5’ Splice Site Mutations in tau Associated with the Inherited Dementia FTDP-17 Affect a Stem-Loop Structure That Regulates Alternative Splicing of Exon 10*

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Missense and splice site mutations in the microtubule-associated protein tau gene were recently found associated with fronto-temporal dementia and parkinsonism linked to chromosome 17 (Poorkaj et al. (1998) Ann. Neurol. 43, 815–825; Hutton et al. (1998) Nature 393, 702–705; Spillantini et al. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7737–7741). The mutations in the 5’ splice site of exon 10 were shown to increase the ratio of tau mRNAs containing exon 10 and thus the proportion of Tau protein isoforms with 4 microtubule binding repeat domains, although how this increase leads to neurodegeneration is presently unclear. The mechanism by which these mutations increase tau exon 10 splicing was not determined, although the mutations were predicted to disrupt a potential stem-loop structure that was likely involved in the regulation of exon 10 alternative splicing. Here we describe in vitro splicing assays and RNA structural analysis that demonstrate that the mutations do indeed act through disruption of the stem-loop structure and that the stability of this secondary structure feature at least partially determines the ratio of tau exon 10+/– transcripts. In addition, we provide evidence that the stability of the stem-loop structure underlies the alternative splicing of this exon in other species.

The microtubule-associated protein Tau plays an important role in the polymerization and stabilization of neuronal microtubules (for review, see Ref. 4). Tau is thus crucial to both maintenance of the neuronal cytoskeleton and axonal transport (4). Abnormal intraneuronal inclusions, termed neurofibrillary tangles, composed of Tau are a feature of the pathology in several neurodegenerative conditions (5) including Alzheimer’s disease, Pick’s disease, fronto-temporal dementia (6), progressive supra-nuclear palsy, and the Lytico and Bodig diseases of Guam.

The tau gene is localized to chromosome 17q21 (7) and consists of 15 exons (8) of which 11 encode the six major Tau protein isoforms in human brain. The six different Tau isoforms are generated by alternative splicing of exons 2, 3, and 10 (9). Exons 9–12 encode four microtubule-binding domains that are imperfect repeats of 31 or 32 residues (10). Alternative splicing of exon 10 gives rise to Tau isoforms with 3 (exon 10–) or 4 (exon 10+) microtubule binding domains (11). The recent identification of four missense (1, 2) and four splice site mutations (2, 3) in the tau gene, associated with fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (6), has demonstrated that Tau dysfunction can lead to neuronal cell death and is not simply a secondary consequence of neurodegenerative disease.

FTDP-17 (6) is inherited as an autosomal dominant condition characterized clinically by behavioral, cognitive, and motor disturbance (many cases of this disease continue to be described clinically as “Pick’s disease”). The age of onset is highly variable but is usually 45–65 years. At autopsy, patients with FTDP-17 display pronounced fronto-temporal atrophy with neuronal cell loss, gray and white matter gliosis, and superficial cortical spongiform changes. In addition, virtually all FTDP-17 cases have abnor-mal intraneuronal Tau inclusions, with glial Tau inclusions present in some families (6, 12). The morphology and isoform composition of the Tau filaments that compose the inclusions also varies in the different families (12). The identification of different mutations in the tau gene has largely explained the variability in Tau pathology observed in FTDP-17 (2, 3, 13). Families with missense (2, 14) (P301L, N279K) or splice site mutations (2, 3) that affect exon 10, and thus 4 repeat Tau isoforms, have intraneuronal and glial Tau inclusions consisting predominantly of four repeat Tau isoforms. The Tau filaments in these cases have a longer periodicity than the paired helical filaments that comprise the neurofibrillary tangles observed in AD (12, 13). In contrast, families with missense mutations (G272V, V337M, and R406W) outside of exon 10 (1, 2), that affect all Tau isoforms, have neuronal inclusions (glial inclusions are absent) that are composed of all six Tau isoforms and are made up of filaments identical to the paired helical filaments observed in AD (12, 13, 15).

All but one of the reported (1, 2, 14) missense mutations (G272V, N279K, P301L, and V337M) associated with FTDP-17 occur within the microtubule binding domains of Tau and four missense mutations (G272V, P301L, V337M, and R406W) have been demonstrated to disrupt the interaction between Tau and the microtubules in vitro (16, 17). In contrast, the splice site mutations in FTDP-17 affect alternative splicing of exon 10

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similar exon trapping results were obtained when additional intronic sequence was included. tau RT-PCR product sizes produced by mRNAs containing exon 10 constructs (not shown) containing that are incorporated into the artificial mRNAs. Mutagenesis was performed on the basic constructs as required for each experiment. Extended bp) from the human immunodeficiency virus sequences cloned into the in vivo with a 2–6-fold increase in the ratio of exon 10 to exon 9 splice site mutations are associated 9 splice site mutations were also capable of increasing the transcripts that are incorporated into the artificial mRNAs. Mutagenesis was performed on the basic constructs as required for each experiment. Extended exon 10 constructs (not shown) containing ~1 kilobase pair of flanking 5’ and 3’ intronic sequence were also generated in pSPL3b to ensure that similar exon trapping results were obtained when additional intronic sequence was included. B, schematic splicing diagrams displaying the RT-PCR product sizes produced by mRNAs containing tau exon 10 (246 bp) and mRNAs with tau exon 10 skipped (153 bp).

such that an increased proportion of Tau exon 10+ transcripts are generated; this leads to an increase in Tau isoforms with four, as opposed to three, binding repeats (2, 3). The mechanism by which this increase leads to neurodegeneration and FTDP-17 is presently unclear; however, it demonstrates that the ratio of four repeat to three repeat isoforms is crucial to the correct functioning of Tau (2, 3). This is consistent with the observation that alternative splicing of exon 10 is developmentally regulated with three repeat Tau alone, in the absence of four repeat Tau, present in fetal brain tissue in multiple species (11, 18). The predominance of three repeat Tau during neuronal development has clear significance for the likely function of these isoforms in adult brain perhaps implying that human neurons require three repeat Tau to maintain plasticity (4). In addition, the ratio of four to three repeat Tau isoforms varies markedly in different species with a slight predominance of three repeat Tau present in adult human brain (11), while in adult mouse neurons only four repeat Tau is observed (18). The ratio of Tau isoforms also varies in different neuronal populations with the granule cells of the human dentate gyrus being reported to contain only three repeat Tau isoforms (11).

We previously employed RT-PCR analysis of FTDP-17 brains to demonstrate that the 5’ splice site mutations are associated in vivo with a 2–6-fold increase in the ratio of exon 10+ to exon 10− tau mRNA (2). In addition, we utilized an exon trapping protocol (2, 19) as a splicing assay to demonstrate in vitro that the 5’ splice site mutations were also capable of increasing the incorporation of tau exon 10 into artificial transcripts. However while both RT-PCR analysis of FTDP-17 brains and splicing assays demonstrated that the 5’ splice site mutations act by increasing the incorporation of exon 10 into tau mRNAs, neither method indicated the mechanism by which the mutations affected splicing (2). Examination of the intronic sequence downstream of exon 10 revealed that each of the mutations was predicted to disrupt a potential stem-loop structure that was likely involved in the regulation of exon 10 alternative splicing by competing with the U1 snRNP for binding to the 5’ splice site (2). Stem loop structures have previously been implicated in regulating the selection of alternative 5’ splice sites (20) and distant branch points (21) and also in the tissue-specific splicing of the chicken β-tropomyosin exons 6A and 6B (22, 23).

In this study we again utilize in vitro exon trapping (splicing) assays (2, 19) to test the hypothesis that the stability of the potential stem-loop structure in the 5’ splice site of tau exon 10 at least partially determines the ratio of tau exon 10+/- transcripts and that the splice site mutations act by disrupting this structure. We also examined the sequence of the exon 10 5’ splice site in the tau gene from bovine, rabbit, rat, mouse, and two other primates to determine if this pre-mRNA structure might regulate alternative splicing in other mammals. Finally, we utilize RNA secondary structure analysis to demonstrate that a stem-loop is present in the normal 5’ splice site of tau exon 10 and that this structure is disrupted by the FTDP-17 splice site mutations.

EXPERIMENTAL PROCEDURES

Generation of Exon Trapping Constructs—Mutant and wild-type versions of tau exon 10 were amplified from the DNA of the patients with the FTDP-17 +13, +14, and +16 splice site mutations (residues numbered from the exon 10 5’ splice site) and from an unaffected individual. PCR products contained exon 10 and flanking intron sequence at either end (Fig. 1). PCR products were cloned into the splicing vector pSPL3b using XhoI and PstI sites incorporated into the amplification products. Mutant and wild-type constructs were identified by sequence analysis. Site-directed mutagenesis was performed on these constructs using the Transformer site-directed mutagenesis kit (CLONTECH). Mutagenic primers for introducing the +17(G/T)+18(T/G) “extended stem” mutations were generated for the wild-type construct and for each of the three FTDP-17 splice site mutant (+13, +14, and +16) constructs. Also generated were mutagenic primers for the mutations for the complementary rescue analysis (see Figs. 2–5 for list of mutations). A selection primer was used in each mutagenesis reaction to remove a single HpaI site from the pSPL3b vector. For each reaction vector DNA was denatured at 100 °C, mismatch and selection primers were annealed and T4 DNA polymerase, and T4 DNA ligase were used to synthesize the second strand. Each mutant was transformed into BMH 71-18 mutS Escherichia coli and the plasmid DNA isolated using the Wizard miniprep kit (Promega). The DNA was treated with HpaI as a primary selection process and then re-transformed into DH10B. Colonies were screened by PCR and restriction enzyme digestion, and the identity of each mutant was confirmed by sequencing.

Wild-type and FTDP-17 mutant (+13, +14, and +16) exon trapping constructs containing additional flanking intronic sequence (1 kb) on either side of tau exon 10 were also generated in pSPL3b to ensure that the short intronic sequences used in the original constructs (Fig. 1) were not artifically affecting splicing assay results. These constructs were generated by performing PCR on control genomic DNA with primers (5’-CACCCCTCGAGGAGACGTTCACCTGACTG-3’ and 5’-GCGGATCCATGCTCTGCAACTTC-3’) designed to more distant
5’ and 3’ intronic sequences (GenBank). Amplification was performed with the high fidelity PCR system (Boehringer Mannheim). The 2082-bp PCR products were cloned into pSPORT1 using XhoI and BamHI sites incorporated into the amplification products and sequenced. Mutagenesis was then performed, as described above, on the wild-type exon 10 splice site with a mouse-specific (9F, 5’-CCACAAATACGCCAGGATGC-3’) and human specific primers (9F, 5’-CTCAGGAGGATGC-3’) and 11R, 5’-CGTCTTCTGGTGTCACGTTG-3’). PCR was performed (30 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 45 s with a final 72°C extension phase for 10 min). Mouse-specific and human-specific PCRs were analyzed on agarose gels; products corresponding to exon 10 mRNA gave a band at 297 bp. A complete description of the generation and neuropsychological analysis of these human tau transgenic mice will be published separately.

RNA Structural Mapping and Gel Migration Analysis—Wild-type and mutant (+8, +13, +14, +16, and 10-bp stem) constructs containing exon 10 and flanking intronic sequence (Fig. 1) were cloned into pBluescript(KS-+) using XhoI and PstI. The plasmid DNA was linearized with BamHI to generate a template for in vitro transcription with T3 RNA polymerase. In vitro transcription was performed using the Ribonax system (Promega) according to manufacturer’s instructions. RNase digests were performed using 4 μg of RNA and 0.08 unit of V1 double strand-specific RNase (Amersham Pharmacia Biotech) or 0.5 unit of T1 single strand-specific RNase (Ambion) for 15 min at 55°C in a 25-μl reaction, using the manufacturers’ recommended buffers. Control samples for each RNA were treated identically, to the RNase digests, but enzyme was omitted. Samples were then immediately purified by phenol/chloroform extraction and resuspended in 15 μl of diethyl pyrocarbonate-treated water. RNA recovery was assessed UV absorption at 260 nm. Primer extension was performed on 1 pmol of digested RNA using 50,000 cpm of 32P-labeled oligonucleotide (5’-CCGGGCTGCAGACACCTC-3’) and Superscript II reverse transcriptase (Life Technologies, Inc.) in a 20-μl reaction according to manufacturer’s instructions. The RNA template was digested with RNase H (Life Technologies, Inc.), and the resultant primer extension products were isopropyl alcohol precipitated, washed in 70% ethanol, and resuspended in 5:1 formamide/Blue Dye loading mix. Sequencing reactions of the wild-type construct were performed using 32P-labeled primer sequencing system (Amersham Pharmacia Biotech) and the same, unlabeled, oligonucleotide as was used for the primer extension reactions. Primer extension samples were run on a 6% polyacrylamide sequencing gel alongside the sequencing reactions and the results visualized by autoradiography. Approximate equal gel loading was verified by comparison of bands generated from full-length cDNA products from control reactions.

To further demonstrate the effect of the FTDP-17 splice site mutants on RNA secondary structure, the variable migration of in vitro transcribed wild-type and mutant (+13, +14, +16, and 10-bp extended stem) RNA transcripts was analyzed on both nondenaturing 4% Meta- fomamide/blue dye loading mix. Sequencing reactions of the wild-type construct were performed using 32P-labeled primer sequencing system (Amersham Pharmacia Biotech) and the same, unlabeled, oligonucleotide as was used for the primer extension reactions. Primer extension samples were run on a 6% polyacrylamide sequencing gel alongside the sequencing reactions and the results visualized by autoradiography. Approximate equal gel loading was verified by comparison of bands generated from full-length cDNA products from control reactions.

RESULTS AND DISCUSSION

Mutations That Disrupt the Predicted Stem-Loop Increase the Incorporation tau Exon 10—To demonstrate that mutations that disrupt the predicted stem-loop in the 5’ splice site increase splicing of tau exon 10 in vitro a series of mutations were introduced into exon trapping constructs. Mutations were made at positions –2, –1, +3, +11, +13, +14, and +16 (Fig. 2) relative to the 5’ splice site that are predicted to lie within the stem, while mutations at +8 and +9 affected loop residues that were not expected to affect the stability of the stem-loop. Mutations at +3 (G to A), +13 (A to G), +14 (C to T), and +16 (C to T) were those that had been shown previously to increase splicing of exon 10 in vivo and to be pathogenic in FTD-17 (2, 3). Exon trapping transfections were performed in triplicate on each of the mutant constructs as well as on the wild-type construct.

The results of these experiments (Fig. 2) demonstrated that each of the FTD-17-associated splice mutants (+3, +13, +14, and +16) significantly increased the incorporation of tau exon 10.
10 into the artificial transcripts generated by the exon trapping assay, compared with the wild-type constructs consistent with previous reports of the effects of these mutants in this in vitro system (2). In addition artificial mutants at +2 (G to A), −1 (T to C), and +11 (T to C), which are predicted to disrupt the stem-loop, also increased splicing of exon 10 relative to the wild-type construct. Mutations at −2 and +3 are also predicted to increase the stability of U1 snRNP binding to the 5′ splice site (Fig. 5), which has also been shown to increase incorporation of an alternatively spliced exon (24). The result of this double effect on the splicing mechanism is that these mutations (+2 and +3) cause the greatest increase in exon 10 incorporation of the mutations tested (Fig. 2, ratios >10). The +3 (G to A) FTDP-17 splice site mutation (3) is thus predicted to increase U1 snRNP binding as well as disrupt the stem-loop structure, suggesting that both factors are likely to play a role in the mechanism of this pathogenic mutation.

In contrast, mutations at +8 and +9 had little impact on exon 10 splicing in this assay relative to the wild-type construct (Fig. 2). This was expected since these mutants occur within the loop region and therefore are not predicted to affect the stability of the stem-loop structure. The small increase in splicing observed with the +8 mutation probably reflects the small increase in U1 snRNP binding stability to the 5′ splice site that is predicted to be generated by this change. Taken together the results from this series of experiments are entirely consistent with the hypothesis that the predicted stem-loop structure at the 5′ splice site plays a major role in regulating the alternative splicing of tau exon 10 in vitro. However the precise ratio of exon 10+ to 10− mRNA is likely to be affected by other factors such as the stability of U1 snRNP binding to the 5′ splice site. Indeed it is significant that mutations that were predicted to reduce U1 snRNP base pairing at positions +3 (G to C), +4 (A to G), and +5 (G to C) resulted in the total loss of exon 10 splicing (Fig. 5). These results, while uninterpretable in terms of demonstrating the role of the stem-loop, do indicate the minimal U1 snRNP binding stability required for this splice site to remain functional in vitro.

Increasing the Stability of the Predicted Stem-Loop Reduces the Incorporation tau Exon 10—To further test the hypothesis that the stability of the predicted stem-loop at least partially determines the ratio of exon 10+ to exon 10− tau mRNAs, we generated wild-type and mutant (+13, +14, and +16) constructs in which residues +17/+18 downstream of the exon 10 5′ splice site were converted from GT to TG by site-directed mutagenesis (Fig. 3). This had the effect of increasing the length of the “stem” from 6 to 10 bp (although the exact structure of the stem-loop has yet to be determined), since residues +19 and +20 also pair with −5 and −6 (Fig. 3), significantly increasing the stability of the potential stem-loop structure in the 5′ splice site sequence. Exon trapping was performed in triplicate on each of the 4 constructs (wild-type and +13, +14, and +16 splice site mutants) with the extended (10 bp) stem-loop sequence as well as on wild-type and mutant constructs with the normal (6 bp) stem-loop (Fig. 3).

As before, each of the FTDP-17-associated splice mutants significantly increased the incorporation of tau exon 10 into the artificial transcripts generated by the exon trapping assay, compared with the wild-type constructs. This increase was present regardless of whether comparisons were between wild-type and mutant constructs with the normal 6 bp or with the extended 10 bp stem-loop structure (Fig. 3). The mutation with the greatest effect on splicing in both the 6- and 10-bp constructs was the +14 mutation (observed in the disinhibition dementia parkinsonism amyotrophy complex family (25)), which also causes the greatest loss in stem-loop stability (2) of the three mutations tested.

Increasing the length of the stem from 6 to 10 bp, and thereby the stability of the stem-loop structures, consistently resulted in a dramatic reduction in the ratio of exon 10+ to exon 10− transcripts, resulting from increased skipping of exon 10. This effect was observed in both the wild-type construct and in each of the three FTDP-17 mutant constructs (Fig. 3). Thus increasing the stability of the stem-loop in this manner reduced the incorporation of exon 10 consistent with the stem-loop structure regulating alternative splicing of this exon. The observed reduction in the incorporation of exon 10 with the extended stem (10 bp) constructs effectively rescues the effect of the FTDP-17 mutations by restabilizing the disrupted stem-loop. However as mentioned earlier the FTDP-17 mutants even with the 10 bp stem continued to have a higher exon 10+/− mRNA ratio than observed in the equivalent 10-bp stem wild-type construct (Fig. 3). Again this result is consistent with the hypothesis that the overall stability of the stem-loop dete-
FIG. 3. Tau exon 10 alternative splicing in vitro is regulated by the stability of the stem-loop. A, normal (6 bp) and extended (10 bp) stem-loops are shown with the location of FTDP-17 splice site mutations (+13, +14, and +16). The 10-bp stem-loop was generated by mutation of residues +17/+18 (shaded box). B, the results of exon trapping analysis with the 6- and 10-bp stem-loop constructs demonstrated that increasing the stability of the stem-loop resulted in a reduction in the ratio of exon 10+ (246-bp product) to exon 10− (153-bp product) mRNAs in wild-type (WT) and mutants (+13, +14, and +16). Note that the mutants (+13, +14, and +16) consistently increased the proportion of exon 10+ mRNA compared with wild-type (WT) regardless of whether comparisons were made among 6- or 10-bp stem-loop constructs. The +14 mutant gives the largest increase in exon 10+/− ratio consistent with this mutation producing the greatest reduction in 6-bp stem-loop stability (2). Molar ratios and S.D. values (shown beneath gel lanes) were calculated from three independent transfections.

As predicted the artificial mutations at −1 and +16 resulted in an increase in the proportion of exon 10+ mRNA (ratios 3.16 and 1.55, respectively), consistent with destabilization of the stem-loop (Fig. 4). The −1/+15 rescue construct reduced exon 10 splicing to levels significantly lower (ratio 0.44) than those observed with wild-type constructs (ratio 0.82). This reflects destabilization of the stem-loop by the opposing +15 mutation, which gives a stem-loop that is more stable than with the wild-type sequence (Fig. 4). The exception is low exon 10+/− ratio observed with the −1/+15 rescue (it was the lowest ratio observed where splicing was not abolished) probably reflected the generation of three consecutive G-C base pairs, over the splice site, which would be expected to significantly increase stem-loop stability. The +16−2 rescue construct also reduced splicing significantly (ratio 1.32) relative to the +16 mutant construct (ratio 1.55) consistent with restoration of the stem-loop. However the rescue construct still gave a higher exon 10+/− ratio than wild-type (0.82). The reason for this difference is unclear although it may reflect a subtle effect on either stem-loop stability, U1 snRNP binding or another part of the splicing process produced by replacing a purine residue with a pyrimidine (G to C) at position −2.

Eoxon trapping analysis with the −1, +16 mutants, and −1/+15, +16−2 rescues (Fig. 4) yielded results that are consistent
that the two rescue constructs would show reduced incorporation of exon 10 relative to the +16 and +3 mutants alone. However, neither rescue construct significantly reduced the splicing of exon 10, compared with the +3 and +16 mutant constructs, and indeed the −2/+16 construct displayed a small (1.8-fold), but significant, increase in the proportion of exon 10 mRNA (Fig. 5). The explanation for these results is likely to be a combination of two factors: first both of the complementary “rescue” constructs have stem-loops that are significantly less stable than in the wild-type construct, with a G-C pair replaced with A-T; second the +3 FTDP-17 mutant and the −2/+16 rescue (to the FTDP-17 +16 mutant) are both predicted to increase the base pairing of the 5′-splice site with the U1 snRNP (Fig. 5). Increased binding of the U1 snRNP to the 5′ splice site would be expected to increase splicing of exon 10 (24), independent of stem-loop stability. In both cases, the overall result is that the restoration of base pairing in the rescue constructs (+3/+12 and −2/+16) is insufficient to significantly reduce exon 10 splicing compared with the FTDP-17 mutants (+3 and +16).

Analysis of the tau Exon 10 5′ Splice Site in Other Species Confirms the Role of Pre-mRNA Secondary Structure in the Regulation of Alternative Splicing of This Exon—The results from the exon trapping analysis demonstrate that the effect of the FTDP-17-associated 5′-splice site mutations on the alternative splicing of tau exon 10 is mediated through destabilization of the predicted stem-loop structure. However exon trapping is an artificial system that does not fully replicate the alternative splicing of tau exon 10 in vivo. Therefore to determine if the predicted stem-loop structure was also likely to play a role in the regulation of tau exon 10 alternative splicing in vivo, we examined the sequence of the 5′ splice site in a range of other mammalian species (human, rhesus, marmoset, bovine, rabbit, rat, and mouse).

The sequence of the 5′ splice site in primates (human, rhesus, and marmoset) and bovine had an identical predicted stem-loop structure with a 6-bp stem and a 6-base loop region (Fig. 6). In contrast in each of the rodent species (rabbit, rat, and mouse) some part of the sequence that makes up this structure was absent (Fig. 6), resulting in a predicted stem-loop of reduced stability. In the rabbit the +11 residue was not conserved (C in rabbit, U in human), resulting in a predicted 5-bp stem and 7-base loop, which mimics the artificial +11 (T to C) mutation employed in exon trapping studies. In the rat residue +13 is not conserved (G in rat and A in human), which mimics the +13 (A to G) FTDP-17 mutation, and results in a 6-bp stem with an internal mismatch (G-U). In the mouse both the +13 and the +16 residues are not conserved. Thus the order of predicted stem-loop stability in the different mammalian species analyzed is primates/bovine > rabbit > rat > mouse (Fig. 6). In order to relate the stability of the stem-loop in the 5′ splice site to the alternative splicing of exon 10, we performed RT-PCR analysis on RNA isolated from the brains of the different species (human frontal lobe, rabbit, rat, and mouse). The results of this analysis demonstrated clearly (Fig. 6) that there was an inverse relationship between the predicted stability of the stem-loop in the sequence of the splice site in each species and the ratio of tau exon 10+/− mRNAs, thus the order of different species in exon 10+/− ratio was: mouse > rat > rabbit > human frontal lobe (bovine and other primates not tested). The exon 10+/− ratios observed in the human, rabbit, and rat brain RNA by RT-PCR are also highly similar to the ratios obtained through in vitro exon trapping studies performed with equivalent tau exon 10 constructs: wild-type, +11 (T to C) and +13 (A to G), respectively (Fig. 2). The results are consistent with the predicted stem-loop structure in the 5′
splicing of exon 10 regulating the alternative splicing of this exon in multiple species and that the stability of this structure at least partially determines the proportion of exon 10 mRNA. It should be noted, however, that other factors in addition to the stem-loop must also be involved in the regulation of exon 10 alternative splicing, since only three repeat Tau (exon 10−) is observed in the mammalian fetal brain (18). One possible explanation for this phenomenon is that an inhibitory splicing factor is present in fetal brain that is absent in the majority of adult neurons; previous studies have also suggested that thyroid hormone expression regulates the generation of four repeat Tau during brain development (26).

In the mouse, tau exon 10− mRNA was not detected by RT-PCR analysis of whole brain RNA, in agreement with previous reports of the Tau isoform composition in adult mouse brain (18). The large difference in Tau isoform composition between adult human and mouse brains (and other rodents) is likely to reflect, if not underlie, some fundamental, undetermined differences in the functions and characteristics of neurons from these species.

**Transgenic Mice Expressing the Human tau Gene Demonstrate Alternative Splicing of Exon 10 Consistent with the Involvement of Cis-acting Elements in Regulation of This Splice Event**— Alternative splicing of exon 10 was investigated in the brains of transgenic mice expressing a human PAC (P1-derived artificial chromosome) (~200 kb) transgene containing the entire human tau gene (TG). RT-PCR analysis with human and mouse tau-specific primers was performed on RNA isolated from the brains of adult transgenic and littermate control animals. This analysis (Fig. 6C) demonstrated that splicing of tau exon 10 in the endogenous mouse gene was unaffected in the transgenic animals (only exon 10+ mouse tau RNA was observed). In contrast, with human-specific primers products corresponding to both exon 10+ and exon 10− mRNA were detected in the brains of transgenic mice expressing the human tau gene (Fig. 6C). This demonstrated that human-like alternative splicing of exon 10 was occurring in pre-RNA generated from the human transgene. Control animals gave no RT-PCR product with the human-specific primers. Western blot analysis of Tau protein in the transgenic mouse brains also demonstrated the presence of human Tau isoforms, generated by alternative splicing of pre-mRNA, that are absent in endogenous mouse Tau protein (not shown).

The fact that the human tau transgene generates pre-mRNA that undergoes alternative splicing of exon 10 similar to that seen in the human brain (although the exon 10+/− ratio is somewhat altered in these mice) clearly suggests that cis-acting sequence elements specific to the human gene regulate alternative splicing of exon 10 (Fig. 6C). This is obviously consistent with the hypothesis that the stem-loop sequence in the 5′ splice site of human exon 10, which is absent in the mouse gene, is at least partially involved in the regulation of alternative splicing of exon 10.

**RNA Structural Mapping Confirms the Presence of a Stem-Loop Structure in the 5′ Splice Site of tau Exon 10 That Is Disrupted by the FTDP-17 Splice Site Mutations**—RNA secondary structure prediction analysis with the program RNAFOLD (28) suggested two possible structures of similar stability for the region around the 5′ splice site of exon 10 (Fig. 7). In both structures the minimal 6-bp stem-loop is maintained, however, beyond this region it is possible for either a short second stem to form (structure 1) or a lateral stem-loop (structure 2). The structures are of similar stability, because they are both formed with an identical 9-base sequence (ACACGUCCC) that is re-

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**Fig. 6. Analysis of tau exon 10 alternative splicing in other mammals and transgenic mice.** **A**, aligned 5′ splice site sequences. **B**, RT-PCR analysis of tau exon 10 alternative splicing in human frontal lobe and rabbit, rat, and mouse whole brain RNA. PCR was between exon 9 and exon 11 (exon 10+ products are 367 bp and exon 10− products are 274 bp), and the identity of PCR products was confirmed by sequencing. The results demonstrate an inverse relationship between stem-loop stability and tau exon 10+/− mRNA ratio. In Humans (most stable stem-loop) the lowest tau exon 10+/− RNA ratio is observed while in mouse (least stable stem-loop) exon 10− mRNA could not be detected. **C**, RT-PCR analysis of tau exon 10 alternative splicing in transgenic mice expressing the entire human tau gene (TG) and in littermate controls (Non-TG). PCR was performed on RNA isolated from adult mouse brains between exon 9 and exon 11. Primers were designed to be human- or mouse-specific. Mouse tau-specific primers gave only exon 10+ products (390 bp), demonstrating that splicing of the endogenous mouse gene is unaffected by the transgene. In contrast, human tau pre-mRNA in the transgenic mice was shown to undergo exon 10 alternative splicing similar to that observed in human Tau. This result is consistent with cis-elements in the human gene (such as the stem-loop) regulating alternative splicing of this exon.
Fig. 7. RNA secondary structure predictions for the tau exon 10 5′ splice site. Proposed structures for the tau exon 10 pre-mRNA 5′ splice site. The minimal 6-bp stem-loop in addition to alternative structures 1 and 2 predicted using RNAFOLD (28) are shown. Structure 1 ($\Delta G = -11.9 \text{Kcal/mol}$) is similar to that proposed in Ref. 3 with additional base pairing in the lower stem region; structure 2 ($\Delta G = -12.8 \text{Kcal/mol}$) is similar in stability to structure 1 but with an alternative lateral stem-loop. A repeated ACACGUCCC sequence equidistant from the splice site (−14 to −22 and +13 to +21, indicated by boxes) allows for the formation of either structure 1 or 2 with similar stability.

Repeated at virtually equidistant positions from the 5′ splice site (−14 to −22 and +13 to +21). The significance of this repeated sequence is unclear however its position on either side of the splice site would suggest that it may be involved in the regulation of alternative splicing of exon 10 in some manner possibly as a binding site for a splicing factor. The FTDP-17 mutations +13, +14, and +16 will alter this repeat sequence, and thus it is possible that this might be an additional mechanism by which these mutations affect the alternative splicing of exon 10 beyond the disruption of the predicted stem-loop structure that is the subject of this study.

Initial characterization of the effects of the FTDP-17 splice site mutants on the secondary structure of tau pre-mRNA was performed by examining the migration of in vitro transcribed RNAs containing exon 10 and flanking intronic sequences (247 bases) on denaturing and nondenaturing agarose gels (Fig. 8A). RNAs containing wild-type exon 10, the +8, +13, +14, +16 splice site mutants and the extended (10 bp) stem-loop (Fig. 3) were analyzed. All five in vitro transcribed RNAs ran as single bands with identical migration on denaturing (formaldehyde/agarose) gels consistent with each RNA being the same size (247 bases). In contrast, migration on nondenaturing agarose gels differed significantly between different RNAs (Fig. 8A). The wild-type and +8 mutant RNAs gave essentially identical migration patterns consistent with the +8 mutation occurring in the predicted loop region and therefore not significantly affecting the stability of the stem-loop. The wild-type and +8 RNAs migrated as a doublet band corresponding to one major product and to one slower minor product, suggesting that these RNAs exist in at least two confirmations. This is consistent with the prediction of the RNAFOLD analysis that there are two possible secondary structures for this region with similar stability (Fig. 7). In contrast, the FTDP-17 splice mutant (+13, +14, and +16) RNAs, which are predicted to disrupt the stem-loop, were observed as a single major band with faster migration than the wild-type RNA. This is consistent with these mutations altering RNA secondary structure presumably resulting in a more flexible molecule. Weaker minor products in the FTDP-17 mutant RNAs (+13, +14, and +16) are also visible that appear to co-migrate with the wild-type RNA major product, suggesting that a proportion of these mutant RNA molecules maintain the wild-type secondary structure (Fig. 8A). Interestingly these minor products are strongest with the FTDP-17 +16 mutation, which also produces the smallest increase in exon 10 to 10− RNA ratio in the exon trapping studies, compared with the +13 and +14 mutations (Figs. 2 and 3). The extended (10 bp) stem RNA migrated as a single band however this product displayed marginally slower migration compared with the major wild-type product consistent with the presence of a more stable stem-loop creating a less flexible molecule. The data from this study are completely consistent with the presence of a dynamic stem-loop structure in the exon 10 RNAs that is disrupted by the FTDP-17 splice site mutants but which is stabilized by the lengthening of the...
predicted 6-bp stem, to 10 bp.

To further investigate the likely pre-RNA secondary structure around tau exon 10, we performed mapping analysis with RNase enzymes that recognize double-stranded (V1 RNase) and single stranded (T1 RNase) RNA (20). The sites of cleavage were mapped by primer extension with an oligonucleotide complementary to a sequence downstream of the region to be studied (see “Experimental Procedures”). In vitro transcribed RNAs containing exon 10 (wild-type, +14 mutant, 10-bp stem-loop mutant) were subject to this analysis (Fig. 8B).

The extended 10-bp stem RNA gave the strongest signals for V1 (double-stranded) digestion in regions predicted to be double-stranded in the extended 10-bp stem-loop (Fig. 8B). The V1 digestion was strongest at residues (−3 to +3) in the 5’ side of the stem. Wild-type RNA also gave significant V1 signals in the predicted double strand region, on both the 5’ and 3’ sides of the stem-loop, although the shorter stem was reflected in the absence of bands at +17 and +18. In contrast, markedly weaker V1 signals were observed from the +14 mutant RNA in the predicted stem region. This is consistent with the proposed disruption of the wild-type secondary structure in this region by the +14 mutation. Interestingly, however, additional V1 products that are specific to the +14 mutant RNA are observed in the predicted loop region (+9, +10), suggesting that this mutation may cause the formation of an alternative, and presumably less stable, secondary structure feature in this region.

Digestion with T1 enzyme gave little obvious difference between each of the RNAs (strong bands in the 10-bp stem-loop, although the shorter stem was reflected in the absence of bands at +17 and +18. In contrast, markedly weaker V1 signals were observed from the +14 mutant RNA in the predicted stem region. This is consistent with the proposed disruption of the wild-type secondary structure in this region by the +14 mutation. Interestingly, however, additional V1 products that are specific to the +14 mutant RNA are observed in the predicted loop region (+9, +10), suggesting that this mutation may cause the formation of an alternative, and presumably less stable, secondary structure feature in this region. Digestion with T1 enzyme gave little obvious difference between each of the RNAs (strong bands in the 10-bp stem-loop, although the shorter stem was reflected in the absence of bands at +17 and +18. In contrast, markedly weaker V1 signals were observed from the +14 mutant RNA in the predicted stem region. This is consistent with the proposed disruption of the wild-type secondary structure in this region by the +14 mutation. Interestingly, however, additional V1 products that are specific to the +14 mutant RNA are observed in the predicted loop region (+9, +10), suggesting that this mutation may cause the formation of an alternative, and presumably less stable, secondary structure feature in this region. Digestion with T1 enzyme gave little obvious difference between each of the RNAs (strong bands in the 10-bp stem-loop, although the shorter stem was reflected in the absence of bands at +17 and +18. In contrast, markedly weaker V1 signals were observed from the +14 mutant RNA in the predicted stem region. This is consistent with the proposed disruption of the wild-type secondary structure in this region by the +14 mutation. Interestingly, however, additional V1 products that are specific to the +14 mutant RNA are observed in the predicted loop region (+9, +10), suggesting that this mutation may cause the formation of an alternative, and presumably less stable, secondary structure feature in this region.

The results from the secondary structure mapping analysis are not sufficiently clear to enable a prediction of the precise structure of the stem-loop. Indeed results from RNAFOLD predictions and gel migration analyses would suggest that there may be multiple (at least two) conformations formed in this region. However the mapping results from different RNAs, wild-type and mutant (+14 and 10-bp stem), are clearly consistent with the presence of a stem-loop, which is disrupted by the FTDP-17 +14 mutation. This conclusion is further supported by the gel migration studies of different tau exon 10 wild-type and mutant RNAs (described above).

Concluding Remarks—Our results demonstrate that alternative splicing of tau exon 10 (in humans and other mammals) is at least partially regulated by a stem-loop structure that forms in the pre-mRNA at the 5’ splice site. In addition, the ratio of exon 10+/−/− mRNAs is largely determined by the stability of this structure. Stem-loop formation at the 5’ splice site of exon 10 is likely to compete with the binding of specific factors required for the early stages of splicing assembly, most likely the U1 snRNP, after transcription has occurred. Inserted stem-loops were shown previously in vivo to block U1 snRNP binding to a 5’ splice site in the yeast RP51A gene (27). Formation of the stem-loop at the 5’ splice site of tau exon 10 will therefore block exon 10 definition and result in skipping of exon 10 in tau mRNA. Further experiments are needed to confirm the role of the U1 snRNP in this mechanism; however, sequence changes in the 5’ splice site, which are predicted to alter the stability of U1 snRNP binding, result in altered exon 10 splicing in vitro independent of the of stem-loop stability. The exact structure of the stem-loop region has yet to be determined with two different confirmations predicted by RNAFOLD (28) analysis (Fig. 7). It is thus clear that the stem-loop structure plays a major role in the regulation of tau exon 10 alternative splicing however additional levels of splicing control may also be mediated through the 5’ splice site. In particular, a 9-base region that is repeated at equidistant positions from the splice site may also be involved in splicing regulation possibly by providing a binding site for a splicing factor.

The 5’ splice site mutations that are associated with FTDP-17 (2, 3) act by disrupting this stem-loop, which leads to increased incorporation of exon 10 in tau mRNA. This in turn leads to an increase in the proportion of Tau isoforms with four microtubule-binding domains, although how this leads to neurodegeneration is yet to be determined. The identification of splice site mutations in tau associated with FTDP-17 has demonstrated the significance of Tau isoform composition to neuronal function. In addition, these mutations in tau are the first shown to cause human disease through disruption of pre-mRNA secondary structure that regulates alternative splicing.

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