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Complex Regulation of Tau Exon 10, Whose Missplicing Causes Frontotemporal Dementia

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Abstract: Tau is a microtubule-associated protein whose transcript undergoes complex regulated splicing in the mammalian nervous system. Exon 10 of the gene is an alternatively spliced cassette that is adult-specific and that codes for a microtubule binding domain. Recently, mutations that affect splicing of exon 10 have been shown to cause inherited frontotemporal dementia (FTDP). In this study, we establish the endogenous expression patterns of exon 10 in human tissue; by reconstituting naturally occurring FTDP mutants in the homologous context of exon 10, we show that the *cis* determinants of exon 10 splicing regulation include an exonic silencer within the exon, its 5′ splice site, and the relative affinities of its flanking exons to it. By cotransfections in vivo, we demonstrate that several splicing regulators affect the ratio of tau isoforms by inhibiting exon 10 inclusion. **Key Words:** Microtubule-associated protein tau—Expression pattern of regulated isoforms—Microtubule binding domain—Regulation of alternative splicing—Frontotemporal dementia.
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Alternative splicing is a versatile and widespread mechanism for generating multiple mRNAs from a single transcript (reviewed by Krämer, 1996; Manley and Tacke, 1996; Grabowski, 1998; López, 1998). Splicing choices are regulated by both tissue/cell type and developmental stage; the mRNAs arising from such processing produce functionally diverse protein isoforms.

Splicing occurs in two transesterification reactions, with the participation of the spliceosome, a large complex of proteins and small RNAs [small nuclear riboproteins (snRNPs)] (reviewed by Sharp, 1994; Reed, 1996). The snRNPs play an important role in exon definition and splice site hierarchies (Black, 1995; Reed, 1996). In addition to the invariant 5′ and 3′ splice sites, another splicing signal is the branch point, normally located 30–50 nucleotides upstream of the 3′ splice site. The branch point is the site of formation of the first splicing intermediate (known as the lariat because of its unusual chemical configuration), and it is usually followed by a polypyrimidine (polyY) tract (Smith and Nadal-Ginard, 1989; Reed, 1996).

A major unanswered question in the splicing field is what distinguishes a cryptic splicing site from an authentic one (reviewed by Black, 1995). Such a distinction is important because mammalian splice sites are loosely defined with respect to sequence and thus redundant in the genome. The exon definition model (in which the snRNPs are postulated to recognize exon boundaries by attachment at the 3′ splice site of an exon and scanning downstream for a 5′ splice site within a certain distance) has provided a partial explanation for splice site authentication (Berget, 1995). However, in alternative splicing, splice sites that adhere to the consensus sequences are either used or bypassed when the primary transcript is processed (Smith et al., 1989).

When alternatively spliced genes are expressed in inappropriate contexts, they are not spliced constitutively, but produce one of the possible mRNAs, called the default mode (Smith et al., 1989). This finding implies that many alternative splicing decisions rely partly or wholly on *cis* determinants, that is, they are intrinsically dictated by sequence location or identity and do not require specific *trans* regulators.

Numerous in-depth studies of *cis* determinants of alternative splicing have shown that exon behavior can be

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Abbreviations used: CMV, cytomegalovirus; FTDP, frontotemporal dementia with parkinsonism; FTDP-17, frontotemporal dementia with parkinsonism associated with chromosome 17; hnRNP protein, heterogeneous nuclear protein; NT2, N-Tera2 human teratocarcinoma cells; polyY, polypyrimidine; SDS, sodium dodecyl sulfate; SKN, SK-N-SH human neuroblastoma cells; snRNP, small nuclear riboprotein; SR proteins, serine/arginine-rich proteins; SSC, saline–sodium citrate.

dictated by exon length, agreement of the splicing sites with the consensus, location of the branch point, and length and composition of the associated polyY tract. Suboptimal splice sites and/or displaced branch points lead to regulation of splice site selection, apparently via intrinsic hierarchies defined by complementarity of splice sites and branch points to the snRNPs (reviewed by Manley and Tacke, 1996; Grabowski, 1998; López, 1998).

In an increasing number of systems, exon inclusion is achieved by use of purine-rich "enhancer" sequences within the regulated exons (reviewed by Black, 1995). However, full regulation in some cases is conferred by exonic silencers or distal intronic elements (Huh and Hynes, 1993, 1994; Amendt et al., 1995; Staffa and Cochrane, 1995; Del Gatto et al., 1996, 1997; Kawamoto, 1996; Staffa et al., 1997).

On the *trans* side of regulation, no mammalian splicing regulators have yet been found that are exclusively tissue-, stage-, or exon-specific, in marked contrast to transcription. Instead, mammalian splicing regulators mostly belong to two superfamilies, the serine/arginine-rich (SR) and heterogeneous nuclear (hnRNP) proteins, neither of which is exclusively involved in alternative splicing (Chabot, 1996; Manley and Tacke, 1996; Valcárcel and Green, 1996). The former are also components of the spliceosome, whereas the latter are also involved in pre-mRNA transport, RNA stability, and translational regulation.

Both SR and hnRNP proteins apparently function by binding to pre-mRNA regulatory sequences. Inasmuch as their mode of action can be generalized, SR proteins tend to promote exon inclusion and usually act on exonic enhancers or splice sites (Valcárcel and Green, 1996); conversely, hnRNP proteins tend to inhibit exon inclusion and may bind to sites in either exons or introns (reviewed by Chabot, 1996; Krämer, 1996). More recently, a few mammalian splicing factors have been isolated that show limited tissue specificity [KSRP, SWAP, and htra2 (Sarkissian et al., 1996; Min et al., 1997; Nayler et al., 1998; Stamm et al., 1999)]. Nevertheless, it appears that the exquisite calibration of mammalian alternative splicing is primarily achieved by distinct and regulated ratios of members of the SR and hnRNP superfamilies.

Tau is a microtubule-associated protein enriched in axons of mature and growing neurons (Binder et al., 1986; Kempf et al., 1996). Tau has also been found in the cell nucleus (Wang et al., 1993), in the distal ends of growing neurons (DiTella et al., 1994; Black et al., 1996), in oligodendrocytes (LoPresti et al., 1995), and in muscle (Wei and Andreadis, 1998). Hyperphosphorylated, microtubule-dissociated tau protein is the major component of neurofibrillary tangles, a hallmark of several neurodegenerative diseases (reviewed by Spillantini and Goedert, 1998).

Tau is encoded by a single copy gene (Neve et al., 1986; Himmler, 1989). Tau transcripts of 2, 6, and 9 kb are differentially expressed in the nervous system, depending on stage of neuronal maturation and neuron type (Drubin et al., 1988; Goedert et al., 1989*a*,*b*, 1992; Himmler et al., 1989; Couchie et al., 1992; Wang et al., 1993). The 2-kb tau mRNA is localized to the nucleus (Wang et al., 1993). The 9-kb tau transcript is restricted to the retina and PNS (reviewed by Nuñez and Fischer, 1997).

The tau transcripts undergo complex, regulated alternative splicing: Six of the 16 tau exons are regulated cassettes, and the gene uses two alternative polyadenylation sites, which correspond to the 2- and 6-kb tau mRNA (Lee et al., 1988; Goedert et al., 1989*a*,*b*, 1992; Himmler, 1989; Himmler et al., 1989; Kosik et al., 1989; Andreadis et al., 1992, 1995; Couchie et al., 1992; Sadot et al., 1994).

The N terminus of the tau protein is acidic and interacts with the plasma membrane (Brandt et al., 1995), although the specific molecules with which it interacts are not yet known. The C terminus of the tau protein is basic and contains four imperfect repeats (encoded by exons 9–12) that act as microtubule binding domains (Himmler et al., 1989; Lee et al., 1989).

Exon 10 is a cassette that codes for an additional microtubule binding domain. Its inclusion increases the affinity of tau for microtubules and the stability of the tau–microtubule interaction (Lee et al., 1989). Splicing of exon 10 is under developmental and cell type-specific regulation. The exon is adult-specific in both the rat and the human (Goedert et al., 1989*a*,*b*; Kosik et al., 1989) but with a crucial difference that becomes relevant in neurodegeneration: In adult rodents, exon 10 becomes constitutive (Kosik et al., 1989; A. Andreadis, unpublished data). In contrast, in adult humans exon 10 remains regulated in the CNS (Goedert et al., 1989*b*; present study). The difference most likely arises from the details of the *cis* sequences flanking exon 10 in various organisms (Grover et al., 1999), which in turn affect the regulation exerted by *trans* factors.

Recent findings have established that missplicing of tau exon 10 can cause inherited frontotemporal dementia with parkinsonism (FTDP) associated with chromosome 17 (FTDP-17), almost certainly by disturbing the normal tau isoform ratio (Clark et al., 1998; Hutton et al., 1998; Spillantini et al., 1998; Hasegawa et al., 1999). The FTDP pedigree mutations initially clustered at or near the 5′ splice site of exon 10, giving rise to the hypothesis that its splicing is partly modulated by a putative hairpin loop, whose formation inhibits interaction with the U1 snRNP (Hutton et al., 1998; Grover et al., 1999). However, other laboratories, including ours, have shown that exon 10 splicing is also affected by an exonic sequence that is identical to a silencer motif found in the HIV *tat* gene (D'Souza et al., 1999; present study).

In this report we present the detailed expression profile of exon 10 in human tissues and investigate the in vivo behavior of splicing constructs that contain this exon. Our study shows that exon 10 has a default phenotype of inclusion and that both its flanking exons are involved in its regulation. We demonstrate that the distal regions of

TABLE 1. *Primers used in PCR*

Primer name	Length	Orientation	Location	Sequence
For wild-type and mutant constructs				
HT10S450	26	Sense	471 nucleotides upstream of exon 10	GGCTTGAGAACAGCCGCAGGGAGTTC
HT10RS	25	Sense	117 nucleotides upstream of exon 10	GAATTCGAGCAAGTAGCGGGTCCAG
HT10RN	24	Antisense	90 nucleotides downstream of exon 10	GAATTCTATGCAGTGTCTCGCAAG
HT10N400	24	Antisense	408 nucleotides downstream of exon 10	AGTCTCAGGTGCCACCACCTTCAG
HT11S500	26	Sense	324 nucleotides upstream of exon 11	GTCTGGGTCTTGCACAATGACAATGG
HT11N	24	Antisense	Within exon 11	ATGTTGCCTAATGAGCCACACTTG
ENH10S[a]	26	Sense	Within exon 10	GCAGATAATTAAGAAGAAGCTGGATC
ENH10N[a]	26	Antisense	Within exon 10	Reverse complement of ENH10S
SIL10S[a]	27	Sense	Within exon 10	GAAGCTGGATCTGAGCAACGTCCAGTC
SIL10N[a]	27	Antisense	Within exon 10	Reverse complement of SIL10S
10M5S[a]	27	Sense	Overlapping the exon 10 5′ splice site	CCGGGAGGCGGCAATGTGAGTACCTTC
10M5N[a]	27	Antisense	Overlapping the exon 10 5′ splice site	Reverse complement of 10M5S
10M14S[a]	35	Sense	In the proximal 3′ intron of exon 10	GGCAGTGTGAGTACCTTCATACGTCCCATGCGCCG
10M14N[a]	35	Antisense	In the proximal 3′ intron of exon 10	Reverse complement of 10M14S
10M16S[a]	35	Sense	In the proximal 3′ intron of exon 10	GGCAGTGTGAGTACCTTCACATGTCCCATGCGCCG
10M16N[a]	35	Antisense	In the proximal 3′ intron of exon 10	Reverse complement of 10M16S
10C16S[a]	35	Sense	In the proximal 3′ intron of exon 10	GGCAATGTGAGTACCTTCACATGTCCCATGCGCCG
10C16N[a]	35	Antisense	In the proximal 3′ intron of exon 10	Reverse complement of 10C16S
For RT-PCR				
HT9SmS	24	Sense	Within tau exon 9	GGGAGCCCAAGAAGGTGGCAGTGG
1017HT	30	Antisense	Within tau exon 13	TAGCGTGGCGAGCTGGGGCGAGTCTACCAT
INS1	30	Sense	End of insulin exon 1	CAGCTACAGTCGGAAACCATCAGCAAGCAG
INS3	30	Antisense	Within insulin exon 3	CACCTCCAGTGCCAAGGTCTGAAGGTCACC

[a] The point mutations in these are shown in Fig. 4A.

the introns that flank exon 10 do not appear to contain any splicing information, whereas alterations within the exon and in the proximal region of its downstream intron alter its splicing regardless of the identity of the flanking exons. Lastly, we show that several known splicing regulators also affect the ratio of exon 10 isoforms in vivo.

MATERIALS AND METHODS

Plasmid construction and mutagenesis

The human tau genomic fragments originated from cosmid or λ clones (Andreadis et al., 1992). The parental vector used for cloning was pSVIRB (Andreadis et al., 1993, 1995; Wei and Andreadis, 1998). pSVIRB contains an additional intron between fused SV40/insulin exon 1 and insulin exon 2 that is not shown in the figures but that served as an internal splicing control.

Tau chimeric constructs were produced by standard cloning methodology or by PCR, insertion into pKS(+) Bluescribe (Stratagene), and subsequent cloning by directional ligation (Sambrook et al., 1989). The most frequently used primers for PCR are listed and shown in Table 1. All inserts generated by PCR were sequenced, to ensure absence of mutations.

The following minigene constructs were generated and are diagramatically shown in Figs. 2A and 3A.

SV10 contains tau exon 10 and the proximal portions of its flanking introns (117 bp upstream and 90 bp downstream) inserted into SVIRB. SV9/10 has the 3′ 161 nucleotides of tau exon 9 and ~1.5 kbp of its downstream intron fused to insulin exon 2. SV10/11 has the 5′ 68 nucleotides of tau exon 11 and 324 nucleotides of its upstream intron fused to insulin exon 3. SV9/10/11 contains both the exon 9 and 11 fusions. All three are identical to SV10 with respect to the exon 10 insert.

The SV10L series constructs (SV10L, SV9/10L, SV10L/11, and SV9/10L/11) are identical to their SV10 homologues except that they contain longer portions of the introns flanking exon 10 (471 bp upstream and 408 bp downstream).

To generate the mutant constructs, whole-plasmid mutagenesis was performed on SV10 or SV9/10L/11 using the QuikChange Site Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions except that the PCR products were digested with *Dpn*I overnight. The resulting plasmids were sequenced to verify the presence of the desired mutations and the absence of undesirable ones. The primers

used for mutagenesis are listed in Table 1, and the alterations to wild-type exon 10 are shown in Fig. 4A.

The cDNA of putative splicing regulator Nova (Buckanovich and Darnell, 1997) was isolated from an adult human brain cDNA 5′-rapid amplification of cDNA ends-ready library (Clontech), placed into vector pCRII (InVitrogen), sequenced, and subsequently placed into expression vector pCI-neo under the control of the cytomegalovirus (CMV) promoter. Production of the Nova protein was confirmed by the coupled transcription–translation reticulocyte system (Promega). Eukaryotic expression vectors bearing other splicing regulator cDNAs expressed them from the following promoters: CMV [hnRNPA1, U2AF65, htra2, and SR proteins 20, ASF/SF2, 30c, SC35, 40, 55, and 75 (Yang et al., 1994; Screaton et al., 1995; Cáceres et al., 1997; Nayler et al., 1998)], adenovirus major late [PTB (Patton et al., 1991)], and SV40 early [SWAP (Lemaire et al., 1999)].

Cell culture, transfections, and RNA preparation

COS (monkey kidney) and N-Tera2 cells (human teratocarcinoma, henceforth called NT2 cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. SK-N-SH cells (human neuroblastoma, henceforth called SKN cells) were maintained in Eagle's minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum.

Plasmid DNA was purified by cesium chloride banding (Sambrook et al., 1989). Plasmids were introduced into cells by the lipofection method (LT1; Panvera).

Plates (100 mm in diameter) that had reached ~30% confluence were transfected with 10 μg of construct DNA or 5 μg of each plasmid for cotransfections. The medium was changed 16 h after transfection, without glycerol shock. Total RNA was isolated 48 h posttransfection by the TRIZOL method (Life Technologies).

RT and PCR procedures

RT and PCR procedures were performed with (a) human poly(A)$^+$ RNA (Clontech) to establish the expression profile of endogenous tau exons and (b) total RNA from transfected cells to examine the behavior of the splicing constructs.

For PCR analysis of RNA, 5 μg of total RNA from transiently transfected cells or 1 μg of poly(A)$^+$ RNA was reverse-transcribed using random hexamer primers and 200 units of reverse transcriptase (RNase H$^-$ Superscript; Life Technologies), in a total volume of 20 μl for 1 h at 42°C. Part of this reaction mix (5 μl) was then diluted to a final volume of 50 μl, the concentrations of the buffer and deoxynucleotide triphosphates were adjusted for PCR, and the mixture was amplified for 22 cycles. The conditions were as follows: for the endogenous gene, denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min; for the constructs, denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min.

The primer pair used for the endogenous exon 10 pattern was HT9SmS/1017HT (Table 1). In the case of transfected cells, the primers (INS1 and INS3; Table 1) were chosen to amplify only products arising from the constructs.

The RT-PCR experiments were done twice with the poly(A)$^+$ RNAs and with total RNAs from three independent transfections, to ensure reproducibility. The RT-PCR product of SV10 whose length differed in size from that of spliced vector pSVIRB was cloned and sequenced. For the data in Figs. 2–5, the percentage of the 10$^+$ isoform was calculated by scanning the bands from three independent transfections and measuring the areas under the curves (by the IPLab Gel program from Scanalytics).

For the expression constructs, the predicted sizes of the 10$^-$/10$^+$ RT-PCR products are as follows, in nucleotides: 295/388 for SV10 and SV10L; 288/381 for SV9/10 and SV9/10L; 343/436 for SV10/11 and SV10/11L; and 336/429 for SV9/10/11 and SV9/10L/11. Thus, the first four constructs give rise to products of almost identical length, as do the last four.

Blotting and hybridization

RT-PCR products from human poly(A)$^+$ RNAs were electrophoresed on a 6% acrylamide/1× TBE (Tris/borate/EDTA) gel. The gel was denatured in 1.5 *M* NaCl/0.5 *M* NaOH for 30 min, renatured in 1.5 *M* NaCl/0.5 *M* Tris (pH 7.2) for 30 min, electroblotted in the presence of 10× saline–sodium citrate (SSC) on Nytran membranes (pore size, 0.20 μm; Schleicher and Schuell), and baked for 60 min at 80°C.

The membrane was prehybridized for at least 3 h at 42°C in a mixture of 50% deionized formamide, 5× SSC, 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml heat-denatured herring sperm DNA. To visualize the products, a probe was used that contained tau exon 9 and its proximal downstream intron (identical to the fragment used in the SV9 construct series).

The probe was labeled using the Amersham random priming kit and [α-^{32}P]dCTP (catalogue no. PB.10205; Amersham). Hybridization was carried out overnight (>17 h) at 42°C in the same solution to which the heat-denatured probe (~5 × 10^6 cpm/ml) was added. Following overnight hybridization, the membrane was washed (15 min at room temperature in 2× SSC/0.1% SDS, 15 min at 52°C in 0.1× SSC/0.1% SDS, and 15 min at 65°C in 0.1× SSC/0.1% SDS), wrapped in Saran wrap, and exposed against Kodak X-Omat film at −70°C in cassettes with intensifying screens.

RT-PCR products from transfected constructs were electrophoresed on 3% NuSieve agarose (FMC Bioproducts)/1× TAE gels.

Cloning and sequencing of PCR products and mutant constructs

Construct intermediates generated by PCR and the primary RT-PCR procedure of SV10 were denatured for 2 min at 90°C in "forward heat" buffer (Sambrook et al., 1989), phosphorylated in the presence of kinase "forward" buffer, 100 m*M* ATP, and 10 units of T4 polynucleotide kinase (New England Biolabs), electrophoresed on a low-melt agarose gel, extracted from the agarose by treatment with β-agarase (New England Biolabs), and ligated into pKS(+) (Stratagene) or pCRII (InVitrogen).

Plasmid DNA was prepared from 5 ml of saturated cultures using Qiagen Tip-20's and then sequenced by the modified dideoxynucleotide method (Sanger et al., 1977; Biggin et al., 1983) with Sequenase version 2.0 (U.S. Biochemicals) and [α-^{35}S]dATPαS (catalogue no. SJ.1304; Amersham). The sequencing ladders were resolved on denaturing (8.3 *M* urea) 6% polyacrylamide gels and analyzed using the GCG software (Devereux et al., 1984).

RESULTS

Expression patterns of exon 10 are very similar to those of exon 2

We previously analyzed the expression of tau exons 2, 3, and 6 (Wei and Andreadis, 1998). To begin investigating the regulation of tau exon 10 splicing, it was

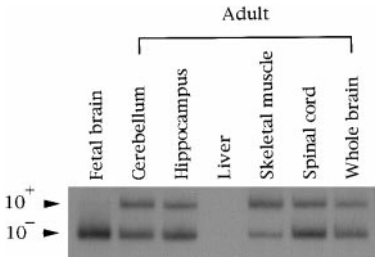

FIG. 1. Expression profile of human tau exon 10 in human fetal brain and various adult human tissues. The RT-PCR products are from poly(A)$^+$ RNAs of the tissues indicated. The primer pair used was 9SmS/1017HT.

important to establish its endogenous expression profile because comparisons between expression profiles often give clues to underlying mechanisms.

RT-PCR was done on poly(A)$^+$ RNA from fetal human brain and various adult human tissues that are known to express tau (cerebellum, hippocampus, skeletal muscle, spinal cord, and whole brain; liver served as a negative control; all RNAs came from Clontech and consisted of pools from nine to 95 individuals). The PCR cycles were kept low to give a true representation of the relative amounts of the various spliced products.

Exon 10 is absent from fetal brain but present in various ratios in all other tissues except liver (Fig. 1). Cerebellum and whole brain show essentially identical patterns of roughly 1:1 10^+ to 10^-, whereas hippocampus shows slightly more of the 10^- isoform. Skeletal muscle and spinal cord show complementary patterns: The former favors 10^+, whereas the latter favors 10^-.

This expression profile is remarkably similar to that of tau exon 2 (Wei and Andreadis, 1998). Both exons are adult-specific (Goedert et al., 1989*a*,*b*; Kosik et al., 1989) and are also constitutively included in the 9-kb tau mRNA (Couchie et al., 1992; Goedert et al., 1992).

The default pattern of exon 10 is inclusion, independent of the length of the flanking introns

In nonneuronal COS cells, exon 10 is almost entirely included if flanked by insulin exons (Fig. 2B, two left lanes). This remains unaltered whether the construct contains the short (SV10) or long (SV10L) version of the insert, strongly suggesting that any intronic splicing determinants of exon 10 are near the exon itself.

The behavior of exon 10 shifts slightly in SKN and NT2 cells, reflecting their respective endogenous exon 10 pattern, which is 10^- major, 10^+ minor for SKN cells and exclusively 10^- for NT2 cells (A.A., unpublished data). In SKN cells there is a discernible amount of 10^-, although the species is decidedly minor compared with 10^+ (Fig. 2B, two middle lanes). In NT2 cells the 10^- isoform becomes dominant for SV10L (Fig. 2B, two right lanes). Thus, although the exon 10 *cis* determinants are largely local, regulation of the default splicing clearly depends on cell-specific *trans* factors. Such factors may differ between SKN and NT2 cells, given the distinct provenance of the two lines (PNS and CNS, respectively).

Both flanking exons are involved in regulation of exon 10 splicing

Because the default behavior of exon 10 in our hands differs from that of the endogenous exon and from results of exon trapping assays (Clark et al., 1998; Hutton et al., 1998; D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999), we wanted to investigate the influence of flanking exons on regulation of exon 10. Therefore, we tested the behavior of constructs that contained exon 10 and either or both of its native flanking exons, 9 and 11.

Both exons 9 and 11 shift the relative ratios of the various exon 10 splicing products (Fig. 3). The effect of exon 9 is most obvious in the SKN and NT2 cells, which produce considerable amounts of the 10^- isoform if exon 10 is flanked by heterologous exons; conversely, the effect of exon 11 is most obvious in the COS cells, which produce 10^+ exclusively if exon 10 is flanked by heterologous exons (as seen in Fig. 2B).

Inclusion of prefused exon 9 resulted in exclusive production of the 10^+ isoform in all three cell types independently of whether the constructs contained the short or long version of exon 10 (SV10 or 10L vs. SV9/10 or 9/10L; Fig. 3B, leftmost four lanes in all three panels). Conversely, inclusion of prefused exon 11 resulted in a ratio of $10^+/10^-$ of roughly 1:1 in SKN and NT2 cells (construct SV10 or 10L vs. SV10/11 or 10L/11; Fig. 3B, two leftmost lanes vs. fifth and sixth lanes from the left in the two bottom panels). Inclusion of both prefused exons resulted in a relative ratio of the 10^+

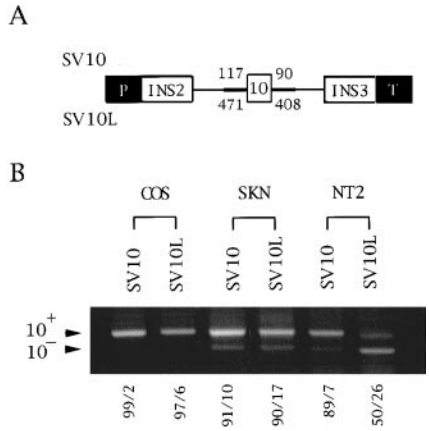

FIG. 2. The default pattern of exon 10 is inclusion, but its expression is modulated by the cellular environment. **A:** Schematic representation of expression constructs SV10 and SV10L. P represents the SV40 early promoter; T represents the insulin terminator. The small intron between the insulin 1 and 2 exons is not shown for the sake of clarity. The numbers on each side of tau exon 10 show how many nucleotides of its flanking introns are present. **B:** RT-PCR of the expression constructs in nonneuronal (COS), neuroblastoma (SKN), and teratocarcinoma (NT2) cells. The primer pair used was INS1/INS3. The numbers underneath each construct indicate the percentage of 10^+ isoform (left side of slash; averaged from three independent transfections, with the decimals rounded off) and the value range (right side of slash).

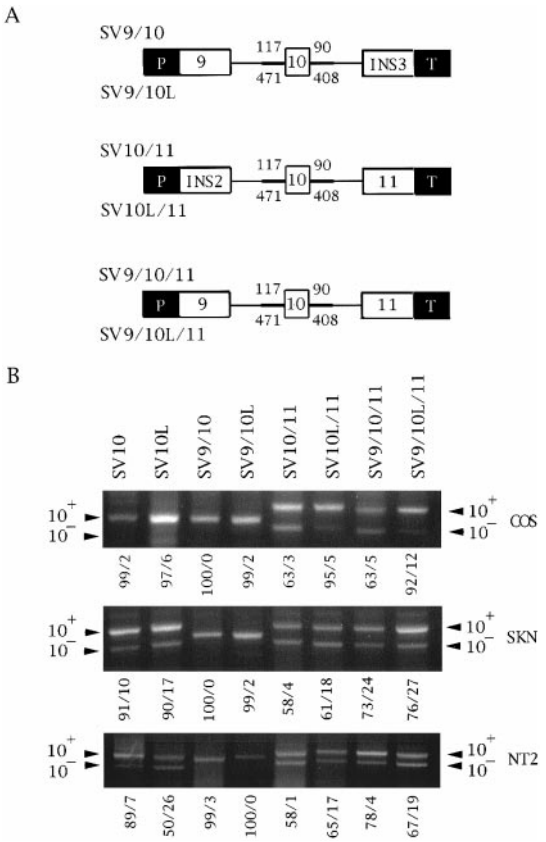

FIG. 3. The flanking exons of tau exon 10 are involved in its splicing regulation. **A:** Schematic representation of expression constructs that contain the short and long version of exon 10 flanked by various combinations of homologous and heterologous exons. The fusions between insulin 2 and exon 9 or tau exon 11 and insulin 3 are not shown for the sake of clarity. Conventions for the constructs are as in Fig. 2A. **B:** RT-PCR of the expression constructs in nonneuronal (COS; **top panel**) neuroblastoma (SKN; **middle panel**), and teratocarcinoma (NT2; **bottom panel**) cells. The predicted sizes of the $10^-/10^+$ RT-PCR products are as follows: 295/388 for SV10 and SV10L; 288/381 for SV9/10 and SV9/10L; 343/436 for SV10/11 and SV10/11L; and 336/429 for SV9/10/11 and SV9/10L/11. Thus, the products of the first four constructs are essentially identical in size, as are the products of the last four. The primer pair used was INS1/INS3. The percentage of 10^+ isoform is tabulated as in Fig. 2B.

product less than that seen in the SV10 or SV9/10 constructs but more than that seen in the SV10/11 constructs (Fig. 3B, two rightmost vs. the other six lanes in all three panels).

There is an interesting difference in the behavior of the SV9/10L/11 construct versus its SV10 counterpart: In COS and SKN cells, the 10L construct shifts the isoform ratio in favor of 10^+ (Fig. 3B, two rightmost lanes in the two top panels); in NT2 cells, the reverse occurs—namely, the 10L construct shifts the ratio toward 10^- (Fig. 3B, four rightmost lanes in the bottom panel). This suggests that distal regions of the introns that flank exon 10 may exert a secondary effect on splicing by binding cell-specific *trans* factors.

Splicing of exon 10 is modulated by its 5′ splice region and a silencer element within the exon

Several colleagues have investigated the splicing behavior of exon 10 mutations in exon trapping assays that use heterologous exons (D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999). We decided to extend their observations by examining the behavior of selected mutations in the 9/10L/11 background—that is, in the presence of large fragments of the introns flanking exon 10 and of homologous flanking exons.

Thus, we recreated several mutations (Fig. 4A) within the exon [ENH, the N279K mutation that creates a purine-rich enhancer; SIL, the L284L silent mutation that destroys a *tat*-like silencer; and M5, the S305N mutation that creates a 5′ splice site capable of much better binding to the U1 snRNP than the wild-type (Clark et al., 1998; D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999)] and within the proximal 3′ intron [M14 and M16, intron positions +14 and +16, respectively (Hutton et al., 1998)] as well as the "compensatory" mutation to the +16 mutant (C16), which would restore the structure of the putative hairpin loop structure forming around the 5′ splice site of exon 10 (Grover et al., 1999).

In the context of the 9/10L/11 construct, all the mutants shifted splicing exclusively to the 10^+ isoform (Fig. 4B), consistent with the previous findings (Hutton et al., 1998; D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999). The SIL and M5 mutants indicate that both the silencer motif and the 5′ splice site of exon 10 are involved in its regulation. The M14, M16, and C16

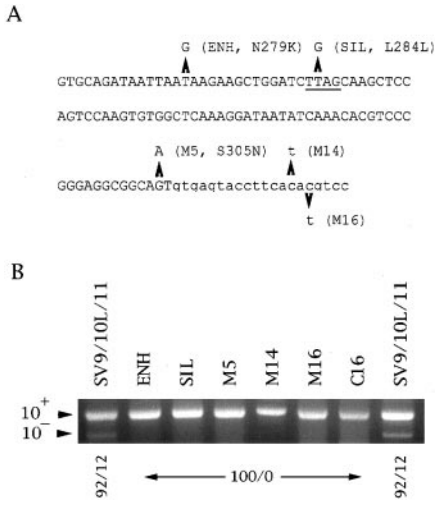

FIG. 4. Splicing of exon 10 is affected by exonic elements and by its 5′ splice site and proximal intron region. The exon sequence is in uppercase; the intron is in lowercase. **A:** Sequence of exon 10 and its proximal downstream intron. Point mutations are indicated, as well as their resulting missense mutations, if any. The underlined sequence is the motif homologous to the *tat* silencer. **B:** RT-PCR of the mutant constructs in nonneuronal (COS) cells. The primer pair used was INS1/INS3. Mutant C16 is the double mutant combining M5 and M16. The percentage of 10^+ isoform is tabulated as in Fig. 2B.

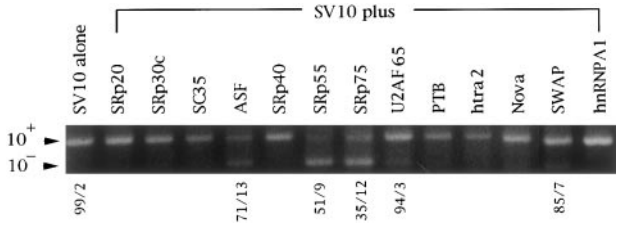

FIG. 5. Several splicing regulators affect the expression of exon 10. The RT-PCR products are from 1:1 cotransfections of SV10 with the factors indicated in COS cells. The primer pair used was INS1/INS3. The percentage of 10^+ isoform is tabulated as in Fig. 2B; numbers are shown only for regulators that alter exon 10 splicing.

mutants additionally point to the involvement of the proximal 3′ intronic region in regulation of exon 10.

The compensatory mutation C16 did not restore exon 10 splicing to its original ratio (Fig. 4B, second lane from the right). There are three possible explanations for this behavior: (a) This mutation restores a base pair to the putative loop, but it also generates the S305N mutant, which by itself affects exon 10 splicing. (b) The resulting A-T base pair cannot restore the original stability of the hairpin loop, being weaker than its G-C wild-type counterpart. (c) The region binds one or more factors without ever forming such a loop, in which case C16 does not act as a compensatory mutation, but as a primary one that weakens the binding of the factor(s).

Several splicing regulators act as inhibitors of exon 10 inclusion

Both the developmental profile (Fig. 1) and the default behavior (Fig. 2) of tau exon 10 point almost conclusively to regulation by at least one inhibitor. To narrow down possible candidates, we did cotransfection experiments with exon 10 constructs and known splicing regulators.

To uncover inhibition, we examined the effect of splicing regulators on SV10, which is constitutively included in COS cells (Fig. 2). Our results establish that SV10 is affected by the constitutive factors ASF/SF2, SRp55, SRp75, and U2AF65 and the specific regulator SWAP (Fig. 5). Each of these shifts the ratio from the exclusive 10^+ phenotype to varying degrees of inclusion of the 10^- isoform. These shifts are also seen in SV9/10L/11 (data not shown), although they are less dramatic because this construct expresses a fair amount of 10^- isoform (Figs. 3B and 4B).

DISCUSSION

Expression profile of tau exon 10: implications for developmental regulation

Tau exon 10 clearly has an expression pattern similar to that of tau exon 2 and very different from that of tau exons 3 and 6 (Wei and Andreadis, 1998; Fig. 1, present study). On the other hand, previous studies of exons 2, 3, and 10 have shown that in human adult tau they occur in all possible six combinations (Goedert et al., 1989*a*). Therefore, the various regulated tau exons are uncoupled both spatially and temporally in terms of both splicing regulation and domain function.

The fact that exon 10 shows a default splicing pattern of inclusion (Fig. 2) lends strong if indirect support to the theory that it is primarily modulated by an inhibitor. This hypothesis is additionally strengthened by the behavior of naturally occurring mutations, which invariably favor its inclusion (Clark et al., 1998; Hutton et al., 1998; Spillantini et al., 1998; D'Souza et al., 1999; Hasegawa et al., 1999; Fig. 4, present study).

The tau constructs show higher exon 10 inclusion in cell lines compared with both the endogenous pattern and results of other laboratories (Clark et al., 1998; Hutton et al., 1998; D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999). The difference from the endogenous gene could be due to the fact that the inhibitory factor(s) are present in rate-limiting amounts and hence cannot adequately suppress inclusion of exon 10 from the transfected gene.

The difference from results of other laboratories is almost certainly due to the details of the constructs; the other researchers have used vector pSPL3, whose tat splice sites have been shown to discriminate against cassette exons (Andreadis et al., 1993). Our work uses either the insulin exons of pSVIRB or the homologous tau exons 9 and 11. In fact, these particular shifts in exon 10 behavior confirm the involvement of flanking exons in its splicing regulation.

Exon 10 splice sites and FTDP-17 mutations

The default inclusion of exon 10 is surprising because neither of the exon's splice sites conforms to the respective consensus sequence (Andreadis et al., 1992): The 3′ splice site is aag/G compared with the canonical yag/G (y = pyrimidine); even weaker is the 5′ splice site, AGT/gtgagt—only five of nine nucleotides agree with the canonical CAG/gtaagt (Andreadis et al., 1992). Such deviation from the consensus has been seen in other neuron-specific exons (Stamm et al., 1994).

The hierarchy of 3′ splice site strengths is CAG∼TAG>AAG>>GAG in in vivo and in vitro experiments (reviewed by Reed, 1996). Nevertheless, this does not appear to result in weak splicing of exon 10. On the other hand, there is no doubt that the 5′ splice site of exon 10 is involved in its regulation, as witnessed by the shift toward the 10^+ isoform seen both in FTDP pedigrees with mutations in that region and in constructs containing these mutations (D'Souza et al., 1999; Grover et al., 1999; Hasegawa et al., 1999; Fig. 4, present study). Regulation via the 5′ splice site is far less common than via the branch point/3′ splice site, with the most prominent example being exon 18 of N-CAM (first studied by Tacke and Goridis, 1991).

It has been suggested that the 5′ splice site of exon 10 can be sequestered into a hairpin loop that hinders interaction with the U1 snRNP (Clark et al., 1998; Grover et al., 1999; Hutton et al., 1998). This theory is difficult

to distinguish from the alternative possibility of a factor binding to this region because all mutations that weaken the putative loop also increase complementarity to the 5′ end of U1.

Unlike human tau exon 10, its rodent counterpart becomes constitutive in adult rats and mice (Kosik et al., 1989; A. Andreadis, unpublished data). In this connection, it is interesting that the 5′ splice site of exon 10 is not conserved among the human and the mouse (Grover et al., 1999), pointing to at least one region where regulation of this exon differs among species. This crucial difference in the behavior of exon 10 in rodents may well correlate with the repeated failure to find neurofibrillary tangles in aged mice or rats as well as the difficulty of inducing them artificially in transgenic models.

Role of exonic silencers and enhancers

Exon 10 contains a region (Fig. 4A) that, based on the behavior of mutant ENH (D'Souza et al., 1999; Fig. 4B, present study), is a latent enhancer. That is, a single nucleotide change turns this region into a purine-rich sequence, simultaneously increasing inclusion of exon 10. Such regions are known to bind SR proteins that activate splicing of otherwise weak exons (reviewed by Black, 1995). The exon also contains a motif that by both sequence inspection (Fig. 4A) and the behavior of mutant SIL (D'Souza et al., 1999; Fig. 4B, present study) is an active silencer. This is another indication that one of the primary *trans* determinants of exon 10 splicing must be an inhibitor.

In contrast to the lack of conservation of the 5′ splice site of exon 10 between the human and the rodent, the silencer motif is conserved—as is indeed most of the exon, showing a divergence of only two of 93 nucleotides (Couchie et al., 1992; Goedert et al., 1992). Thus, the inhibitory factor that dictates exclusion of exon 10 in fetal tissues may well bind to this element.

Exonic silencers are much rarer and less well understood than exonic enhancers. The motif found within exon 10 has been shown to regulate splicing of the HIV *tat* gene in concert with an enhancer (Amendt et al., 1995; Staffa and Cochrane, 1995). This silencer seems to block early spliceosome assembly (Si et al., 1998). In this respect, it is interesting that a latent enhancer is just upstream of the silencer motif in tau exon 10 (Clark et al., 1998; D'Souza et al., 1999; Fig. 4A, present study).

Exonic silencers have also been found to affect the splicing of the EDA fibronectin exon (Caputi et al., 1994; Staffa et al., 1997) and the SAM exon of fibroblast growth factor 2 (Del Gatto et al., 1996, 1997). In the latter case, the silencer seems to exert its effect by recruiting hnRNPA1 (Del Gatto-Konczak et al., 1999). However, hnRNPA1 does not influence splicing of tau exon 10 (Fig. 5).

Role of flanking sequences

Flanking exons do not play a role in most alternative splicing systems. Prominent exceptions are fibronectin exon EIIIB (Huh and Hynes, 1993, 1994), the mutually exclusive exons in myosin light chain 1/3 (Gallego and Nadal-Ginard, 1990) and β-tropomyosin (Guo and Helfman, 1993), and tau exon 6 (Wei and Andreadis, 1998).

Tau exon 10 also falls in this small category. The behavior of the constructs in Fig. 3 indicates that the two flanking exons play opposite roles with respect to the inclusion of exon 10: Exon 9 promotes exon 10 splicing, whereas exon 11 suppresses it.

In this context, it is interesting that exon 9 also has a relatively weak 5′ splice site [six of nine nucleotides in agreement with the consensus (Andreadis et al., 1992)], whereas exon 11 has a perfect 3′ splice site [four of four (Andreadis et al., 1992)]. Thus, splicing in this region may also be partly regulated by "weighing" of splice site strengths.

For cassette exons, intronic regulatory elements tend to be relatively local. In the c-*src* N1 exon, the regulatory regions are within 60 nucleotides of the exon (Chan and Black, 1995). Exons EIIIB of fibronectin and K-SAM of the fibroblast growth factor receptor 2 are unusual in having regulatory elements located 520 and ~1,000 nucleotides away, respectively (Huh and Hynes, 1993, 1994; Del Gatto et al., 1997). Proximity of *cis* splicing regulatory elements also seems to be the case with exon 10 because the constructs in the 10 and 10L series behave similarly to a first approximation (Figs. 2 and 3). However, the 10 and 10L series constructs differ subtly depending on cellular context; therefore, it is possible that distal elements may be involved in fine-tuning the final isoform ratio.

Inhibitors of exon 10 splicing

There can be little doubt that at least one, and possibly two, inhibitory regulators modulate splicing of exon 10. As discussed previously, one may act on the *tat*-like silencer during fetal development; the other may act on the 5′ splice site during adulthood. Until now, no tissue- or stage-specific mammalian splicing factors have been isolated, although KSRP (Min et al., 1997), SWAP (Denhez and Lafyatis, 1994), and htra2-β (Nayler et al., 1998) show preferential tissue/cell type expression.

In our search for regulators that inhibit exon 10 splicing, we found that the following molecules suppress the inclusion of this exon: the SR proteins ASF/SF2, SRp55, and SRp75; the U2AF65 subunit, which acts in association with the U2 snRNP and interacts with the branch point during the very first step of splicing (Lin and Patton, 1995; Valcárcel et al., 1996); and SWAP (Fig. 5).

Oddly enough, two factors that usually act as splicing inhibitors, hnRNPA1 (reviewed by Chabot, 1996) and PTB (reviewed by Valcárcel and Gebauer, 1997), do not affect splicing of exon 10. Because the proximal upstream intron of exon 10 (branch point/polyY tract) has not been implicated in its regulation so far, the neutrality of PTB is understandable, although the effect of U2AF65 is correspondingly unexpected. Conversely, the neutrality of hnRNPA1 is puzzling because this protein is invariably involved in cases of regulation via 5′ splice

site selection, a mode indisputably operating on exon 10 (Mayeda et al., 1993; Chabot et al., 1997).

Of the SR proteins that affect exon 10 splicing, SRp75 is the most interesting because it is highly enriched in brain (Zahler et al., 1993). The scientific literature on the actions of SR proteins is vast and growing fast—especially in the crucially important field of neuron-specific alternative splicing (reviewed by Grabowski, 1998); however, there is no hard and fast rule at this point that would help predict the effect of an SR protein on any alternatively spliced exon. Binding sites for SR proteins are redundant in sequence and invariably bind more than one member of the superfamily (Liu et al., 1998; reviewed by Valcárcel and Green, 1996). Thus, details of how these proteins act on exon 10 will require further studies.

The most interesting of the molecules that affect exon 10 splicing is SWAP: It suppresses splicing of at least two other exons besides exon 10—fibronectin IICS and exon 4 of CD45 (Sarkissian et al., 1996; Lemaire et al., 1999). Furthermore, it is most highly expressed in brain and placenta (Denhez and Lafyatis, 1994). Thus, its temporal and spatial distribution and its effect make it a strong candidate as one of the putative inhibitors of exon 10.

Tau exon 10 and frontotemporal dementia

The recent findings have firmly placed tau and, specifically, its splicing directly upstream of the process that causes dementia. This makes tau one of only two genes that are known to cause disease if their alternative splicing is disturbed, even while they produce wild-type proteins. The other is the Wilms tumor gene, in which incorrect isoform ratios result in Frasier syndrome, characterized by gonadal dysgenesis and early renal failure (Barbeaux et al., 1997; Klamt et al., 1998). Coincidentally, the Frasier syndrome mutations also cluster on the 5′ splice site of a cassette exon.

Several experts believe that FTDP-17 is the second most common dementia after Alzheimer's (Wilhelmsen, 1998). Although FTDP-17 shows such clinical variability that it has often been misdiagnosed (Pasquier and Petit, 1997; Wilhelmsen, 1998), its molecular causes are remarkably uniform: In the afflicted pedigrees analyzed thus far it predominantly shows mutations in tau exon 10, although a few pedigrees carry mutations in tau exons 9, 12, and 13 (G272V, V337M, and R406W, respectively) that influence either microtubule binding or protein conformation (Hasegawa et al., 1998; Poorkaj et al., 1998).

The exon 10 mutations fall in two categories—those that influence microtubule bindings, such as P301L (Hasegawa et al., 1998), and those that alter the ratio of exon 10 isoforms (all the ones discussed in this work; Clark et al., 1998; Hutton et al., 1998; Spillantini et al., 1998; Hasegawa et al., 1999). In this connection, it is interesting that the FTDP-17 neurofibrillary tangles in pedigrees that show exon 10 missplicing differ in their detailed morphology from those found in missense pedigrees (Spillantini and Goedert, 1998).

It is intriguing that this disturbance in the relative isoform abundance should result in tangle formation and hence neuronal death. Given that the inclusion of exon 10 gives rise to four-repeat tau with its increased affinity for microtubules, perhaps the overabundance of this isoform leads to microtubule networks that are too rigid and incapable of rapid adjustments. What the FTDP results do show is that tangles are sufficient to initiate the cascade of events leading to neurodegeneration.

Thus, continued work on the basic molecular biology of the tau molecule may give us the tools to combat not only FTDP, but also other types of dementia (for example, Alzheimer's disease, Pick's disease, and progressive supranuclear palsy). These diseases vary widely in both clinical phenotype and brain pathology, but they share tangles as an invariable defining characteristic (Spillantini and Goedert, 1998).

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