Determinants of 4-Repeat Tau Expression

COORDINATION BETWEEN ENHANCING AND INHIBITORY SPLICING SEQUENCES FOR EXON 10 INCLUSION*

Mutations in the tau gene are pathogenic causing autosomal dominant frontotemporal dementia with Parkinsonism-chromosome 17 type (FTDP-17). Some mutations in tau exon 10 (E10) and immediately adjacent sequences cause disease by altering E10 splicing. To determine the mechanism of normal E10 splicing regulation and how FTDP-17 mutations alter splicing, mutational analysis of E10 was performed. The results show that E10 contains a complex array of both enhancer and inhibitor cis-acting elements that modulate usage of a weak 5' splice site. The 5' end of E10 contains a previously unrecognized multipartite exon splicing enhancer (ESE) composed of an SC35-like binding sequence, a purine-rich sequence, and an AC-rich element. Downstream of this ESE is a purine-rich exon splicing inhibitor. Intronic sequences immediately downstream of E10 also are inhibitory. The results support an alternative model in which I10 inhibitory sequences appear to function as a linear sequence. The cis-elements described are not redundant, and all appear required for normal E10 splicing. Results with double mutations demonstrate that the ESE and the intrinsic inhibitory element collaborate to regulate splicing. Thus splicing of tau E10 is regulated by a complex set of cis-acting elements that span nearly the entire exon and also include intronic sequences.

Tau is a microtubule-associated protein that in vitro promotes microtubule assembly and stability. In vivo, tau is important in neuronal morphogenesis, axon polarity, and axonal transport (1, 2). In the central nervous system, tau is expressed primarily in neurons, although it is also present in glial cells at lower levels. During central nervous system development, the gene encoding tau (tau) is highly regulated by alternative exon splicing. In the human fetal brain, a single tau isoform is produced consisting of exons 1, 4, 5, 7, 9, and 11–13 (3). In the adult human central nervous system, 6 tau isoforms are made from the same exons plus alternative splicing of exons 2, 3, and 10 (4). tau exon 10 (E10)\(^1\) encodes a microtubule-binding motif that is repeated 3 times in tau isoforms lacking E10 sequences (3 repeat or 3R tau) and 4 times when E10 is present (4R tau).

Mutations in tau cause frontotemporal dementia with Parkinsonism-chromosome 17 type (FTDP-17) (5–7), a group of autosomal dominantly inherited neurodegenerative disorders with varied clinical and neuropathologic phenotypes. A common consequence of most if not all tau mutations is abnormal filamentous aggregates of tau in central nervous system neurons and in some cases in glial cells (8). For some mutations, the aggregated tau is present as paired helical filaments in neurofibrillary tangles. These tangles are composed of all 6 tau isoforms and are structurally and biochemically indistinguishable from those found in Alzheimer’s disease (AD) (9, 10). For other FTDP-17 mutations, tau is present as paired straight filaments (11) or paired helical filaments with different dimensions than those of AD filaments (12, 13). Also the isoform ratio can be different from that found in AD.

tau missense mutations fall into 2 categories as follows: mutations that alter protein function, and mutations that affect alternative splicing of E10. Mutations G272V (E9), P301L, P301S, ∆280K (E10), V337M (E12), and R406W (E13) reduce the affinity of tau binding to microtubules (14) and/or reduce the ability of tau to promote microtubule assembly when compared with normal tau (11, 14–17). Thus, these mutations alter tau protein function. Since E9, E12, and E13 are constitutively included in tau transcripts, mutations in these exons affect all tau isoforms, whereas E10 mutations P301L and P301S only affect 4R tau function. E10 mutations N279K and S305N do not alter the ability of tau to interact with microtubules but do affect the regulation of E10 splicing (15, 18). In splicing assays, the N279K and S305N mutations result in almost constitutive inclusion of E10. Similarly, the L284L E10 silent mutation and intron 10 (I10) mutations immediately adjacent to the 3' end of E10 also increase E10 inclusion (6, 15). Another FTDP-17 mutation, an in-frame 3-base deletion in E10 (∆280K), is unique in that it alters tau protein function and also completely abolishes E10 inclusion in tau transcripts (15, 17). The effect of ∆280K on splicing is presumed to be the disease-causative mechanism since, with E10 being completely excluded, no ∆280K protein would be produced. The mutations that affect E10 splicing cause FTDP-17 by altering the 4R3R tau ratio, and in some cases, the elevated 4R tau produced has the normal tau amino acid sequence (e.g. L284L and I10 mutations; Fig. 1) (14, 19). As temporal dementia with Parkinsonism-chromosome 17 type; I9, intron 9; I10, intron 10; ISS, intronic splicing silencer; MCS, multiple cloning site; PPE, polyurine enhancer; SR, Arg/Ser-rich splicing factors; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair; HIV, human immunodeficiency virus; snRNP, small nuclear ribonucleoprotein.

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\^ The abbreviations used are: E10, exon 10; 3R, 3-repeat tau; 4R, 4-repeat tau; ACE, A/C-rich enhancer; AD, Alzheimer’s disease; ESE, exon splicing enhancer; ESS, exon splicing silencer; FTDP-17, frontotemporal dementia with Parkinsonism-chromosome 17 type; I9, intron 9; I10, intron 10; ISS, intronic splicing silencer; MCS, multiple cloning site; PPE, polyurine enhancer; SR, Arg/Ser-rich splicing factors; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; bp, base pair; HIV, human immunodeficiency virus; snRNP, small nuclear ribonucleoprotein.

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expected, when E10 usage is increased, the aggregated tau formed has excess 4R tau isoforms (7, 14, 19). Different mutations that increase E10 usage result in different clinical and neuropathologic phenotypes, presumably because these mutations affect different cis-acting regulatory elements controlling E10 splicing. Thus, understanding the mechanisms that regulate E10 usage is important to understanding FTDP-17.

Our previous work examining the effects of FTDP-17 mutations on tau E10 splicing indicated that multiple cis-acting sequences regulate E10 alternative splicing (15). An additional intrinsic inhibitory element immediately adjacent to the end of E10 in I10 was revealed by other FTDP-17 mutations (6, 7). Here we examine exon and intron sequences that control tau E10 inclusion. We performed mutation and deletion studies to identify splicing regulatory sequences that control E10 splicing and to explore the functional interactions between elements in orchestrating E10 usage. Our results reveal that there are at least 4 cis-acting regulatory sequences within E10 that are non-redundant and that function cooperatively to regulate E10 inclusion. These E10 cis-elements interact with a novel 110 inhibitory element that potentially acts by forming a complex secondary structure, although evidence that the inhibitory element appears to function as a linear sequence is presented here. Correct regulation of tau E10 is the result of contributions from each of these elements.

**Experimental Procedures**

**Plasmid Construction and DNA Mutagenesis—**Vector pSPL3 (Life Technologies, Inc.) contains an HIV genomic fragment with truncated tat exons 2 and 3 inserted into rabbit β-globin coding sequences (20). The resulting hybrid exons in pSPL3 are globin E12-tat exon 2 and tat exon 3-globin E3 separated by more than 2.5 kilobase pairs of tat intron sequence. pSPL3 contains a multiple cloning sequence (MCS) around exon 3 used in previous work on the effects of FTDP-17 mutations on tau E10 splicing showed that mutations at nucleotides 15–18 within a purine-rich region in E10 and a mutation at nucleotide 30 altered splicing of this exon (15). To identify the regulatory elements affected by these mutations and to reveal other potential regulatory sequences, we generated in-frame 3 nucleotide deletions from the beginning of E10 exon to nucleotide 33 (E1–11). Nine nucleotide deletions were generated from nucleotide 34 to the end of the exon (E12–E17) (Fig. 1). Additional substitutions were used to identify critical nucleotides and to determine if effects seen with deletions were due to removal of critical sequences or caused by changes in distances between other cis-acting elements. Splicing was assayed by inserting the normal or modified E10 into 31 nucleotides of I9 and 51 nucleotides of I10 (Fig. 1) between HIV tat exons 2 and 3. When the normal E10 is assayed in COS-7 cells, 45% of the transcripts contain E10 (Fig. 1). The cis-acting regulatory elements identified are described individually below.

**Purine-rich ESE Element—**Deletions E25 to E37 severely reduce E10 inclusion (Fig. 1) indicating the presence of an ESE sequence. This ESE is designated the polypurine enhancer (PPE) because of the high purine content of this sequence (AAGAAGCTG) and is the cis-acting element affected by FTDP-17 mutations N279K and Δ280K. E25 and E36 are structurally equivalent and the same as FTDP-17 mutation Δ280K. This deletion results in no detectable E10+ transcript. The downstream flanking deletion E37 reduces the splicing ratio, whereas E38 does not, thus defining the 3’ limits of the PPE. The 5’ end of the PPE is more difficult to define. Deletions E35 and E44 result in higher than normal E10 inclusion, suggesting that these sequences may inhibit function of the PPE.

The sequence requirements for PPE function were explored with single nucleotide substitutions alone or in combination with the E25 deletion (Fig. 2). Mutation 15T→G (FTDP-17 mutation N279K) increases E10 incorporation to 79%. The 15T→G change possibly acts by increasing the number of GAR

**RNA Structure Analyses—**RNA secondary structures and free energies were predicted using the GCG version of the MFOLD program (22). The free energies of the most stable structures computed for each sequence were compared.

**Results**

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**Regulation of Tau Exon 10 Splicing**

**17701**

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sequences (R is a purine) from 1 to 2 when compared with the normal sequence or by increasing the number of AAG repeats from 2 to 3. Both GAR and AAG repeats are known to enhance splicing in other systems (23, 24). Compatible with both explanations is that ED5, which eliminates E10 inclusion, deletes the lone GAR repeat and 1 of the 2 normal AAG repeats. When an A substitution is used rather than a G (15T→A), E10 inclusion is increased but to a lesser extent than with the 15T→G change (61 compared with 79%, respectively). Likewise, in double mutants when the normal function of the PPE is severely compromised by ED5, the G substitution gives much higher E10 incorporation than the A substitution (71 versus 16%, respectively). In the context of the deletion, the G substitution, like the normal sequence, maintains 1 GAR repeat and 2 AAG repeats, whereas the A substitution yields only 1 AAG. Thus, although both mutations increase the number of consecutive purines in this region by 3 nucleotides, clearly the types of purine and thus the specific sequence of the element are critical.

AC-rich ESE Element—Deletions ED9 and ED10 were de-
signed to disrupt the region of E10 affected by FTDP-17 mutation L284L (30T→C). This mutation alters the sequence TTAG, which acts as an ESS sequence in HIV-1 tat exon 3 (21). These deletions were expected to increase splicing due to deletion of the putative TTAG silencer. However, unlike mutation 30T→C that increases E10 inclusion (87%, Fig. 2), these deletions either gave near normal splicing (E1-D9, 51%) or substantially reduced E10 inclusion (E1-D10, 17%; Fig. 1). E1-D11 and E1-D12, like E1-D10, also reduce the amount of E10+ produced (6 and 33%, respectively). Thus, in the tau E10 context, unlike in HIV-1 tat exon 3, TTAG does not function as an ESS. Rather, the sequence in the E1-D9-E1-D12 region contains an ESE that may be an AC-rich enhancer (ACE) (25), particularly when the 30T→C mutation is present. This region of E10 resembles ACE elements observed in other systems (Fig. 2) (26). In agreement with this hypothesis, transitions within the TTAG sequence that increase the AC content (29T→C and 32G→A), like 30T→C, increase E10 inclusion with the T→C substitutions yielding the largest increase. However, 31A→G, which decreases the AC content, also increases E10 inclusion. Thus the sequence requirements for this ESE are more complex than simply a high AC content.

Additional cis-Acting Sequences in E10—Deletions E1-D14 and E1-D15 markedly increase E10 inclusion (69 and 90%, respectively, Fig. 1), suggesting removal of a previously unknown inhibitory element at the 3' end of the exon. Flanking deletions resulted in either slightly elevated (E1-D10) or slightly reduced (E1-D16 and E1-D17) E10 incorporation, indicating that the putative inhibitory element is primarily confined to the E1-D14/E1-D15 segment. A series of point mutations (14a, 15a, and 15b, Fig. 3) were introduced into the same region replacing the clusters of As with G, C, and T nucleotides. Each different substitution as
well as combinations of substitutions increased E10 incorporation (Fig. 3). These point mutations show that the EΔ14/EΔ15 region contains a purine-rich ESS and that the effects seen with these mutations are not simply due to changes in E10 length or altering the proximity of other cis-acting elements. Purine-rich ESSs downstream of an ESE are also present in the human fibronectin EDA exon (27) and in HIV tat exons 2 and 3 (28, 29).

Another potential regulatory region is at the 5′ end of E10. EΔ1 dramatically reduces E10 inclusion to 12% (Fig. 1). This deletion alters the sequence TGGCAT that resembles the degenerate binding consensus TGCNGYY for the SR protein SC35 (30). Deletion of the AT-rich sequences in EΔ3 and EΔ4 results in increased E10 inclusion suggesting that either an inhibitory sequence has been removed or that a reduction in the distance between the SC35-like and the purine-rich ESE sequences is important for both to function.

**Role of the 5′ Splice Site in E10 Inclusion**—The E10 5′ splice site is predicted to be weak because the sequence (GTgtgagt) differs from the optimal consensus sequence (AGgtgagt) for U1/U6 snRNP binding (31). The role of the 5′ splice site and immediately adjacent exon sequences was investigated using single nucleotide substitutions in the 5′ splice site and by replacing tau E10 sequences with the 3′ end of human β-globin E2. The β-globin E2 sequences used were the last 10 nucleotides of the exon and the first 6 nucleotides of the intron (the entire 5′ splice site). This constitutively spliced middle exon was chosen because its 5′ splice site can easily be converted to that of tau E10 by altering the −1 position. Predictably, the hybrid construct spliced to completion as it contains a strong 5′ splice site and adjacent E10 sequences.

**Construct | Sequence | % E10+ | SD | p value**
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The 5′ Splice Site-ISS Interaction—Intronic FTDP-17 mutations (E10+3g→a, E10+12c→t, E10+13a→g, E10+14c→t, and E10+16c→t; Fig. 1) immediately adjacent to the 5′ splice site dramatically increase E10 inclusion, demonstrating that this region is an ISS element. One proposed mechanism for how this ISS functions is that the normal sequence results in a spliced middle exon, whereas the FTDP-17 mutation 92G→A enhances E10 inclusion by disrupting base pairing in this stem-loop. Three different stem-loops have been proposed, each containing differ-
different lengths of E10 sequences (Fig. 6). The longer versions extend into E10 raising the possibility that the inhibitory element includes both exon and intron sequences. However, EΔ17 reduces E10 inclusion to 26% (Fig. 1), a result that is inconsistent with these exon sequences being part of an inhibitory element. Also, substitutions -4Δ-9 and -5Δ-9 (Fig. 4) introduced into the E10 sequences immediately adjacent to the 5’ splice either did not alter E10 inclusion (-4Δ-9) or only slightly increased E10 inclusion to 58%. The substituted nucleotides were mostly transitions, chosen so as not to base pair with their corresponding partners in I10. These substitutions and the EΔ17 deletion would be expected to disrupt the lower portions of the 25- and 30-nucleotide stem-loop structures (Fig. 6A) without disrupting the 18-nucleotide structure at the top. These results show that either secondary structure is not important for the inhibitory activity of the ISS or that only the shortest stem-loop is important for regulation.

Interactions between cis-Acting Elements—The interactions between different regulatory sequences were examined by comparing E10 splicing in constructs with mutations in single elements to constructs with mutations in two different elements. The EΔ5 mutation was used to abolish function of the PPE. FTDP-17 mutation 92G→A or E10+3g→a was used to strengthen the 5’ splice site. Double mutants 92G→A/EΔ5 and E10+3g→a/EΔ5 yielded high levels of E10 inclusion (80 and 87%, respectively) demonstrating that in the presence of a strong 5’ splice site, the PPE is not required for efficient E10 splicing (Fig. 5). When the FTDP-17 ISS mutations E10+12c→t, E10+12g→c, and E10+16c→t were combined with EΔ5, E10 inclusion was reduced compared with the results when only the ISS mutations were present. Thus the ISS and the PPE must collaborate to regulate E10 inclusion. Removal of the PPE with the EΔ5 mutation had varied effects when combined with the different ISS mutations. The EΔ5 mutation with E10+12c→t or E10+14c→t only modestly reduced E10 inclusion compared with results for only the single E10+12c→t or E10+14c→t mutations; E10 inclusion was still greater (59 and 65%, respectively) than seen with the normal E10 sequence. In contrast, for double mutants containing E10+13a→g and E10+16c→t, E10 inclusion was dramatically reduced (28 and 26%, respectively) to levels below those seen with the normal sequence. These results imply that not all nucleotides within the ISS are equivalent. The PPE also interacts with the ACE element. The effect of the FTDP-17 mutation 3OT→C that strengthens the AC-rich ESE and enhances E10 inclusion is modulated when the PPE is abolished in the double mutant 3OT→C/EΔ5 (Fig. 2).

Comparison of Human and Mouse E10 Splicing—Mice and humans regulate E10 splicing differently. In fetal brain in both organisms, E10 is not incorporated into tau transcripts. In adult mouse brain, all tau transcripts have E10, and only 4R tau protein is produced. In contrast, in adult human brain, both E10+ and E10− tau transcripts and 3R and 4R tau protein are found in roughly equal proportions (14). E10 is highly conserved between these two species with only 3 nucleotides being different (Fig. 1A), and the 5’ and 3’ splice sites are identical. However, the adjacent intronic regions are more divergent (Fig. 7). To assess the role of these divergent sequences on E10 regulation, a wild-type mouse splicing template (mWT) was generated that is analogous to the human sequences used here and is comprised of mouse E10 (E3M) flanked by 30 and 51 nucleotides from mouse I9 (I9M) and I10 (I10M), respectively. In COS-7 cells, the efficiency of E10 inclusion (34%) was somewhat less than the human equivalent.

To evaluate the effects of mouse and human intronic sequence differences on splicing, constructs were generated with different combinations of human and mouse sequences (Fig. 7). These combinations permitted evaluation of the role of I10 and E10 sequence differences separately. Mouse I10 sequences (I10M) are substantially more inhibitory than I10H sequences; when human I9 and E10 (I9H/E10H) are coupled to I10M, splicing is reduced (compare hN to I9H/E10H/I10M; 45 and 5%, respectively). Conversely, when mouse I9 and E10 sequences are coupled to I10H, E10 incorporation is increased (compare mWT to I9HM/E10M/I10H; 34 and 61%, respectively). The same strong inhibition by I10M is also seen when mouse and human divergent
nucleotides (TA and AC, respectively) at positions 84 and 87 in E10 are exchanged (compare results from I9M ЕGАСC110M and I9M ЕGАСC110H).

Exon sequence differences between human and mouse tau also affect splicing. When in hN, E10 nucleotide 57 is changed from A found in humans to G found in mouse (I9M ЕGАСC110H), and E10 inclusion is increased from 45 to 89%. This result is consistent with an ESS element in the E10 region of E10 (Fig. 3). When mouse E10 nucleotides at positions 84 and 87 were inserted into hN, splicing was reduced to 14% (compare hN to I9M ЕGАСC110H, Fig. 7). Human nucleotides at positions 84 and 87 were also replaced individually to determine which is responsible for the decrease in splicing. At position 87, when the human C nucleotide was replaced with the mouse A, little change in splicing was observed (compare hN to I9M ЕGАААC110H, 45 versus 50%, respectively, Fig. 7). In contrast, the single nucleotide replacement of the human A at position 84 with either the mouse T or another pyrimidine (C) resulted in a dramatic reduction in splicing to 11 and 5%, respectively (compare the mouse T or another pyrimidine (C) resulted in a dramatic reduction in splicing to 11 and 5%, respectively (compare hN to I9M ЕGАCСC110H, Fig. 7). Thus, nucleotide 84 is critical, and the reduction in splicing observed when this nucleotide is changed is consistent with results seen with E14 and E15 (Fig. 1) and suggests that a stimulatory element is present immediately upstream of the end of E10. However, presently we cannot exclude the possibility that deletions E14 and E15 affect splicing by bringing the E10 ESS closer to the 5’ splice site.

When human nucleotides were inserted into mWT at positions 84 and 87, E10 inclusion was reduced (compare mWT to I9M ЕGАСC110M, 34 versus 20%, respectively). However, the inhibitory effects of the substituted nucleotides were overridden when the downstream intron was human (compare I9M ЕGАСC110M to I9M ЕGАСC110H, 20 and 93%, respectively). In contrast, no change in splicing occurred on replacing the mouse G at nucleotide 57 for the human A at this position (compare mWT to I9M ЕGАААC110M, 34 and 35%, respectively). Presumably the strong inhibitory effect of mouse 110 overrides other elements (e.g., the ESS) in mouse E10. The mouse and human I9 sequences used here also differentially affect E10 splicing. The mouse I9 sequence is more permissive for E10 inclusion when compared with the human I9 sequence (compare I9M ЕGАААC110M to I9M ЕGАААC110M, 35 and 3%, respectively).

DISCUSSION

The ratio of tau E10+ to E10− transcripts is regulated by a complex combination of intronic and exonic sequences. Nearly the entire exon is involved in controlling the inclusion of E10 in tau transcripts. The in vivo importance of this complex regulation is revealed by FTDP-17 mutations that disrupt critical cis-acting elements within or closely flanking E10. In the adult under normal conditions, the E10+/E10− ratio is close to 1 (14). Autosomal dominant FTDP-17 splicing mutations that only affect 1 copy of the tau gene, elevate this ratio to 2:1 (e.g., N279K mutation) (14, 19) or 3:1 (I10 mutations) (6, 7) or decrease this ratio to 1:3 (Δ280K mutation) (15, 17). The consequence of these relatively subtle changes is onset of severe neurodegenerative disease in mid-life. The work described here demonstrates that the regulation of E10 splicing is complex involving both ESE and ESS elements within the exon. In addition, intronic sequences immediately adjacent to the 3’ end of E10 inhibit splicing. Other more distant intronic sequences not studied here also affect E10 splicing.

The deletion and substitution analysis described above demonstrates that tau E10 splicing is regulated by a multipartite ESE composed of at least three functional motifs as follows: a potential 5’ SC35-like binding element, a central purine-rich enhancer (PPE), and a 3’ ACE-like element. In addition, results from deletions E3 and E4 show that the sequence between the SC35-like element and the PPE element is critical for splicing regulation and is a potential inhibitory element. Deletions in each ESE motif reduce E10 inclusion indicating that all three ESE motifs are required for the level of E10 inclusion observed for normal tau. Thus, these individual ESE components are not functionally redundant. Whereas only the PPE element appears to be absolutely required for E10 splicing as shown by results from the E5S/E6 deletion, correct regulation of E10 splicing requires that all elements be present for exon definition. Double mutation experiments show that when the PPE is silenced by the E5S deletion, either other enhancer sequences must be strengthened or inhibitory sequences weakened before E10 can be recognized. Although in the present work, the function of ESE was defined in COS-7 cells, this ESE clearly functions in vivo because the N279K mutation in this ESE results in an increase of 4R tau (19). This increase in the 4R/3R ratio at the protein level is consistent with this mutation causing increased E10 inclusion and resulting FTDP-17. Also, two other mutations in the ESE (Δ280K and L284L) cause FTDP-17.

The PPE element has a core requirement for GAR or possibly AAG repeats. The mechanism by which the FTDP-17 mutation N279K (15T→G) causes increased E10 inclusion is somewhat difficult to define because this mutation is at the border between the PPE and a potential inhibitory element defined by
deletions EΔ3 and EΔ4. This mutation appears to act by extending the purine tract of the PRE as either an A or G at position 15 increases E10 inclusion. The G at position 15 is clearly more effective than an A as seen when either are assayed alone or in combination with the EΔ5 deletion (Fig. 2). The normal T at position 15 may negatively modulate the activity of PPE. Others (23) have shown that a T within a purine-rich enhancer sequence can be inhibitory.

The splicing enhancing effects of the FTDP-17 mutation L284L were originally hypothesized to be due to disruption of an ISS with the core sequence of TTAG (15). This prediction was based on previous work (21) showing that this core sequence inhibits splicing of HIV tat exon 3. However, deletions EΔ9 and EΔ10 that span the TTAG sequence, rather than increasing E10 inclusion, do not alter or actually reduce E10 inclusion, respectively (Fig. 1). Deletions EΔ10 and EΔ11 reduce splicing, demonstrating that this region contains an enhancer element. Mutations introduced to alter each nucleotide of TTAG all increase E10 inclusion (Fig. 2). These results suggest that the TTAG inhibitory sequence is directly adjacent to an enhancer region (ACE, Fig. 1).

The multipartite ESE and the inhibitory sequences within E10 act in the context of a weak 5' splice site. When the 5'
Regulation of Tau Exon 10 Splicing

TABLE I
Stability of RNA secondary structures

| Construct | Secondary structure free energies (ΔG) for sequences of different lengths | Percent E10 retained |
|-----------|---------------------------------------------------------------|---------------------|
|           | 49 nt | 30 nt | 25 nt | 18 nt | Single mutation | Double mutation (+E5Δ) |
| hN        | −14.6 | −9.2  | −8.4  | −6.5  | 45              | 0                   |
| EΔ17      | −10.5 | −8.4  | −8.4  | −6.5  | 26              |                     |
| −4A−9     | −15.8 | −8.4  | −8.4  | −6.5  | 40              |                     |
| −5A−9     | −15.8 | −8.4  | −8.4  | −6.5  | 58              |                     |
| Glo−1     | −15.9 | −8.4  | −8.4  | −6.5  | 39              |                     |
| E10+12−4t| −12.4 | −7.8  | −6.2  | −4.3  | 92              | 59                  |
| E10+13−8t| −12.5 | −7.9  | −6.3  | −4.4  | 91              | 28                  |
| E10+14−8t| −12.5 | −6.7  | −6.3  | −4.4  | 93              | 65                  |
| E10+16−8t| −13.0 | −7.8  | −6.8  | −5.4  | 89              | 26                  |
| mWT       | −16.4 | −10.4 | −7.5  | −4.3  | 34              |                     |
| Δ9GΔNTP10Δ6| −15.1 | −10.0 | −8.4  | −6.5  | 14              |                     |
| Δ9GΔNTP10Δ| −15.1 | −10.0 | −8.4  | −6.5  | 60              |                     |

Splicing is strengthened either by mutations that bring the sequence closer to the U1/U6 snRNP hybridization consensus sequence (e.g. FTDP-17 mutations 92G→A) or when the splice site is replaced by a β-globin splice site known to give constitutive splicing (e.g. WTGlo), E10 inclusion is nearly complete. When a strong splice site is present, destruction of the ESE element that acts as a single-stranded sequence and binds a U1/U6 snRNP complex portends the stem-loop hypothesis is that substitutions in nucleotides closer to the U1/U6 snRNP hybridization consensus (32) and as predicted by free energy calculations (22). This result appears to argue against the stem-loop hypothesis although another interpretation is that E10+3 and 92G→A result in a strong splice site that over-rides the silencing effects of the stem-loop. As shown here, a strong 5’ splice site is dominant over other cis-acting elements that control E10 splicing. E10 mutations that disrupt the longer possible stem-loop structures (−4A−9, −5AΔ, E17, and the β-globin sequences used in Glo−1) do not significantly alter splicing. Thus the sequences immediately 5’ to the end of the exon do not participate in regulation, and the longer stem-loop structures are probably not involved in E10 regulation.

Evidence against the short 18-nucleotide stem-loop being a regulatory structure comes from splicing experiments with FTDP-17 mutations E10+12c→t, E10+13a→g, E10+14c→t, and E10+16c→t in combination with the EΔ5 mutation. Simply, these mutations increase splicing so that E10 is nearly constitutively included, which correlates with the expected destabilization of the stem-loop. However, when EΔ5 is present, splicing is dramatically variable, ranging from 65% for EΔ5/E10+14c→t to 26% for EΔ5/E10+16c→t, even though these I10 mutants would produce stem-loops with similar free energies (Table I). Thus, for these double mutants, splicing does not correlate with stem-loop stability for any of the structures described in Fig. 6 and Table I. Other less-direct evidence against the stem-loop hypothesis is that when mouse sequences are used either alone or in hybrid constructs with human sequences, splicing and predicted stem-loop free energies also do not correlate with E10 splicing levels (Fig. 6 and Table I). The conclusion from our work is consistent with an alternative possibility for E10 splicing regulation, where the linear sequence starting at I10 position 12 and extending at least to nucleotide 18 and perhaps farther is an ISS.

E10 splicing is regulated differently in mice and humans. Whereas no E10+ is produced in fetal brain of either species, in adult mouse brain only E10+ mRNA is generated, whereas in adult human brain approximately equal amounts of E10+ and E10− transcripts are made. The factors controlling the switching from fetal to adult pattern of regulation is unknown, as is the reason for the differences in regulation between mouse and human in adult brain. Hutton and co-workers (33) suggested that the difference in regulation between these two species is due to the fact that a stem-loop forms at the E10/I10 junction in human but not mouse pre-mRNA. The lack of a stem-loop in mouse would result in constitutive E10 inclusion in mouse brain. As shown in Fig. 6, the mouse sequence can form an E10/I10 junction stem-loop that is different from the human
structure and is less stable ($\Delta G = -4.3$ and $-6.5$ kcal, respectively, for the shortest structures). The lower stability of the mouse stem-loop would be consistent with more E10 inclusion in the adult mouse. However, when hN and mWT are compared in COS-7 cells, the mouse construct actually results in slightly lower levels of E10 inclusion (Fig. 7). Also, mouse I10 ISS sequences are more inhibitory than the human ISS sequence. This observation is not consistent with the E10/I10 junction being the mechanistic difference between mouse and human splicing regulation. However, the possibility remains that this ISS may act differently in adult neurons, compared with COS-7 cells. A more likely explanation is that the constructs used here lack a regulatory element(s) that controls the switch between fetal and adult patterns and the element(s) that controls E10+/E10− ratios in the adult brain. This interpretation is supported by the fact that when the human and mouse E10 constructs used here are assayed in rat fetal neuron cultures, approximately equal amounts of E10+ and E10− transcripts are made. In contrast, the endogenous rat tau gene almost exclusively produces E10− transcripts in the same cultured cells, suggesting that additional regulatory elements have been deleted in the abbreviated hN and mWT constructs.

The work described here shows that there are at least five different regulatory elements within tau E10 and an inhibitory element in the intronic sequences immediately downstream of this exon. Other work2 indicates that other intronic sequences also control E10 splicing. FTDP-17 mutations alter function of three of these elements (the PPE, ACE, and the I10 ISS), also control E10 splicing. FTDP-17 mutations alter function of this exon. Other work2 indicates that other intronic sequences are more inhibitory than the human ISS sequence.

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