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Tau exon 10, whose missplicing causes frontotemporal dementia, is regulated by an intricate interplay of *cis* elements and *trans* factors

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Abstract

Tau is a microtubule-associated protein whose transcript undergoes complex regulated splicing in the mammalian nervous system. In humans, exon 10 of the gene is an alternatively spliced cassette which is adult-specific and which codes for a microtubule binding domain. Mutations that affect splicing of exon 10 have been shown to cause inherited frontotemporal dementia (FTDP). In this study, we reconstituted naturally occurring exon 10 FTDP mutants and classified their effects on its splicing. We also carried out a comprehensive survey of the influence of splicing regulators on exon 10 inclusion and tentatively identified the site of action for several of these factors. Lastly, we identified the domains of regulators SWAP and hnRNPG, which are required for regulation of exon 10 splicing.

Keywords: alternative splicing, *cis* regulatory element and *trans* splicing regulator, frontotemporal dementia, MAP tau, regulated isoform.

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Alternative splicing is a versatile and widespread mechanism for generating multiple mRNAs from a single transcript (Grabowski 1998; Graveley 2001). Splicing choices are spatially and temporally regulated and the ensuing mRNAs produce functionally diverse proteins, contributing significantly to proteomic complexity (Graveley 2001; Black 2003).

Splicing is carried out by the spliceosome, a large and dynamic complex of proteins and small RNAs (Neubauer *et al*. 1998). A major question in splicing, and an obvious point of regulation, is how the spliceosome recognizes authentic splicing sites. The rules governing splice site selection are not fully understood; combinatorial control is used to enable precise recognition of the short and degenerate splice sites (Smith and Valcárcel 2000). Despite the high fidelity of exon recognition *in vivo*, it is currently impossible to accurately predict alternative exons (Thanaraj and Stamm 2003).

Numerous studies of *cis* determinants of alternative splicing have shown that splice site selection occurs via hierarchies defined by complementarity of splice sites, branch points and their associated polypyrimidine tracts to their cognate snRNPs (Grabowski 1998; Smith and Valcárcel 2000). In an increasing number of systems, exon inclusion is

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Abbreviations used: AD, Alzheimer's disease; CELF, CUG-BP- and ETR-3-like factor; FCS, fetal calf serum; (k)bp, (kilo)base pairs; FTDP-17, frontotemporal dementia with Parkinsonism associated with chromosome 17; hnRNP, heterogeneous nuclear protein; htra, human transformer; KSRP, KH-type splicing regulatory protein; MAP, microtubule-associated protein; nt, nucleotides; NFT, neurofibrillary tangle; NT2, N-Tera2 human teratocarcinoma cells; polyY, polypyrimidine; PTB, polypyrimidine tract binding protein; RRM, RNA recognition motif; R/S, arginine/serine; SKN, SK-N-SH human neuroblastoma cells; SLM, Sam68-like mammalian protein; snRNPs, small nuclear riboproteins; SR, proteins, serine/arginine-rich proteins; SWAP, suppressor of white apricot protein; U2AF, U2snRNP auxiliary factor.

achieved by use of exonic enhancers (Blencowe 2000; Cartegni *et al.* 2002). Their mutation can result in human disease by causing aberrant splicing (Stoilov *et al*. 2002; Faustino and Cooper 2003). However, regulation is sometimes conferred by exonic silencers or intronic elements (Chabot *et al*. 1997; Staffa and Cochrane 1995; Del Gatto *et al*. 1996; Staffa *et al*. 1997; Si *et al*. 1998; Gao *et al*. 2000; Ladd *et al*. 2001).

On the *trans* side of regulation, mammalian-splicing regulators mostly belong to two superfamilies, the SR/SR-like and hnRNP proteins, neither of which is exclusively involved in alternative splicing (Dreyfuss *et al*. 2002; Graveley 2000). The former are also components of the spliceosome, whereas the latter are also involved in prem-RNA transport, RNA stability and translational regulation. Several mammalian splicing factors are enhanced in or restricted to neurons: htra2β3 (Nayler *et al*. 1998), KSRP (Min *et al*. 1997), Nova1 (Buckanovich and Darnell 1997), nPTB (Markovtsov *et al*. 2000; Polydorides *et al*. 2000), SWAP (Sarkissian *et al*. 1996). Nevertheless, it appears that the exquisite calibration of mammalian alternative splicing is primarily achieved by SR and hnRNP proteins that show distinct ratios in tissues and during development, despite their ubiquitous distribution (Kamma *et al*. 1995; Hanamura *et al*. 1998).

Tau is a microtubule-associated protein enriched in axons of mature and growing neurons (Kempf *et al*. 1996). Tau is also found in the cell nucleus (Wang *et al*. 1993), in the distal ends of growing neurons (DiTella *et al*. 1994; Black *et al*. 1996), in oligodendrocytes (Gorath *et al*. 2001) and in muscle (Wei and Andreadis 1998). Hyperphosphorylated, microtubule-dissociated tau protein is the major component of neurofibrillary tangles, a hallmark of several neurodegenerative diseases (Spillantini and Goedert 1998).

Tau is encoded by a single copy gene (Himmler 1989). It produces three transcripts of 2, 6 and 9 kb which are differentially expressed and localized in the nervous system, depending upon stage of neuronal maturation and neuron type (Goedert *et al*. 1989a, 1989b; Wang *et al*. 1993; Nuñez and Fischer 1997). The three tau transcripts undergo complex alternative splicing: six of the 16 tau exons are regulated cassettes (Andreadis *et al*. 1992; Goedert *et al*. 1989a, 1989b; Himmler 1989; Himmler *et al*. 1989; Kosik *et al*. 1989; Gao *et al*. 2000).

The N-terminus of the tau protein interacts with the plasma membrane (Brandt *et al*. 1995). The C-terminus of the tau protein contains four imperfect repeats (encoded by exons 9–12) which act as microtubule binding domains (Lee *et al*. 1989). Exon 10 is a cassette that codes for a complete additional microtubule-binding domain. Its inclusion increases the affinity of tau for microtubules (Lee *et al*. 1989). Splicing of exon 10 is under developmental and cell type-specific regulation. Exon 10 is adult-specific in rodents and humans (Goedert *et al*. 1989a, 1989b; Kosik *et al*. 1989) but with a crucial difference relevant to neurodegeneration: in adult rodents, exon 10 becomes constitutive (Kosik *et al*. 1989). In contrast, in adult humans exon 10 remains regulated in the central nervous system (Goedert *et al*. 1989b; Gao *et al*. 2000). The difference most likely arises from the details of the *cis* sequences flanking exon 10 in various organisms (Grover *et al*. 1999; Poorkaj *et al*. 2001), which in turn affect the regulation exerted by *trans* factors.

Investigations of dementia pedigrees have established that missplicing of tau exon 10 can cause inherited frontotemporal dementia with parkinsonism (FTDP-17), almost certainly by disturbing the normal tau isoform ratio (reviewed in Ingram and Spillantini 2002). The FTDP mutations initially clustered around the 5′ splice site of exon 10, engendering the hypothesis that its splicing is partly modulated by a putative hairpin loop, which inhibits interaction with the U1 snRNP (Hutton *et al*. 1998; Grover *et al*. 1999). However, other laboratories, as well as ours, have shown that exon 10 splicing is affected by additional intronic and exonic sequences (D'Souza *et al*. 1999; D'Souza and Schellenberg 2000, 2002; Gao *et al*. 2000; Stanford *et al*. 2003). A number of *trans* acting factors regulating these sequences have been identified (Gao *et al*. 2000; Jiang *et al*. 2003) and it has shown that their action is regulated by phosphorylation (Hartmann *et al*. 2001).

In a previous study (Gao *et al*. 2000), we showed that splicing of exon 10 is influenced by cell type, the identity of its flanking exons, sequences within and near the exon itself and several splicing regulators. In this report we show that exon 10 splicing is influenced by the extent of flanking introns and by additional *cis* sequences. We also systematically survey splicing regulators for their effect on exon 10 splicing and focus on two of them, SWAP and hnRNPG.

Materials and methods

Plasmid construction and mutagenesis

The human tau genomic fragments originated from cosmid or λ clones (Andreadis *et al*. 1992). The starting exon 10 construct was SV9/10L/11 (Fig. 1a; Gao *et al.* 2000). We generated SP/10L by isolating an EcoRI fragment from SV9/10L/11 containing exon 10 and its flanking introns and inserting it into pSPL3 (Invitrogen). Both constructs contain tau exon 10, 471 bp of its upstream intron and 408 bp of its downstream intron. The flanking exons are: SV9/10L/11, tau exons 9 and 11; SP/10L, tat exons 2 and 3. The additional intron lengths shown in Fig. 1(a) are from the vectors.

Constructs reproducing several FTDP pedigree mutations (ENH, SIL, M5 and M14) had been created in SV9/10L/11 by directed mutagenesis (Gao *et al*. 2000). Constructs bearing additional FTDP pedigree or scanning mutations were also originally created in SV9/10L/11 (Δ5, Δ11, N296N, M3). The primers used for mutagenesis are listed in Gao *et al*. (the former set) or in Table 1 (the latter set) and the alterations to wild-type exon 10 are shown in Fig. 2(a). To generate the mutant constructs, whole-plasmid mutagenesis was performed on SV9/10L/11 using the QuikChange mutagenesis kit

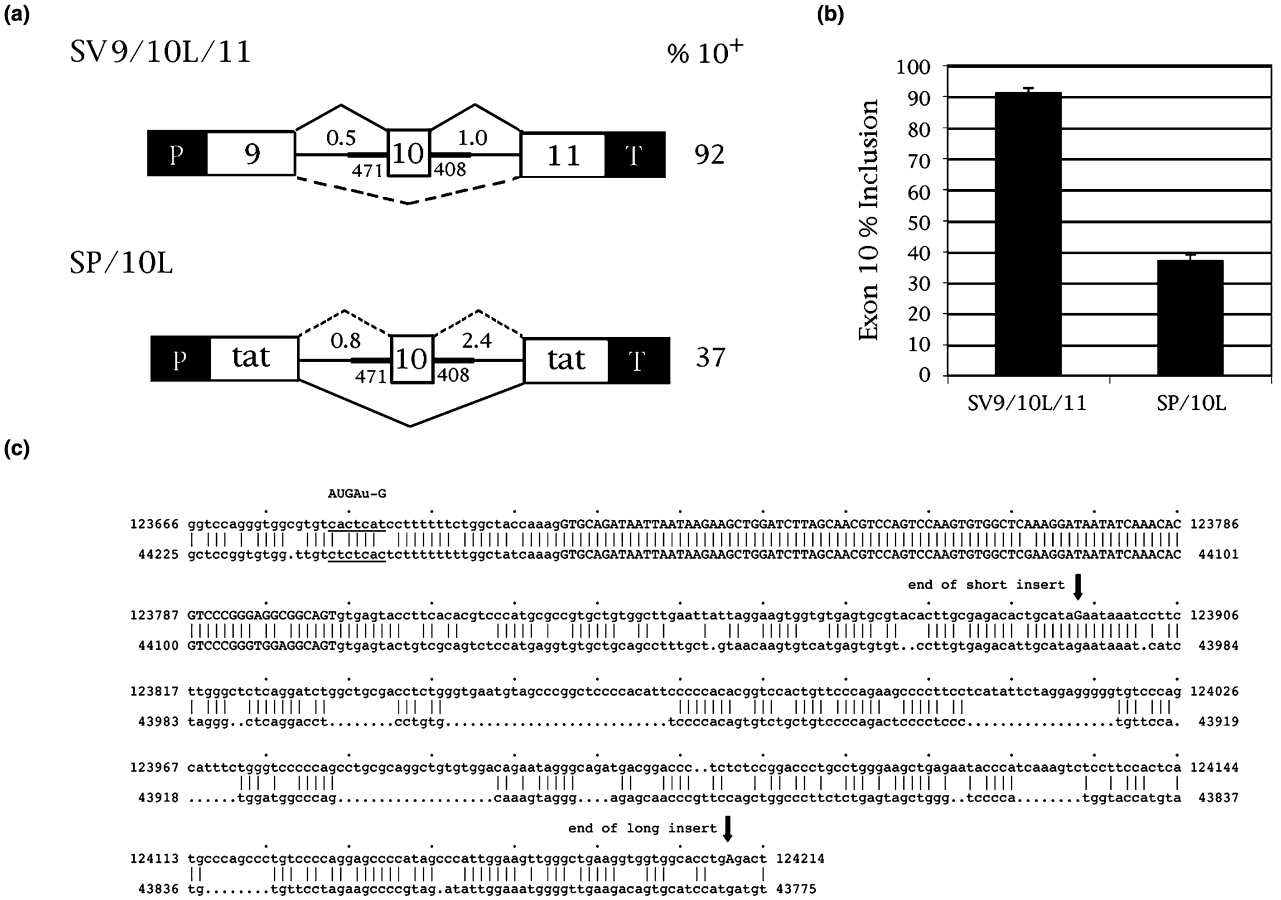

Fig. 1 The behavior of human tau exon 10 depends on its flanking exons. (a) Schematic representation of expression constructs SV9/10L/11 and SP/10L. P and T represent the vector promoters and terminators. Introns and exon junctions in the vector portions of the constructs are not shown for the sake of clarity. The numbers on each side of exon 10 show (above) how many kilobases of flanking introns are present and (below) the extent of native introns flanking exon 10. Major splicing pathways are shown by solid, minor by dashed lines. The numbers to the right of each construct indicate percentage of exon 10 inclusion in COS cells. (b) RT-PCR of SV9/10L/11 and SP/10L transfected into COS cells. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program. Primer pairs: INS1/INS3 for SV9/10L/11, SPL-LS/SPL-LN for SP/10L. (c) Sequence comparison of tau exon 10 and its flanking introns between human (top line) and mouse (bottom line). Exonic sequences are in uppercase, intronic in lowercase. The putative branch point is shaded; above it is shown the complementarity to the U2 snRNA (by uppercase letters). The sequence numbering is from the full-length genomic sequences of the human and mouse tau gene, accession numbers AC091628.gb_pr and AC091629.gb_ro, respectively.

(Stratagene) as previously described (Arikan *et al*. 2002). The resulting plasmids were sequenced to verify the presence of the desired mutations and the absence of undesirable ones. All mutants were recreated in SPL3 by digestion with EcoRI and conventional ligation.

Vectors bearing splicing factor cDNAs expressed them from the following promoters: CMV for hnRNPA1, hnRNPG, htra2β1, Nova1, SLM1, SLM2, CELF3, CELF4, U2AF, nPTB; SR proteins 20, ASF/SF2, 30c, 9G8, SC35, 40, 55, 75 (Yang *et al*. 1994; Screaton *et al*. 1995; Cáceres *et al*. 1997; Nayler *et al*. 1998; Gao *et al*. 2000); adenovirus major late for PTB (Patton *et al*. 1991); SV40 early for SWAP (Lemaire *et al*. 1999); Maloney leukemia virus LTR for KSRP (Min *et al*. 1997). Previous western blots as well as *in vivo* cotransfections (described in the references just listed) have shown that these constructs express functional proteins.

SWAP deletion mutants ΔS, ΔST, ΔH, ΔBB and RS have been previously described [Lemaire *et al*. 1999; shown in Fig. 5(a)]. Rat hnRNPG deletion mutants were generated by amplifying the fragments diagrammed in Fig. 6(a) by PCR and then subcloning them into pEGFP-C2 (Clontech).

Cell culture, transfections and RNA preparation

Monkey kidney (COS) cells were maintained as previously described (Gao *et al*. 2000). Plasmid DNA was prepared by cesium chloride banding or Qiagen Tip-50 s and introduced into cells by the lipofection method (LT1, Panvera). Total RNA was isolated by the TRIzol method (Invitrogen).

Table 1 Primers used in PCR

Name	Length	Orientation	Location	Sequence
Primers used for mutant constructs*				
N296NS	29	Sense	Within exon 10	GGCTCAAAGGATAACATCAAACACGTCCC
N296NN	29	Antisense	Within exon 10	Reverse complement of N296NS
10Δ5S	30	Sense	Within exon 10	GTGCAGATAATTAATAAGCTGGATCTTAGC
10Δ5N	30	Antisense	Within exon 10	Reverse complement of 10Δ5S
10Δ11S	30	Sense	Within exon 10	AAGCTGGATCTTAGCGTCCAGTCCAAGGTG
10Δ11N	30	Antisense	Within exon 10	Reverse complement of 10Δ11S
10M3S	31	Sense	In proximal 3′ intron of exon 10	GGGAGGCGGCAGTGTAAGTACCTTCACACGT
10M3N	31	Antisense	In proximal 3′ intron of exon 10	Reverse complement of 10M3S
Primers used for RT-PCR				
SPL-LS	27	Sense	Within tat exon	TCTGAGTCACCTGGACAACCTCAAAGG
SPL-LN	27	Antisense	Within tat exon	ATCTCAGTGGTATTTGTGAGCCAGGGC

*The point mutations in these are shown in Fig. 2(a).

Reverse transcription and PCR reactions

For PCR analysis of RNA, 5 μg of total RNA from transiently transfected cells were reverse transcribed using RNAase H^- Superscript (Invitrogen), in 20 μL for 1 h at 42 °C. Three microliters of this reaction mix were then added to two Ready-to-Go PCR beads (Pharmacia) in a total volume of 50 μL and the mixture was amplified for 25 cycles. The PCR conditions were: for SV constructs and primer pair INS1/INS3, denaturation 94 °C/1 min, annealing 65 °C/1 min, extension 72 °C/1 min; for SP constructs and primer pair SPL-LS/SPL-LN, the single change was that annealing occurred at 60 °C.

Some PCR amplifications were done using the Promega priming kit and [α-^{32}P]dCTP. Unlabeled RT-PCR products were electrophoresed on 3% Nusieve agarose (Amresco)/1 × TAE gels and visualized by ethidium bromide. Radioactive RT-PCR products were electrophoresed on 5% acrylamide/1 × TBE gels, which were dried, then exposed against Kodak film at −70 °C overnight.

The RT-PCR experiments were carried out with total RNAs from at least 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 Laboratory analysis software from Scanalytics. For the expression constructs, the predicted sizes of the $10^-/10^+$ RT-PCR products are: 336/429 for SV9/10L/11 and 270/363 for SP/10L.

Results

The identity of flanking exons and the length of flanking introns influence exon 10 splicing

Comparison of the behavior of exon 10 constructs from our work and from other laboratories led us to conclude that splicing of exon 10 is strongly influenced by the identity of its flanking exons. If exon 10 is flanked either by insulin exons 2 and 3 or its own native exons 9 and 11, it is almost 100% included in COS cells (Gao *et al*. 2000). However, if the same fragment is inserted into the exon-trapping vector pSPL3, exon 10 inclusion drops significantly (Fig. 1b). This result is not surprising, since the tat exons of pSPL have been shown to discriminate against alternatively spliced exons (Andreadis *et al*. 1993).

Most interestingly, flanking intron length also becomes a player in exon 10 splicing in neuroblastoma cells (Gao *et al*. 2000) or in the SPL background. The constructs of all other laboratories, which are based on SPL3 and contain flanking introns of ∼100 nucleotides native to exon 10, show 50% exon 10 inclusion (Clark *et al*. 1998; Hutton *et al*. 1998; D'Souza *et al*. 1999; Grover *et al*. 1999; Hasegawa *et al*. 1999). Our construct SP/10L, which is also based on SPL3 but contains flanking introns of ∼400 nucleotides native to exon 10, shows 37% exon 10 inclusion (Fig. 1 and the leftmost lane in Figs 2–6).

Recreation of FTDP mutations indicates that the splicing of exon 10 is modulated by a multitude of *cis* elements within or near the exon

Several colleagues have investigated the splicing behavior of exon 10 mutations in the context of short flanking introns and heterologous (SPL) flanking exons (D'Souza *et al*. 1999; Grover *et al*. 1999; Hasegawa *et al*. 1999). In our previous study of exon 10 (Gao *et al*. 2000) we extended their observations by examining several of these mutants in the 9/10L/11 background; that is, in the presence of large native flanking introns and homologous flanking exons. Here we examine previous and additional *cis* mutants in the presence of large flanking introns and heterologous (SPL) flanking exons. This particular combination is interesting because it renders the system most sensitive to fluctuations and allows weighing of contributions from discrete elements.

Thus, we recreated several mutations within exon 10 and its proximal 3′ intron, shown in Fig. 2(a). All these mutations, with the exception of Δ11, were originally found

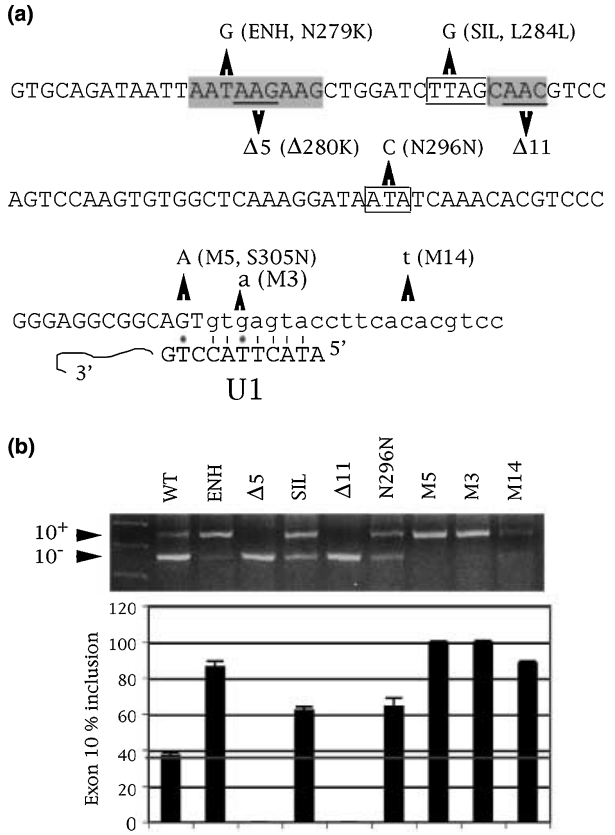

Fig. 2 Human tau exon 10 contains a bevy of *cis* elements involved in its splicing regulation. (a) The sequence of exon 10 and its proximal downstream intron. Exonic sequences are in uppercase, intronic in lowercase. Point mutations are indicated, as well as their resulting missense mutations, if any. Deletions are underlined. The boxed regions define enhancers (gray) or silencers (white). Also shown is the complementarity of the 5′ splice site with the U1 snRNA. (b) RT-PCR of the mutant constructs transfected in COS cells. The identities of the spliced species are indicated. Primer pair: SPL-LS/SPL-LN. The gray bar across the graph shows the level of the wild type construct SP/10L. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program.

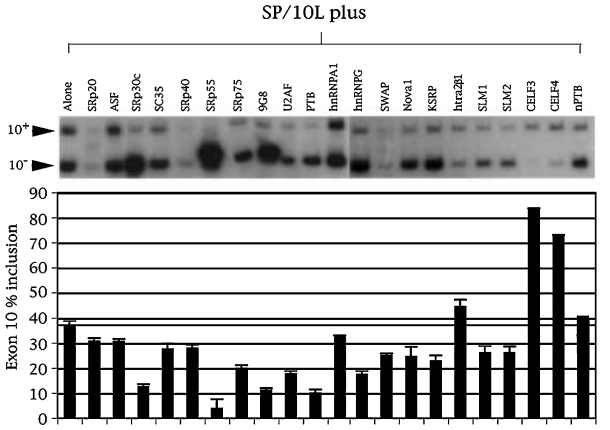

Fig. 3 Several splicing regulators affect the expression of exon 10. The RT-PCR products are from 1 : 1 cotransfections of SP/10L with the factors indicated in COS cells. Primer pair: SPL-LS/SPL-LN. The gray bar across the graph shows the level of SP/10L in the absence of factors. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program.

in FTDP pedigrees (Clark *et al*. 1998; Hutton *et al*. 1998; D'Souza *et al*. 1999; Grover *et al*. 1999; Hasegawa *et al*. 1999; reviewed by Ingram and Spillantini 2002). Mutants Δ5 and Δ11 have been shown to strongly decrease inclusion of exon 10, whereas the rest increase inclusion of exon 10 to varying extents. These effects are seen in both the FTDP pedigrees and in constructs incorporating the mutations.

In our previous work, we examined mutants ENH, SIL, M5 and M14 (Gao *et al*. 2000). These mutants shifted splicing exclusively to the 10^+ isoform whether exon 10 was flanked by insulin or tau exons and regardless of the length of the flanking introns.

The SP/10L configuration allows us to discriminate more finely among mutational effects. Figure 2(b) shows that the two mutations proximal to the 5′ splice site of exon 10 (M5 and M3) still shift splicing exclusively to the 10^+ isoform. These two mutants create a 5′ splice site capable of stronger binding to the U1 snRNP than the wild type. Mutant M14, located further downstream in the intron, increases exon 10 inclusion less strongly, followed in relative effect by exonic mutants ENH, SIL and N296N (Fig. 2). On the other hand, mutations Δ5 and Δ11 completely obliterate the 10^+ isoform. All these results are in complete agreement with observations of other investigators (Hutton *et al*. 1998; D'Souza *et al*. 1999; Grover *et al*. 1999; Hasegawa *et al*. 1999).

Thus, two regions within exon 10 (one defined by ENH and Δ5, the other by Δ11) act as strong splicing enhancers. This is not unexpected, since their sequences correspond, respectively, to GAR and ACE splicing enhancer motifs. The N296N and SIL (L284L) mutants define relatively weak exonic silencers. Moreover, the effect of the SIL-defined silencer is dependent on context: recreating the TTAG sequence within constitutive tau exon 7 has no effects on its splicing (data not shown). Finally, the region overlapping the 5′ splice site of exon 10 and defined by mutants M5, M3 and M14 exerts a strong silencing effect on exon 10 splicing.

Several splicing regulators modulate exon 10 inclusion

Both the developmental profile (Goedert *et al*. 1989a; Himmler *et al*. 1989; Kosik *et al*. 1989; Wei and Andreadis 1998) and the default behavior of tau exon 10 (Gao *et al*. 2000) point to regulation by at least one inhibitor. In our previous work, we concentrated exclusively on identifying splicing regulators which inhibit exon 10 splicing. However, the behavior of exon 10 in adult PNS and its regulation by enhancers also suggests activating factors. To identify

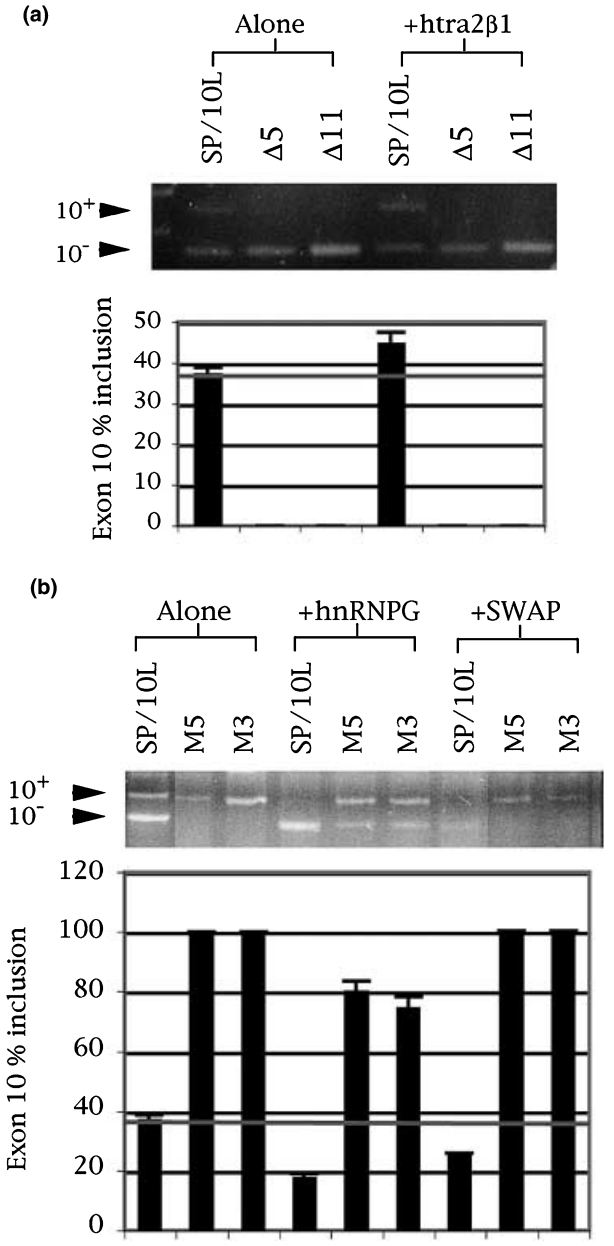

Fig. 4 Interaction of the strongest *cis* elements of exon 10 with selected *trans* regulators. The RT-PCR products come from 1 : 1 cotransfections of wild-type and mutated exon 10 constructs with the factors indicated. Mutations which define enhancers were paired with an activator, whereas mutations which define silencers were paired with two inhibitors. RT-PCRs from COS cells of (a) htra2β1 with mutants Δ5 and Δ11 (b) hnRNPG and SWAP with mutants M5 and M3. Primer pair: SPL-LS/SPL-LN. The gray bar across the graph shows the level of SP/10L in the absence of mutations or factors. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program.

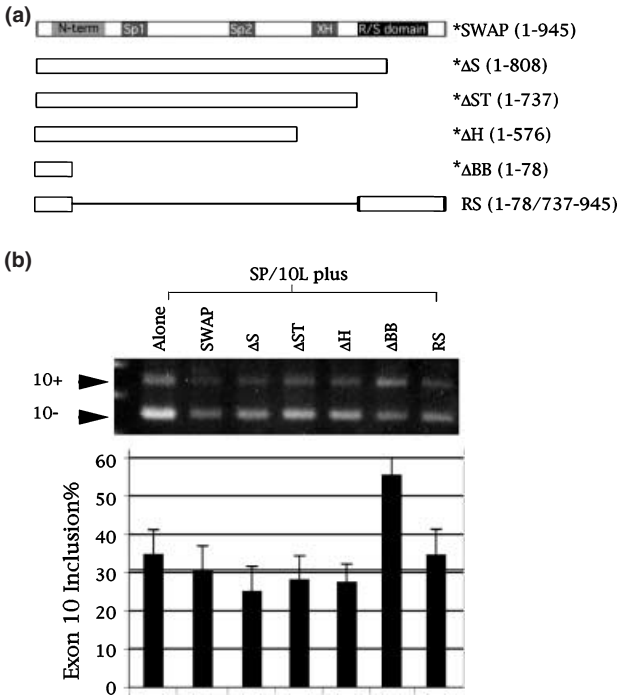

Fig. 5 The surp domains of SWAP are required for regulation of exon 10. The RT-PCR products come from 1 : 1 cotransfections of SP/10L with SWAP deletion mutants. (a) Diagram of the SWAP deletion variants (adapted from Lemaire *et al.* 1999). The long N-terminal homology (N-term), surp homology regions (SP), additional downstream conserved region (XH) and RS domains are indicated. Numbers in parentheses show the amino acid composition of the variants. The mutants which influence exon 10 splicing are accompanied by asterisks. (b) RT-PCR of the cotransfections of SP/10L with SWAP mutants in COS cells. Primer pair: SPL-LS/SPL-LN. The gray bar across the graph shows the level of SP/10L in the presence of full length SWAP. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program.

candidates in each category, we did cotransfection experiments using known splicing regulators and construct SP/10L, whose behavior allows characterization of both activators and inhibitors.

Our previous work showed that inclusion of exon 10 is inhibited by constitutive factors ASF/SF2, SRp55, SRp75, U2AF65 and the tissue-specific regulator SWAP (Gao *et al.* 2000) and by hyperphosphorylation (Hartmann *et al.* 2001). Our present work extends the factor list and shows that most of the splicing regulators inhibit inclusion of exon 10 to varying degrees (Fig. 3). Seven factors act as strong inhibitors (more than 50% downshift): the ubiquitous factors SRp30c, SRp55, SRp75, 9G8, U2AF, PTB and hnRNPG. Three factors act as activators: htra2β1, CELF3 and CELF4 (Fig. 3). All the other factors tested have no effect or act as weak-to-moderate inhibitors. The results are consistent with our previous results (Gao *et al.* 2000) and with the work

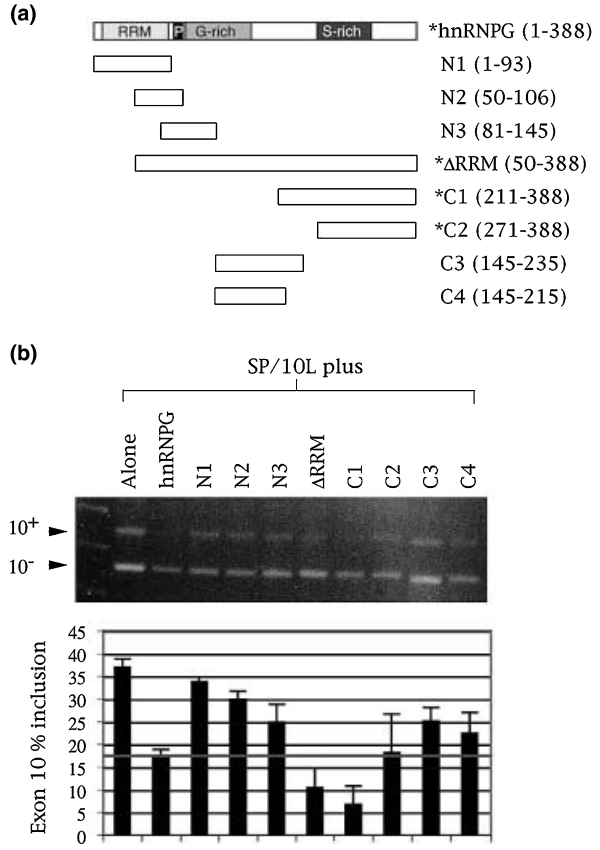

Fig. 6 The RRM domain of hnRNPG is not required for regulation of exon 10. The RT-PCR products come from 1 : 1 cotransfections of SP/10L with hnRNPG deletion mutants. (a) Diagram of the hnRNPG deletion variants. The RRM (RRM), proline rich (P), glycine-rich (G) and serine-rich (S) regions are indicated. Numbers in parentheses show the amino acid composition of the variants. The mutants which influence exon 10 splicing are accompanied by asterisks. (b) RT-PCR of the cotransfections of SP/10L with hnRNPG mutants in COS cells. Primer pair: SPL-LS/SPL-LN. The gray bar across the graph shows the level of SP/10L in the presence of full-length hnRNPG. The percentage exon inclusion was calculated by scanning the bands from at least three independent transfections and measuring their areas using the OneDscan analysis program.

from the Wu laboratory (Jiang *et al*. 2003), which confirms that htra2β1 activates splicing of exon 10.

Combinations of mutant constructs and regulators pinpoint possible binding sites for factors within exon 10

After we had determined both *cis* elements and *trans* factors that influence the splicing of tau exon 10, we carried out 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 *cis* mutant, that defines a possible binding site for the factor. We concentrated on four *cis* mutants (Δ5, Δ11, M5 and M3) and three factors (htra2β1, hnRNPG and SWAP). We selected these specific *cis* mutants because they have the strongest effect on exon 10 behavior. We chose the *trans* factors for different reasons. For htra2β1 we had a strong putative candidate binding site (Jiang *et al*. 2003; our results). HnRNPG is a major binding partner and antagonist of htra2β1 (Venables *et al*. 2000; Hofmann and Wirth 2002; Nasim *et al*. 2003). Finally, the expression and regulatory profile of SWAP make it a strong candidate for being the inhibitor of exon 10 splicing in fetal brain: it invariably inhibits splicing (Sarkissian *et al*. 1996; Lemaire *et al*. 1999) and is highly expressed in brain and placenta (Denhez and Lafyatis 1994).

Δ5 and Δ11 define enhancers (Fig. 2), so we cotransfected them with htra2β1, which activates exon 10 splicing (Fig. 3). Htra2β1 loses its ability to increase exon 10 inclusion in both mutants (Fig. 4a). Htra2β1 is known to bind purine-rich RNA motifs and Δ5 eliminates most of a purine-rich region. These results, in conjunction with findings of the Wu laboratory (Jiang *et al*. 2003), establish Δ5 as the binding site for htra2β1.

Δ11 eliminates an ACE motif (Fig. 2). Htra2β1 is influenced by this mutation, as it is no longer able to promote exon inclusion. This suggests that htra2β1 may interact with factors which recognize this motif. To control for nonspecific effects, we did qualitative cotransfections of Δ5 and Δ11 with CELF3. CELF3 is not expected to influence these two mutants, because the CELF proteins are known to bind to intronic elements. Indeed, CELF3 still activates exon 10 splicing in both mutants, as expected (data not shown).

M5 and M3 define a silencing element (Fig. 2), so we cotransfected them with hnRNPG and SWAP, which inhibit exon 10 splicing (Fig. 3). HnRNPG still inhibits exon 10 inclusion in these mutants, implying it either does not bind near the 5′ splice site of the exon or it does not modulate exon 10 splicing by direct binding (Fig. 4b, three center lanes). However, SWAP has lost its ability to decrease exon 10 inclusion in both mutants (Fig. 4b, three right-hand lanes). Hence, SWAP may bind close to the 5′ splice site of the exon or interact with a factor that does. Alternatively, the increased affinity of both mutants to the U1 snRNP may render them insensitive to SWAP regulation, regardless of the location of its binding site.

Domains of SWAP and hnRNPG required for inhibition of exon 10 splicing

As discussed in the previous section, we believe that regulation of exon 10 splicing by hnRNPG and SWAP is particularly relevant and therefore we wanted to analyze their mechanism of action in more detail. Two panels of SWAP and hnRNPG deletion mutants allowed us to do the obverse experiment from that described above, namely, combine SWAP and hnRNPG mutants with wild type exon 10, in an effort to delineate regions of the proteins required for exon 10 regulation.

SWAP is a protein highly conserved during evolution, which contains two surp motifs along its length and an R/S domain at its C-terminus (Fig. 5a; Denhez and Lafyatis

1994). R/S domains are known to promote formation of splicing factor complexes, whereas it has been suggested that surp domains interact with both proteins and RNA. Mutants ΔS and ΔST, which, respectively, lack the distal part of or the entire R/S domain, and mutant ΔH, which additionally lacks a homologous region upstream of the R/S domain, inhibit exon 10 splicing to the same extent as full-length SWAP (Fig. 5b). Mutant RS, which still includes the R/S domain but contains an internal deletion which removes the surp domains conserved between mammalian SWAP and its Drosophila ortholog, does not inhibit exon 10 splicing. Additionally, staining has shown that mutant RS does not enter the nucleus (Lafyatis *et al.,* unpublished observations). Surprisingly, mutant ΔBB, which lacks both the R/S and surp domains, activates exon 10 splicing, almost certainly by titrating out another factor. The results suggest that the surp domains are required for SWAP to exert its effect on exon 10 splicing but that the R/S domain and the homology domain upstream of it are dispensable. The pattern implies that SWAP may modulate splicing of exon 10 through its surp domains either by direct binding to the exon 10 premRNA or by interacting with other splicing regulators.

HnRNPG is a hnRNP closely related to RBMY and hnRNPG-T which binds to several splicing factors, among them htra2β1, SRp30c, SLM2 and SAM68, as well as to itself and the related protein RBM (Venables *et al*. 2000; Hofmann and Wirth 2002). It is composed of an N-terminal RRM, followed by a short proline stretch and a glycine and serine-rich region in the C-terminus that also contains three RGG repeats. To determine which parts of the protein are responsible for the action on exon 10, we analyzed deletion variants of hnRNPG. Deleting the RRM had no effect on hnRNPG action and resulted in ∼12% exon 10 inclusion which is comparable with full-length hnRNPG. In agreement with this finding, the N-terminal fragments N1 (which composes the RRM), N2 and N3 had no significant influence on exon 10 splicing, suggesting that the C-terminus of the protein is important for function. We therefore analyzed four different C-terminal parts: C1 to C4. The variants C1 and C2 reduced exon 10 inclusion, whereas C3 and C4 had only a small effect (Fig. 6). This implicates the serine-rich region and the ∼60 amino acids downstream of it. Since the action of hnRNPG is independent of its ability to bind RNA via its RRM, the likeliest mechanism is sequestration of other factors, probably htra2β1 that binds to a purine-rich enhancer in exon 10.

Discussion

Flanking exons and introns influence the splicing pattern of exon 10

Flanking exons rarely play a role in alternative splicing systems. Prominent exceptions are fibronectin exon EIIIB (Huh and Hynes 1993), β-tropomyosin (Guo and Helfman 1993) and tau exon 6 (Wei and Andreadis 1998). Tau exon 10 also falls in this small category. Our previous study (Gao *et al*. 2000) showed that the two native flanking exons play opposite roles with respect to the inclusion of exon 10: exon 9 promotes exon 10 splicing, whereas exon 11 suppresses it. In that work, we also showed that the insulin exons of vector SVIRB drive almost complete inclusion of exon 10 in COS cells. In the present study, we show that if exon 10 is placed between the tat exons of vector SPL which discriminate against alternatively spliced exons (Andreadis *et al*. 1993), this results in 37% inclusion of exon 10 in COS cells (Figs 1 and 2–6, left-most lane). These results cumulatively confirm that splicing of exon 10 is sensitive to the identity of its flanking exons.

Other labs have reported that exon 10 constructs in SPL3 result in 50% inclusion in COS cells (Clark *et al*. 1998; Hutton *et al*. 1998; D'Souza *et al*. 1999; Grover *et al*. 1999; Hasegawa *et al*. 1999). The difference between their constructs and ours is the extent of the native flanking introns: in SP/10L ∼500 nucleotides of native flanking intron are present (Fig. 1a), whereas in the constructs of other labs ∼100 nucleotides of the native flanking introns were included. In Fig. 1(c), the 3′ extent of the inserts is denoted by vertical arrows and the designations 'short' and 'long' insert. Several studies confirm that inclusion of exon 10 invariably decreases if the construct contains increasing lengths of flanking introns, regardless of *trans* context or identity of flanking exons (D'Souza and Schellenberg 2000, 2002; Gao *et al*. 2000; Poorkaj *et al*. 2001). This behavior has been seen with other regulated exons (Li *et al*. 2003); although very distant regulatory elements are a formal possibility, a likelier explanation is that longer introns may act as temporary sinks for splicing factors, thereby inhibiting the formation of productive spliceosomal complexes.

For cassette exons, intronic regulatory elements tend to be relatively local. Unusual exceptions are exons EIIIB of fibronectin and K-SAM of the FGF receptor 2 and tau exon 2, which have regulatory elements located ∼500, ∼1000 and ∼300 nt away from the exon, respectively (Huh and Hynes 1993; Del Gatto *et al*. 1997; Li *et al*. 2003). The difference in the behavior of tau exon 10 'short' and 'long' constructs suggests that elements missing from the former but within the latter may be involved in fine-tuning the final 10 isoform ratio. This hypothesis is consistent with the recent discovery of a possible regulatory element located 177 nt upstream of exon 10 (Sobrido *et al*. 2003). The sequence shown in Fig. 1(c) contains the full extent of high homology between human and mouse tau around exon 10. As can be seen in Fig. 1(c), SP/10L contains three islands of conservation downstream of exon 10 missing from the 'short' constructs.

The 5′ splice site of exon 10 and its involvement in FTDP-17 mutations

The exhaustively studied behavior of the M5, M3 and M14 mutants indicates that the region that overlaps the 5′ splice

site of exon 10 exerts a strong silencing effect (Clark *et al*. 1998; Hutton *et al*. 1998; D'Souza *et al*. 1999; Grover *et al*. 1999; Gao *et al*. 2000). More recent findings, from both constructs and FTDP pedigrees, indicate that this silencer is accompanied by an adjacent downstream splicing enhancer (D'Souza and Schellenberg 2000; Stanford *et al*. 2003).

Two theories have been put forward to explain the mechanism of the region proximal to the 5′ splice site of exon 10: the sequence may form an stem/loop structure which hinders interaction with the U1 snRNP (Clark *et al*. 1998; Hutton *et al*. 1998; Grover *et al*. 1999) or it may bind an splicing inhibitor (D'Souza *et al*. 1999; D'Souza and Schellenberg 2000, 2002). The two theories are difficult to distinguish, because all mutations that weaken the putative loop also increase complementarity to the 5′ end of U1. Two results, however, argue in favor of the second theory: Compensatory mutations for position + 16 do not completely restore the wild-type splicing ratio (Grover *et al*. 1999) and mutant M14 increases exon 10 inclusion yet lies outside the range of U1 interaction (Fig. 2). Also, the predicted free energy of ~9 kcal/mol of the proposed stem/loop structure (Hutton *et al*. 1998; D'Souza and Schellenberg 2000) is too low for stability *in vivo* (Roberts *et al*. 1998; Solnick and Lee 1987).

In contrast to the human scenario, tau exon 10 becomes constitutive in adult rats and mice (Kosik *et al*. 1989). In this connection, it is interesting that the region around the 5′ splice site of exon 10 is conserved only partially among human and mouse, diverging past position + 8 (Fig. 1c; Grover *et al.* 1999). Given the strong effects of mutants in positions + 14 to + 29 (Hutton *et al*. 1998; D'Souza *et al*. 1999; D'Souza and Schellenberg 2000, 2002; Gao *et al*. 2000; Stanford *et al*. 2003), this must be at least one region where regulation of this exon differs among species. This crucial difference in the behavior of exon 10 in rodents may well correlate with the repeated failure to find neurofibrillary tangles in aged rodents as well as the difficulty of inducing them artificially in transgenic models.

The role of exonic silencers and enhancers

The behavior of the *cis* mutants establishes that exon 10 contains two exonic enhancers: a GAR-like one defined by ENH (N279K) and Δ5 (Δ280K) and an ACE-like one defined by Δ11 (Fig. 2; Gao *et al*. 2000; D'Souza and Schellenberg 2000). Exonic enhancers act by binding splicing regulators, which activate splicing of otherwise weak exons (Coulter *et al*. 1997; Cartegni *et al*. 2002). Indeed, the GAR-like enhancer of exon 10 interacts with htra2β1, a regulator which recognizes purine-rich regions (Hofmann *et al*. 2000; Nayler *et al*. 1998) and activates exon 10 splicing (Figs 3 and 4a; Jiang *et al.* 2003). The identity of the partner for the ACE-like enhancer is not yet known: although htra2β1 no longer activates exon 10 splicing in the Δ11 mutant (Fig. 4a), neither does any other factor which activates splicing of exon 10 (data not shown).

The exon also contains two motifs, defined by mutants SIL (L284L) and N296N, which act as silencers (Fig. 2b; D'Souza *et al.* 1999; Gao *et al*. 2000). Both silencers are relatively weak (Fig. 2) and the one defined by mutant SIL is context-dependent: it no longer acts as a silencer if embedded in a constitutive exon (data not shown). The TTAG motif defined by mutant SIL has been shown to regulate splicing of the HIV tat gene in concert with an enhancer by blocking early spliceosome assembly (Staffa and Cochrane 1995; Si *et al*. 1998).

Exonic silencers also affect the splicing of the EDA fibronectin exon (Caputi *et al*. 1994; Staffa *et al*. 1997) and the SAM exon of fibroblast growth factor 2 (Del Gatto *et al*. 1996, 1997). In the latter case, the silencer seems to exert its effect by recruiting hnRNPA1 (Del Gatto-Konczak *et al*. 1999). However, hnRNPA1 influences splicing of tau exon 10 either weakly (Fig. 3) or not at all (Gao *et al*. 2000), depending on construct details.

Possible sites and modes of action of factors which regulate exon 10 splicing

The default splicing behavior of exon 10 is inclusion (Gao *et al*. 2000). Yet the majority of the SR and hnRNP proteins we tested inhibit exon 10 inclusion to some extent (Fig. 3); this collective behavior is unusual, although consistent with the hypothesis that exon 10 must be primarily regulated through inhibition. It is possible that some of the weak inhibitors become such by titrating away other spliceosomal components. The factors that show a strong influence on exon 10 splicing in cotransfections are the likeliest to be the real regulatory agents *in vivo*. SRp30c, SRp55, SRp75, 9G8, U2AF, PTB and hnRNPG strongly inhibit exon 10 splicing, whereas htra2β1, CELF3 and CELF4 activate it (Fig. 3).

As discussed in the previous section, htra2β1 apparently activates splicing of exon 10 by binding to the purine-rich enhancer defined by mutants ENH and Δ5 (Figs 3 and 4a; Jiang *et al.* 2003). Of the strong inhibitors of exon 10, SRp30c and SRp55 interact with htra2β1 (Young *et al*. 2002; Tran *et al*. 2003) and hnRNPG interacts with SRp30c and htra2β1, antagonizing the action of the latter (Hofmann and Wirth 2002; Venables *et al*. 2000; Nasim *et al*. 2003). Thus, hnRNPG, SRp30c and SRp55 may act in a complex, which titrates out htra2β1 through the C-terminal domain of hnRNPG. The observation that the C-terminal region of hnRNPG inhibits exon 10 splicing (Fig. 6) strengthens this possibility. Sequestration of htra2β1 by hnRNPG is enhanced by phosphorylation caused by STY/CLK1. Not surprisingly, exon 10 is predominantly skipped in the presence of CLK1 (Hartmann *et al*. 2001).

The exact mechanism of SWAP is not clear, though our results (Figs 4b and 5) suggest that it may bind to the region close to the 5′ splice site of exon 10 through its surp domains and its presence may antagonize the action of the U1 snRNP.

Oddly, two factors which usually suppress splicing, hnRNPA1 (Yang *et al*. 1994) and PTB (Valcárcel and Gebauer 1997), affect exon 10 splicing only in certain contexts: neither inhibits exon 10 splicing if the exon is flanked by strong exons or short introns (Gao *et al*. 2000). If exon 10 is flanked by weak exons, the two become inhibitors; hnRNPA1 a weak one and PTB a strong one (Fig. 3). The weak effect of hnRNPA1 is puzzling, because this protein is invariably involved in cases of regulation via 5′ splice site selection, a mode indisputably operating on exon 10 (Mayeda *et al*. 1993; Chabot *et al*. 1997).

The effect of PTB is puzzling for a different reason: it usually affects exons with weak or oddly located branch points (Valcárcel and Gebauer 1997). Mutated constructs (D'Souza and Schellenberg 2002) and sequence comparisons (Fig. 1c) indicate that the −28 to −22 region upstream of exon 10 contains a sequence reasonably complementary (4/6) to the beginning of the U2 snRNA, which is almost certainly the branch point for exon 10. This is a strong branch point in terms of both sequence and location; furthermore, its associated polyY sequence lacks the CUU motifs recognized by PTB (Singh *et al*. 1995; Lin and Patton 1995).

There is, however, an alternative possible explanation for the behavior of PTB: in some systems, it regulates splicing by binding to elements downstream of the regulated exon (Cote *et al*. 2001). The downstream intron of exon 10 shows several conserved islands. One of these falls just after the cutoff of most exon 10 constructs (Fig. 1c, shown as the 'short' insert) and contains CUU motifs, as well as CUG/GUG motifs recognized by the CELF protein family, whose members also regulate splicing by binding to intronic elements (Ladd *et al*. 2001; Charlet *et al*. 2002). This may explain the lack of PTB effect on exon 10 constructs with short flanking introns (Gao *et al*. 2000) and, obversely, the effects of both PTB and CELF proteins on exon 10 constructs with longer flanking introns (Fig. 1c). The opposing effects of PTB and the CELF proteins on exon 10 splicing (Fig. 3) are consistent with their antagonistic behavior in other systems (Charlet *et al*. 2002).

Tau exon 10 and frontotemporal dementia

The accumulation of abnormal tau filaments into tangles is a pathological hallmark of many neurodegenerative diseases, including AD. Formation of NFTs is an early event in the dementia cascade, and the number of NFTs correlate with disease severity. In several neurodegenerative diseases, now collectively termed tauopathies, tau pathology appears to be directly responsible for neuronal death and hence development of the clinical dementia manifestations (reviewed by Ingram and Spillantini 2002; the diseases include frontotemporal dementia with Parkinsonism, Pick's disease, corticobasal degeneration and progressive supranuclear palsy).

The characterization of the FTDP-17 pedigrees firmly placed tau and, specifically, its splicing directly upstream of the process that causes dementia. Several experts believe that FTDP-17 is the second most common dementia after Alzheimer's (Wilhelmsen 1998). Although FTDP-17 shows such clinical variability that is has often been misdiagnosed (Pasquier and Petit 1997; Wilhelmsen 1998), its molecular causes are remarkably uniform: in the afflicted pedigrees analyzed thus far it predominantly shows mutations in tau exon 10, although srbrtsl pedigrees carry mutations in tau exons 1, 9, 11, 12 and 13 which influence either microtubule binding or protein conformation (Ingram and Spillantini 2002). The exon 10 mutations fall into two categories: those which influence microtubule binding, such as P301L, and those which alter the ratio of exon 10 isoforms.

It is intriguing that disturbance in the relative tau isoform abundance should result in tangle formation and hence neuronal death. This correlates with the finding that, whereas tau null mice are viable (though affected in muscular strength and cognition; Harada *et al*. 1994; Ikegami *et al*. 2000), mice which overexpress tau develop severe neuropathies or gliopathies regardless of the details of the tau constructs (reviewed by Gotz 2001). This implies that tauopathies are a variant of a dosage disease, like chromosomal trisomies. One possibility that would accommodate the behavior of all tau mutants is that tau polymers (whether with itself or with other ligands) may require a precise stoichiometry of isoforms to correctly discharge one or more of their functions, which extend beyond interaction with microtubules. What the FTDP results do show is that tangles are sufficient to initiate the cascade of events leading to neurodegeneration.

Our work shows that splicing factors influence the ratio of exon 10 isoforms; therefore, their spatial and temporal balance in brain may be relevant in predicting or preventing tauopathies. Co-transfections in COS cells, which act as 'neutral' background, are standard tools in the splicing field and very useful in categorizing factors and tentatively assigning binding sites. However, the definitive deciphering of operating mechanisms on regulated exons is complicated by the combinatorial nature of splicing regulation, the involvement of ubiquitous factors in tissue-specific splicing decisions and the sequence redundancy of splicing factor binding sites (reviewed by Smith and Valcárcel 2000). Future *in vitro* work will determine which factors, in addition to htra2β1 (Jiang *et al*. 2003), are the primary determinants of exon 10 splicing and how they exert their influence.

Continued work on the basic molecular biology of the tau molecule may give us the tools to comprehend and combat not only FTDP, but also other types of dementia. These diseases vary widely both in clinical phenotype and brain pathology, but they share tangles as an invariable defining characteristic (Spillantini and Goedert 1998).

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