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Tau Exons 2 and 10, Which Are Misregulated in Neurodegenerative Diseases, Are Partly Regulated by Silencers Which Bind a SRp30c·SRp55 Complex That Either Recruits or Antagonizes htra2β1*

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Tau is a microtubule-associated protein whose transcript undergoes complex regulated splicing in the mammalian nervous system. Exon 2 modulates the tau N-terminal domain, which interacts with the axonal membrane. Exon 10 codes for a microtubule binding domain, increasing the affinity of tau for microtubules. Both exons are excluded from fetal brain, but their default behavior is inclusion, suggesting that silencers are involved in their regulation. Exon 2 is significantly reduced in myotonic dystrophy type 1, whose symptoms include dementia. Mutations that affect exon 10 splicing cause frontotemporal dementia (FTDP). In this study, we investigated three regulators of exon 2 and 10 splicing: serine/arginine-rich (SR) proteins SRp55, SRp30c, and htra2β1. The first two inhibit both exons; htra2β1 inhibits exon 2 but activates exon 10. By deletion analysis, we identified splicing silencers located at the 5′ end of each exon. Furthermore, we demonstrated that SRp30c and SRp55 bind to both silencers and to each other. In exon 2, htra2β1 binds to the inhibitory heterodimer through its RS1 domain but not to exon 2, whereas in exon 10 the heterodimer may sterically interfere with htra2β1 binding to a purine-rich enhancer (defined by FTDP mutation E10-Δ5 = Δ280K) directly downstream of the silencer. Increased exon 10 inclusion in FTDP mutant ENH (N279K) may arise from abolishing SRp30c binding. Also, htra2β3, a naturally occurring variant of htra2β1, no longer inhibits exon 2 splicing but can partially rescue splicing of exon 10 in FTDP mutation E10-Δ5. This work provides interesting insights into the splicing regulation of the tau gene.

Alternative splicing is a versatile and widespread mechanism for generating multiple mRNAs from a single transcript (1, 2). Splicing choices are spatially and temporally regulated, and the ensuing mRNAs produce functionally diverse proteins, contributing significantly to proteomic complexity (2, 3).

Splicing is carried out by the spliceosome, a large and dynamic complex of proteins and small RNAs (4, 5). 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 and "weighing" of splice element strength are used to enable precise recognition of the short and degenerate splice sites (6). Despite the high fidelity of exon recognition *in vivo*, it is currently impossible to accurately predict alternative exons (7). Exonic and intronic enhancers and silencers are involved in splicing regulation (8, 9). Their mutation can result in human disease by causing aberrant splicing (10, 11).

On the *trans* side of regulation, mammalian splicing regulators mostly belong to two superfamilies, the serine/arginine-rich (SR)[1] proteins and the heterogeneous ribonuclear proteins (hnRNPs), neither of which is exclusively involved in alternative splicing (12, 13). 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, among them htra2β3, a splicing variant of htra2β1 (14). Nevertheless, it appears that the exquisite calibration of mammalian alternative splicing is primarily achieved by SR and hnRNP proteins, which show distinct tissue and developmental ratios, despite their ubiquitous distribution (15, 16).

Tau is a microtubule-associated protein enriched in axons of mature and growing neurons (17), although it is also found in other cell compartments and types (18). Hyperphosphorylated, microtubule-dissociated tau protein is the major component of neurofibrillary tangles, a hallmark of several neurodegenerative diseases (18, 19).

The tau gene produces three transcripts that undergo complex alternative splicing: 6 of the 16 tau exons are regulated cassettes (18). The N terminus of the tau protein interacts with the plasma membrane (20). The structure and function of the tau N terminus are modulated by cassette exons 2 and 3. The C terminus of the tau protein contains four imperfect repeats (encoded by exons 9–12) that act as microtubule binding domains (21).

Exons 2 and 10 are adult-specific in rodents and humans but with a crucial difference relevant to neurodegeneration: in adult rodents, exon 2 remains regulated, but exon 10 becomes

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[1] The abbreviations used are: SR, serine/arginine-rich; hnRNP, heterogeneous ribonuclear protein; RRM, RNA recognition motif; RS, arginine/serine-rich; FTDP, frontotemporal dementia; GST, glutathione *S*-transferase; RT, reverse transcription.

TABLE I
Primers used in PCR

For creation of deletions and point mutations, we used pairs of these primers and their reverse complements (not shown). The carets in the sequence of the first three primers show the location of the deletion. The mutations in primer M280S are shown in bold. In Fig. 1*A*, the deleted regions are *underlined*, and the point mutations are indicated by *arrows*. S, sense; A, antisense.

Name	Strand	Location	Sequence
Deletion and point mutation constructs			
2-Δ1S	S	Within exon 2	CACTGTATGTGTTCCAGA^CTGCAGACCCCCACTGAGG
2-Δ2S	S	Within exon 2	CTGTATGTGTTCCAGAATCTCCC^CCCCACTGAGGACGGATCTGAGG
2-Δ3S	S	Within exon 2	CCAGAATCTCCCCTGCAGACCCC^GGATCTGAGGAACCGGGCTC
2-Δ4S	S	Within exon 2	CTGCAGACCCCCACTGAGGAC^AACCGGGCTCTGAAACCTCTG
2-Δ5S	S	Within exon 2	CCACTGAGGACGGATCTGAGG^GCTCTGAAACCTCTGATGCTAAG
2-Δ6S	S	Within exon 2	CTGAGGACGGATCTGAGGAACCGG^ACCTCTGATGCTAAGAGCAC
2-Δ7S	S	Within exon 2	CGGATCTGAGGAACCGGGCTCTGAAAC^CTAAGAGCACTCCAACAGCGG
2-Δ8S	S	Within exon 2	GGGCTCTGAAACCTCTGATGCT^TCCAACAGCGGAAGGTGGGC
2-Δ9S	S	Within exon 2	CCTCTGATGCTAAGAGCACTC^GAAGGTGGGCCCCCCTTCAGACG
10-Δ3/4S	S	Within exon 10	CTGGCTACCAAAGGTGCAGA^AAGAAGCTGGATCTTAGCAACGTCC
10-Δ8/9S	S	Within exon 10	GTGCAGATAATTAATAAGAAGCTG^AGCAACGTCCAGTCCAAGTGTGGC
10-Δ15S	S	Within exon 10	GTCCAAGTGTGGCTCAAAGGAT^CACGTCCCGGGAGGCGGCAGTG
M280S	S	Within exon 10	GTGCAGATAATTAATAA**T**AA**T**CTGGATCTTAGCAA
In vitro constructs, PCR and sequencing			
2PCS	S	Exon 2 5′ intron	TGTTCCAGCTGTTTCCACAGGGAG
2PCN	A	3′ end of tau exon 2	CTTCCGCTGTTGGAGTGCTCTTAG
HTI2	A	Exon 2 3′ intron	GCTCCCACCACGCTGTCCTGCAAAGCACCG
HT10PS	S	Exon 10 5′ intron plus EcoRI site	GAATTCGAGCAAGTAGCGGGTCCAG
HT10N90	A	Exon 10 3′ intron	ACTGCCGCCTCCCGGGACGTGTTTG
RT-PCR			
SPL-LS	S	In SPL3 vector	TCTGAGTCACCTGGACAACCTCAAAGG
SPL-LN	A	In SPL3 vector	ATCTCAGTGGTATTTGTGAGCCAGGGC
SVP2	S	In SPL3 vector	CTGAGCTATTCCAGAAGTAGTGAGGAGGC

constitutive (22–24). In contrast, in adult humans exon 10, like exon 2, remains regulated in the central nervous system (23, 25). The difference most likely arises from the details of the *cis* sequences flanking exon 10 in various organisms (26, 27), which in turn affect the regulation exerted by *trans* factors.

Altered splicing factor ratios suppress exon 2 splicing in myotonic dystrophy type 1, a multisystemic disorder whose symptoms include dementia (28). Altered splicing regulation of tau exon 10 can cause inherited frontotemporal dementia (FTDP) with Parkinsonism (17), almost certainly by disturbing the normal tau isoform ratio (18, 19). Thus, the splicing regulation of these two exons is directly relevant to neurodegeneration.

Both exons show a splicing default pattern of inclusion (25, 29), are affected by intronic and exonic sequences (25–27, 29–35), and are regulated by several splicing factors, most acting as inhibitors (25, 27, 36–38). In this report we show that these two tau exons are partially regulated by exonic silencers located at their respective 5′ ends, which bind splicing factors SRp30c and SRp55. A third factor, htra2β1, acts in opposite fashion on the two exons: it inhibits exon 2 by binding to the SRp30c·SRp55 heterodimer through its RS1 domain but activates exon 10 by binding to an exonic enhancer located directly downstream of the silencer that recognizes the SRp30c·SRp55 heterodimer. In the case of exon 10, htra2β1 and the SRp30c·SRp55 complex may sterically interfere with each other.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The starting constructs were SP/2L (Fig. 1*A*) and SP/10L (Fig. 1*C*), in which tau genomic fragments are inserted in vector pSPL3 (Invitrogen). SP/2L contains tau exon 2 and 1 kilobase pair of the upstream and downstream native introns flanking exon 2 (30). Construct SP/10L contains tau exon 10, 471 bp of its upstream intron, and 408 bp of its downstream intron (27). Using directed mutagenesis, as previously described (27, 30), we created nine deletions that scan exon 2 in SP/2L (E2-Δ1 to E2-Δ9; Fig. 1*A*) and three deletions and a double point mutation in exon 10 (E10-Δ2/3/4, E10-Δ8/9, and E10-Δ15, following the nomenclature of D'Souza and Schellenberg (32), and point mutation M280 in the M3 background (Fig. 1*C*) (24)). The mutations are diagrammed in Fig. 1, *A* and *C*, and the mutagenic primers are listed in Table I. The deletions were verified by PCR (using primers 2PCS and HTI2 for exon 2 and primers HT10PS and HT10N90 for exon 10; Table I) and by sequencing.

To generate riboprobes, we created constructs 81+2PCS/2PCN and E10+30. 81+2PCS/2PCN contains tau exon 2 plus 150 nucleotides of its upstream intron replacing part of the intron and second exon of *in vitro* splicing vector 81/AdML (39). E10+30 contains all of tau exon 10 and 30 nucleotides of its downstream intron inserted into vector pGEM-TE (Promega). We also made deletions E2-Δ1, E2-Δ2, and E2-Δ4 (Fig. 1*A*) in 81+2PCS/2PCN and deletion E10-Δ3/4 (Fig. 1*C*) in E10+30.

Protein Expression in Cells and Bacteria and GST Pull-down Assays—For eukaryotic expression, cDNAs were expressed from the following promoters: cytomegalovirus (CMV) for htra2β1, SRp30c, SRp55, and the variants of htra2β1 and SRp30c (14, 40, 41); and SV40 for the variants of SRp55 (42). Western blots show that the factor variants express stable proteins in equivalent amounts (data not shown).

For protein interactions *in vitro*, htra2β1 and SRp55 were cloned into pGEX-4T1 and pGEX-4T3, respectively (Amersham Biosciences). SRp30c (a generous gift from Dr. Chris Lorson) was in vector pRSET-c (Invitrogen). The recombinant proteins were produced and purified from *Escherichia coli* strain BL21 (Stratagene) according to the vendor's instructions. For protein and RNA pull-downs, htra2β1 and SRp55 were cloned into pFLAG-CMV-6c, and SRp30c was cloned into pFLAG-CMV-6b (Sigma). Protein expression was verified by using anti-FLAG M2 monoclonal antibody (Sigma) on Western blots of crude lysates from cells transfected with each construct.

1 μl of a GST-factor fusion or GST alone was incubated with 1 μl of SRp30c-His or with 1 μl of htra2β1-FLAG in 0.5 ml of loading buffer (1% Triton X-100, 10% glycerol, 0.25 M NaCl, 1 mM EDTA, and 50 mM Tris, pH 7.5) for 1 h at 4 °C and then incubated with 10 μl (bed volume) of glutathione-agarose beads (Sigma) for 1 h at 4 °C. The beads were washed three times with 500 μl of wash buffer (0.05% Triton X-100, 10% glycerol, 50 mM NaCl, and 50 mM Hepes, pH 7.5). The retained proteins were run on 12% SDS-PAGE and immunoblotted with mouse monoclonal antibodies as primary antibodies (anti-His (Invitrogen) at 1:3000 or anti-FLAG M2 (Sigma) at 1:2500) and goat anti-mouse IgG (Zymed Laboratories Inc.) at 1:10,000 as secondary antibody.

Cell Culture, Transfections, and RNA Preparation—Monkey kidney (COS) and human epithelial (HeLa) cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Plasmid DNA was prepared by Qiagen Tip-50s and introduced into cells by lipofection (LT1; Panvera). Total RNA was isolated by the TRIzol method (Invitrogen).

Reverse Transcription and PCRs—We performed PCR analysis of RNA as previously described (30) with primer pair SPL-LS/SPL-LN or SVP2/SPL-LN (Table I). The isoform ratio was calculated by scanning the bands from three independent transfections using the One-Dscan program and the Scanalytics IPLab software.

Riboprobe Generation, UV-cross-linking, and RNA-protein Immunoprecipitation—We transcribed 81+2PCS/2PCN, E10+30, and their variants *in vitro* using the Promega transcription kit, [^{32}P]CTP, [^{32}P]UTP, or both (Amersham Biosciences) and RNA polymerase T3 and T7, respectively (Promega).

For UV-cross-linking, exon 2 riboprobes and GST fusions of htra2β1, SRp30c, and SRp55 were incubated at room temperature for 20 min in binding buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 50 mM KCl, and 1 μg of tRNA). Reaction mixtures were pre-chilled on ice and irradiated with UV light (254 nm) for 10 min. The samples were digested with RNase A at 37 °C for 1 h and run on 12% SDS-PAGE. The gels were fixed, dried, and subjected to autoradiography.

For RNA pull-downs, equal counts of the riboprobes were added to lysates from HeLa cells transfected with htra2β1, SRp30c, and SRp55 FLAG fusions. The vectors were used as negative controls. We immunoprecipitated the proteins using anti-FLAG monoclonal antibody conjugated with agarose (Sigma) according to the Novagen instructions and counted the radioactivity of the precipitated pellet in a beta scintillation counter.

RESULTS

Scanning of Exon 2 by Deletions Uncovers an Exonic Silencer—Because the default behavior of exon 2 and 10 is inclusion (25, 29), the most likely explanation for their exclusion in fetal brain (18) is that in that tissue an inhibitory protein recognizes a silencer sequence on each exon. In our previous work we found one weak silencer within exon 2 by point mutagenesis (30). However, this element is context-specific and is not influenced by factors that strongly inhibit exon 2 splicing (30), suggesting that the point mutation may strengthen an enhancer rather than disrupt a silencer. In this study, we decided to employ a strategy used successfully for tau exon 10 (37, 38) by creating nine deletions that scan exon 2 (Fig. 1*A*).

Fig. 1*B* shows the splicing behavior of the nine deletions. Two (E2-Δ7 and E2-Δ8) leave exon 2 splicing essentially unchanged. Four (E2-Δ3, E2-Δ5, E2-Δ6, and E2-Δ9) show a significant decrease of exon 2 inclusion, and one (E2-Δ4) shows a moderate decrease of exon 2 inclusion, suggesting that these deletions excise exonic enhancers; E2-Δ4 and E2-Δ5 partly overlap an enhancer previously defined by a point mutation (E2–6, shown in Fig. 1*A*) (30). Finally, two (E2-Δ1 and E2-Δ2) increase exon 2 inclusion, indicating that the first ~20 residues of exon 2 act as a silencer.

In the case of exon 10, we found that the regions defined by E10-Δ2/3/4, E10-Δ8/9, and E10-Δ15 act as splicing silencers (Fig. 2*C*, *lanes 3*, *5*, and *7*; Fig. 2*D*, *lane 3*), in agreement with previous reports (26). Conversely, mutant M280 defines an enhancer (Fig. 4*E*, *lane 1*). This mutant is the obverse of mutant ENH (N279K) that extends a purine-rich region that binds htra2β1 (27, 37) (Fig. 3*B*, *lane 1*; Fig. 4*D*, *lane 1*). Deletion E10-Δ5 (also denoted Δ280K) overlaps mutation M280 and almost completely abolishes exon 10 splicing (27, 31) (Fig. 4*F*, *lane 1*). We believe that inclusion of exon 10 in the M280 mutant would be even lower in the absence of the compensating M3 mutation, which affects a silencer (26, 27, 31).

Combinations of Deletion Constructs and Regulators Pinpoint Possible Binding Sites for SRp30c, SRp55, and htra2β1 within Exons 2 and 10—Our previous work showed that SRp30c and SRp55 strongly inhibit exon 2 splicing (30), whereas htra2β1 strongly inhibits exon 2 (30) but moderately activates exon 10 (27). This situation gives an ideal handle for comparing how the three factors distinctly regulate two similarly behaved exons. We first did a preliminary correlation by co-transfection experiments: we paired our exon 2 and 10 mutants with each member of the trio.

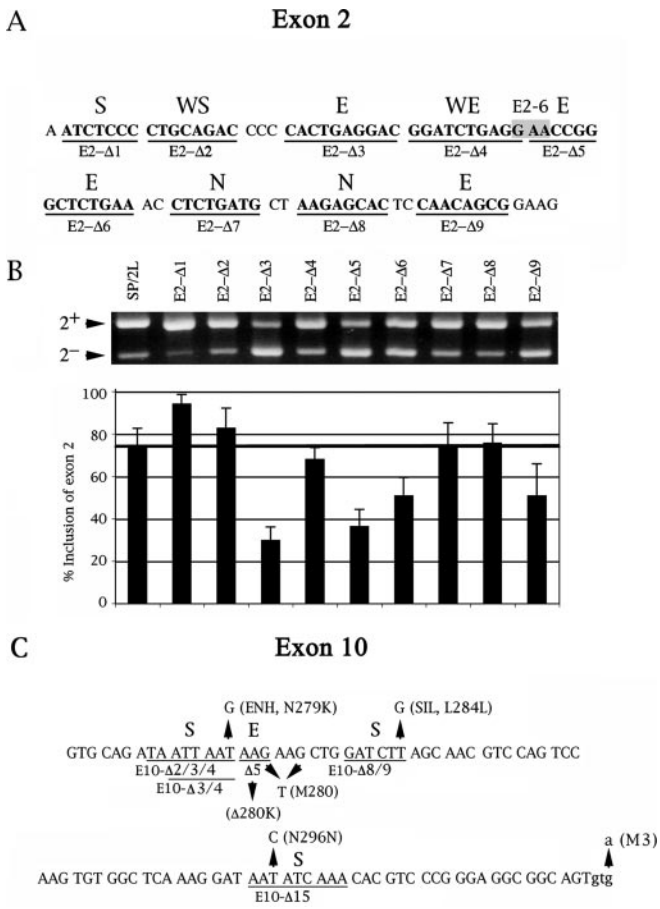

FIG. 1. **Exon 2 contains an extended silencer at its 5′ end, whereas exon 10 has several silencers throughout its length.** *A*, scanning deletions within exon 2. Deleted nucleotides are in *bold*, and each deletion is *underlined*. Shown above each deletion is its action, if any: (*W*)*E* = (weak) enhancer, (*W*)*S* = (weak) silencer, *N* = none. E2–6 defines a purine-rich enhancer from previous work (30). *B*, RT-PCR of the mutated exon 2 constructs 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. The percentage of exon inclusion was calculated by scanning the bands from three independent transfections and measuring their areas using the OneDscan analysis program. *C*, silencers and an enhancer within exon 10. The silencer defined by E10-Δ2/3/4 and the purine-rich enhancer defined by FTDP mutation E10-Δ5 (Δ280K) are indicated. Also shown are the M280 double mutation, two additional deletions (E10-Δ8/9 and E10-Δ15) that define silencers, FTDP point mutations that overlap the silencers (N279K, L284L, and N296N), and the M3 FTDP mutation that is the background of M280.

All three factors (SRp30c, SRp55, and htra2β1) lose their ability to inhibit exon 2 splicing in mutants E2-Δ1 and E2-Δ2 (Fig. 2, *A* and *B*), implying that they all exert their effect through this extended silencer. SRp55 additionally loses its ability to influence exon 2 splicing when co-transfected with deletion E2-Δ4 (Fig. 2*B*), although E2-Δ4 has minimal effect on exon 2 splicing (Fig. 1).

The silencer defined by E2-Δ1 and E2-Δ2 is not a canonical htra2β1 binding site: htra2β1 binds to purine-rich and purine-enriched regions (43, 44), whereas this region of exon 2 is pyrimidine-rich (Fig. 1*A*). On the other hand, deletion E2-Δ2 contains a sequence (TGCAGA) close to the consensus recognition site for SRp55, according to the ESEfinder program (9).

Similarly, both SRp30c and SRp55 lose their ability to inhibit exon 10 splicing in mutant E10-Δ2/3/4 (Fig. 2, *C* and *D*), implying that they exert their effect through this region. Neither of the two other exon 10 silencer deletions relieves inhibi-

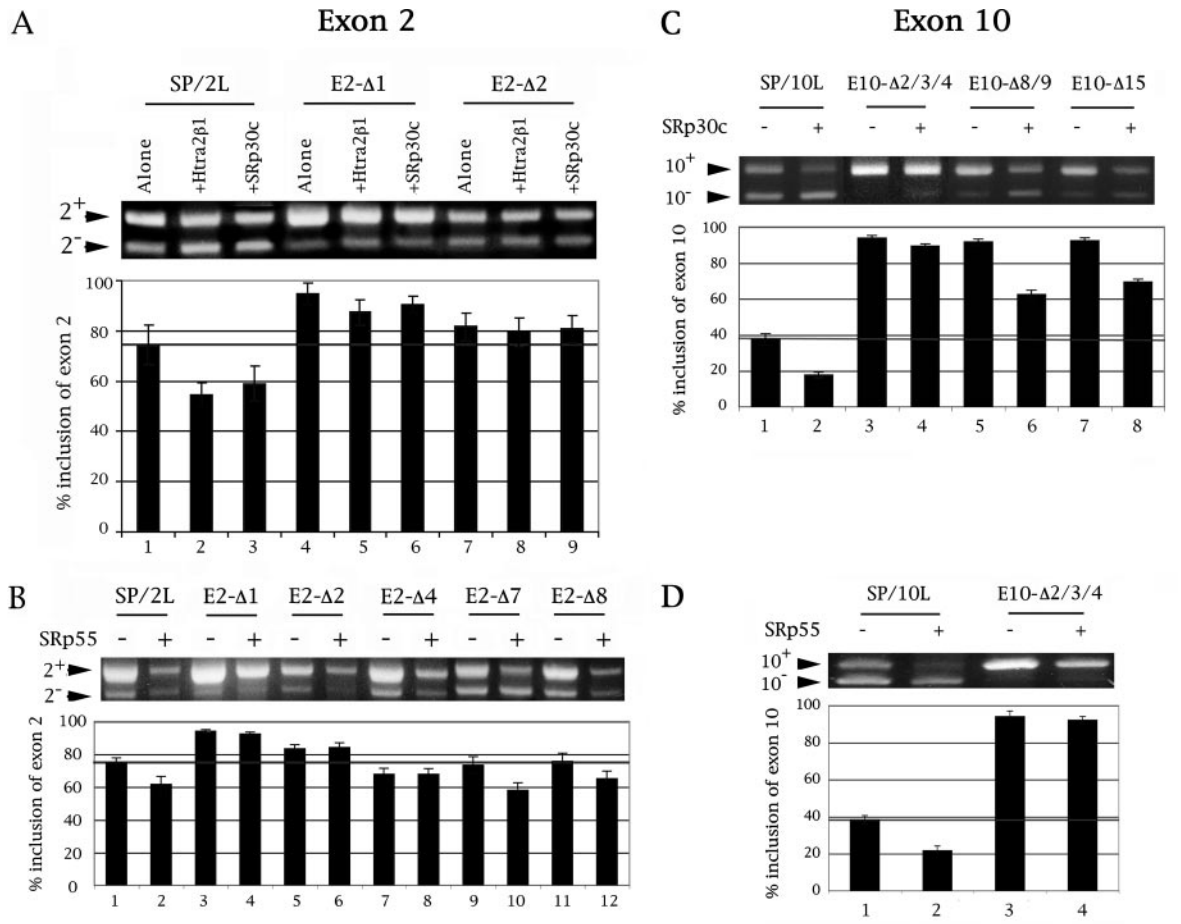

FIG. 2. **Action of splicing factors htra2β1, SRp30c, and SRp55 on exons 2 and 10.** *A* and *B*, splicing factors htra2β1, SRp30c (*A*), and SRp55 (*B*) inhibit exon 2 splicing through the region defined by deletions E2-Δ1 and E2-Δ2; SRp55 is also influenced by deletion E2-Δ4. *C* and *D*, splicing factors SRp30c (*C*) and SRp55 (*D*) influence exon 10 splicing through deletion E10-Δ2/3/4. *A*–*D*, RT-PCR of wild-type and deleted SP/2L or SP/10L in COS cells in the absence and presence of htra2β1, SRp55, and SRp30c. The RT-PCR products come from 1:1 co-transfections of exon constructs with the three factors in COS cells. Exon ratio calculations and graph conventions are as described in Fig. 1. Primer pair: *A* and *C*, SPL-LS/SPL-LN; *B* and *D*, SVP2/SPL-LN.

tion by SRp55 or SRp30c (Fig. 2*C* shows the SRp30c results; the SRp55 data are not shown). Interestingly, E10-Δ2/3/4 is directly upstream of a purine-rich enhancer defined by FTDP mutation Δ280K (E10-Δ5), which binds htra2β1 in its role as an activator of exon 10 splicing (27, 37).

To discover which regulatory action is dominant on exon 10, we did dosage co-transfections of SRp55, SRp30c, and htra2β1 with wild-type exon 10 as well as mutant ENH (Fig. 3), which changes a nucleotide right at the boundary between the silencer and the enhancer (Fig. 1*C*). The results suggest that the two inhibitors "trump" the activator, a finding consistent with the fact that htra2β1 activates exon 10 splicing rather weakly (Ref. 24 and this work). In the wild type, 0.5 μg of each inhibitor dominate 1 μg of htra2β1 (Fig. 3*A*). SRp55 still inhibits mutant ENH, although more of it is required to achieve full inhibition (Fig. 3*B*, *lanes 2–4*), but SRp30c no longer inhibits ENH (Fig. 3*B*, *lane 6*). This result strongly implies that 1) the altered nucleotide in ENH comprises an integral part of the SRp30c binding site and 2) the two inhibitors act independently.

Domains of Splicing Factors Required for Regulating the Splicing of Exons 2 and 10—To determine which domains of the three splicing factors are required for exon 2 and 10 regulation, we next co-transfected wild-type exon 2 and 10 with variants of htra2β1, SRp55, and SRp30c. Figs. 4*A*, 5*A*, and 6*A* diagram the variants we used for the assays; the functional domains of these three factors are designated according to the conventions of the splicing field: RRM for RNA recognition motif domain(s) and RS for the serine/arginine-rich domain(s).

The N-terminal RS domain of htra2β1 is required for inhibition of exon 2 splicing, but the C-terminal one is dispensable (Fig. 4*B*, *lanes 4* and *5*). The RRM domain of htra2β1 cannot inhibit exon 2 by itself (Fig. 4*B*, mutant RRM, *lane 3*). These results imply that htra2β1 does not bind directly to exon 2.

Unlike htra2β1, the SRp55 RS domain is not required for suppression of exon 2 splicing; in fact, SRp55 mutant RS, which only contains the RS domain, activates exon 2 splicing (Fig. 5*B*, *lane 8*). Its RRM2 domain is sufficient to suppress exon 2 splicing as strongly as the wild type, whether it is alone or with RRM1 or RS (Fig. 5*B*, *lanes 3*, *6*, and *7*). Finally, SRp30c no longer suppresses splicing of exon 2 when it is truncated down to either its RNA or protein binding segment (Fig. 6*B*).

These results strongly suggest that SRp55 modulates exon 2 splicing by direct binding to the mRNA, whereas htra2β1 influences exon 2 splicing through a protein interaction mediated by its N-terminal RS domain. This result is consistent with the fact that the exon 2 silencer contains a consensus site for SRp55 but not for htra2β1. SRp30c requires its entire length to regulate exon 2, implying that it binds directly to exon 2 but must also interact with SRp55, htra2β1, or both to discharge its function.

The RRM domain of htra2β1 suffices to activate exon 10 splicing and is a slightly better activator than the full-length htra2β1 (Fig. 4*C*, mutant RRM, *lane 3*). In the presence of the

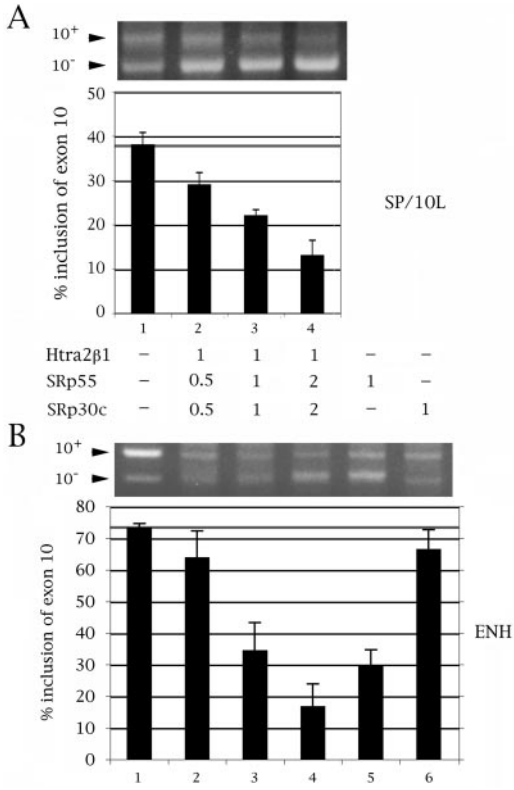

FIG. 3. **Inhibitors SRp55 and SRp30c are dominant over activator htra2β1, but mutant ENH is no longer inhibited by SRp30c.** RT-PCRs from co-transfections of 1 μg of (*A*) SP/10L and (*B*) ENH with varying amounts of the three factors in COS cells. The *numbers* in the *middle* show the amount of factor plasmid added (in micrograms). Exon ratio calculations, graph conventions, and primers are as described in Fig. 1. Primer pair: SPL-LS/SPL-LN.

RRM domain, the N-terminal RS domain cannot activate exon 10 splicing (Fig. 4*C*, mutant ΔRS2, *lane 4*), whereas the C-terminal RS domain activates exon 10 splicing as strongly as full-length htra2β1 (Fig. 4*C*, mutant ΔRS1, *lane 5*). Mutant ENH responds to the htra2β1 variants weakly because of its high inclusion of exon 10 (Fig. 4*D*), whereas mutant M280 responds more strongly than wild type (~3x rather than ~1.5x; Fig. 4*E*); nevertheless, the relative order of activation strength for both mutants remains the same as that for wild type. Finally, neither full-length htra2β1 nor the RRM domain can activate mutant E10-Δ5 and ΔRS2 slightly inhibits it, but ΔRS1 still activates it strongly (Fig. 4*F*). These results imply that 1) htra2β1 still binds to the mutated but still purine-rich M280 site and 2) its RS2 domain is crucial for the activation.

The two RRM domains of SRp55 combined and either RRM domain, alone or in combination with the RS domain, inhibit splicing of exon 10 (Fig. 5*C*, *lanes 3–7*). RRM2, alone or with the RS domain, is as good an inhibitor as the full-length SRp55 (Fig. 5*C*, *lanes 6* and *7*), whereas RRM1 inhibits only partially (Fig. 5*C*, lanes 4 and 5). The RS domain alone activates splicing of exon 10 (Fig. 5*C*, *lane 8*). The behavior of SRp55 suggests that RRM2 is the primary binding partner of exon 10. SRp30c loses its ability to suppress splicing of exon 10 when it is truncated down to either its RNA or protein binding segment (Fig. 6*C*). Like SRp55, the RS domain alone of SRp30c activates splicing of exon 10 (Fig. 6*C*, *lane 3*). These results strongly imply that SRp55 and SRp30c, like htra2β1 (32), modulate exon 10 splicing by direct binding to the mRNA.

RNA and Protein Pull-downs Elucidate How Splicing Factors htra2β1, SRp55, and SRp30c Modulate Exon 2 and Exon 10 Splicing—To further dissect the mechanism of action of the three inhibitory factors on exons 2 and 10, we investigated whether htra2β1, SRp55, or SRp30c associates with the exon 2 and 10 RNAs in cell lysates or *in vitro* and whether they interact with each other in GST pull-down assays.

Consistent with the results of the factor variant co-transfections with exon 2 (previous section, Figs. 4*B*–6B), htra2β1 does not associate with the wild-type exon 2 riboprobe (Fig. 7*A*, *lanes 10–12*), whereas both SRp55 and SRp30c strongly associate with it, the latter showing stronger binding than the former (Fig. 7*A*, *lanes 3* and *7*). Both deletions of the silencer region reduce the ability of SRp30c to bind by 50% (Fig. 7*A*, *lanes 8* and *9*), correlating very well with the co-transfection results (Fig. 2*A*, *lanes 6* and *9*). Deletion E2-Δ2 also greatly decreases binding to SRp55 (Fig. 7*A*, *lane 5*), as expected from the co-transfection results (Fig. 2*B*, *lane 6*). However, deletions E2-Δ1 and E2-Δ4 show enhanced binding to SRp55 (Fig. 7*A*, *lanes 4* and *6*), even though SRp55 no longer inhibits exon 2 splicing in the presence of these mutants (Fig. 2*B*, *lanes 4* and *8*). The Western blots of the immunoprecipitated proteins (Fig. 7*B*) show that htra2β1 is equivalently expressed and is immunoprecipitated as efficiently as the other two factors, further confirming the absence of htra2β1 binding in exon 2. The findings support the model that SRp30c binds directly to exon 2 through a site that overlaps deletions E2-Δ1 and E2-Δ2; SRp55 binds to the region defined by E2-Δ2; finally, htra2β1 exerts its influence through protein-protein interactions mediated by one of its RS domains.

These conclusions are strengthened by the UV-cross-linking experiments (Fig. 7, *C* and *D*). Fig. 7*C* shows that htra2β1 does not bind to any of the riboprobes (wild-type exon 2 or deletions E2-Δ1 and E2-Δ2), whereas SRp30c binds strongly to wild-type exon 2, but not to the two deletions. These results are completely consistent with the behavior of the two factors in RNA pull-downs (Fig. 7*A*).

The SRp55 profile is slightly complicated by the fact that during cross-linking the protein degrades into two major species (Fig. 7*D*). Taking this into account, SRp55 shows a complex but explicable cross-linking profile. The behavior of the *top band*, which is full-length SRp55, is congruent with the RNA pull-down results (Fig. 7*A*): it binds to wild-type exon 2 and binds even more strongly to deletion E2-Δ1, but does not bind to deletions E2-Δ2 and E2-Δ4. The fact that the full-size SRp55 band does not bind to E2-Δ4 when purified, whereas SRp55 binds strongly to E2-Δ4 in cell lysates, almost certainly means that the effect of SRp55 on E2-Δ4 is mediated by yet another factor.

The *bottom band* in Fig. 7*D* is truncated SRp55. The truncation, combined with the lack of phosphorylation in bacteria, may allow the positively charged RS domain of SRp55 to interact nonspecifically with the negatively charged riboprobe. Still, it binds more strongly to E2-Δ1 than to the wild type and binds most weakly to deletion E2-Δ2, which lacks the SRp55 consensus binding site.

Also consistent with the factor variant co-transfections with exon 10, htra2β1, SRp55, and SRp30c directly bind to the exon 10 riboprobe (Fig. 7*E*, *lanes 3*, *4*, and *6*). Of the three factors, SRp30c binds strongly (~10× compared with the control vector), SRp55 binds moderately (~5×), and htra2β1 binds weakly (~2.5×). The relative binding strength of htra2β1 is consistent with results from other laboratories (32, 33). SRp55 binds weakly to the E10-Δ3/4 deletion, and SRp30c does not bind at all to it (Fig. 7*E*, *lanes 5* and *7*), correlating exactly with the co-transfection results (Fig. 2, *C* and *D*, *lanes 4*).

To confirm our suspicion that the three factors can act as a complex, we did co-immunoprecipitations of the factors in pairs. Fig. 8, *A* and *B*, shows that htra2β1 interacts with SRp55 and that SRp30c interacts with both SRp55 and htra2β1.

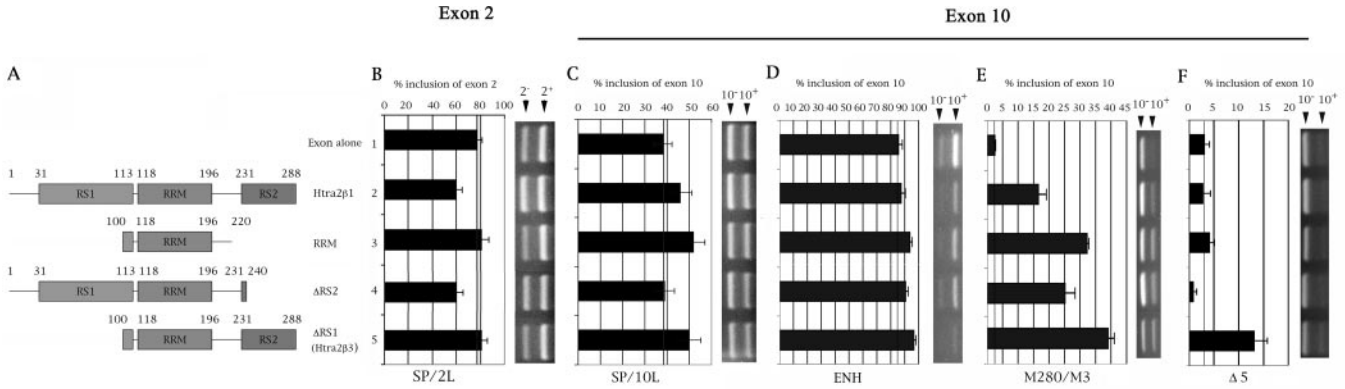

FIG. 4. **The N-terminal RS domain (RS1) of htra2β1 is required for inhibition of exon 2 splicing, whereas its C-terminal RS domain (RS2) is crucial for activation of exon 10 splicing.** *A*, diagram of htra2β1 deletion variants. The RRM motifs and RS regions are indicated. *Numbers* show the amino acid composition of the variants. *B*, RT-PCR products come from 1:1 co-transfections of construct SP/2L with the htra2β1 deletion variants in COS cells. *C*–*F*, RT-PCRs from 1:1 co-transfections of constructs (*C*) SP/10L, (*D*) E10-Δ5, (*E*) ENH, and (*F*) M280/M3 with the htra2β1 deletion variants in COS cells. Exon ratio calculations and graph conventions are as described in Fig. 1. Primer pair: SPL-LS/SPL-LN.

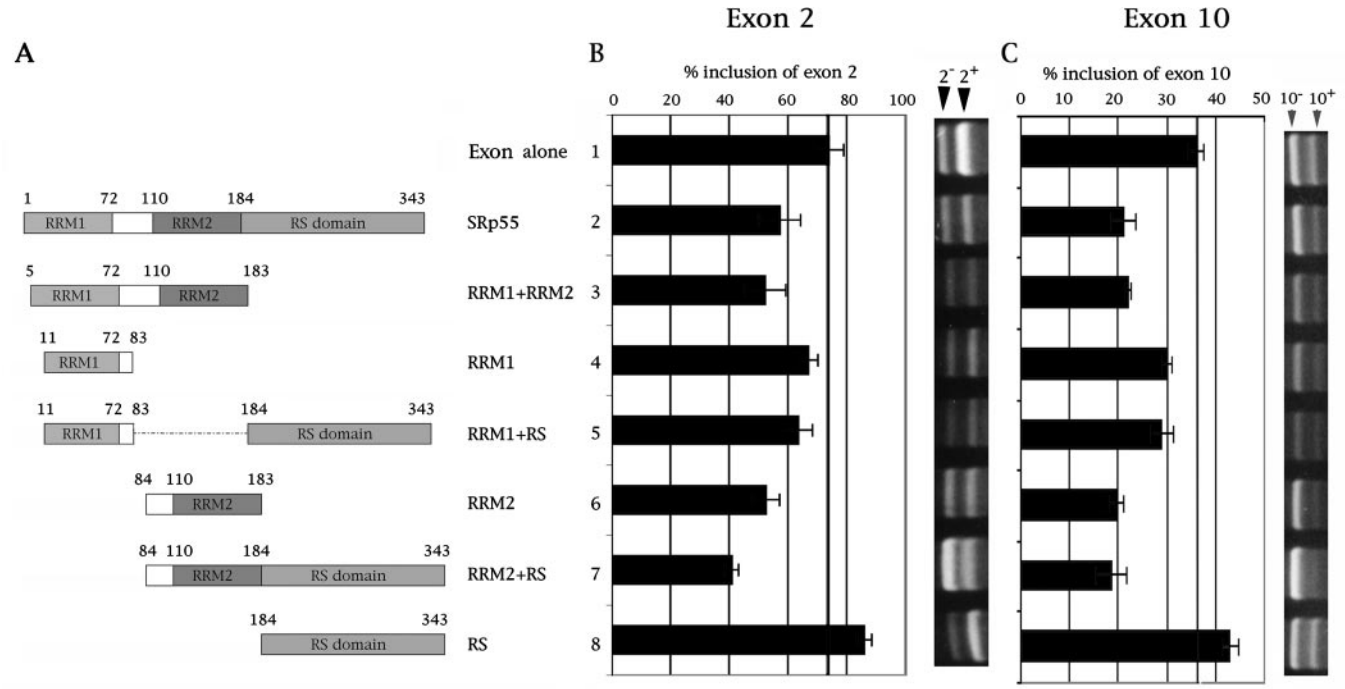

FIG. 5. **The RRM2 domain of SRp55 is required for inhibition of exon 2 and exon 10 splicing.** *A*, diagram of SRp55 deletion variants. Drawing conventions are as described in Fig. 4. The *dotted line* in mutant RRM1+RS indicates that the intervening region has been deleted. RT-PCR products come from 1:1 co-transfections of the SRp55 deletion variants in COS cells with constructs SP/2L (*B*) and SP/10L (*C*). Exon ratio calculations and graph conventions are as described in Fig. 1. Primer pair for *B* and *C*: SVP2/SPL-LN.

DISCUSSION

One of the Regulatory Elements of Tau Exon 2 Is an Exonic Silencer Modulated by a Complex Consisting of htra2β1, SRp55, and SRp30c—In our present foray we discovered that exon 2 contains a silencer at its 5′ end (defined by deletions E2-Δ1 and E2-Δ2; Fig. 1*B*). Our assays (Figs. 2 and 4–8) further indicate that this element exerts its effect by binding a complex comprising htra2β1, SRp55, and SRp30c, which binds to exon 2 via the two latter proteins.

Exonic silencers are postulated to work by recruiting inhibitory factors or by blocking spliceosome assembly (45). Our results suggest that SRp30c and SRp55 directly bind to the E2-Δ1/Δ2 and E2-Δ2 elements, respectively, because at least one of their RRM domains is required for exon 2 exclusion. The activation of exon 2 splicing by the RS mutant of SRp55 has been seen with RS mutants of other SR proteins (40) and suggests that overexpression of this domain sequesters interacting inhibitors (in the case of exon 2, htra2β1).

Besides tau exons 2 and 10, htra2β1, SRp55, and SRp30c can either activate or inhibit splicing of other alternatively spliced exons, including tau exon 3 (46–50). Htra2β1 activates splicing by binding to purine-rich or -enriched motifs (37, 43, 44). However, in exon 2, it does not exert its effect through direct binding, although exon 2 has a purine-rich enhancer defined by mutants E2-Δ6 and E2-Δ5 (Fig. 1, *A* and *B*). Instead, htra2β1 no longer inhibits exon 2 in mutants E2-Δ1 and E2-Δ2 (Fig. 2*A*), and its N-terminal RS domain is required for this activity (Fig. 4*B*). The naturally occurring variant htra2β3 lacks this RS domain and is predominantly expressed in brain after day E18 in the rat (14). This suggests that the relative inclusion of exon 2 during development may partly depend on htra2β isoform ratios: the increase of the 2^+ tau isoform in adulthood correlates with the increase of htra2β3, which cannot inhibit exon 2 splicing as htra2β1 can.

SRp30c and SRp55 are known to interact with htra2β1 (Fig. 8) (44, 48, 51). Like htra2β1, SRp55 and SRp30c no longer inhibit

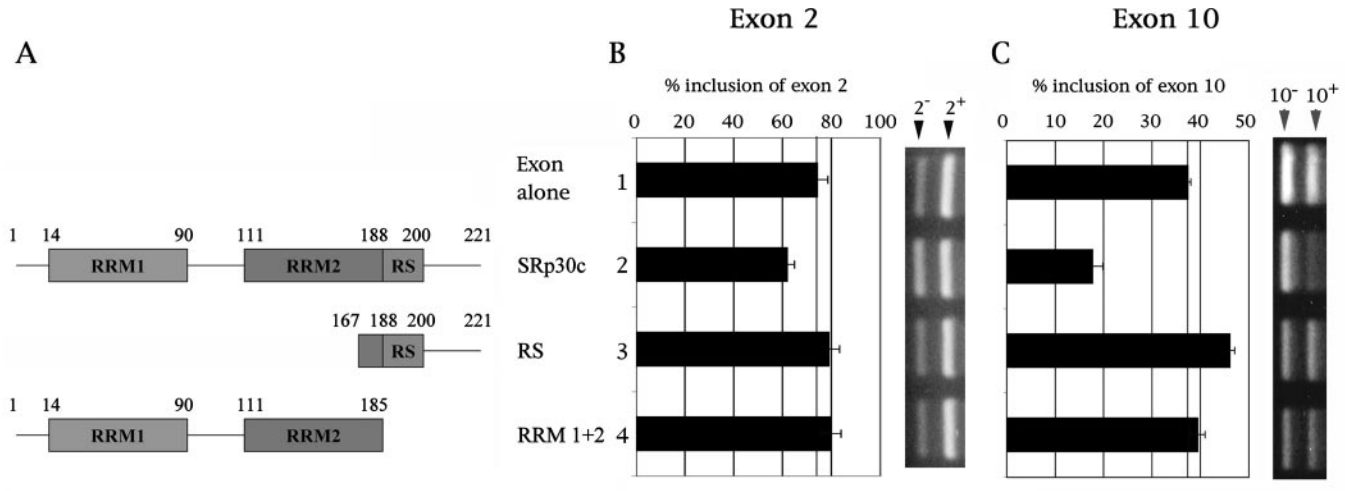

FIG. 6. **The full length of SRp30c is required for inhibition of exon 2 and 10 splicing.** *A*, diagram of SRp30c deletion variants. Drawing conventions are as described in Fig. 4. RT-PCR products come from 1:1 co-transfections of the SRp30c deletion variants in COS cells with constructs SP/2L (*B*) and SP/10L (*C*). Exon ratio calculations and graph conventions are as described in Fig. 1. Primer pair: SPL-LS/SPL-LN.

exon 2 splicing in deletions E2-Δ1 and E2-Δ2 (Fig. 2, *A* and *B*). However, unlike htra2β1, the RRM domains of the two factors are indispensable for their inhibitory activity (Figs. 5*B* and 6*B*).

Therefore, it appears that the three factors inhibit exon 2 splicing by forming a complex that recognizes the E2-Δ1/Δ2 region through the RRM domains of SRp30c and the RRM2 domain of SRp55. Htra2β1 exerts its influence by binding to both SR proteins via its N-terminal RS domain (RS1). SRp30c is the core element of the inhibitory complex acting on exon 2, and it binds to E2-Δ1 and part of E2-Δ2. E2-Δ2 is an SRp55 consensus site (9) and would be expected to bind SRp55 very strongly, yet SRp55 is the weakest inhibitor of exon 2 among the three factors in cellular assays (Fig. 2*A*, *lanes 2* and *3*; Fig. 2*B*, *lane 2*). Binding of SRp30c partly masks the perfect binding site of SRp55 in E2-Δ2, thereby decreasing the influence of SRp55 on exon 2. In deletion E2-Δ1, SRp30c can no longer bind and therefore allows strong binding of SRp55.

The regulatory complex may inhibit exon 2 splicing by sterically interfering with the U2 small nuclear riboprotein that would form directly upstream of the silencer, at the 3′ splice site of exon 2. However, the strong SRp55 binding in deletion E2-Δ4 (Fig. 7*A*) with the concurrent loss of ability of SRp55 to regulate exon 2 splicing in that deletion (Fig. 2*B*) suggests that this region regulates exon 2 splicing by binding yet another factor that interacts with SRp55. A final player is the purine-rich enhancer defined by mutants E2-Δ5 and E2–6 (Fig. 1*A*) that may sterically interfere with the action of E2-Δ4 by binding an activator.

In our previous work (30), we tentatively proposed that htra2β1, SRp30c, and SRp55 may act as a complex that inhibits exon 2 splicing, possibly by binding to a then-unidentified silencer. In the present study, we identify the silencer and confirm that it is regulated by an htra2β1·SRp55·SRp30c complex. In Fig. 9*A*, we show an updated model of exon 2 splicing regulation that incorporates this new knowledge.

One of the Regulatory Elements of Tau Exon 10 Is an Exonic Silencer Recognized by SRp55 and SRp30c—Previous work (25, 27, 31–34) showed that tau exon 10 contains several silencers. Mutations in them appear in FTDP pedigrees: N279K, L284L, and N296N, which occur within E10-Δ2/3/4, E10-Δ8/9, and E10-Δ15, respectively (Fig. 1*C*) (25, 27, 32). Our assays (Figs. 2–7) indicate that the silencer defined by E10-Δ2/3/4 exerts its effect by directly binding a complex composed of SRp55 and SRp30c.

The region directly upstream of E10-Δ2/3/4 defines a consensus SRp55 binding site (TGCAGA) according to the ESEfinder (9). Yet SRp55 binds to E10-Δ2/3/4 and not to its consensus site, which jibes with the fact that the latter region acts as an enhancer (32).[2] SRp30c also appears to bind directly to the E10-Δ2/3/4 region, which consists exclusively of A and T residues. This composition resembles the 5′ half of the hnRNPA1 CE9 intronic element, to which SRp30c binds as a splicing inhibitor (46). The weak residual binding of SRp55 to E10-Δ3/4 (Fig. 7*E*, *lane 5*) indicates that the region defined by E10-Δ2 is part of its binding site. The abolition of SRp30c splicing inhibition in mutant ENH (N279K) (Fig. 3*B*) confirms that it binds downstream from SRp55. This mutation overlaps the beginning of E10-Δ5, which defines the start of a purine-rich enhancer that binds htra2β1.

Previous work (27, 37) led to the conclusion that htra2β1 activates splicing of exon 10 by binding to the purine-rich enhancer defined by region E10-Δ5/6. Our work suggests that the increase of exon 10 inclusion in mutant ENH may arise not from strengthening the binding of htra2β1 (Fig. 4*D*) but rather from abolishing the binding of SRp30c (Fig. 3*B*, *lane 6*). The behavior of SRp55 and SRp30c indicates that the two inhibit splicing of tau exon 10 by binding to a region (E10-Δ2/3/4) directly adjacent to the htra2β1 site (E10-Δ5/6), thereby probably sterically interfering with the activator. The two inhibitors can inhibit exon 10 individually, as shown by the behavior of mutant ENH (Fig. 3*B*).

SRp30c and SRp55 apparently inhibit exon 10 splicing by binding to the E10-Δ2/3/4 region through their RRM domains. SRp30c binds downstream of SRp55, and the RRM2 of SRp55 is the primary binding component for that factor. As is the case with exon 2, splicing of exon 10 is also activated by the RS mutants of SRp55 and SRp30c, strengthening the theory that overexpression of this domain may relieve inhibition by sequestering partnering factor(s).

Htra2β1 activates exon 10 splicing through direct binding to a region adjacent to the SRp55/SRp30c binding site, but it can be outcompeted easily by the two inhibitors. The RRM domain alone of htra2β1 is a better activator than the full-length factor (Fig. 4*C*, *lane 3*), implying that SRp30c may interfere with htra2β1 by interacting with the RS1 domain of htra2β1. Finally, the RS2 domain of htra2β1 is crucial for activation of

[2] Y. Wang, L. Gao, and A. Andreadis, unpublished results.

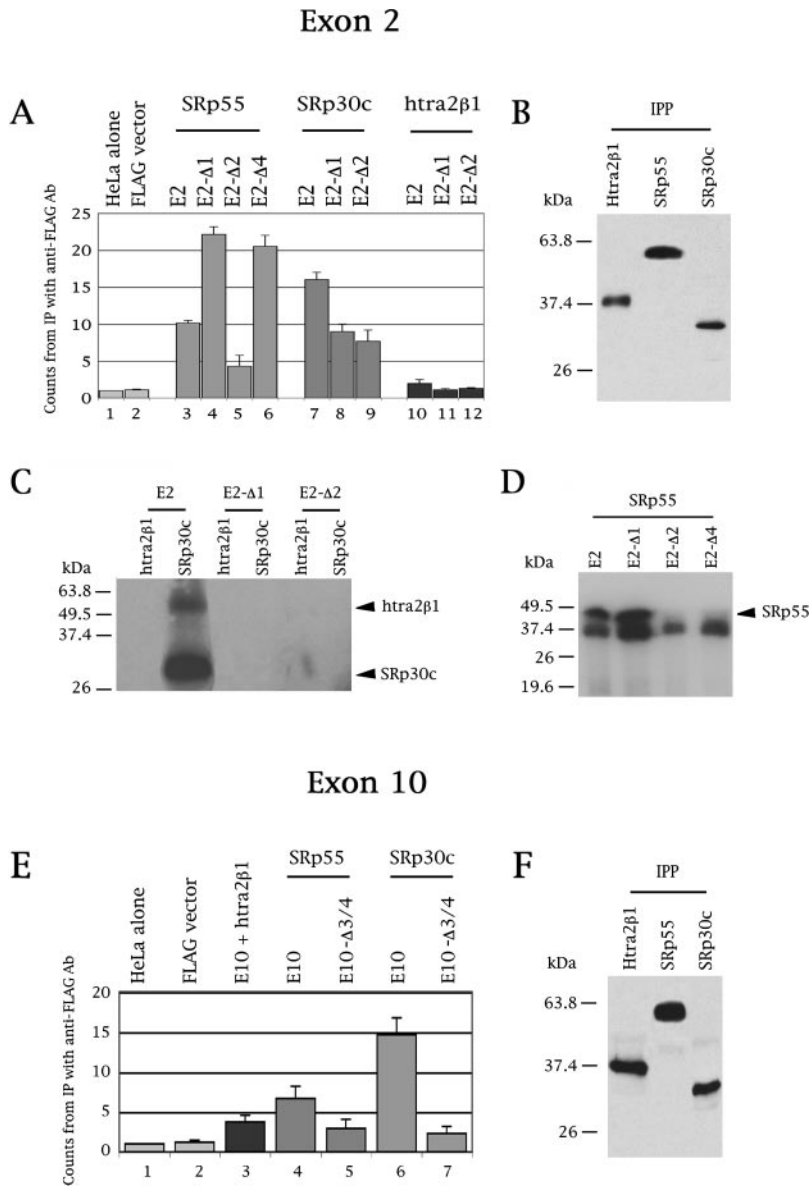

FIG. 7. **The behavior of htra2β1 differs in the regulation of exons 2 and 10.** *A* and *E*, ^{32}P-labeled riboprobes containing exon 2 or 10 were incubated with extracts from HeLa cells transfected with plasmids of FLAG-tagged factors, and they were immunoprecipitated by anti-FLAG monoclonal antibody. Amounts of each SR protein bound to RNA of wild-type and mutant tau exon 2 (*A*) or exon 10 (*E*). Values were calculated relative to the nonspecific binding of the mock vector transfection (means ± S.D. of three analyses). *B* and *F*, Western blots of immunoprecipitated proteins (*IPP*) from cell lysates that were transfected with each FLAG-tagged factor by anti-FLAG antibody in the presence of riboprobes for (*B*) exon 2 and (*F*) exon 10. Transfection efficiency and expression levels of each tagged SR protein are equivalent. *A* and *B*, SRp30c and SRp55, but not htra2β1, bind directly to tau exon 2 pre-mRNA in HeLa cells, and their binding is altered in deletion mutants E2-Δ1, E2-Δ2, and E2-Δ4. *C* and *D*, SRp55 and SRp30c, but not htra2β1, bind directly to the exon 2 pre-mRNA in UV-cross-linking assays. GST versions of the three proteins were UV-cross-linked to wild-type and deleted (E2-Δ1, E2-Δ2, and E2-Δ4) exon 2 riboprobes. Cross-linking of (*C*) SRp30c and htra2β1 and (*D*) SRp55. The *top band* in *D* is full-length SRp55. The *bottom band* in *D* is a truncated degradation product, discussed under "Results." *E* and *F*, htra2β1, SRp55, and SRp30c bind directly to tau exon 10 pre-mRNA in HeLa cells, and the binding of the latter two is abolished in mutant E10-Δ3/4.

exon 10 because its combination with the RRM gives the strongest activation regardless of the strength of the RNA binding site (Fig. 4, *C*–*F*, *lane 5*). Interestingly, htra2β1 mutant ΔRS1 can still activate exon 10 deletion Δ5, suggesting that htra2β1 can function even if its binding to exon 10 is greatly weakened. These results, combined with the fact that htra2β1 activates exon 10 relatively weakly, suggest the possible involvement of a co-activator. Alternatively, each of the two RS domains may alter the conformation of htra2β1. Fig. 9*B* shows a model of exon 10 splicing regulation that incorporates these results.

Comparisons of the Splicing Regulatory Mechanisms That Operate on Tau Exons 2 and 10—Tau exons 2 and 10 show intriguing similarities in their expression patterns and behavior. Both are adult-specific, and their relative levels of expression are congruent across different nervous tissues (18). Also, the default splicing behavior of both exons is inclusion (25, 29), yet most SR and hnRNP proteins inhibit their splicing (25, 27, 30). This collective behavior is unusual, although it is consistent with the theory that exons 2 and 10 must be primarily regulated through inhibition.

The cumulative results from our experiments let us formulate speculative models of splicing regulation of tau exons 2 and 10 (Fig. 9, *A* and *B*). Here, too, interesting similarities and contrasts emerge. Both exons have a silencer at or near their 5′ end, although that of exon 2 is pyrimidine-rich, whereas that of exon 10 is AT-rich (Fig. 1, *A* and *C*). In both cases, SRp55 and SRp30c act as an inhibitory heterodimer that binds to the silencer at the 5′ end of each exon. Both domains of SRp30c and the RRM2 domain of SRp55 are required for suppression of either exon, and their RS domains alone moderately activate splicing of both exons, almost certainly through a sequestering effect. However, in exon 2, SRp30c is apparently situated upstream of SRp55 on the silencer, but their relative locations are switched in exon 10. Finally, although htra2β1 acts in opposite ways on the two exons, it shows an interesting parallel nevertheless: htra2β1 mutant ΔRS1 can be considered an "activator" for both exons because it no longer inhibits exon 2 but is the strongest activator of exon 10 among the htra2 mutants. Interestingly, mutant ΔRS1 corresponds to naturally occurring variant htra2β3, which is predominantly expressed in brain and increases during development (14). Thus, the shift toward more htra2β3 favors inclusion of both tau exons, but by different mechanisms.

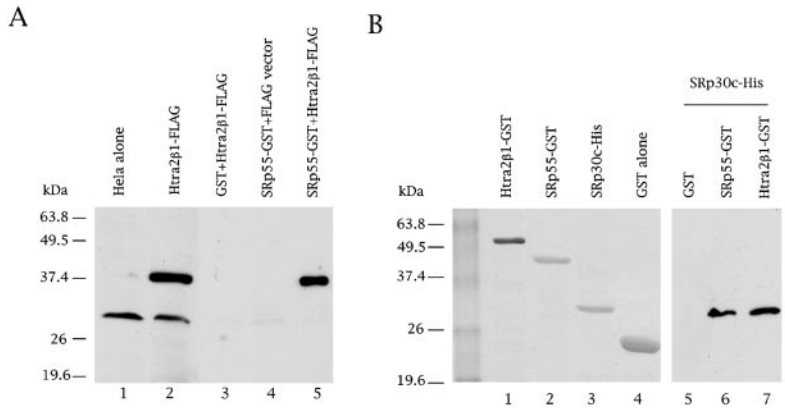

FIG. 8. **SRp55, SRp30c, and htra2β1 interact with each other.** *In vitro* interaction assays were done using recombinant htra2β1-GST, SRp55-GST, and SRp30c-His fusion proteins produced in *E. coli* and FLAG-tagged htra2β1 produced in HeLa cells. *A*, htra2β1 interacts with SRp55 (*lane 5*). htra2β1-FLAG was expressed in HeLa cells (*lane 2*). FLAG and htra2β1-FLAG proteins were incubated with GST and SRp55-GST proteins. GST alone/htra2β1 and FLAG alone/SRp55 were used as the negative controls (*lanes 3* and *4*). *B*, SRp30c interacts with SRp55 and htra2β1 (*lanes 6* and *7*). Fusion proteins were expressed in *E. coli* (*lanes 1–4*). SRp30-His was incubated with GST alone and GST fusion proteins. GST alone was used as the negative control (*lane 5*).

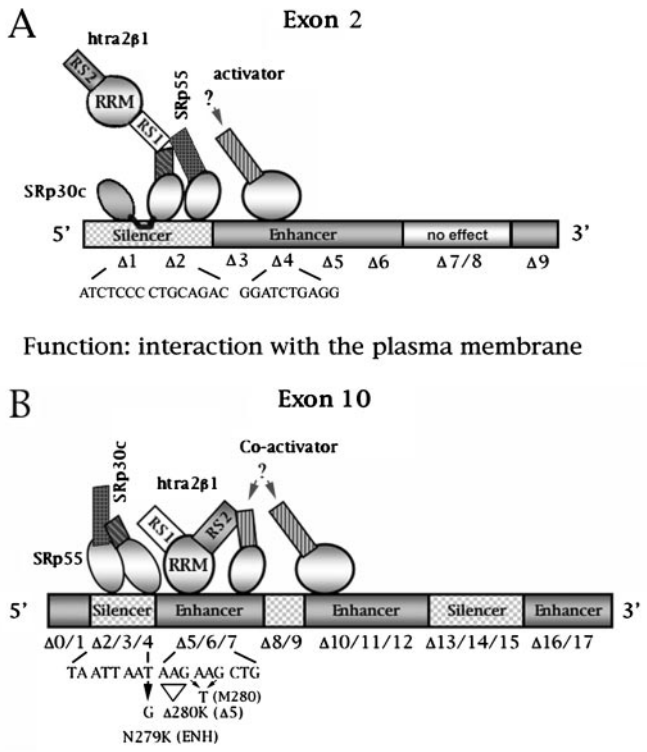

FIG. 9. **Speculative model of splicing regulation for the silencers located at the 5′ end of tau exons 2 and 10.** The cellular functions of the tau domains are shown *below* the diagram. For the exons, *shaded regions* represent enhancers, and *plaid regions* represent silencers (not drawn to scale). For the factors, *circles* represent RRM domains, and *squares* represent RS domains. *A*, exon 2. SRp30c directly binds to regions E2-Δ1 and E2-Δ2. SRp55 directly binds to region E2-Δ2 and indirectly influences regions E2-Δ1 and E2-Δ4 through SRp30c and an unknown activator, respectively. SRp30c, SRp55, and htra2β1 bind to each other through their RS domains (in the case of htra2β1, the RS1 domain). *B*, exon 10. SRp30c and SRp55 bind to region E10-Δ2/3/4, whereas htra2β1 binds to region E10-Δ5/6. The two inhibitors interact with (or sterically interfere with) the RS1 domain of htra2β1; its RS2 domain may interact with a putative co-activator that may bind to either E10-Δ6/7 or E10-Δ10/11/12.

Effects of Modulation of the Tau Projection Domain on the Structure and Function of the Tau Protein—The tau exon 2/3 splicing events are developmentally regulated (18, 22–24). The resulting adult variants (2^+3^-, 2^+3^+) undoubtedly influence the conformation of the tau molecule, given the predominance of acidic residues encoded by the two exons. These changes alter tau interactions with other ligands and hence may regulate tau influence on axonal stability and neuronal plasticity.

The specific functions and ligands of the protein domains encoded by tau exons 2 and 3 are unknown. They are not involved in microtubule binding or spacing, functions that are localized to the C terminus and hinge region of tau and specifically influenced by the presence of exon 10 (18, 53, 54). The N terminus may be important for microtubule bundling because N-terminal deletion mutants display reduced capacity for microtubule bundling (55). The N terminus of the tau protein (also called the projection domain) interacts with the plasma membrane (20) and is phosphorylated by the non-receptor tyrosine kinase *fyn* (56).

Regulation of Tau Exon Splicing and Frontotemporal Dementia—The accumulation of abnormal tau filaments into tangles is a hallmark of many neurodegenerative diseases, including Alzheimer disease. Formation of neurofibrillary tangles is an early event in the dementia cascade, and the number of neurofibrillary tangles correlates with disease severity. In several neurodegenerative diseases, now collectively termed tauopathies, tau pathology is solely and directly responsible for neuronal death and development of the clinical dementia manifestations (19).

The tauopathy pedigrees analyzed thus far predominantly show mutations in tau exon 10, although several pedigrees carry mutations in tau exons 1, 9, 11, 12, and 13 that influence either microtubule binding or protein conformation (19). The exon 10 mutations fall in two categories, those that influence microtubule binding and those that alter the ratio of exon 10 isoforms. However, one atypical tauopathy (myotonic dystrophy type 1) is characterized by tau hyperphosphorylation, formation of intraneuronal aggregates, and significant reduction of tau isoforms containing exon 2 (28). Myotonic dystrophy type 1 arises from a CTG repeat extension at the 3′-untranslated region of the DM protein kinase gene (57). More recent studies indicate that exon 10 is also affected in myotonic dystrophy type 1, although its relative suppression is much less than that of exon 2 (58). Almost certainly, the repeat extension acts as a sink for the CELF family of splicing factors (52) that affect tau exons 2 and 10 (27, 30). Thus, tau isoform ratios can cause neurodegeneration either directly (by *cis* mutations in tau exons) or indirectly (by variations in levels of *trans* factors that regulate tau exons).

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 (19).

Acknowledgment—We thank Dr. Chris Lorson for the generous gift of the SRp30c/RSET construct.

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