (pH 7.2) for 30 min, capillary-blotted with 10× SSC on 0.20 μm Nytran (Schleicher & Schuell) and UV-crosslinked (Stratalinker).

The membrane was prehybridized for at least 3 h at 42 °C in a mixture of 50% deionized formamide, 5× SSC, 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 100 μg/ml heat-denatured herring sperm DNA. To visualize all RT-PCR products, we used a probe from tau exon 1 (HT1NL, shown in Table 1) labeled with T4 polynucleotide kinase (New England Biolabs) and $[^{32}P]\gamma$-ATP (Amersham). The membrane was hybridized to the probe overnight at 42 °C, washed (15 min at room temperature in 2× SSC/0.1% SDS, 15 min at 52 °C in 0.1×SSC/0.1% SDS, 15 min at 65 °C in 0.1×SSC/0.1% SDS) and exposed.

3. Results

3.1. The behavior of the constructs mirrors the endogenous pattern

Fig. 1A shows the status of tau exon 3 in two very commonly employed human cell lines, NT2 teratocarcinoma and SKN neuroblastoma. Exon 3 is not visible in either cell line; this is not surprising, since the isoform containing this exon is decidedly minor even in the fully differentiated adult central nervous system [120] and becomes predominant only in the adult peripheral nervous system [25,41,85].

Consistent with the endogenous behavior, constructs which contain exon 3 reflect the cellular environment in their inclusion ratios of the two exons. The inclusion ratio of exon 3 intrinsically varies depending on the identity of its upstream exon: presence of exon 2 boosts the inclusion of exon 3 [3]. However, within a single construct there is more exclusion of exon 3 in NT2 than in SKN (Fig. 1C).

So although the *cis* determinants of exon 3 are largely local, its splicing regulation clearly depends on cell-specific *trans* factors—differing in NT2 and SKN, which derive from the central and peripheral nervous system, respectively.

3.2. Substitution of a strong branch point renders exon 3 constitutive

From previous work we surmised that the primary splicing defect of exon 3 was a weak branch point/3′ splice site region [3]. To dissect this further, we asked how exon 3 behaves if it acquires a strong heterologous branch point and/or 3′ splice site.

Substitution of the strong α-tropomyosin region (α-TM; [102]) results in constitutive inclusion of exon 3 (Fig. 2B, construct SVΔ2/3TM). To pinpoint the element responsible for this shift, we generated two 'mix-and-match' mutants: SVΔ2/$3TM_{BP}$ contains the α-TM branch point but the 3′ splice site of tau exon 3; SVΔ2/$3TM_{SS}$ contains the inverse combination. Construct SVΔ2/$3TM_{BP}$ shows constitutive inclusion of exon 3 whereas construct SVΔ2/$3TM_{SS}$ shows complete exclusion (Fig. 2B, lanes labeled with the name of the corresponding construct). This definitively pinpoints the branch point as the major determinant of exclusion for tau exon 3. However, as is the case with other regulated exons, our subsequent results show that additional *cis* elements modulate the behavior of tau exon 3.

3.3. Mutagenesis uncovers the presence of enhancers and silencers within tau exon 3

To locate possible *cis* regulatory elements within exon 3, we scanned the exons for possible regulatory motifs, and altered several of these by directed mutagenesis. We zeroed in on purine-rich ('GAR') or A/C rich ('ACE') sequences which act as splicing enhancers [26] or on CTAG/TTAG/CAAG/CAGG motifs, which behave as splicing silencers in several systems [2,17,27,29,30,38,100,109,110]. The sequences we changed are shown in Fig. 3A.

To locate regulatory sequences in exon 3, we used construct SVf2/3. SVf2/3 can be utilized to find either enhancers or silencers, since it shows considerable inclusion of exon 3. Of the seven mutants that we generated, three (E3-3, E3-4 and E3-6) are neutral, two (E3-5 and E3-7) pinpoint exonic enhancers and two (E3-1 and E3-2) exonic silencers (Fig. 3B; E3-3, E3-4 and E3-6 are not shown, since they do not alter splicing of exon 3).

The strongest effects are E3-2, which effectively reverses the wild type $3^+/3^-$ ratio (Fig. 3B, second lane from the left) and E3-7, which totally excludes exon 3 (Fig. 3B, second lane from the right). Both results are expected: E3-2 alters a TTAG sequence, which is known to act as a silencer in tat and tau exon 10 [2,27,38,110]; E3-7 decreases the complementarity of the 5′ splice site of exon 3 to the U1 snRNP (from CAGgtgagg to CGGgtgagg; the perfect complement is CAGgtaagt).

The pattern of these mutants indicates strongly that splicing regulation depends not just on specific nucleotide sequences but on local context as well. This is not surprising, since binding sites for splicing regulators are redundant [48,73,118].

3.4. Several splicing regulators modulate inclusion of exon 3

Both the constitutive inclusion of tau exon 3 in adult peripheral nervous system [85] and its weak default inclusion in non-neuronal and neuroblastoma cells ([3],

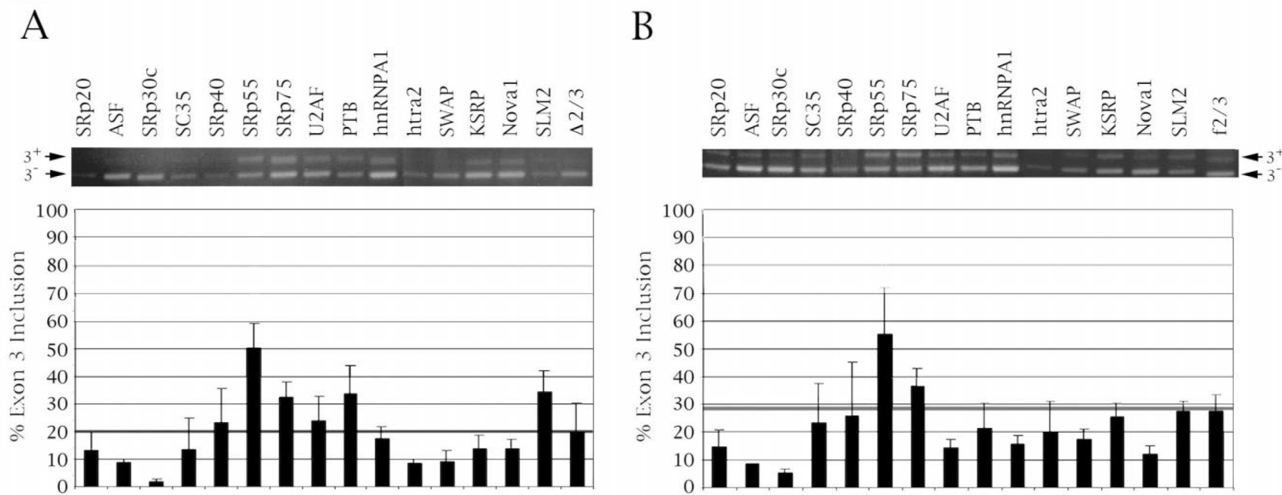

Fig. 4. Several splicing regulators affect the expression of exon 3. The RT-PCR products come from 1:1 co-transfections of tau splicing constructs with the factors indicated. % Exon inclusion was calculated as in Fig. 3B. The gray bar across the length of the graphs shows the expression level of the construct in the absence of factors. The identities of the spliced species are indicated. Primer pair: INS1/INS3. Co-transfections in COS cells of exon 3 constructs (A) Δ2/3 and (B) f2/3.

this work) point to regulation by at least one activator. To narrow down possible candidates, we did co-transfection experiments with exon 3 constructs and splicing regulators.

We examined the effect of splicing regulators on both SVΔ2/3 and SVf2/3 in COS cells (Fig. 4A and B, respectively). SC35 and SRp40 show little effect; SRp20, ASF, SRp30c, SWAP, htra2β1 and Nova1 act as inhibitors; SRp55 and SRp75 as activators. Finally, U2AF, PTB, hnRNPA1, KSRP and SLM2 show construct-specific effects: U2AF and PTB act as activators in SVΔ2/3 but as inhibitors in SVf2/3; hnRNPA1 inhibits exon 3 inclusion only in SVf2/3 while KSRP inhibits exon 3 inclusion only in SVΔ2/3; and SLM2 promotes exon 3 inclusion strongly in SVΔ2/3 but very weakly in SVf2/3.

Co-transfections of SVΔ2/3 in NT2 cells showed results qualitatively similar to those in COS (data not shown), consistent with the fact that in NT2 cells both the endogenous and transfected tau exhibit almost exclusively the 3^- pattern (Fig. 1A and C). Finally, in contrast with other studies [87], overexpression of PTB did not cause missplicing of exon 3.

3.5. Combination of mutant constructs and regulators pinpoint possible binding sites for the factors within exon 3

After we determined *cis* elements and *trans* factors that influence the splicing of tau exon 3, we did a preliminary scan for correlations between enhancers/activators and silencers/inhibitors. If a factor which influences the splicing of the wild type ceases to do so for a mutant, the mutated region defines a possible binding site for the factor.

We paired mutants E3-1 and E3-2, which define silencers, with exon 3 splicing inhibitors, concentrating on those which are tissue specific (htra2β1, Nova1, SWAP). The behavior of E3-1 remains unchanged in co-transfections with htra2β1 (Fig. 5A), whereas the same happens to E3-2 in the presence of SWAP (Fig. 5B). Thus, these two mutants are possible binding sites for the respective two factors.

We also paired mutants E3-5 and E3-7, which define enhancers, with SRp55, SRp75 and Sam68-like mammalian protein (SLM2) which activate exon 3 splicing. E3-5 shows no change in the presence of the factors (Fig. 5C), suggesting that it does not define a binding site for any of these factors. On the other hand, SLM2 only partly reverses the effect of E3-7 (Fig. 5D), so E3-7 may directly or indirectly bind SLM2.

4. Discussion

4.1. The suboptimal exon 3 branch point region and its regulators

Branch points can sometimes be located by comparison to the consensus [90] or by comparing aligned sequences across species. Neither strategy works for tau exon 3. The upstream intron of exon 3 contains two potential branch-points (Fig. 2C, top strand; [3]). BP-d, which adheres to the consensus and is followed by a long polyY tract, is far upstream. Conversely, BP-p, located within the usual interval for branch points, is a poor match for the consensus and lacks an associated polyY stretch.

Moreover, comparison of the human and mouse introns upstream of exon 3 shows that these two putative branch points for the human exon 3 [3] are not conserved in the mouse (Fig. 2C). Yet tau exon 3 is identically regulated in human and mouse; hence, they must have the same branch

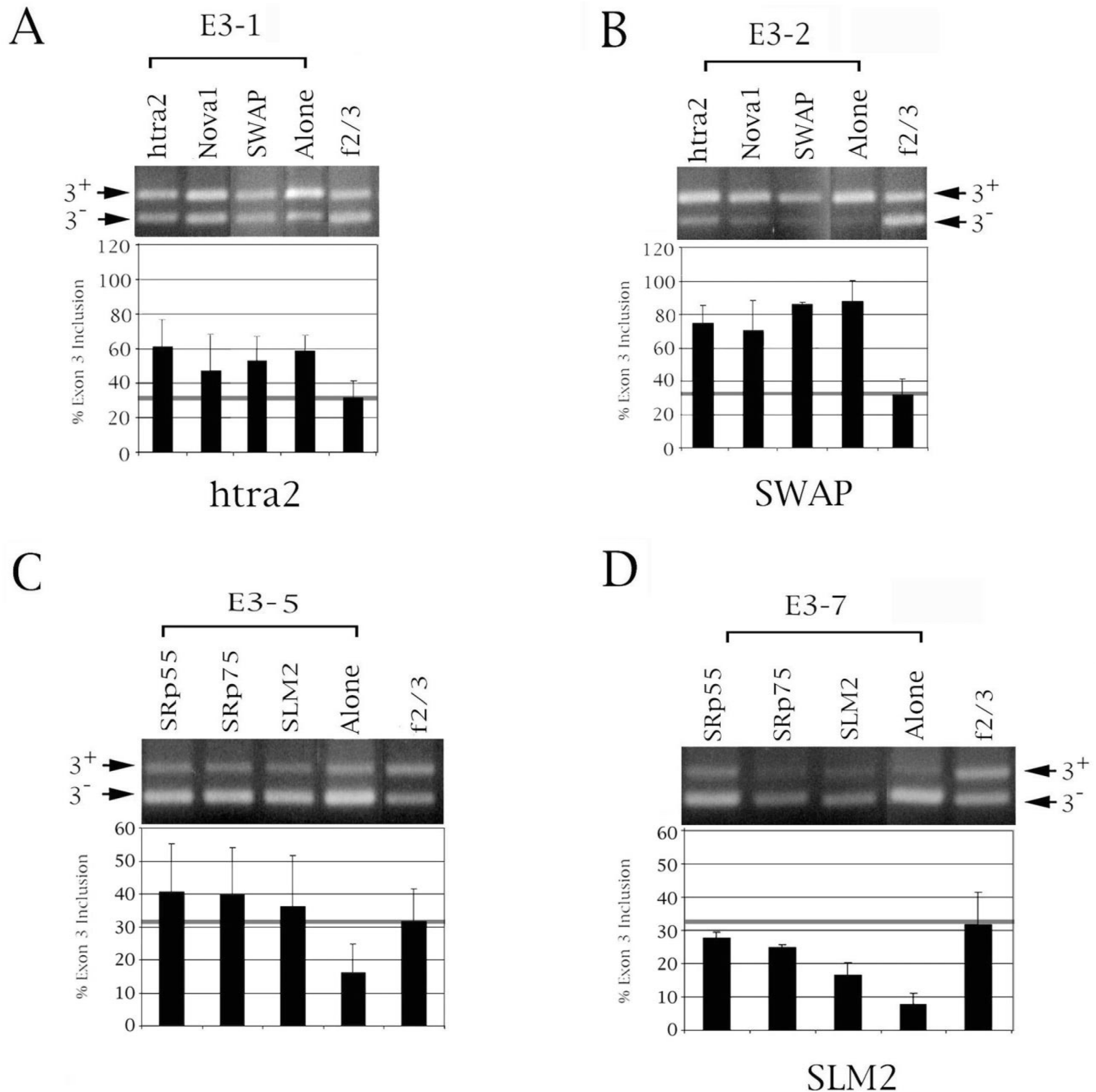

Fig. 5. Interaction of *cis*-acting elements of exon 3 with selected *trans* regulators of the same exons. The RT-PCR products come from 1:1 co-transfections of wild type and mutated splicing constructs with the factors indicated. Mutations which define enhancers were paired with activators, whereas mutations which define silencers were paired with inhibitors. Shown below each panel is the tentatively assigned splicing factor, if any. % Exon inclusion was calculated as in Fig. 3B. The gray bar across each graph shows the level of the wild type. The identities of the spliced species are indicated. Primer pair: INS1/INS3. Co-transfections of three inhibitors of exon 3 with mutants (A) E3-1 and (B) E3-2. Co-transfections of three activators of exon 3 with mutants (C) E3-5 and (D) E3-7.

point. So tau exon 3 may be that rare but documented phenomenon, an exon which does not use A as its lariat point [1,52].

Splicing regulation by weak or oddly situated branch points is a well-documented mechanism, especially for neuron- or muscle-specific genes [45,72,84,102–104,106,121]. In such cases regulation often proceeds by competition between PTB and U2AF, which recognize the polyY tract and branch point, respectively.

PTB is involved in the regulation of several neuronal-specific splicing events: the N1 exon of c-src, the EN exon of clathrin light chain B, exon 5 of the NMDA receptor NR1 subunit and the 24-nt exon of the $GABA_A$ $\gamma 2$ subunit [6,19,124]. U2AF has been implicated in neuronal-specific splicing of APP [115]. In the list of alternatively spliced systems examined so far, which includes tau exon 6 [121], PTB almost invariably favors exon skipping while U2AF favors exon utilization [116,117].

U2AF and PTB influence the behavior of tau exon 3 but, uniquely, the two factors behave identically (Fig. 4A, B):

they increase inclusion of exon 3 if its inclusion is negligible (SVΔ2/3) but decrease it if its inclusion is considerable (SVf2/3). The other unexpected reversal is that when they activate exon 3 splicing, PTB is the better activator (Fig. 4A), whereas when they inhibit it, U2AF is the better inhibitor (Fig. 4B). This unusual behavior may arise if the construct splicing shifts stem from transient interactions resulting from the overexpression of the specific factor. The difference in effect on SVΔ2/3 versus SVf2/3 cannot be ascribed to the presence of exon 2 in the latter: in SVf2/3, exon 2 is prespliced to the upstream insulin exon and therefore cannot act as a 'sink' for branch point factors such as U2AF or PTB.

For binding, U2AF prefers U-rich stretches interrupted by Cs, whereas the PTB consensus site is UCUU embedded within a polyY tract [6,87,101]. No U-rich tracts exist in the intron upstream of tau exon 3; instead, it contains several very C-rich tracts (Fig. 2C, [3]). Thus, the true regulator of the exon 3 branch point may well be neither PTB nor U2AF. One candidate is the newly discovered brain-specific PTB [79,88], whose mode of action differs from that of PTB in subtle but significant ways.

4.2. The complex interaction of exon 3 exonic cis elements and their putative trans regulators

For exon 3, the presence of additional inhibitory elements beyond the weak branch point underscores the tight control exerted on the expression of this exon. The present work demonstrates that exon 3 is also affected by splicing regulators. Our results show two unexpected wrinkles: (1) some regulators which influence exon 3 splicing exhibit construct-specific effects (besides U2AF and PTB, discussed above, these include hnRNPA1, KSRP and SLM2); (2) other regulators which influence exon 3 splicing show novel behavior (KSRP and Nova1, previously found to act invariably as splicing activators, inhibit inclusion of exon 3).

4.2.1. Enhancers and activators

Most exonic splicing enhancers belong to the purine-rich ('GAR') category [2,109,112,114]. More recently, A/C rich sequences ('ACE') were also defined as splicing enhancers by SELEX and functional analysis [26]. Both enhancer classes presumably exert their effect by binding SR proteins [12,24].

Neither of the two enhancers found in exon 3 (mutants E3-5, E3-7, Fig. 3) belongs to an obvious class, although E3-5 is within a relatively purine-rich region which nevertheless does not adhere to the GAR pattern. E3-7 cannot be considered to define an enhancer per se, because the effect of that mutant can be ascribed to weakening of the 5′ splice site of exon 3.

Among the SR proteins, SRp55 and SRp75 promote exon 3 splicing. SRp75 is the least studied SR protein but in the case of tau it is potentially the most interesting, because it is highly enriched in brain [123]. Also interesting is the observation that SRp55 and SRp75 inhibit tau exon 10, which is intrinsically strong [38] yet the same two SR proteins activate tau exon 3, which is intrinsically weak [3]. However, neither factor appears to bind to the sites defined by mutants E3-5 and E3-7 (Fig. 5C, D).

SLM2, which activates exon 3 in its SVΔ2/3 configuration (Fig. 4A), belongs to the Sam68 family of STAR (=signal transduction and activation of RNA) proteins and is highly enriched in brain, muscle and testis [34,113]. STAR proteins function as adaptors between tyrosine kinase signal transduction cascades and RNA splicing. SLM2 binds to purine-rich regions and generally acts as a splicing activator [34,113]. Yet SLM2 apparently does not bind to the purine-rich enhancer defined by E3-5 (Fig. 5C), though it may be peripherally involved in the region of E3-7 (Fig. 5D).

4.2.2. Silencers and inhibitors

Exonic silencers are much rarer and less well understood than exonic enhancers. They are postulated to work by either recruiting inhibitory factors or by blocking spliceosome assembly [2,21,31,100,109].

The TTAG motif within exon 3 (mutant E3-2) has been shown to regulate splicing of both tau exon 10 and exon 3 of the HIV tat gene [27,38,110]. In tau exon 3, mutagenesis of that motif practically reverses the behavior of the exon (Fig. 3B). The TTAG sequence is conserved between human and bovine tau but becomes CTAG in rodent tau; however, CTAG also acts as a silencer in exon 2 of the HIV tat gene [99]. So this motif may function as a silencer across species, despite the sequence variation.

Silencers of similar sequences also affect splicing of the EDA fibronectin exon [17,109] and the SAM exon of fibroblast growth factor 2 [29,30]. In the latter, the silencer seems to act by recruiting hnRNPA1 [31]. Yet hnRNPA1 inhibits exon 3 only in its SVf2/3 configuration (Fig. 4B); furthermore, hnRNPA1 does not influence splicing of tau exon 10, which contains a TTAG motif with silencing activity [27,38].

Of the tissue-specific regulators, SWAP, Nova1, KSRP and htra2β1 inhibit splicing of exon 3 (Fig. 4). SWAP is primarily expressed in brain and placenta [32] and is a universal inhibitor: it suppresses splicing of at least three other exons: tau exon 10, fibronectin IICS and exon 4 of CD45 [38,71,96]. Its temporal and spatial distribution and its behavior make it a strong candidate as an native inhibitor of exon 3 and our work tentatively assigns its site of action to the region defined by E3-2 (Fig. 5B).

Neuronal-specific Nova1 activates splicing of two receptor pre-mRNAs, glycine alpha2 exon 3A and $GABA_A$ exon γ2L [60]. Thus, its inhibition of exon 3 splicing is novel. However, exon 3 does not contain the putative binding site for Nova1, UCAY [61], nor does Nova1 bind any of our exon 3 mutants. Either Nova1 recognizes

additional unidentified sequences, or it does not bind directly to exon 3.

KSRP, a protein which contains a KH domain like Nova1, has been defined as an activator of the N1 exon of the src gene [81] and thus its inhibitory effect on tau exon 3 is novel. Htra2β1 shows variable behavior in other systems—it activates exon 7 of SMN2 [56] but inhibits the EN exon of clathrin [111]—and in tau it may bind to the region defined by mutant E3-1 (Fig. 5A).

In theory, full splicing regulation of tau exon 3 could be achieved by a single rate-limiting adult-specific activator present in excess in the peripheral nervous system. However, the behavior of wild-type exon 3 constructs (Fig. 1C) implies that both NT2 and SKN contain a factor which inhibits splicing of exon 3 and is overcome in SKN by the extra copies of the construct. Alternatively, SKN might contain an activator of exon 3 which is absent in NT2. This is consistent with the elevated ratio of the 3^+ isoform in the peripheral nervous system (which is the provenance of SKN). However, such an activator should act on the endogenous tau as well, but SKN shows no 3^+ isoform (Fig. 1A). Undoubtedly, our tentative assignation of binding sites will change as we delve deeper into the system, especially because binding sites for splicing factors are redundant in both sequence and partners [48,73,118].

4.3. Effects of modulation of the tau projection domain on the structure and function of the tau protein

With the recent direct linkage of tau splicing regulation to the cause of frontotemporal dementia with Parkinsonism [40], interest has quickened over the tau variants. Tau exons 2 and 3 are also of intrinsic interest in the splicing regulation field, because they fall in the very rare ‘incremental combinatorial’ category, in which the downstream exon of an alternatively spliced pair never appears by itself, but all other combinations are allowed. Such a pattern has been documented in only two other genes: exons 7 and 8 of the amyloid precursor protein [44,94] and neuron-specific exons N1 and N2 of src [89].

Regulation of exon 3 splicing may have repercussions on tau specific localization and in both normal and abnormal brain function. In the adult nervous system, the 3^+ isoform tau in oligodendrocytes is present in vanishingly low amounts [46,91], whereas it is abundant in peripheral neurons [85]. In rat hippocampus, 3^+ mRNAs are expressed only in mature neurons that have established their connections [23]. The 3^+ tau isoform has increased phosphorylation of Thr39 in the domain encoded by exon 1 [50] and Alzheimer’s brains show elevated amounts of the 3^+ isoform [74]. Phosphorylation is critical in establishing neuronal polarity by forming a gradient of progressively less phosphorylated tau down the axon [77] and equally critical in the formation of neurofibrillary tangles, which predominantly consist of hyperphosphorylated tau which has lost its ability to bind microtubules [107].

The function of the protein domain encoded by tau exon 3 is unknown. It is not involved in microtubule binding, which is localized to the C-terminus of tau [55,70] nor in microtubule spacing, which is dictated by the hinge domain of tau [20,37,63]. The N-terminus of the tau protein interacts with the plasma membrane [13], although the specific interacting ligands have not yet been identified. However, the function of the projection domain must be specific to mammals, because it is not conserved in the tau analogues of yeast, *Drosophila* or *Caenorhabditis elegans* [16,39,59,80].

The present study has uncovered sequences and factors which fine-tune the configuration of the tau projection domain. However, definitive assignments of mode and place of action will require further work in vivo and in vitro.

Acknowledgements

This work was supported by NIH RO1 grant NS38051 (A.A.), an Arthritis Foundation Medical Science Grant (R.L.), Deutsche Forschungsgemeinschaft SFB 473 (S.S.), and the Medical Research Council (G.S.). We want to thank our colleagues for their generous gifts of factor cDNAs: Drs. Joanne Yeakley and Javier Cáceres for ASF, James Patton for PTB, Benoit Chabot for hnRNPA1 and Christopher Smith for SV-αTM.

References

[1] G.J. Adema, K.L. van Hulst, P.D. Baas, Uridine branch acceptor is a *cis*-acting element involved in regulation of the alternative processing of calcitonin/CGRP-1 pre-mRNA, Nucleic Acids Res. 18 (1990) 5365–5373.

[2] B.A. Amendt, Z.H. Si, C.M. Stoltzfus, Presence of exon splicing silencers within human immunodeficiency virus type 1 tat exon 2 and tat-rev exon 3: Evidence for inhibition mediated by cellular factors, Mol. Cell. Biol. 15 (1995) 4606–4615.

[3] A. Andreadis, J.A. Broderick, K.S. Kosik, Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons, Nucleic Acids Res. 23 (1995) 3585–3593.

[4] A. Andreadis, W.M. Brown, K.S. Kosik, Structure and novel exons of the human tau gene, Biochemistry 31 (1992) 10626–10633.

[5] A. Andreadis, P.E. Nisson, K.S. Kosik, P.C. Watkins, The exon trapping assay partly discriminates against alternatively spliced exons, Nucleic Acids Res. 21 (1993) 2217–2221.

[6] M. Ashiya, P.J. Grabowski, A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart, RNA 3 (1997) 996–1015.

[7] S.M. Berget, Exon recognition in vertebrate splicing, J. Biol. Chem. 270 (1995) 2411–2414.

[8] M.D. Biggin, T.J. Gibson, G.F. Hong, Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination, Proc. Natl. Acad. Sci. USA 80 (1983) 3963–3965.

[9] L.I. Binder, A. Frankfurter, L.I. Rebhun, The distribution of tau in the mammalian central nervous system, J. Cell Biol. 101 (1986) 1371–1378.

[10] D.L. Black, Finding splice sites within a wilderness of RNA, RNA 1 (1995) 763–771.

[11] M.M. Black, T. Slaughter, S. Moshiach, M. Obrocka, I. Fischer, Tau is enriched on dynamic microtubules in the distal region of growing axons, J. Neurosci. 16 (1996) 3601–3619.

[12] B.J. Blencowe, Exonic splicing enhancers: Mechanism of action, diversity and role in human genetic diseases, Trends Biochem. Sci. 25 (2000) 106–110.

[13] R. Brandt, J. Léger, G. Lee, Interaction of tau with the neural plasma membrane mediated by tau's amino-terminal projection domain, J. Cell Biol. 131 (1995) 1327–1340.

[14] R.J. Buckanovich, J.B. Posner, R.B. Darnell, Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system, Neuron 11 (1993) 657–672.

[15] J.F. Cáceres, T. Misteli, G.R. Screaton, D.L. Spector, A.R. Krainer, Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity, J. Cell Biol. 138 (1997) 225–238.

[16] V. Cambiazo, M. Gonzalez, R.B. Maccioni, DMAP-85: a tau-like protein from *Drosophila melanogaster* larvae, J. Neurochem. 64 (1995) 1288–1297.

[17] M. Caputi, G. Casari, S. Guenzi, R. Tagliabue, A. Sidoli, C.A. Melo, F.E. Baralle, A novel bipartite splicing enhancer modulates the differential processing of the human fibronectin EDA exon, Nucleic Acids Res. 22 (1994) 1018–1022.

[18] B. Chabot, Directing alternative splicing: cast and scenarios, Trends Genet. 12 (1996) 472–478.

[19] R.C. Chan, D.L. Black, The polypyrimidine tract binding protein binds upstream of neural cell-specific c-src exon N1 to repress the splicing of the intron downstream, Mol. Cell. Biol. 17 (1997) 4667–4676.

[20] J. Chen, Y. Kanai, N.J. Cowan, N. Hirokawa, Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons, Nature 360 (1992) 674–677.

[21] S.L. Chew, L. Baginsky, I.C. Eperon, An exonic splicing silencer in the testes-specific DNA ligase III beta exon, Nucleic Acids Res. 28 (2000) 402–410.

[22] L.N. Clark, P. Poorkaj, Z. Wszolek, D.H. Geschwind, Z.S. Nasreddine, B. Miller, D. Li, H. Payami, F. Awert, K. Markopoulou, A. Andreadis, I. D'Souza, V.M. Lee, L. Reed, J.Q. Trojanowski, V. Zhukareva, T. Bird, G. Schellenberg, K.C. Wilhelmsen, Pathogenic implications of mutations in the tau gene in pallido-ponto-nigral degeneration and related neurodegenerative disorders linked to chromosome 17, Proc. Natl. Acad. Sci. USA 95 (1998) 13103–13117.

[23] J. Collet, L. Fehrat, H. Pollard, L. Ribas de Pouplana, G. Charton, A. Bernard, J. Moreau, Y. Ben-Ari, M. Khrestchatisky, Developmentally regulated alternative splicing of mRNAs encoding N-terminal tau variants in the rat hippocampus: Structural and functional implications, Eur. J. Neurosci. 9 (1997) 2723–2733.

[24] T.A. Cooper, W. Mattox, The regulation of splice-site selection, and its role in human disease, Am. J. Hum. Genet. 61 (1997) 259–266.

[25] D. Couchie, C. Mavilia, I.S. Georgieff, R.K. Liem, M.L. Shelanski, J. Nuñez, Primary structure of high molecular weight tau present in the peripheral nervous system, Proc. Natl. Acad. Sci. USA 89 (1992) 4378–4381.

[26] L.R. Coulter, M.A. Landree, T.A. Cooper, Identification of a new class of exonic splicing enhancers by in vivo selection, Mol. Cell. Biol. 17 (1997) 2143–2150.

[27] I. D'Souza, P. Poorkaj, M. Hong, D. Nochlin, V.M. Lee, T.D. Bird, G.D. Schellenberg, Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements, Proc. Natl. Acad. Sci. USA 96 (1999) 5598–5603.

[28] R. Daoud, M. Da Penha Berzaghi, F. Siedler, M. Hubener, S. Stamm, Activity-dependent regulation of alternative splicing patterns in the rat brain, Eur. J. Neurosci. 11 (1999) 788–802.

[29] F. Del Gatto, M.C. Gesnel, R. Breathnach, The exon sequence TAGG can inhibit splicing, Nucleic Acids Res. 24 (1996) 2017–2021.

[30] F. Del Gatto, A. Plet, M.C. Gesnel, C. Fort, R. Breathnach, Multiple interdependent sequence elements control splicing of a fibroblast growth factor receptor 2 alternative exon, Mol. Cell. Biol. 17 (1997) 5106–5116.

[31] F. Del Gatto-Konczak, M. Olive, M.C. Gesnel, R. Breathnach, HnRNP A1 recruited to an exon in vivo can function as an exon splicing silencer, Mol. Cell. Biol. 19 (1999) 251–260.

[32] F. Denhez, R. Lafyatis, Conservation of regulated alternative splicing and identification of functional domains in vertebrate homologs to the Drosophila splicing regulator, suppressor-of-white-apricot, J. Biol. Chem. 269 (1994) 16170–16179.

[33] J. Devereux, P. Haeberli, O. Smithies, A comprehensive set of sequence analysis programs for the VAX, Nucleic Acids Res. 12 (1984) 387–395.

[34] M. Di Fruscio, T. Chen, S. Richard, Characterization of Sam68-like mammalian proteins SLM-1 and SLM-2: SLM-1 is a src substrate during mitosis, Proc. Natl. Acad. Sci. USA 96 (1999) 2710–2715.

[35] M. DiTella, F. Feiguin, G. Morfini, A. Cáceres, Microfilament-associated growth cone component depends upon tau for its intracellular localization, Cell. Motil. Cytoskeleton 29 (1994) 117–130.

[36] D.G. Drubin, S. Kobayashi, D. Kellogg, M. Kirschner, Regulation of microtubule protein levels during cellular morphogenesis in nerve growth factor-treated PC12 cells, J. Cell Biol. 106 (1988) 1583–1591.

[37] T.F. Frappier, I.S. Georgieff, K. Brown, M.L. Shelanski, Tau regulation of microtubule–microtubule spacing and bundling, J. Neurochem. 63 (1994) 2288–2294.

[38] Q.S. Gao, J. Memmott, R. Lafyatis, S. Stamm, G. Screaton, A. Andreadis, Complex regulation of tau exon 10, whose missplicing causes frontotemporal dementia, J. Neurochem. 74 (2000) 490–500.

[39] M. Goedert, C.P. Baur, J. Ahringer, R. Jakes, M. Hasegawa, M.G. Spillantini, M.J. Smith, F. Hill, PTL-1, a microtubule-associated protein with tau-like repeats from the nematode *Caenorhabditis elegans*, J. Cell Sci. 109 (1996) 2661–2672.

[40] M. Goedert, M.G. Spillantini, Tau mutations in frontotemporal dementia FTDP-17 and their relevance for Alzheimer's disease, Biochim. Biophys. Acta 1502 (2000) 110–121.

[41] M. Goedert, M.G. Spillantini, R.A. Crowther, Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system, Proc. Natl. Acad. Sci. USA 89 (1992) 1983–1987.

[42] M. Goedert, M.G. Spillantini, R. Jakes, D. Rutherford, R.A. Crowther, Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease, Neuron 3 (1989) 519–526.

[43] M. Goedert, M.G. Spillantini, M.C. Potier, J. Ulrich, R.A. Crowther, Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain, EMBO J. 8 (1989) 393–399.

[44] T.E. Golde, S. Estus, M. Usiak, L.H. Younkin, S.G. Younkin, Expression of beta amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR, Neuron 4 (1990) 253–267.

[45] C. Gooding, G.C. Roberts, G. Moreau, B. Nadal-Ginard, C.W. Smith, Smooth muscle-specific switching of alpha-tropomyosin mutually exclusive exon selection by specific inhibition of the strong default exon, EMBO J. 13 (1994) 3861–3872.

[46] M. Gorath, T. Stahnke, T. Mronga, O. Goldbaum, C. Richter-Landsbert, Developmental changes of tau protein and mRNA in cultured rat brain oligodendrocytes, Glia 36 (2001) 89–101.

[47] P.J. Grabowski, Splicing regulation in neurons: tinkering with cell-specific control, Cell 92 (1998) 709–712.

[48] B.R. Graveley, Sorting out the complexity of SR protein functions, RNA 6 (2000) 1197–1211.

[49] B.R. Graveley, Alternative splicing: increasing diversity in the proteomic world, Trends Genet. 17 (2001) 100–107.

[50] J.A. Greenwood, C.W. Scott, R.C. Spreen, C.B. Caputo, G.V. Johnson, Casein kinase II preferentially phosphorylates human tau isoforms containing an amino-terminal insert. Identification of threonine 39 as the primary phosphate acceptor, J. Biol. Chem. 269 (1994) 4373–4380.

[51] A. Hanamura, J.F. Cáceres, A. Mayeda, B.R. Franza Jr., A.R. Krainer, Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors, RNA 4 (1998) 430–444.

[52] K. Hartmuth, A. Barta, Unusual branch point selection in processing of human growth hormone pre-mRNA, Mol. Cell. Biol. 8 (1988) 2011–2020.

[53] M. Hasegawa, M.J. Smith, M. Iijima, T. Tabira, M. Goedert, FTDP-17 mutations N279K and S305N in tau produce increased splicing of exon 10, FEBS Lett. 443 (1999) 93–96.

[54] A. Himmler, Structure of the bovine tau gene: alternatively spliced transcripts generate a protein family, Mol. Cell. Biol. 9 (1989) 1389–1396.

[55] A. Himmler, D. Drechsel, M.W. Kirschner, D.W. Martin Jr., Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains, Mol. Cell. Biol. 9 (1989) 1381–1388.

[56] Y. Hofmann, C.L. Lorson, S. Stamm, E.J. Androphy, B. Wirth, Htra2-beta1 stimulates an exonic splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2), Proc. Natl. Acad. Sci. USA 97 (2000) 9618–9623.

[57] G.S. Huh, R.O. Hynes, Elements regulating an alternatively spliced exon of the rat fibronectin gene, Mol. Cell. Biol. 13 (1993) 5301–5314.

[58] M. Hutton, C.L. Lendon, P. Rizzu, M. Baker, S. Froelich, H. Houlden, S. Pickering-Brown, S. Chakraverty, A. Isaacs, A. Grover, J. Hackett, J. Adamson, S. Lincoln, D. Dickson, P. Davies, R.C. Petersen, M. Stevens, E. de Graaff, E. Wauters, J. van Baren, M. Hillebrand, M. Joosse, J.M. Kwon, P. Nowotny, P. Heutink et al., Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17, Nature 393 (1998) 702–705.

[59] I. Irminger-Finger, E. Hurt, A. Roebuck, M.A. Collart, S.J. Edelstein, MHP1, an essential gene in *Saccharomyces cerevisiae* required for microtubule function, J. Cell. Biol. 135 (1996) 1323–1339.

[60] K.B. Jensen, B.K. Dredge, G. Stefani, R. Zhong, R.J. Buckanovich, H.J. Okano, Y.Y. Yang, R.B. Darnell, Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability, Neuron 25 (2000) 359–371.

[61] K.B. Jensen, K. Musunuru, H.A. Lewis, S.K. Burley, R.B. Darnell, The tetranucleotide UCAY directs the specific recognition of RNA by the Nova K-homology 3 domain, Proc. Natl. Acad. Sci. USA 97 (2000) 5740–5745.

[62] H. Kamma, D.S. Portman, G. Dreyfuss, Cell type-specific expression of hnRNP proteins, Exp. Cell Res. 221 (1995) 187–196.

[63] Y. Kanai, J. Chen, N. Hirokawa, Microtubule bundling by tau proteins in vivo: analysis of functional domains, EMBO J. 11 (1992) 3953–3961.

[64] S. Kawamoto, Neuron-specific alternative splicing of nonmuscle myosin II heavy chain-β pre-mRNA requires a *cis*-acting intron sequence, J. Biol. Chem. 271 (1996) 17613–17616.

[65] M. Kempf, A. Clement, A. Faissner, G. Lee, R. Brandt, Tau binds to the distal axon early in development of polarity in a microtubule-and microfilament-dependent manner, J. Neurosci. 16 (1996) 5583–5592.

[66] K.S. Kosik, L.D. Orecchio, S. Bakalis, R.L. Neve, Developmentally regulated expression of specific tau sequences, Neuron 2 (1989) 1389–1397.

[67] A. Krämer, The structure and function of proteins involved in mammalian pre-mRNA splicing, Annu. Rev. Biochem. 65 (1996) 367–409.

[68] E.S. Lander, L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A. Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier et al., Initial sequencing and analysis of the human genome, Nature 409 (2001) 860–921.

[69] G. Lee, N. Cowan, M. Kirschner, The primary structure and heterogeneity of tau protein from mouse brain, Science 239 (1988) 285–288.

[70] G. Lee, R.L. Neve, K.S. Kosik, The microtubule binding domain of tau protein, Neuron 2 (1989) 1615–1624.

[71] R. Lemaire, A. Winne, M. Sarkissian, R. Lafyatis, SF2 and SRp55 regulation of CD45 exon 4 skipping during T cell activation, Eur. J. Immunol. 29 (1999) 823–837.

[72] D. Libri, L. Balvay, M.Y. Fiszman, In vivo splicing of the beta tropomyosin pre-mRNA: a role for branch point and donor site competition, Mol. Cell. Biol. 12 (1992) 3204–3215.

[73] H.X. Liu, M. Zhang, A.R. Krainer, Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins, Genes Dev. 12 (1998) 1998–2012.

[74] W.K. Liu, D.W. Dickson, S.H. Yen, Heterogeneity of tau proteins in Alzheimer's disease. Evidence for increased expression of an isoform and preferential distribution of a phosphorylated isoform in neurites, Am. J. Pathol. 142 (1993) 387–394.

[75] A.J. López, Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation, Annu. Rev. Genet. 32 (1998) 279–305.

[76] P. LoPresti, S. Szuchet, S.C. Papasozomenos, R.P. Zinkowski, L.I. Binder, Functional implications for the microtubule-associated protein tau: localization in oligodendrocytes, Proc. Natl. Acad. Sci. USA 92 (1995) 10369–10373.

[77] J.W. Mandell, G.A. Banker, A spatial gradient of tau protein phosphorylation in nascent axons, J. Neurosci. 16 (1996) 5727–5740.

[78] J.L. Manley, R. Tacke, SR proteins and splicing control, Genes Dev. 10 (1996) 1569–1579.

[79] V. Markovtsov, J.M. Nikolic, J.A. Goldman, C.W. Turck, M.Y. Chou, D.L. Black, Cooperative assembly of an hnRNP complex induced by a tissue-specific homolog of polypyrimidine tract binding protein, Mol. Cell. Biol. 20 (2000) 7463–7479.

[80] J.B. McDermott, S. Aamodt, E. Aamodt, PTL-1, a *Caenorhabditis elegans* gene whose products are homologous to the tau microtubule-associated proteins, Biochemistry 35 (1996) 9415–9423.

[81] H. Min, C.W. Turck, J.M. Nikolic, D.L. Black, A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer, Genes Dev. 11 (1997) 1023–1036.

[82] O. Nayler, C. Cap, S. Stamm, Human transformer-2-beta gene (SFRS10): complete nucleotide sequence, chromosomal localization, and generation of a tissue-specific isoform, Genomics 53 (1998) 191–202.

[83] R.L. Neve, P. Harris, K.S. Kosik, D.M. Kurnit, T.A. Donlon, Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2, Brain Res. 387 (1986) 271–280.

[84] P.A. Norton, Polypyrimidine tract sequences direct selection of alternative branch sites and influence protein binding, Nucleic Acids Res. 22 (1994) 3854–3860.
[85] J. Nuñez, I. Fischer, Microtubule-associated proteins (MAPs) in the peripheral nervous system during development and regeneration, J. Mol. Neurosci. 8 (1997) 207–222.
[86] J.G. Patton, S.A. Mayer, P. Tempst, B. Nadal-Ginard, Characterization and molecular cloning of polypyrimidine tract-binding protein: a component of a complex necessary for pre-mRNA splicing, Genes Dev. 5 (1991) 1237–1251.
[87] I. Pérez, C.H. Lin, J.G. McAfee, J.G. Patton, Mutation of PTB binding sites causes misregulation of alternative 3′ splice site selection in vivo, RNA 3 (1997) 764–778.
[88] A.D. Polydorides, H.J. Okano, Y.Y. Yang, G. Stefani, R.B. Darnell, A brain-enriched polypyrimidine tract-binding protein antagonizes the ability of Nova to regulate neuron-specific alternative splicing, Proc. Natl. Acad. Sci. USA 97 (2000) 6350–6355.
[89] J.M. Pyper, J.B. Bolen, Identification of a novel neuronal c-src exon expressed in human brain, Mol. Cell. Biol. 10 (1990) 2035–2040.
[90] R. Reed, Initial splice-site recognition and pairing during pre-mRNA splicing, Curr. Opin. Genet. Dev. 6 (1996) 215–220.
[91] C. Richter-Landsberg, M. Gorath, Developmental regulation of alternatively spliced isoforms of mRNA encoding MAP2 and tau in rat brain oligodendrocytes during culture maturation, J. Neurosci. Res. 56 (1999) 259–270.
[92] E. Sadot, R. Marx, J. Barg, L. Behar, I. Ginzburg, Complete sequence of 3′-untranslated region of tau from rat central nervous system. Implications for mRNA heterogeneity, J. Mol. Biol. 241 (1994) 325–331.
[93] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[94] R. Sandbrink, R. Banati, C.L. Masters, K. Beyreuther, G. Konig, Expression of β-APP mRNA in brain cells, Ann. N. Y. Acad. Sci. 695 (1993) 183–189.
[95] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.
[96] M. Sarkissian, A. Winne, R. Lafyatis, The mammalian homolog of suppressor-of-white-apricot regulates alternative mRNA splicing of CD45 exon 4 and fibronectin IIICS, J. Biol. Chem. 271 (1996) 31106–31114.
[97] G.R. Screaton, J.F. Cáceres, A. Mayeda, M.V. Bell, M. Plebanski, D.G. Jackson, J.I. Bell, A.R. Krainer, Identification and characterization of three members of the human SR family of pre-mRNA splicing factors, EMBO J. 14 (1995) 4336–4349.
[98] P.A. Sharp, Split genes and RNA splicing, Cell 77 (1994) 805–815.
[99] Z. Si, B.A. Amendt, C.M. Stoltzfus, Splicing efficiency of human immunodeficiency virus type 1 tat RNA is determined by both a suboptimal 3′ splice site and a 10 nucleotide exon splicing silencer element located within tat exon 2, Nucleic Acids Res. 25 (1997) 861–867.
[100] Z.H. Si, D. Rauch, C.M. Stoltzfus, The exon splicing silencer in human immunodeficiency virus type 1 tat exon 3 is bipartite and acts early in spliceosome assembly, Mol. Cell. Biol. 18 (1998) 5404–5413.
[101] R. Singh, J. Valcárcel, M.R. Green, Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins, Science 268 (1995) 1173–1176.
[102] C.W. Smith, B. Nadal-Ginard, Mutually exclusive splicing of alpha-tropomyosin exons enforced by an unusual lariat branch point location: Implications for constitutive splicing, Cell 56 (1989) 749–758.
[103] C.W. Smith, J.G. Patton, B. Nadal-Ginard, Alternative splicing in the control of gene expression, Annu. Rev. Genet. 23 (1989) 527–577.
[104] C.W. Smith, E.B. Porro, J.G. Patton, B. Nadal-Ginard, Scanning from an independently specified branch point defines the 3′ splice site of mammalian introns, Nature 342 (1989) 243–247.
[105] C.W. Smith, J. Valcárcel, Alternative pre-mRNA splicing: the logic of combinatorial control, Trends Biochem. Sci. 25 (2000) 381–388.
[106] J. Southby, C. Gooding, C.W. Smith, Polypyrimidine tract binding protein functions as a repressor to regulate alternative splicing of alpha-actinin mutally exclusive exons, Mol. Cell. Biol. 19 (1999) 2699–2711.
[107] M.G. Spillantini, M. Goedert, Tau protein pathology in neurodegenerative diseases, Trends Neurosci. 21 (1998) 428–433.
[108] M.G. Spillantini, J.R. Murrell, M. Goedert, M.R. Farlow, A. Klug, B. Ghetti, Mutation in the tau gene in familial multiple system tauopathy with presenile dementia, Proc. Natl. Acad. Sci. USA 95 (1998) 7737–7741.
[109] A. Staffa, N.H. Acheson, A. Cochrane, Novel exonic elements that modulate splicing of the human fibronectin EDA exon, J. Biol. Chem. 272 (1997) 33394–33401.
[110] A. Staffa, A. Cochrane, Identification of positive and negative splicing regulatory elements within the terminal tat-rev exon of human immunodeficiency virus type 1, Mol. Cell. Biol. 15 (1995) 4597–4605.
[111] S. Stamm, D. Casper, V. Hanson, D.M. Helfman, Regulation of the neuron-specific exon of clathrin light chain B, Brain Res. Mol. Brain Res. 64 (1999) 108–118.
[112] S. Stamm, J. Zhu, K. Nakai, P. Stoilov, O. Stoss, M.Q. Zhang, An alternative-exon database and its statistical analysis, DNA Cell Biol. 19 (2000) 739–756.
[113] O. Stoss, M. Olbrich, A.M. Hartmann, H. Konig, J. Memmott, A. Andreadis, S. Stamm, The STAR/GCG family protein rSLM-2 regulates the selection of alternative splice sites, J. Biol. Chem. 276 (2001) 8665–8673.
[114] O. Stoss, P. Stoilov, R. Daoud, A.M. Hartman, M. Olbrich, S. Stamm, Misregulation of pre-mRNA splicing that causes human diseases, Gene Ther. Mol. Biol. 5 (2000) 9–28.
[115] T. Tsukahara, N. Kunika, T. Momoi, K. Arahata, Regulation of alternative splicing in the amyloid precursor protein (APP) mRNA during neuronal and glial differentiation of P19 embryonal carcinoma cells, Brain Res. 679 (1995) 178–183.
[116] J. Valcárcel, R.K. Gaur, R. Singh, M.R. Green, Interaction of U2AF65 RS region with pre-mRNA branch point and promotion of base pairing with U2 snRNA [corrected], Science 273 (1996) 1706–1709.
[117] J. Valcárcel, F. Gebauer, Post-transcriptional regulation: The dawn of PTB, Curr. Biol. 7 (1997) R705–708.
[118] J. Valcárcel, M.R. Green, The SR protein family: pleiotropic functions in pre-mRNA splicing, Trends Biochem. Sci. 21 (1996) 296–301.
[119] Y. Wang, P.A. Loomis, R.P. Zinkowski, L.I. Binder, A novel tau transcript in cultured human neuroblastoma cells expressing nuclear tau, J. Cell Biol. 121 (1993) 257–267.
[120] M.L. Wei, A. Andreadis, Splicing of a regulated exon reveals additional complexity in the axonal microtubule-associated protein tau, J. Neurochem. 70 (1998) 1346–1356.
[121] M.L. Wei, J. Memmott, G. Screaton, A. Andreadis, The splicing determinants of a regulated exon in the axonal MAP tau reside within the exon and in its upstream intron, Brain Res. Mol. Brain Res. 80 (2000) 207–218.
[122] X. Yang, M.R. Bani, S.J. Lu, S. Rowan, Y. Ben-David, B. Chabot, The A1 and A1b proteins of heterogeneous nuclear ribonucleoparticles modulate 5′ splice site selection in vivo, Proc. Natl. Acad. Sci. USA 91 (1994) 6924–6928.
[123] A.M. Zahler, K.M. Neugebauer, W.S. Lane, M.B. Roth, Distinct functions of SR proteins in alternative pre-mRNA splicing, Science 260 (1993) 219–222.
[124] L. Zhang, W. Liu, P.J. Grabowski, Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB, RNA 5 (1999) 117–130.