Arginine/Serine-rich Protein Interaction Domain-dependent Modulation of a Tau Exon 10 Splicing Enhancer

ALTED INTERACTIONS AND MECHANISMS FOR FUNCTIONALLY ANTAGONISTIC FTDP-17 MUTATIONS Δ280K AND N279K*

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† The abbreviations used are: E10, exon 10; ESE, exon splicing enhancer; FTDP-17, frontotemporal dementia with parkinsonism chromosome 17-type; I10, intron 10; PPE, polyuridine enhancer; RRM, RNA recognition motif; RS, arginine/serine-rich protein interaction domain; siRNA, small interfering RNA; SR, Arg/Ser-rich splicing factors; 3R, three-repeat tau isoforms; 4R, four-repeat tau isoforms; RT, reverse transcription.

Tau exon 10 splicing is altered by autosomal dominant mutations that cause frontotemporal dementia with parkinsonism chromosome 17-type and by unknown mechanisms in other related neurodegenerative disorders. Identifying cis- and trans-regulators of tau exon 10 splicing is therefore crucial for understanding disease mechanisms.

In the central nervous system, the microtubule-associated protein tau is highly expressed in neuronal axons and at lower levels in glial cells. Tau functions in microtubule assembly, influences microtubule stability and is important for neurogenesis, axonal maintenance, and axonal transport (1, 2). In the human tau gene (MAPT), exons 9–12 each encode a 31–32-amino acid imperfect repeat that comprises the microtubule-binding domain of tau. In adult human brain, exons 2, 3, and 10 are alternatively spliced to produce six different tau isoforms (3–5). Exon 10 (E10) inclusion generates isoforms with four microtubule repeats called four-repeat (4R) tau. When E10 is skipped, the result is 3R tau isoforms. The 4R/3R ratio in normal adult brain is ~1. In fetal brain, only the shortest 3R tau isoform is expressed because of constitutive exclusion of exons 2, 3, and 10 (6, 7).

Pre-mRNA splicing regulation is complex requiring multiple interactions between small ribonucleoprotein particles and non-small ribonucleoprotein particle splicing factors with conserved and nonconserved splicing elements in the unprocessed RNA transcript (8). Conserved elements are the 3′ and 5′ splice site signals that demarcate the exon-intron boundary. Nonconserved sequences that promote splicing are called exon splicing enhancers (ESE) and intron splicing enhancers (ISE) (9). Serine/arginine-rich (SR) proteins usually bind to enhancer elements and are essential at multiple steps in the splicing pathway (10). Most alternatively spliced exons contain a weak splice site, whose recognition requires SR factor associations with ESEs and ISEs (11). Alternative splicing is also influenced by splicing inhibitory sequences called exon splicing silencers and intron splicing silencers. An additional function of some enhancers is to counteract the inhibitory effects of neighboring silencer elements (12–17).

For MAPT, cis-regulation of E10 alternative splicing has been extensively studied, where multiple enhancers and silencers regulate use of the weak E10 3′ and 5′ splice sites (14, 18). E10 sequences include three nonredundant, weak ESEs in the first half of the exon that include an SC35-like ESE, a polypurine enhancer (PPE), and an A/C-rich enhancer separated by a central exon splicing silencer from additional ESE sequences at the 3′ end of E10. Intronic sequences immediately downstream of the E10 5′ splice site include an intron splicing silencer and an adjacent intron splicing modulator, which counteracts the intron splicing silencer.

Mutations in MAPT cause frontotemporal dementia with parkinsonism chromosome 17-type (FTDP-17), an autosomal dominant neurodegenerative disease (19–22). FTDP-17 belongs a group of disorders called tauopathies, in which hyperphosphorylated tau forms pathological neuronal aggregates called neurofibrillary tangles. Other tauopathies include Alzheimer disease, progressive supranuclear palsy, corticobasal degeneration, and Pick disease. Over 30 coding and intronic MAPT mutations are known that cause FTDP-17 (23, 24). One class includes missense mutations that alter tau protein function. The second mutation class includes missense, silent, deletion, and intronic mutations that alter E10 splicing by disrupting either the 5′ splice site (14, 21, 25), PPE, A/C-rich enhancer, and exon splicing silencer sequences in E10 (18, 26–29) or the intron splicing silencer and intron splicing modulator sequences in intron 10 (I10) (14, 30). Most splicing mutations increase 4R tau isoforms and alter the 4R/3R ratio from 1 to 2–3. However, mutations Δ280K and E10+19, which decrease E10 inclusion in splicing assays, are expected to reduce the 4R/3R ratio to as low as 0.33.
Silent and intronic mutations do not change the protein sequence of tau but affect E10 splicing and ultimately tau function. Thus, subtle changes in the ratio of normal tau isoforms cause severe neurodegeneration.

Identifying proteins that associate with cis-elements is necessary to elucidate how E10 splicing is regulated. Here we focus on identifying protein interactions with the E10 PPE, a 9-nucleotide purine-rich ESE sequence between E10 positions +16 and +24 (see Fig. 1A). The PPE sequence contains two copies of an AAG motif and one copy of a GAR (where R is a purine) motif that occur frequently in natural, purine-rich ESEs. These motifs are often high affinity binding sites for SR and SR-related proteins. FTDP-17 mutations N279K and Δ280K alter the normal PPE sequence by adding or removing an AAG copy, respectively (see Fig. 1A). Consequently these mutations have opposite effects on E10 splicing, where N279K increases and Δ280K decreases E10 inclusion. Our earlier work predicted that the normal PPE function requires SR factor interactions, which are altered by disease mutations Δ280K and N279K (26). Indeed, the SR-like splicing factor Tra2β is essential for E10 inclusion in splicing assays and associates in vitro with both normal and mutant PPE templates (31). Here we show specific in vitro associations of a trio of factors with the normal and strengthened (mutation N279K) PPE but not with a disrupted PPE (mutation Δ280K) and identify two of these proteins as the SR domain-containing factors SF2/ASF and Tra2β. We corroborate their specific effects on E10 splicing by gene knock-down assays and present in vivo mechanisms for their varied effects on E10 splicing. Overexpression of SF2/ASF rescues the splicing defect in mutation Δ280K. Domain deletion assays in non-neuronal and neuronal cells show that the C-terminal protein-interacting domains of both SF2/ASF and Tra2β are required for E10 splicing. Our data reveal PPE-dependent and PPE-independent roles for SF2/ASF for the use of the weak 3′ and 5′ splice sites, respectively. Thus, SF2/ASF has complex essential and regulatory roles on E10 splicing, whereas the role of Tra2β appears secondary to SF2/ASF.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and DNA Mutagenesis—**Splicing vector hN has been described (18) and contains the entire 93-bp MAPT E10 sequence with 33 and 51 bp of flanking introns (see Fig. 3A). Minigene construct E10AH (see Fig. 2) contains entire MAPT exons 9, 10, and 11 as well as 324 and 559 bp of intron 9 and 110 sequences, respectively, inserted into expression vector pRcRsv (Invitrogen). Intron 9 in E10AH contains 224 bp of sequence immediately 5′ of E9 and 100 bp of sequence immediately 3′ of E10. E10 in E10AH contains 100 bp of sequence immediately 5′ of E10 and 459 bp of sequence immediately 5′ of E11. FTDP-17 mutations Δ280K and N279K were introduced into hN (18) and E10AH by PCR mutagenesis.

SF2/ASF, Tra2β, and hnRNP G Expression Constructs—SF2/ASF cDNA was amplified by RT-PCR from HeLa RNA using primers ASE5′F (5′-AGCTGGATCCATGCGAGGTGGTGCAG-3′) and ASE3′R (5′-CGATCTCGAGTTATGTAACAGGAGGCGGCTC-3′). The PCR product was digested at BamHI and XhoI sites in the primer sequences and inserted into vector pCDNA3.1/Zeo+ (Invitrogen) to generate expression construct SF2/ASF. The Tra2β expression vector contains a cDNA sequence obtained by PCR amplification from vector Htra2β-V5 (32) using primers Tra2b5′F (5′-AGCTGGATCCATGCGAGGTGGTGCAG-3′) and Tra2b3′R (5′-CGATCTCGAGTTATGTAACAGGAGGCGGCTC-3′). The BamHI/XhoI-digested product was inserted into pCDNA3.1/Zeo+. We generated domain deletion constructs by PCR mutagenesis of full-length SF2/ASF and Tra2β expression vectors (see Fig. 4A). Primers used to delete the N-terminal RRM domains from SF2/ASF (construct SF2/ASF-ΔRRM) were SF2dRRMF (5′-AGCTGGATCCATGCGAGGTGGTGCAGGAT- GTA-3′) and SF23′R. For RS domain deletion construct SF2/ASF-ΔRS, the primer pair SF25′F and SF2dRS (5′-CGGCCCTGAGTTATGGGGCCATCAAATTTAACC-3′) was used. The N-terminal RS domain in construct Tra2β-ΔRS1 (see Fig. 4A) was deleted using primers Tra2bRS1 (5′-CGGCCCTGAGTTATGGGGCCATCAAATTTAACC-3′) and Tra2b3′R. The central RRM domain in Tra2β-ΔRRM (see Fig. 4A) was deleted using primers Tra2b5′ and Tra2b3′R with expression construct PCMvHtt1 + 5′ as template (kind gift from William Mattox). In Tra2β-ΔRS2 (see Fig. 4A) the C-terminal RS domain was deleted using primers Tra2b5′ and Tra2bRS2 (5′-CGGCCCTGAGTTATGGGGCCATCAAATTTAACC-3′). Construct hN-RNP-G-V5 is described elsewhere (32).

**Cell Culture, Transfections, and RNA Isolation—**Maintenance and transfection of PC12 cells is described elsewhere (14). HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen). All of the transfections were performed in triplicate with Lipfectamine (Invitrogen) in six-well plates. HeLa cells were transfected with 2 μg of plasmid DNA and 6 μl of Lipofectamine in 1 ml of OptiMEM (Invitrogen) for 5 h at 37 °C (5% CO2), after which 1 ml of Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum was added. For coexpression experiments in PC12 cells, 2.5 μg of total plasmid containing 0.5 μg of an E10 splicing vector and 2 μg (4-fold excess) of either pSKII control vector (Stratagene) or individual splicing factor expression constructs were used with 6 μl of Lipofectamine. Total RNA was isolated with TRIzol reagent (Invitrogen) as previously described (26).

**siRNA Synthesis and Transfection—**Commercially synthesized (Dharmacon Inc.) siCONTROL nontargeting siRNA number 1 and gene-specific siRNA pools were used for endogenous suppression of SF2/ASF and Tra2β. siRNA transfections were optimized in 24-well plates containing cells at 70% confluency using Oligofectamine (Invitrogen) with 25, 50, 75, and 100 nmol of each siRNA in the pool (see Fig. 2A). Maximal inhibition was observed with 100 nmol of each siRNA. Protein was harvested 48–72 h post-transfection, and expression of targeted factors was tested by immunoblot analyses (described below). To test the effects of siRNA treatment on E10 splicing vectors, the cells were seeded into 12-well plates, and siRNA transfections were scaled up. After 24 h, the cells were rinsed in phosphate-buffered saline and fed 450 μl of antibiotic-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. One μg of normal or mutant splicing vector E10AH was mixed with 3 μl of Lipofectamine in 50 μl of OptiMEM and added to each well. Total RNA was isolated 48 h later for RT-PCR analyses with TRIzol reagent (Invitrogen).

**Quantitation of Tau E10 Splicing by RT-PCR—**Tau E10 splicing from transiently transfected splicing vectors was analyzed by RT-PCR assays as described previously (26). E10+ and E10− transcripts were amplified by RT-PCR using vector-specific primer pairs SD6/S2A for hN and pREP/BGHPA2 for E10AH as previously reported (18, 33). Amplified products from hN are 261 bp (E10AH) and 354 bp of E10+ and from E10AH are 447 bp (E10−) and 540 bp (E10+). The products were resolved on 5–7.5% acrylamide gels and quantitated using a Molecular Imager® system (Bio-Rad). For each mutant construct, the values presented are the averages of at least three different transfection experiments with the normal E10 construct transfected in parallel. Statistical comparisons were made using a two-tailed Student’s t test. Criteria for significance were calculated using a Bonferroni correction for multiple comparisons.

**In Vitro RNA Synthesis—**A primer-based strategy was used to in vitro synthesize short, radiolabeled RNAs containing normal or mutant PPE
sequences. Forward and reverse primers were designed such that on annealing they form a double-stranded T7 promoter region upstream of the RNA target to be transcribed. A universal forward primer, T7F3 (5'-GCGCTCTAGATATACGACTCCTATAGGGATATGTTAT-3') containing the core T7 promoter sequence (italics) was used. Nine additional nucleotides upstream of the T7 promoter sequence maximize transcription efficiency, and five additional nucleotides downstream of the promoter serve as a linker that overlaps with the RNA target. The reverse primer contains the reverse complement of the core T7 promoter (underlined) with two sequences (italics) that include the normal or mutant PPE element (bold type). The reverse primers were used: T7PPE (5'-GCGCTCTAGATCCAGCTCTTCTATATCTCCTATAGTGCTAGTAGGCG-3'), T7279K (5'-GCGCTCTAGATTATGTTAGAGGGATATGTTATAGGGCGAATCCAGCTCTTCTATACTCCCTATGTTAGCCTGATTAGCG-3'), and T7Δ280K (5'-GCGCTCTAGATCCAGCTCTTCTATATCTCCTATAGTGCTAGTAGGCGAATCCAGCTCTTCTATACTCCCTATGTTAGCCTGATTAGCG-3'). To anneal universal and reverse primers, 100 ng of each in 0.1 M NaCl was heated to 94 °C for 3 min and cooled at room temperature for 10 min. For in vitro transcription, 1 µl of the annealed primer reaction was added to 19 µl of a transcription mixture containing 1 X T7 polymerase buffer (Invitrogen), 20 mM dithiothreitol, 20 units of RNasin (Promega), 5 µM [32P]αATP or [32P]αGTP (800 Ci/mm), 0.5 mM each of the remaining three NTPs and 50 units of T7 RNA polymerase. The reactions were incubated at 31 °C for 2 h, heat-denatured at 75 °C for 2 min, quenched on ice for 2 min, and treated with 10 units of DNase I at 37 °C for 20 min to remove primers. 7 µl of formamide loading dye was added to samples, heated at 95 °C for 5 min, and resolved on 12% urea-PAGE gels. Full-length transcripts were visualized by autoradiography, excised, and eluted in 400 µl of elution mix (0.5 M ammonium acetate, 2.5 mM EDTA, 0.5% SDS) for 1 h at room temperature. The eluate was ethanol-precipitated and resuspended in 100 µl of RNase-free H2O, and 1 µl was used for quantitation in a scintillation counter.

HeLa Nuclear Extract Preparation, UV Cross-linking, and Affinity Purification—HeLa nuclear extracts were prepared as described (34). The cell pellets were obtained from HeLa-S3 spinner cultures grown at 37 °C for 48 h. The cell pellets were obtained from HeLa-S3 spinner cultures grown at 37 °C for 48 h. Purification in a scintillation counter.

RESULTS

The following experiments were designed to identify the trans-acting proteins that recognize the normal PPE, to determine whether the protein-RNA interaction is disrupted by mutations that eliminate E10 inclusion, and to determine whether PPE mutations that enhance E10 inclusion work by strengthening normal protein-RNA interactions or cause different proteins to interact with the extended PPE. Altered Association of Splicing Factors with Normal and Mutant PPE Sequences—To identify proteins associations, radiolabeled RNA targets containing the normal and mutant PPE sequences (Fig. 1A) were incubated with HeLa nuclear extracts under splicing conditions and treated with UV light to crosslink RNA-protein complexes. Because purine-rich enhancers such as the PPE bind SR proteins, we first tested four different HeLa nuclear extract preparations for SR protein content by immunoblot analysis. The pan-SR monoclonal antibody mAb104 recognizes phosphorylated epitopes within the RS domain of prominent SR protein family members SRp75, SRp55, SRp40, and SRp30 (36) that were consistently observed in all four nuclear extract preparations (Fig. 1B). UV cross-linking experiments reveal three proteins with apparent molecular masses of 52, 45, and 36 kDa that individually cross-link to normal PPE and 279K RNA targets (Fig. 1C). The 45-kDa factor interacts more strongly with 279K than normal PPE templates. In contrast, almost no SR protein cross-links to the Δ280K target, although overexposure of the immunoblot showed very weak signals from the 52- and 36-kDa factors but no signal from the 45-kDa factor (not shown). Similar results were obtained with different HeLa extract preparations. Thus, mutation Δ280K may inhibit E10 splicing by abolishing producive SR protein interactions. The near absence of detectable proteins with Δ280K targets verifies the binding specificity and rules out nonspecific interactions with flanking linker sequences. Candidate proteins for the 45-kDa band are SR factor SRp40 and the SR-like factor Tra2β. Based on molecular size, the candidates for the 36-kDa protein are SF2/ASF (SRp30a), SC35 (SRp30b), and SRp30c.

To identify these proteins, RNA affinity columns were constructed by coupling the normal and mutant RNA sequences to agarose. Columns were incubated with HeLa nuclear extract under splicing conditions as above but without exposure to UV light. Bound proteins were eluted and analyzed by immunoblotting with Tra2β- and SF2/ASF-specific antibodies. We probed for Tra2β because it preferentially binds AAG repeats (38), present in two and three copies, respectively, in normal and 279K templates (Fig. 1A). Consistent with this observation and similar
to the binding profiles in UV cross-linking assays, we identify the 45- and 36-kDa proteins as Tra2β and SF2/ASF, respectively. Tra2β eluted robustly from the 279K column compared with the normal PPE column (Fig. 1D) and SF2/ASF eluted at similar levels from both columns. Interestingly, only SF2/ASF was eluted at very low levels from Δ280K. The in vitro affinity of SF2/ASF and Tra2β for normal PPE and 279K mutant templates correlates well with their match to their respective binding consensus sequences (Fig. 1A). Missense mutation N279K strengthens the SF2/ASF binding consensus and may allow stronger SF2/ASF binding, although the observed amounts of SF2/ASF bound to 279K and normal PPE targets are similar. ESE sequences are usually degenerate to accommodate their presence within diverse coding sequences. This degeneracy allows more than one SR protein to recognize the same ESE (39, 40). Our results show that the normal PPE sequence is an overlapping low affinity site for both SF2/ASF and Tra2β. Other examples have been described where SF2/ASF associates with an ESE that is also recognized by Tra2β (41). The identity of the 50-kDa factor detected in the UV cross-linking experiments is unknown and is being pursued. In addition to mutation N279K (AAT → AAA) at the same nucleotide position strengthened the PPE by increasing the number of consecutive purines to enhance E10 splicing (18). Because these substitutions enhanced E10 splicing to different extents, the purine context is an important determinant of PPE strength. Although the T to A change increases the number of consecutive purines, unlike in N279K, it does not add an extra AAG copy and is less efficient in E10 enhancement, possibly because of a decreased affinity for Tra2β.

Effects of SF2/ASF and Tra2β Depletion on E10 Splicing—To establish their in vivo requirement on E10 splicing, SF2/ASF and Tra2β were depleted individually or together in cell culture using double-stranded siRNAs (42). HeLa cells treated with increasing concentrations of SF2/ASF-specific siRNAs show a significant reduction in endogenous SF2/ASF levels compared with β-tubulin as a control (Fig. 2A, left panel). Similar assays with increasing concentrations of Tra2β-specific siRNAs show almost total reduction in endogenous Tra2β levels (Fig. 2A, middle panel). The gene-specific siRNAs show appropriate target specificity. si-SF2/ASF does not affect Tra2β levels, and si-Tra2β does not affect SF2/ASF levels (Fig. 2A, right panel, lanes 3 and 4). The lack of an effect between mock and siCONTROL-treated cells rules out nonspecific effects that may result from siRNA exposure (Fig. 2A, right panel, lanes 1 and 2). Cells simultaneously exposed to si-SF2/ASF and si-Tra2β show a depletion of both factors (Fig. 2A, right panel, last lane). However, the extent of depletion is lower compared with the individual siRNA treatments, because the gene-specific siRNA concentration in the simultaneous treatment is half that of the individual treatment. The effects of SF2/ASF and Tra2β depletion in non-neuronal HeLa and neuronal PC12 cells were tested on the transiently transfected E10 minigene splicing vector E10AH, which contains MAPT exons 9–11 with shortened intron 9 and 10 sequences (Fig. 2B). E10AH splicing is dramatically reduced by similar amounts from 80% to 53% and 49% in SF2/ASF- and Tra2β-depleted HeLa cells, respectively (Fig. 2C). Similar reductions of E10 levels in SF2/ASF- and Tra2β-depleted PC12 cells (from 76% to 62% and 54%, respectively) confirm that SF2/ASF and Tra2β are required for E10 splicing in vivo. E10AH splicing remains unchanged in HeLa or PC12 cells treated with the control siRNA compared with mock treated cells (Fig. 2D, left panel). Because si-SF2/ASF does not completely deplete endogenous SF2/ASF levels, the extent of E10 splicing inhibition in si-SF2/ASF-treated cells is very likely an underestimate and preliminarily suggests that SF2/ASF is more prominently required for E10 splicing. When HeLa and PC12 cells are simultaneously treated with both si-SF2/ASF and si-Tra2β, E10AH splicing is also reduced by similar levels to 57%, which is not significantly different from the single treatments. These results suggest that SF2/ASF and Tra2β, although essential, may not act synergistically on normal E10 splicing and may substitute for each other.

Overexpression of Candidate Splicing Factors in PC12 and HeLa Cells—The effects of candidate factors on E10 splicing were tested in vivo by overexpressing these proteins in cells cotransfected with either E10AH or another E10 splicing vector hN, in which E10 is inserted in an intron between two heterologous exons (Fig. 3A). E10 splicing in E10AH is similar in both transiently transfected HeLa (76%) and PC12 (82%) cells (Fig. 3B). In both cell types, mutation N279K causes almost constitutive E10 inclusion (90–93%), whereas mutation Δ280K severely inhibits E10 splicing (5–14%). As shown previously for hN, normal E10 inclusion is ~45% in COS-7,

![FIGURE 1. Identification of nuclear factors that bind normal and mutant PPE targets. A, sequences of in vitro transcribed normal (PPE) and mutant (Δ280K,279K) RNA targets. E10 sequences (bold type) contain the nine-nucleotide core PPE element (underlined) and are flanked by 8 nucleotides of linker sequence shown in normal letters. Deletion mutation Δ280K removes an AAG copy (dashed line) from the PPE. Missense mutation N279K (arrow) creates an extra AAG copy and extends the 5'-end of the PPE. To the right are shown differences in the match of normal and mutant PPE sequences to the 8-nucleotide preferred binding consensus for SF2/ASF and in the amounts of AAG or GAR (where R is a purine) repeats favored by Tra2β. B, immunoblot analysis of four separate HeLa nuclear extract preparations (lanes A–D) using monoclonal antibody mAb104, which detects members of the SR family ranging from 30 to 70 kDa. C, autoradiograph of UV cross-linking assays showing 52-, 45-, and 36-kDa factors covalently bound to radiolabeled normal (PPE) and mutant (279K) targets. Associations with Δ280K templates are virtually undetectable. D, immunoblot analysis of HeLa nuclear factors eluted from Δ280K, PPE, and 279K RNA-agarose affinity columns. The Tra2β polyclonal antibody Rb3505 and the SF2/ASF-specific monoclonal antibody identify 45- and 36-kDa bands (arrows, right panel), respectively, from normal and 279K RNA columns. Only SF2/ASF is weakly detected from Δ280K affinity columns.

| PPE Region Target Sequences | Match to SF2/ASF binding site | Number of Tra2β site consensus repeats | (AAG)n | (GAR)n |
|-----------------------------|-------------------------------|---------------------------------------|--------|--------|
| PPE 5′-GGGAGAUAUAUAAAGCGGGCAAGCAGGAAGAGG-3′ | 6/8 | 2x | 1x |
| 279K 5′-GGGAGAUAUAUAAAGCGGGCAAGCAGGAAGAGG-3′ | 7/8 | 3x | 2x |
| Δ280K 5′-GGGAGAUAUAUAAAGCGGGCAAGCAGGAAGAGG-3′ | 3/8 | 1x | - |

![A image with numbers and symbols]
Coexpression of hN with 4-fold excess expression vector SF2/ASF increases E10 inclusion in PC12 cells, whereas overexpressing Tra2 does not affect E10 splicing (Fig. 3). Similar assays do not significantly increase E10 splicing from minigene vector E10AH in mock or siCONTROL-treated HeLa and PC12 cells (left panel) as well as in individual or combined (si-SF2/ASF + si-Tra2β) gene-specific siRNA-treated HeLa (middle panel) and PC12 cells (right panel).

PC12, and rat P1 neurons, 79% for N279K (COS-7 cells), and <5% for Δ280K (COS-7 cells) (14, 18). Similar results for N279K and Δ280K in vector hN are also seen in HeLa and PC12 cells (data not shown).

PC12 does not reveal a functional synergy between these two factors in enhancing E10 splicing as seen with other exons (41). Because SF2/ASF associations with Δ280K targets are almost undetectable in vitro, the enhancement of E10AH/Δ280K splicing in the presence of exogenous SF2/ASF suggests that in vivo, either abnormally high concentrations of SF2/ASF can bind to the Δ280K-shortened PPE or that SF2/ASF has a PPE-independent function, where it binds to other sites present in E10 or interacts with factors already bound to the pre-mRNA. Because the enhancing effect of SF2/ASF overexpression is observed in both minigene and heterologous constructs that contain different flanking exons, the effect appears specific for E10 sequences. Expression of intact and domain deletion constructs (described in the next section) were confirmed by immunoblotting (Fig. 3D). Monoclonal antibody αSF2/ASF recognizes an epitope within the N-terminal 90 residues that is retained in the faster migrating SF2/ASF-ΔRS (Fig. 3D, left panel). The polyclonal antibody Rb3505 recognizes an epitope in the N-terminal 15 residues of Tra2β.

RS Domain-dependent Regulation of E10 Splicing—SR family proteins like SF2/ASF (Fig. 4A) contain modular functional domains that include one or two N-terminal RRM domains and a C-terminal RS domain (10). SR-related proteins like Tra2β contain a central RRM domain flanked on either side by an RS domain (Fig. 4A). To determine whether the regulatory function of a candidate factor is dependent on RNA binding or protein interaction, RRM or RS
domain deletion constructs for SF2/ASF and Tra2β were generated (Fig. 4A). We cotransfected PC12 cells with a 4-fold excess of intact or domain deletion splicing factor constructs and individual mini-gene constructs E10AH, E10AH/Δ280K, or E10AH/279K. 4-fold excess empty vector pSKII was cotransfected with each minigene construct as a control (Fig. 4B, top gel, lanes 1 and 9). As in Fig. 3C, E10 splicing from E10AH (77% E10) is not altered in the presence of excess SF2/ASF (82%) or Tra2β (78%) (Fig. 4B). Deletion of both SF2/ASF RRM domains in SF2-ΔRRM slightly reduces E10AH splicing (from 77% to 67%), whereas deletion of the protein-interacting domain in SF2-ΔRS dramatically reduces E10AH splicing to 53% E10 inclusion. Analyses with Tra2β deletion mutants show that removal of the Tra2β RRM domain (Tra2β-ΔRRM, 77%) or N-terminal RS domain (Tra2β-ΔRS1, 77%) are neutral on E10AH splicing. However, E10AH splicing is significantly reduced (from 78 to 53%; Tra2β-ΔRS2) in the absence of the Tra2β C-terminal RS domain. Similar assays performed with the inefficiently spliced E10AH/Δ280K construct reveal that SF2/ASF overexpression suppresses the splicing defect in E10AH/Δ280K and enhances splicing from 28 to 46% (Fig. 4B, bottom gel). Deletion of the SF2/ASF RRM domain (SF2-ΔRRM) has no effect (28% E10+), whereas deletion of the RS domain (SF2-ΔRS) almost abolishes E10 inclusion (from 28 to 9%). Surprisingly, SF2-ΔRS also dramatically reduces E10 splicing in the efficiently spliced E10AH/279K construct (from 88 to 59%), suggesting that the RS domain of SF2/ASF plays an essential role in normal and N279K-enhanced E10 splicing (Fig. 4B, lanes 9 and 10). Interestingly, the only effect seen in Tra2β expression constructs is a reduction in E10 splicing (from 78 to 60%) when the C-terminal RS2 domain is deleted. Overexpression of intact or domain deletion Tra2β constructs have no significant effect on E10AH/Δ280K splicing, suggesting that E10AH/Δ280K is not responsive to normal or mutant Tra2β overexpression, presumably because Tra2β does not
FIGURE 4. Domain deletion analyses of splicing factors on E10 splicing. A, domain structure of SR factor SF2/ASF with deletions of the entire RRM or RS domains denoted by ΔRRM and ΔRS, respectively. In contrast, the SR-like factor Tra2β has a different domain structure and deletion of the central RRM, and individual flanking RS domains are denoted by ΔRRM as well as ΔRS1 and ΔRS2, respectively. B, autoradiographs of representative E10+ and E10− RT-PCR products obtained by cotransfecting PC12 cells with minigene vectors E10AH (top gel) or E10AH/Δ280K (bottom gel) together with 4-fold excess control vector pSKII (lane 1), intact and domain deletion SF2 (lanes 2–4) as well as Tra2β (lanes 5–8) expression vectors. Lanes 9 and 10 in the top gel correspond to RT-PCR products from E10AH/279K cotransfected with pSKII and SF2-ΔRS. The bar graph shows E10 splicing levels determined by RT-PCR assays from triplicate transfections. The corrected significance criteria used are: p < 0.0125, for E10AH comparisons; p < 0.0166, for E10AH/Δ280K comparisons; and p < 0.025, for E10AH/279K comparisons. C, autoradiographs of coexpression assays performed in PC12 cells are the same as in B but with mutant E10 heterologous constructs hN/279K (top gel) and hN/Δ280K (bottom gel). Quantitation of E10 splicing levels are shown in the bar graph. The corrected significance criteria used are: p < 0.00625 for hN/279K comparisons and p < 0.025 for hN/Δ280K comparisons. The symbols for the significance levels are as described in preceding figure legends.
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When the PPE was disrupted by introducing mutation Δ280K into construct −3T-7T, constitutive splicing was either abolished or severely reduced in COS-7 and PC12 cells, respectively. In contrast, the nearly constitutive E10 splicing achieved by strengthening the weak 5′ splice site using FTDP-17 mutation E10+3 (Fig. 5A) was only marginally reduced (97% to 87%) when the PPE was similarly disrupted. Thus, the normal PPE functions bidirectionally but is primarily required to promote use of the 3′ splice site, allowing it to efficiently establish communication with its weak 5′ splice site partner.

Because protein interactions with Δ280K templates are nearly abolished in vitro, we asked whether altered enhancer dependence of the weak E10 splice sites is mediated through protein interactions with SF2/ASF or Tra2β. RS domain deletion constructs SF2/ASF-ΔRS and Tra2β-ΔRS2 were coexpressed individually with heterologous E10 splicing constructs hN/−3T-7T or hN/E10+3 that contain the normal PPE. As in our previous reports (14, 18), E10 inclusion in the above splicing constructs is almost constitutive (Fig. 5B). Coexpression of SF2/ASF-ΔRS with construct hN/E10+3 severely diminishes E10 splicing (from 97 to 36%). Thus, the constitutive effect of the strong 5′ splice site, although shown previously to be relatively PPE-independent, strongly depends on the SF2/ASF RS domain. Coexpression of SF2/ASF-ΔRS with construct hN/−3T-7T partially reduces E10 splicing (from 98 to 80%). Thus, strengthening the 3′ splice site appears to decrease its dependence on the SF2/ASF RS domain. Because splicing of hN/−3T-7T was previously shown to require an intact PPE, interactions of endogenous SF2/ASF or of the other members in the PPE-binding complex may be strengthened in the presence of a strong 3′ splice site that overrides the effect of SF2/ASF-ΔRS. Similar assays with Tra2β-ΔRS2 had no effect on either splicing vector. These results further support both PPE-dependent and PPE-independent roles for SF2/ASF on E10 splicing regulation. Because both E10 splice sites are normally weak, the requirement for SF2/ASF is expected to be stronger.

**DISCUSSION**

FTDP-17 highlights the association between splicing mutations that affect multiple cis-sequences and the pronounced variability in phenotype that is characteristic of inherited disorders. Productive interactions that positively define E10 are mediated in part by the PPE element we previously identified. Here we identify factors that associate with the PPE and extend our understanding of how the normal PPE functions and how it is disrupted by FTDP-17 mutations Δ280K and N279K.

The strength of an ESE is determined by its affinity for available SR factors and the activity of their RS domains (45). FTDP-17 mutations Δ280K and N279K highlight the physiological relevance of the AAG repeat composition, characteristic of prototypical purine-rich ESEs, on PPE strength and function. Our in vitro binding data provide an explanation for the antagonistic effects of these two mutations on E10 splicing in vivo. Furthermore, our siRNA-mediated depletion assays validate the specificity of in vivo requirement of both SF2/ASF and Tra2β for normal E10 splicing. These results also give the first indication that normal E10 splicing shows a stronger requirement for SF2/ASF than Tra2β, because incomplete depletion of SF2/ASF strongly reduced E10 transcripts to levels achieved only by complete depletion of Tra2β. The second indication is that coexpression of SF2/ASF rather than Tra2β in neuronal PC12 cells dramatically enhanced E10 splicing in heterologous constructs hN and hN/Δ280K (2.5-fold each) as well as in minigene expression constructs E10AH/Δ280K (3-fold). hN, hN/Δ280K, and E10AH/Δ280K splice inefficiently and appear more responsive to excess SF2/ASF in contrast to vectors E10AH and E10AH/Δ279K, which splice almost constitutively. It is interesting that SF2/ASF coexpression in vivo substan-
An earlier report showed that the RS domain of SF2/ASF is required to recruit the U2 small ribonucleoprotein particle-associated heterodimer U2AP\(^{55}\)/U2AF\(^{35}\) to introns that contain a weak 3' splice site (50). Because the requirement for the SF2/ASF RS domain is dependent on the 3' splice site strength, an essential function of SF2/ASF bound to the PPE, which is in close proximity to the 3' splice site, would be to stabilize the association of splicing machinery components with the weak polypyrimidine tract upstream of E10. On the other hand, Tra2\(\beta\) protein interactions are required only for normal E10 splicing but may be dispensable when either splice site is strengthened. Thus, SF2/ASF and Tra2\(\beta\) have distinct roles in E10 splicing where SF2/ASF appears to play a primary essential role, whereas Tra2\(\beta\) plays a regulatory role.

The difference in normal and enhanced tau E10 inclusion may simply reflect a difference in the strength of associations at the enhancer. Because only one factor tethered to an ESE interacts with splicing machinery components (51), the preferential association of SF2/ASF versus Tra2\(\beta\) may present different protein-protein interaction specificities and strengths specified by their RS domains. Thus, E10 splicing may be vulnerable to subtle alterations in the levels and activities of SF2/ASF and Tra2\(\beta\). Our observations highlight the requirement of SF2/ASF for use of the weak E10 3' and 5' splice sites as observed in other mRNAs (46, 52, 53). Further work is required to distinguish between the enhancer-dependent functions of SF2/ASF versus Tra2\(\beta\) and their interaction targets within the splicing complex.

Others have attempted to identify E10 trans-acting factors using templates that encompass most or all of E10 including the PPE (31, 47). N279K targets bound endogenous Tra2\(\beta\) in UV cross-linking assays and exogenously added Tra2\(\beta\) in affinity purification assays (31). In contrast to our results, Tra2\(\beta\) was also affinity-purified from targets with mutation \(\Delta 280K\) or with the entire nine-nucleotide PPE deleted. Another report showed increased immunoprecipitation of exogenous Tra2\(\beta\), SF2/ASF, and SRp35c with N279K targets, although significant associations were also detected with \(\Delta 280K\) (47). Because both studies used almost the entire E10 sequence as a template and because binding in the absence of UV cross-linking was observed with \(\Delta 280K\) and PPE deletion constructs, these experiments cannot distinguish between direct binding to the PPE, indirect binding through factors already bound to the PPE, and the presence of additional SF2/ASF and Tra2\(\beta\) E10-binding sites outside of the PPE. While this manuscript was in preparation, Wang et al. (56) recently reported that Tra2\(\beta\) overexpression enhances E10 splicing from N279K templates by antagonizing SRp55 and SRp30c associations with a regulatory sequence located immediately upstream of the PPE.

The cumulative interactions between multiple cis-elements and trans-factors result in balanced E10 splicing, which maintains a normal 4R/3R ratio of 1. The requirement for multiple ESEs in E10 and the intron splicing modulator in I10 reflects the need to overcome the presence of: weak splice sites, silencer sequences in E10 and I10, and large introns that flank E10. Our results provide a biochemical explanation for the opposite effects of mutations \(\Delta 280K\) and N279K on E10 splicing. The altered associations and distinct regulatory functions of SF2/ASF and Tra2\(\beta\) proteins may contribute to the different clinical phenotypes associated with these splicing mutations. The altered activity (phosphorylation pattern) (54, 55) or expression levels of SF2/ASF and Tra2\(\beta\) in different brain regions provide a pathogenic mechanism for abnormal 4R/3R tau isoform ratios observed not only in FTDP-17 but also in related disorders progressive supranuclear palsy, corticobasal degeneration, and Pick disease. SF2/ASF and Tra2\(\beta\) are thus modifiers of disease. Because tau is involved in over 22 neurodegenerative disorders including Alzheimer disease, revealing normal and aberrant mecha-

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Significant increase in splicing levels when coexpressed with Tra2\(\beta\) Another possibility is that Tra2\(\beta\) in excess, its ability to promote E10 inclusion in E10AH and hN. Clues to SF2/ASF high affinity binding sites upstream of the E10 5' splice site is predicted (not shown) by the ESEfinder program (48). Although the function of these predicted sites requires testing, in vivo analyses in PC12 cells reveal an RNA-dependent function for SF2/ASF on normal E10 splicing. Deletion of the entire SF2/ASF RRM domain marginally decreased E10AH splicing (from 77% to 67%) but had no effect on splicing of E10AH/\(\Delta 280K\) and E10AH/\(279K\). Thus, the RNA binding function of SF2/ASF is required to maintain normal E10 levels in E10AH and, when in excess, its ability to promote E10 inclusion in E10AH and hN. Clues to the PPE-independent functions of SF2/ASF lie in its ability to rescue the splicing defect in constructs E10AH/\(\Delta 280K\) and hN/\(\Delta 280K\). The third indication of the important role for SF2/ASF in E10 splicing is apparent when its RS domain is deleted. SF2-\(\Delta RS\) coexpression not only reduced E10 splicing by similar levels in constructs containing a normal (E10AH) or strengthened (E10AH/\(279K\), hN/\(279K\)) PPE but also drastically diminished splicing (by 68%) in the splicing-deficient construct E10AH/\(\Delta 280K\). Thus, SF2/ASF-mediated protein interactions are a critical function for regulated and enhanced tau E10 splicing.

In contrast to SF2/ASF, only normal E10 showed a slight but nonsignificant increase in splicing levels when coexpressed with Tra2\(\beta\) in vivo (E10AH; Fig. 3C). One possibility is that endogenous levels of Tra2\(\beta\) are functionally adequate in maintaining E10 splicing as suggested by depletion assays. Coexpressing SF2/ASF with Tra2\(\beta\) did not show a synergistic increase in E10 splicing (Fig. 3C), although both factors have been shown to interact in other studies and depletion of either factor alone or together reduced normal E10 splicing by similar levels in vivo (Fig. 2C). Another possibility is that Tra2\(\beta\) mediates its effect by interacting with a limiting factor, which remains to be identified. Also, deletion of the Tra2\(\beta\) RRM domain did not affect E10 splicing, suggesting either that endogenous Tra2\(\beta\) association with the PPE competes efficiently with Tra2\(\beta\)-\(\Delta RRM\) to maintain normal E10 levels or that Tra2\(\beta\)-\(\Delta RRM\) may have an RNA-independent effect and is localized by factors already bound to the PPE. Deletion of only the C-terminal RS2 domain of Tra2\(\beta\) specifically decreased normal E10 splicing in E10AH splicing, indicating a requirement for protein interactions mediated specifically by this domain (Fig. 4B). The C-terminal RS deletion mutants of SF2/ASF and Tra2\(\beta\) may behave in a dominant-negative fashion, where they may occupy the PPE but prevent productive protein interactions. Nevertheless, the strength and type of protein interactions mediated by the RS2 domain of Tra2\(\beta\) are distinct from the RS domain of SF2/ASF because both show different functional specificities in regulating E10 templates with a normal or mutant PPE. Use of a weak splice site is regulated both by the availability of SR factors and the strength of RNA-protein interactions as determined by the sequence of the recognition site. Our results show that the specific requirements for SF2/ASF and Tra2\(\beta\) protein interactions are also distinguished when either the E10 5' or 3' splice site is strengthened. SF2/ASF shows PPE-dependent and PPE-independent functions for the 3' and 5' splice sites, respectively. SF2/ASF protein interactions are critical for use of the weak 3' splice site despite the presence of a strong 5' splice site. Strengthening the 3' splice site almost completely compensates for the lack of SF2/ASF protein interactions with the weak 5' splice site (Fig. 5). Recently, the RS domain of SF2/ASF tethered to an ESE was shown to directly bind the branchpoint sequence region in the upstream intron and promote the assembly of splicing complexes (49).
nisms in tau expression will be valuable not only in understanding its pathogenic role in the central nervous system but also in the potential for designing RNA- or protein-based therapeutics.

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