Mutations in Tau Gene Exon 10 Associated with FTDP-17 Alter the Activity of an Exonic Splicing Enhancer to Interact with Tra2β*

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Mutations in the human tau gene leading to aberrant splicing have been identified in FTDP-17, an autosomal dominant hereditary neurodegenerative disorder. Molecular mechanisms by which such mutations cause tau aberrant splicing were not understood. We characterized two mutations in exon 10 of the tau gene, N279K and Del280K. Our results revealed an exonic splicing enhancer element located in exon 10. The activity of this AG-rich splicing enhancer was altered by N279K and Del280K mutations. This exonic enhancer element interacts with human Tra2β protein. The interaction between Tra2β and the exonic splicing enhancer correlates with the activity of this enhancer element in stimulating splicing. Biochemical studies including in vitro splicing and RNA interference experiments in transfected cells support a role for Tra2β protein in regulating alternative splicing of human tau gene. Our results implicate the human tau gene as a target gene for the alternative splicing regulator Tra2β, suggesting that Tra2β may play a role in aberrant tau exon 10 alternative splicing and in the pathogenesis of tauopathies.

Microtubule-associated protein tau plays an important role in microtubule assembly and stabilization (1–9). Recent studies have shown that, together with MAP1B, tau acts as a regulator of microtubule organization during axonal growth and neuronal migration (reviewed in Ref. 10). Tau protein is encoded by a single gene located on chromosome 17 (11). In the adult human brain, six isoforms of tau protein are produced as a result of alternative splicing of exons 2, 3, and 10 (11–13). Alternative pre-mRNA splicing of exon 10 in the tau gene, either exclusion or inclusion of exon 10, results in proteins with either three or four microtubule-binding repeats (Tau3R or Tau4R). In the normal human adult brain, the ratio of Tau4R to Tau3R is approximately 1, and this delicate balance between Tau4R and Tau3R isoforms appears to be critical for neuronal function in maintaining learning and memory (14–18).

Filamentous tau protein-containing inclusions have been recognized as a hallmark of neuropathology of several neurodegenerative diseases, such as Alzheimer’s disease, Down’s syndrome, several variants of Prion diseases, progressive supranuclear palsy, amyotrophic lateral sclerosis, Pick’s disease, corticobasal degeneration, and sporadic frontotemporal dementias (reviewed in Ref. 19). Recent genetic studies have revealed mutations in the tau gene associated with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), an autosomal dominant hereditary neurodegenerative disorder characterized by behavioral and personality changes as well as muscular rigidity similar to that found in Parkinson’s disease. A major neuropathological characteristic of FTDP-17 is filamentous inclusions containing hyperphosphorylated tau protein in the absence of β-amyloid plaques. Since 1998, several groups have reported the association between mutations in the tau gene and FTDP-17 (15, 16, 20–39). So far, tau mutations associated with various forms of frontotemporal dementia can be classified as two types (14): missense mutations impairing Tau protein function and splicing mutations altering Tau4R/3R ratio. Most missense mutations occur inside or near the microtubule-binding domain. These missense mutations may affect tau function by reducing tau binding to microtubules and promoting the formation of paired helical filament aggregation (16, 25–28, 35, 36, 39–41). On the other hand, splicing mutations, including all intrinsic mutations and several exonic mutations, are associated with changes in Tau4R/3R ratio. These observations suggest that a proper ratio of Tau4R/3R is essential for normal function of Tau in the human brain. It remains unclear how alterations in Tau4R/3R ratio lead to neuronal degeneration and, in some cases, also glial dysfunction and death. However, the discovery of these splicing mutations in the tau gene indicates the critical importance of controlling the balance of different Tau isoforms by alternative splicing for normal brain function.

We are interested in understanding molecular mechanisms underlying tau alternative splicing regulation. Our previous work established a tau minigene system (17). Many of the tau intronic mutations described so far are near the splice donor site (5’ splicing site) of the intron following exon 10. Our biochemical studies support the model that some intronic mutations near the 5’ splice site following exon 10 destabilize a stem-loop structure and lead to enhanced U1 snRNP1 interaction with the 5’ splicing site of exon 10 and thus an increase in splicing between exon 10 and exon 11 and an increase in the ratio of Tau4R to Tau3R (15, 17, 27, 42, 43).

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1 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; Ad, adenovirus; RT, reverse transcriptase; WT, wild type; RNAi, RNA interference; ESE, exonic splicing enhancer.
Graded DNA Technologies, Inc. Anti-SR monoclonal antibody 1H4 was tested on an ABI 373A automatic sequencer using a PRISM Ready reaction genomic fragments and different expression plasmids was carried out (Ad) major late transcription unit. DNA sequence analysis of tau intronic sequences with the first exon region (L1) of the adenovirus –N279K, Del280K were made by inserting this sequence upstream of cytomegalovirus promoter (17). Tau minigene plasmids with N279K and Del280K mutation were made by site-directed mutagenesis with thick line and Del280K mutation were made by site-directed mutagenesis with a RNA-binding domain and a domain rich in serine and arginine (the SR domain), Tra2β, has been identified among the proteins interacting with this exonic enhancer element. The interaction of this Tra2β with the exonic splicing enhancer correlates with the activity of this enhancer element in stimulating tau exon 10 splicing. Finally, down-regulation of Tra2β expression by RNA interference in transfected cells led to a reduction in tau exon 10 inclusion. These observations suggest that human Tra2β may act as an important regulator for facilitating exon 10 inclusion in tau splicing.

EXPERIMENTAL PROCEDURES

Plasmid, Oligonucleotides, and Antibodies—The tau genomic DNA fragments containing exons 9–11 as well as intronic sequences flanking exon 10 were amplified by PCR from the normal adult human or FTDP-17 patient brain genomic DNA to make wild-type or mutant tau exon 10 splicing substrates. Splicing reagents were as follows in a 10-μl reaction: 500 ng of linearized DNA as template, 0.4 mM ATP and CTP, 0.1 mM GTP and UTP, 0.84 mM GpppG cap analogue, 20 μl of RNA polymerase, and 1 unit/μl RNA polymerase. Samples were then treated with 0.1 unit/μl RNase I (Promega) for 15 min and ethanol-precipitated. The full-length transcripts were gel-purified. Synthesis of cold competitor RNAs was in a scaled-up 100-μl reaction with the following modifications: 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 0.42 mM cap analogue, and a trace amount of [α-32P]UTP for quantification. HeLa cell nuclear extracts were prepared according to previously established protocols and contained 20 mg/ml total proteins (37). Splicing reactions were set up and processed as previously (70) except that some batches of nuclear extracts were supplemented with 1 unit of hepatitis B (Sigma). 2–3 fmol of RNA substrates were added, and the samples were incubated at 30 °C for 2 h unless otherwise mentioned. Splicing products were resolved on 8% polyacrylamide, 8 M urea gels. The identity of lariat molecules was determined by perform- ing a debranching reaction in a S100 extract (71). Splicing reactions were set up and processed as previously (72) and followed by gel migration alongside molecular weight markers. The intensity of the products was quantified by the PhosphorImager. In some splicing assays, purified SR protein or U2AF65 or cell lysates containing overexpressed Tra2β or SRp40 was added.

UV Cross-linking and Immunoprecipitation Assays—Splicing reactions were set up as described above except using 20 fmol of RNA substrates. 10-μl aliquots were transferred onto a 96-well microtiter plate and irradiated with 1 J in UV Stratallinker 1800 (Stratagene). For site-specific cross-linking, a specific label was put inside the second AAG sequence following a protocol previously described (74, 75). Various amounts of competitive transcripts were added to demonstrate the specificity of bound proteins. Samples were then treated for 30 min at 37 °C with equal volume of RNase A (5 mg/ml). Radiolabeled cross-linked proteins were boiled for 5 min in 1× SDS-PAGE loading buffer and separated on 12.5% SDS-PAGE. For immunoprecipitation, following the RNase treatment, samples were incubated with 1μg anti-SR monoclonal antibody (ATCC). Protein-A–agarose beads were then added with further incubation and gentle rocking. The RNA-labeled proteins retained on the beads after several washings were eluted and resolved on SDS-PAGE.

Site-specific Label Incorporation and Cross-linking—Human tau exon 10 DNA with N279K mutation was in vitro transcribed into RNA with trace-labeled [α-32P]UTP. 40 pmol of synthesized RNA was annealed to 60 pmol of chiomer oligonucleotide (O-Methyl-TTCTTICUUC2′-O-Methyl-5′) and cleaved with RNase H. The 3′-half RNA was treated with calf intestinal phosphatase and labeled with [γ-32P]ATP. The 5′-half RNA was ligated with 3′ half RNA using a DNA ligation kit (Roche Applied Science) by bridge oligonucleotide (5′-GTTGCAATCTCACTGATGATGCTTCCAGCTTCTTCTTAATTATCTGC-ACCCCTGG-3′). 30,000 cpm of RNA was incubated with 20–40 μg of HeLa cell nuclear extracts for cross-linking. Various amounts (0, 4, and 40 pmol) of competitor oligonucleotide (5′-GTTGCAATAATGGAAGCTGCGATG-3′) corresponding to the enhancer element were added.

RNA-Protein Interaction Assays—40 fmol of biotin-conjugated corresponding RNA was added to TauEx10–11dEx5, TauEx10–11dEx280K, TauEx10–11wt, and TauEx10–11N279K were incubated for 30 min on ice with various amounts of cell lysates prepared from HEK293 cells transiently transfected with a Tra2β-Myc expression construct. After affinity selection with streptavidin-agarose beads (Sigma), the beads were washed four times in cold phosphate-buffered saline with 75 mM NaCl and 0.1% Nonidet P-40. Proteins associated with the biotinylated RNAs were dissolved in sample buffer and analyzed by Western blotting using 9E10 anti-Myc monoclonal antibody (Babco).

U snRNP Blocking Assays—Partial inhibition of U snRNAs was achieved by incubation with 2′-O-methyl oligoribonucleotides as described (17). 2′-O-methyl oligoribonucleotides (U1, 8 μM; U2, 0.3 μM; U3, 12 μM; U4, 13 μM) were added to U snRNA-containing reactions at 30 °C for 10 min prior to the addition of RNA substrates. Incubations were then carried on for 1.5 h. Splicing products were resolved on 8% denaturing gels.

U1 snRNAP RNase H Protection Assay—The TauEx10–11 RNA substrate (3 fmol) was added to a 12.5 μl of splicing reaction containing mock/tr2β/1 snRNP-particles. Unlabeled HeLa cell extract S100 (53) and incubated for 15 min at 30 °C. Oligonucleotides (20 pmol) directed against exon 10 5′ splice sites (17) were then added along with 0.2 units of RNase H (Invitrogen), and the incubation continued for 10 min at 37 °C. Resulting RNA fragments were resolved on 6% polyacryl- amide, 8 M urea gels (76, 77). Protein or SR proteins were then added (37). Tra2β protein-containing complexes were immunoprecipitated with 293 cells transfected with Tra2β-Myc expression plasmid.

RNA Interference Experiments—An RNA interference assay was carried out in HEK293 cells as described (79). The oligonucleotides were custom-synthesized by Dharmacor (Boulder, CO).
An Exonic Splicing Enhancer Located in Exon 10 of the Human Tau Pre-mRNA—The N279K mutation in exon 10 of the tau gene was reported to affect exon 10 splicing (Fig. 1A) (20, 27). It was proposed that a T to G change in the N279K mutation might improve an exonic splicing enhancer because of the sequence change from AA TAAGAAG to AAGAAG. Consistent with the hypothesis that this AG-rich region acts as a splicing enhancer, is the discovery of the Del280K mutation that changes the sequence from AA TAAGAAG to AATAAGAAG. To test the exonic splicing enhancer hypothesis, we made use of a tau minigene construct containing the upstream exon 9, the 93-bp exon 10 splicing unit and first exon region (L1) of the Ad major late transcription unit are shown in gray boxes. The sizes of the corresponding exons and introns are indicated above the respective regions (in base pairs).

RESULTS

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FIG. 1. A, positions of reported mutations in exon 10 of the human tau gene. 5’s, 5′ splice site. 3’s, 3′ splice site. The sequence deleted from TauEx10–11d5(WT) to generate TauEx10–11d5(dEn) is indicated by the thick black line. B, schematics of a series of tau minigene constructs. The genomic DNA fragments containing exons 9, 10 (WT, N279K, Del280K, and dEn), and 11 as well as intronic sequences flanking exon 10 were inserted in mammalian expression vector pcDNA3 under the control of the cytomegalovirus promoter (PcMv). The AG-rich region in exon 10 is indicated by the black box. The caspase-2 (casp2) exon 9 and 10 splicing unit and first exon region (L1) of the Ad major late transcription unit are shown in gray boxes. The sizes of the corresponding exons and introns are indicated above the respective regions (in base pairs).

A

B

C

FIG. 2. N279K mutation enhances exon 10 splicing. A, alternative splicing of exon 10 from transfected WT or N279K mutant tau minigenes. The tau minigene constructs were transfected into HeLaRB, HEK293, and N2a cells. The splicing products expressed from the transfected minigenes were detected by RT-PCR with primers specific to the transfected tau minigenes. The positions of exon 10-containing and exon 10-skipping splicing products are as indicated. B, in vitro splicing of TauEx9–11d5 (WT or N279K) (lanes 1–6), TauEx9–10d5 (WT or N279K) (lanes 7 and 8), and TauEx10–11d5 (WT or N279K) (lanes 9 and 10) RNA substrates. 32P-Labeled pre-mRNA substrates were incubated in HeLa nuclear extracts under the splicing conditions. The reactions shown in lanes 3–6 contained different amounts of the exogenous purified U2AF65 protein. Positions of pre-mRNA and splicing products are as indicated. C shows the quantification using a PhosphorImager of splicing products expressed as the ratio of exon 10-containing mRNA (Ex10+) to exon 10-skipping mRNA (Ex10−) in splicing reactions with TauEx9–11d5 substrate (lanes 1–6). In the absence of exogenous U2AF65, the HeLa nuclear extract did not produce a sufficient amount of exon 10-containing splicing products that were detectable by the PhosphorImager; therefore, quantification was only carried out for splicing reactions containing the supplemented U2AF65 protein.
molecular mechanism underlying the effect of N279K mutation, we set up an in vitro splicing system using HeLa cell nuclear extracts. Although the three-exon-containing splicing substrate TauEx9–11d5 (either WT or N279K) pre-mRNAs were not spliced efficiently in vitro (Fig. 2B, lanes 1 and 2) (17), TauEx10–11d5 transcripts containing exon 10 and exon 11 underwent efficient splicing in the presence of the same HeLa nuclear extracts under the same conditions (Fig. 2B, lanes 7 and 8). This allowed us to compare the efficiency of the splicing of exon 10 between the wild-type and N279K mutant pre-mRNA transcripts. N279K mutation significantly increased the efficiency of exon 10 splicing in TauEx10–11d5 constructs. Similar to TauEx9–11d5 pre-mRNA, the TauEx9–10d5 (either WT or N279K mutant) substrates also showed a very low efficiency of splicing (Fig. 2B, lanes 9 and 10). This may be due to the weak 3′ splicing site upstream of exon 10 as predicted from the nucleotide sequence. Because U2AF65 can stimulate splicing of substrates containing weak 3′ splice sites, we attempted to improve the in vitro splicing efficiency by adding purified recombinant U2AF65 protein in the splicing reaction (Fig. 2B, lanes 3–6). Indeed, the addition of U2AF65 improved the splicing efficiency of TauEx9–11d5 to a level high enough to allow the detection of in vitro splicing products. Using these complemented splicing reactions, we observed that, similar to that in transfected cells and in the brain tissue of FTDP-17 patients, N279K mutation in exon 10 significantly increased the inclusion of exon 10 (Fig. 2B, compare lane 4 with lane 3 and lane 6 with lane 5, with the quantification of the ratio between exon 10-containing and exon 10-skipping mRNA shown in Fig. 2C). These results clearly indicate that the N279K mutation stimulates the inclusion of the exon 10.

The Mutations in the AG-rich Region of Exon 10 Affect Both Upstream and Downstream Splicing Units—The observation that N279K mutation increases exon 10 splicing supports the activity of an AG-rich exonic splicing enhancer. To gain insights into the mechanism of action of this regulatory element, we constructed several minigenes with this tau exon 10 AG-rich element located in either the 5′ exon or 3′ exon of single-intron pre-mRNA substrates (Fig. 1B). We first tested TauEx9–10d5 minigenes containing wild-type sequence (AATGAGAAAG) or the N279K mutation (AAGAAGAAG) in exon 10. As described before, splicing of TauEx9–10d5 transcripts was inefficient, with only a low level of splicing products detectable in N279K mutation (not in WT substrate) when purified U2AF65 protein was not added to the splicing reactions (Fig. 3A, lanes 1 and 2). It was clear that N279K mutation increased the efficiency of splicing between exon 9 and exon 10, as compared with the minigene containing the wild-type sequence (Fig. 3, A and C). When one copy of AAG trinucleotide was deleted as in the Del280K mutant (AATGAGGAAG instead of the wild-type AATGAGAAAG sequence), the efficiency of splicing between exon 9 and 10 was reduced below a detectable level even in the presence of exogenous U2AF65 protein (data not shown). These results indicate that the AG-rich element in exon 10 acts to promote splicing between exon 9 and exon 10 and that the T to G nucleotide change in N279K mutant further increases the activity of the AG-rich exonic splicing enhancer element. We then tested whether the upstream exon 9 sequence was required for the activity of the AG-rich exonic enhancer present in exon 10. The upstream exon 9 with an associated 5′ splice site was substituted with the first exon region (L1) of the Ad major late transcription unit. In this chimeric splicing substrate lacking the native tau exon 9, N279K mutation also increased the splicing between exon 10 and the upstream heterologous exon (Fig. 3, B and C), demonstrating that the AG-rich element in tau exon 10 was functionally active in a heterologous context. Thus, the AG-rich element, when residing in the downstream exon, acts to stimulate the splicing to the upstream exon either in the native tau exon 9–10 splicing unit or in the heterologous context.

We then asked whether this AG-rich element could affect splicing to the downstream exon. TauEx10–11d5 pre-mRNA containing N279K mutation in exon 10 showed a significantly increased splicing efficiency as compared with the wild-type TauEx10–11d5 pre-mRNA (Fig. 3, D lanes 2 and 5 compared with lanes 1 and 4 and F; compare the levels of mRNA products Ex10–11, Ex10, and lariat intermediates). Del280K mutant (with one copy of AAG deleted in the AG-rich element) showed reduced splicing as compared with the wild-type pre-mRNA (Fig. 3, D, lanes 3 and 6 and F). These two mutations showed similar, albeit less dramatic, effects on the splicing to the downstream exon when the AG-rich element was fused to exon 9 of a caspase-2 exon 9–10 splicing cassette (44), namely N279K increasing and Del280K decreasing the splicing efficiency (Fig. 3, E and F). Taken together, these in vitro results indicate that this AG-rich element inside tau exon 10 is an exonic splicing enhancer that functions in a bidirectional manner to facilitate splicing of exon 10 with both the upstream and the downstream exons. This element is also active in certain heterologous contexts. Mutations found in FTDP-17 patients inside this AG-rich element directly affect splicing of both exon 9–10 and exon 10–11 splicing units. Bidirectional splicing enhancers have been described that contain multiple elements with different sequences affecting splicing in different directions. However, to our knowledge, our observation with tau exon 10 splicing provides an example in which a simple AG-rich splicing enhancer acts in a bidirectional manner to promote splicing of both upstream and downstream splicing units.

Trans-acting Splicing Factor Tra2β Interacts with Tau Exon 10 Splicing Enhancer—To identify proteins associated with this exonic splicing enhancer, we made additional plasmids and employed a UV cross-linking assay. As shown in Fig. 1, plasmids were made for synthesizing RNAs containing either WT or mutant exon 10 sequences including N279K and Del280K as described before. In addition, another mutant, dEn, was constructed in which the entire AG-rich enhancer was deleted. α32P-labeled RNAs corresponding to exon 10 of dEn, Del280K, WT, and N279K constructs were synthesized, and these transcripts contained 0, 1, 2, and 3 copies of AAG in the AG-rich element, respectively (Fig. 1). The wild-type or mutant RNA transcripts were incubated with HeLa nuclear extracts and then subjected to UV cross-linking. After treatment with RNase A, proteins cross-linked to RNA were resolved on SDS-PAGE. Among the cross-linked proteins, a complex of ~40 kDa was detected with WT and N279K but at a low level in the reaction with Del280K RNA (Fig. 4A). The level of the 40-kDa cross-linking species detected in the UV cross-linking assay correlated with the number of AAG repeats present in exon 10, with the highest level in the reaction containing N279K mutant (three copies of AAG) and in a decreasing order in WT (2 copies), Del280K (1 copy), and almost undetectable in dEn (0 copies of AAG) RNA-containing reactions (Fig. 4A, as marked by the black arrow). A 42-kDa protein and other proteins (as marked by the white arrowheads above the 40-kDa band), however, were present at a similar level in reactions with different RNA substrates (Fig. 4A). Because SR and SR-like proteins are known to bind to exonic sequences where they act to enhance the splicing of the adjacent intron, we focused our search on SR domain-containing splicing regulators. A monoclonal antibody (1H4) was used, which recognized a number of SR domain-containing proteins. Immunoprecipitation exper-
ment with this monoclonal antibody, following the above described UV cross-linking, revealed several proteins, including one of ~40 kDa in size which interacted with N279K RNA more efficiently than with wild type exon 10 RNA (Fig. 4B). Since the monoclonal antibody 1H4 specifically recognizes SR domain-containing proteins, this result suggests that the 40-kDa protein may contain serine-arginine-rich sequences.

The UV cross-linking pattern also suggests that the 40-kDa protein may interact with tau RNA at or near the AG-rich region. To confirm that the 40-kDa protein indeed interacted with the AG-rich element, we placed a site-specific label inside the AG-rich sequence element (Fig. 4D, at the G residue marked with an asterisk) and then performed the UV cross-linking experiment as described under “Experimental Procedures.” In the reactions using the tau RNA transcript containing the N279K mutation, a 40-kDa protein was one of the most prominent cross-linking species detected (Fig. 4C), indicating that the 40-kDa protein indeed interacted with this AG-rich region of N279K mutant transcript. In the presence of an increasing concentration of unlabeled cold competing transcripts, the intensity of several cross-linked bands including the 40-kDa protein was reduced, suggesting that these proteins interact specifically with the tau transcript at the AG-rich region. In addition to the 40-kDa species, two other protein species with gel mobility of ~65 and 100 kDa were also detected to be competed away by the unlabeled competing transcript (as marked by an asterisk on the left side of the gel; Fig. 4C). In addition, another protein of ~120 kDa in size showed
increased binding in the presence of competing transcript. The functional significance of these proteins in tau exon 10 splicing will be further investigated in our future study. We chose to initially follow up the 40-kDa protein because this protein appeared to interact specifically with tau pre-mRNA in a manner dependent on AAG sequence.

Among the known human SR domain-containing proteins, SRp40 and Tra2β are ~40 kDa in size. SRp40 is a member of a highly conserved family of splicing factors called SR proteins (reviewed in Refs. 54, 55, and 65). Tra2β is a human homologue of Drosophila splicing regulator Tra2, also containing an RNA-binding domain and an RS domain (67). In vitro SELEX studies demonstrated that SRp40 and Tra2β bind to purine-rich exonic sequences (45, 46). We therefore tested whether the 40-kDa protein identified in UV cross-linking experiments was Tra2β or SRp40. Because of the difficulty we encountered in preparing functionally active purified recombinant Tra2β and SRp40 proteins, we used an alternative approach, expressing these proteins, we used an alternative approach, expressing these proteins in HEK293 cells by transient transfection. Biotinylated TauEx10–11 RNAs corresponding to dEn, Del280K, WT, or N279K RNAs were incubated under splicing conditions with increasing amounts of cell lysates containing transiently transfected Myc-tagged Tra2β or HA-tagged SRp40. After affinity selection with streptavidin-agarose beads, the bound proteins were detected by Western blotting using anti-Myc antibody (for Tra2β-Myc) or anti-HA antibody (for SRp40). The intensity of Tra2β protein pulled down by dEn, Del280K, or N279K RNA in A was quantified, respectively, and compared with that pulled down by WT RNA. The numbers shown below the gel in A represent the relative quantification using the band intensity in the wild type (lanes 5 or lane 6) as 1.00 in the presence of either 0.5 μl (lanes 1, 3, 5, and 7) or 1 μl (lanes 2, 4, 6, and 8) of Tra2β-expressing cell lysates.
siently expressed SRp40, amounts of SRp40 affinity-selected using the same series of TauEx10–11 RNAs did not show any significant differences (Fig. 5B). The level of expression of SRp40 was comparable with that of Tra2β in the lysates of transfected cells (data not shown). These results demonstrate that Tra2β could specifically interact with the AG-rich element inside exon 10 and that mutations found in FTDP-17 patients affect the binding of Tra2β to this element.

In order to evaluate the functional significance of Tra2β in tau pre-mRNA splicing, we tested whether Tra2β was able to increase the splicing of exon 10 when added in vitro. It was reported that Tra2β alone was not sufficient to activate the splicing in S100 extracts of a mouse IgM pre-mRNA containing a binding site for Tra2β as a splicing enhancer (46). We saw similar results in S100 extracts using tau minigene constructs (see Fig. 8B, lanes 11 and 12). To overcome this problem, we incubated TauEx10–11 splicing substrates (WT or N279K) with cell lysates containing transiently transfected Myc-tagged Tra2β in the presence of a limiting amount of nuclear extracts. We used exon 10 and 11 splicing substrate to set up the in vitro splicing assay because the in vitro splicing efficiency of either exon 9–11 or exon 9 and 10 transcripts was too low, as described earlier. The addition of Tra2β protein-containing cell lysates further increased splicing efficiency above that of mock cell lysates (Fig. 6A, compare lanes 3 and 4 with lanes 1 and 2 and lanes 9 and 10 with lanes 7 and 8). Furthermore, the difference in splicing efficiency between substrates with WT sequence and N279K mutation was more obvious in the presence of cell lysates containing overexpressed Tra2β protein (Fig. 6, A (compare lanes 7 and 8 with lanes 5 and 6) and B). This is consistent with the higher level of Tra2β associated with N279K transcript (Figs. 4 and 5). Although SRp40 is also able to increase the splicing efficiency (Fig. 6A, compare lanes 5 and 6 with lanes 1 and 2 and lanes 9 and 12 with lanes 7 and 8), it stimulated splicing of substrates with WT sequence and the N279K mutation at almost the same level (Fig. 6B). The results described above indicate that Tra2β protein is a specific splicing activator interacting with the AG-rich element of tau exon 10. The mutations improving or disrupting the exonic splicing enhancer may affect the binding of Tra2β, thereby increasing or decreasing the inclusion of exon 10 as in the case of N279K and Del280K mutations, respectively.

Tra2β Increases the U1 snRNP-dependent Complex Formation at the 5' Splice Site of Exon 10—To dissect the mechanism by which Tra2β promotes the splicing of tau exon 10, we used U snRNP inactivation assays (17, 47). U1, U2, U5, or U6 snRNPs were partially blocked by using specific individual 2′-O-methyl oligoribonucleotides at appropriate concentrations to treat HeLa nuclear extracts prior to the splicing reactions. When either U2 snRNPs (Fig. 7, lanes 3 and 4), U5 snRNPs (Fig. 7, lanes 5 and 6), or U6 snRNPs (Fig. 7, lanes 7 and 8) were partially blocked, both wild type and N279K RNA splicing were partially inhibited to a similar extent as compared with the splicing reactions containing the mock-treated nuclear extracts (Fig. 7, lanes 9 and 10). However, when U1 snRNP was partially blocked, splicing of wild-type tau was significantly decreased, whereas the splicing of N279K mutation-containing pre-mRNA was barely affected (Fig. 7, lanes 1 and 2). Quantification of the exon 10 splicing under these conditions shows that in the presence of U1 snRNP-partially blocked splicing extract, the ratio of N279K mutant splicing product to that of the wild type splicing product is increased as compared with that in the presence of mock-treated splicing nuclear extract. Partial blocking of other U snRNPs important for splicing, including U2, U5, and U6, did not show the same effect (Fig. 7B). This result suggests that U1 snRNP may play an impor-
tantal role in differential recognition of tau WT and N279K RNA during splicing.

As described earlier, Tra2β could specifically bind to the AG-rich element of exon 10 and activate tau exon 10 splicing. We further asked whether Tra2β could increase the formation of U1 snRNP-dependent early spliceosome complexes on the 5′ splice site of exon 10 and compared the wild-type with N279K mutant in this assay. A specific U1 protection assay was performed as described before (17). TauEx10–11 RNA substrate (WT or N279K) was added to a 12.5-μl splicing reaction using mock- or U1 snRNP-partially depleted HeLa cell cytosolic S100, purified SR proteins, and cell lysates with overexpressed Tra2β protein. Following the incubation, the oligonucleotide complementary to the 5′ splice site of exon 10 (17) was added along with RNase H (0.4 units), and the incubation was continued for another 15 min at 37°C. The RNA cleavage products were then analyzed by gel electrophoresis. Positions of the U1-protected pre-mRNA and the RNase H cleavage products are indicated on the right. Either SR protein mixture or transiently expressed Tra2β protein increased the formation of U1 snRNP-dependent complex when N279K mutant pre-mRNA was used (lanes 10 and 12). In vitro splicing of TauEx10–11 RNA substrate (WT or N279K). Mock- or U1 snRNP-partially depleted HeLa cell cytosolic S100, SR proteins, and cell lysates containing overexpressed Tra2β protein were incubated with WT or N279K mutant pre-mRNA transcript under splicing conditions. Splicing products were detected after gel electrophoresis. The asterisk indicates the position of a nonspecific cleavage product generated by hybridization of the U1 RNA-specific oligonucleotide on the tau pre-mRNA.
SRp40 RNAi-treated cells (Fig. 9, C and D). The treatment by the same Tra2β oligonucleotide did not affect alternative exon inclusion in other genes examined such as caspase-2 (data not shown), suggesting relative specificity of Tra2β in regulation of tau gene alternative splicing. Consistent with the lower level of Tra2β protein associated with the wild-type tau transcript as compared with the N279K transcript (Figs. 4 and 5), the effect of Tra2β reduction on wild-type tau exon 10 splicing was detectable but less dramatic (data not shown).

To confirm that Tra2β gene is indeed expressed in the human brain, we used a previously established RT-PCR assay (17) with cDNA samples prepared from the frontal cortex, the major region affected in FTDP-17 patients. Specific Tra2β signals were detected in the cDNA samples prepared from both fetal and adult brain tissues (Fig. 10). Consistent with our observation, a recent study was published while this manuscript was in preparation, documenting the expression of Tra2β in the rat brain (80). Therefore, Tra2β gene is expressed in the brain, where the balance of different tau alternative splicing isoforms is crucial for proper neuronal function.

Taken together, our results establish tau pre-mRNA as one of targets for Tra2β splicing regulator and support a role of Tra2β in the regulation of alternative splicing of exon 10 in the tau gene. The in vivo significance of Tra2β in regulation of tau alternative splicing will be further investigated in our future study using approaches such as gene knock-out.

**DISCUSSION**

In this study, we report characterization of an exonic spacing enhancer, located in exon 10 of the human tau gene with AATAAGAG as the wild-type sequence. This AG-rich region contains the sequence in which mutations associated with FTDP-17 have been identified. Specifically, two mutations have been found inside this enhancer element, N279K (AA-GAAGAAG) and Del280K (AATAAG). We made an additional mutant, dEn, lacking the entire AG-rich element. By systematically comparing the splicing efficiency, protein-RNA interaction profiles, and the splicing complex formation between the wild type and mutant transcripts, we conclude that these mutations alter the activity of this splicing enhancer by increasing (for N279K) or decreasing (for Del280K) interaction with protein factors interacting with this AG-rich element. Among the trans-acting factors interacting with this AG-rich element, we have identified human Tra2β protein. N279K mutation stimulates, whereas Del280K or dEn mutations reduce, the binding of Tra2β to the AG-rich exonic enhancer. The interaction of Tra2β protein with this exonic element correlates well with the copy number of AAG repeats inside the exonic enhancer. Furthermore, down-regulation of endogenous Tra2β expression using RNA interference led to a reduction in tau exon 10 splicing. Our study suggests that Tra2β or Tra2β-like protein(s) may play an important role in affecting the delicate balance of tau isoforms and in pathogenesis of tauopathies involving aberrant tau exon 10 alternative splicing. Further investigation is necessary to determine the spectrum and specificity of individual splicing activators such as Tra2β. Nonetheless, the down-regulation of endogenous Tra2β reduced the tau exon 10 inclusion in a relatively specific manner, and such a treatment partially reverses the exon 10 splicing effect caused by N279K mutation.
Our results suggest a new direction for designing therapy for certain FTDP patients (such as those carrying the N279K mutation) based on down-regulation of certain specific splicing activators for tau exon 10 splicing.

The importance of tau exon 10 alternative splicing has been demonstrated by a number of genetic studies that link the mutations affecting exon 10 splicing in the human tau gene to the development of FTDP-17 (15, 16, 20, 23, 26–28, 37, 38, 48–50). These mutations are located either in the intron downstream of exon 10 or inside exon 10. Our previous study has demonstrated that some intronic mutations act at the level of U1 snRNP binding to the 5′ splice site of exon 10 during the earliest step of spliceosome assembly (17). Exonic mutations inside exon 10 have been studied by several groups (15, 16, 20, 25–29, 37, 50–62). Some of the exonic mutations have been shown to affect the microtubule binding properties of Tau protein(s) (14, 16, 25, 30, 37, 40), whereas other exon 10 mutations alter the Tau4R/Tau3R ratio presumably by influencing exon 10 splicing (26, 27, 37). However, the molecular mechanisms underlying the splicing mutations inside exon 10 were not understood.

Exonic splicing enhancers (ESEs) are sequence elements within exons that promote pre-mRNA splicing. A number of ESEs have been identified in tissue-specific or developmentally regulated exons, which typically have weak splice sites and require the ESE for exon inclusion. As shown by our study, the upstream 3′ splice site of exon 10 is weak. Utilization of this suboptimal 3′ splice site was only detectable in the in vitro splicing assay when additional U2AF65 protein was provided in the splicing reactions. The presence of an ESE in exon 10, together with a suboptimal 3′ splice site, provides a fine-tuning mechanism for the regulation of exon 10 inclusion in certain tissues or at certain developmental stages. It is conceivable that during fetal stages of human development, tau exon 10 inclusion is repressed because of either insufficient levels of splicing activator(s) or the presence of trans-acting factors that counteract the activity of embryonically expressed splicing activators. It is equally possible that the fetal pattern of tau exon 10 splicing (almost exclusively exon 10 skipping) is a result of the combination of the above mentioned mechanisms. We are actively investigating these possibilities using both molecular and biochemical approaches.

The sequence motif for ESEs can be divergent, although purine-rich motifs represent one of the best characterized elements. Often ESEs function by interacting with SR domain-containing splicing regulators (reviewed in Refs. 53–57). ESEs were initially identified by their ability to activate upstream 3′ splice sites (58–60). Recent studies also identified splicing enhancers that stimulate downstream 5′ splice site utilization (61–63). There have also been reports of bidirectional enhancer elements (62, 63), and these elements contain multiple domains with distinct regions functioning to facilitate splicing of either upstream or downstream exons. In our study, we have used biochemical approaches to identify a simple AG-rich element that acts bidirectionally to promote exon 10 splicing with both exon 9 and exon 11. We show that one of the important trans-acting factors involved in the function of this exonic splicing enhancer is human Tra2β protein that acts as an alternative splicing activator. Using a transient transfection assay, we were able to express Tra2β in a functionally active form at a level comparable with that detected in the rat brain tissue by Western blotting (data not shown). Similar to previous studies, our results show that Tra2β by itself is not sufficient to complement S100 cytoplasmic extracts for the splicing activity in the manner that certain essential splicing factors of the SR family do (46). It is likely that Tra2β protein acts in concert with certain basic essential splicing factors to activate tau exon 10 splicing. Furthermore, Tra2β protein interacts with tau pre-mRNA by binding to the AG-rich exonic splicing enhancer, as shown by site-specific UV cross-linking experiments. The addition of the transiently expressed Tra2β protein into the in vitro splicing reaction further stimulates exon 10 splicing, and such stimulatory effect is more pronounced in the presence of the N279K mutation. Thus, Tra2β acts as a splicing activator in tau exon 10 splicing. The bidirectional activity of tau gene exonic splicing enhancers and involvement of alternative splicing regulators, in addition to essential splicing factors, make the similarity more obvious between splicing enhancers and transcription enhancers.

SR domain-containing splicing factors play important roles in mammalian pre-mRNA splicing and alternative splicing regulation (for recent reviews, see Refs. 54, 55, 57, 64, and 65). In addition to interacting with pre-mRNAs via their RNA recognition motifs, these splicing factors have been proposed to mediate interactions both across introns and across exons (59). More recently, SR proteins have been reported to support basal AT-AC splicing and to stimulate exonic splicing enhancer important for AT-AC intron splicing (60). Tra2β is an SR domain-containing protein initially identified as one of the human homologues of Drosophila SR protein transformer 2 (Tra2) (67). Systematic biochemical experiments demonstrated that human Tra2β is a splicing activator interacting with exonic enhancer sequences rich in A and G or GAA repeats rather than a constitutive splicing factor (46). Tra2β has been reported to activate the splicing of exon 7 of survival of motor neuron genes when overexpressed by transfection (68). The mechanism by which Tra2β activates splicing was not clear. Our UV cross-linking and RNase H cleavage/U1 snRNP protection experiments show that increased exon 10 splicing in the tau N279K mutant is associated with increased Tra2β binding to the exonic splicing enhancer and that the addition of Tra2β promotes the formation of U1 snRNP-dependent complex at the 5′ splice site of exon 10. Protein-protein interaction between Tra2β and a U1 snRNP protein U1 70K has been observed, and the functional significance of such interaction in promoting U1 binding will be further investigated. These results suggest that one mechanism by which Tra2β acts to stimulate exon 10 splicing may be by facilitating U1 snRNP binding to the 5′ splice site of exon 10.

A previous study reported that overexpression of human Tra2β in a chimeric tau minigene system had no effect on human tau gene alternative splicing (69). It is possible that in this previous study, the failure to detect the effect of Tra2β was a result of using the artificial chimeric tau minigene rather than the native tau minigene (69). Alternatively, the presence of endogenous Tra2β was high enough to mask the effect of overexpression. Our systematic biochemical analyses of the interaction between Tra2β and the AG-rich splicing enhancer in exon 10 and results from Tra2β down-regulation experiments strongly suggest an important role of Tra2β in tau exon 10 alternative splicing regulation. A definitive in vivo role of Tra2β in this alternative splicing event awaits future exploration using strategies such as targeted gene deletion.

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