

# ETR-3 Represses Tau Exons 2/3 Inclusion, a Splicing Event Abnormally Enhanced in Myotonic Dystrophy Type I

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Altered splicing of transcripts, including the insulin receptor (IR) and the cardiac troponin (cTNT), is a key feature of myotonic dystrophy type I (DM1). CELF and MBNL splicing factor members regulate the splicing of those transcripts. We have previously described an alteration of Tau exon 2 splicing in DM1 brain, resulting in the favored exclusion of exon 2. However, the factors required for alternative splicing of Tau exon 2 remain undetermined. Here we report a decreased expression of *CELF* family member and *MBNL* transcripts in DM1 brains as assessed by RT-PCR. By using cellular models with a control- or DM1-like splicing pattern of *Tau* transcripts, we demonstrate that ETR-3 promotes selectively the exclusion of Tau exon 2. These results together with the analysis of Tau exon 6 and IR exon 11 splicing in brain, muscle, and cell models suggest that DM1 splicing alteration of several transcripts involves various factors. © 2006 Wiley-Liss, Inc.

**Key words:** myotonic dystrophy; splicing; microtubule-associated Tau; insulin receptor; ETR-3

Myotonic dystrophy type I (DM1) is a multisystemic disorder with neurological symptoms including mental retardation, sleep disorders, behavioral changes, alterations in brain structure, and cognitive defects, as well as molecular abnormalities (Chang et al., 1993; Vermersch et al., 1996; Ishii et al., 1996; Meola, 2000; Nardone et al., 2000; Di Costanzo et al., 2002; Meola et al., 2003; Modoni et al., 2004). DM1 is caused by the expansion of an unstable CTG repeat located in the 3'-untranslated region of the *myotonic dystrophy protein kinase* gene (*DMPK*; Brook et al., 1992). *DMPK* is widely expressed, in particular in skeletal muscle and heart, but also in brain (Lam et al., 2000; Jiang et al., 2004; Sarkar et al., 2004). Clinical expression of the disease seems to be caused by a pathogenic RNA gain-of-function mechanism in which the CUG repeats alter cellular functions, including alternative splicing. Alternative splicing of transcripts such as *cTNT*, *IR*, *Tau*, and *muscle-specific chloride channel-1* (*CIC-1*) is altered in several organs (e.g., heart, skeletal muscle, brain; Philips et al.,

1998; Savkur et al., 2001; Sergeant et al., 2001; Charlet et al., 2002; Mankodi et al., 2002; Jiang et al., 2004; Leroy et al., 2005). These transcripts encode proteins having a key role in the organs affected by pathology.

Together, nuclear foci composed of mutated RNA and alterations of splicing are characteristic features found in both muscle and brain of DM1 patients (Taneja et al., 1995; Jiang et al., 2004). These common pathology-related features suggest a unique mechanism of splicing alteration of the transcripts and organs affected in DM1. To understand the mechanisms leading to those splicing alterations, splicing regulatory factors have been investigated. Members of two protein families, CELF (CUG-BP and ETR-3-like factors) and MBNL (muscleblind-like factors), might be responsible for the missplicing of several transcripts observed in DM1. The six CELF family members (CUG-BP1, ETR-3, CELF3–6) promote cTNT exon 5 inclusion (Ladd et al., 2001, 2004; Han and Cooper, 2005), and CUG-BP1 protein level is increased in DM1 muscle (Savkur et al., 2001; Timchenko et al., 2001). The role of CELF family members in the DM1 missplicing is supported by results from transgenic mice expressing either a negative dominant mutant of CELF4 protein (Ladd et al., 2005) or overexpression of CUG-BP1 protein (Ho et al., 2005). MBNL proteins also regulate splicing, apparently as antagonists to CELFs (Ho et al., 2004), and localize to nuclear foci in DM1 (Fardaei et al., 2001, 2002; Jiang et al., 2004). Furthermore, invalidation of MBNL expression also reproduces DM1 pathology and missplicing of several transcripts in mice (Kanadia et al., 2003).

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Such a relationship between MBNL and CELF dysfunction has not been investigated with regard to Tau splicing. In adult human brain, six Tau mRNAs are mainly expressed that differ from each other by the inclusion or exclusion of exons 2, 3, and 10 (Goedert et al., 1988, 1989a,b). In DM1 brains, a preferential exclusion of Tau exons 2/3, and 10 from *Tau* transcripts, results in the main expression of the isoform lacking the three possible inserts (Jiang, 1984; Vermersch et al., 1996; Sergeant et al., 2001; Jiang et al., 2004). More recently, we demonstrated altered splicing of Tau exon 6 (Leroy et al., 2005), a minor alternative cassette in brain (Wei and Andreadis, 1998). Major *trans*-regulatory factors involved in the Tau exons 2, 3, or 6 splicing events are unknown. In the present study, we first investigated the expression of *CELF* and *MBNL* transcripts in DM1 brain and then studied the *trans*-regulatory function of two CELF members, ETR-3 and CELF4, on Tau exons 2 and 3 splicing comparative with its action on IR exon 11.

## MATERIALS AND METHODS

### Human Brain and Muscle Tissues

Brain tissues were obtained from the Department of Neurology, Lille, France, in accordance with the local ethics committee protocol. DM1 brain tissue samples were obtained after autopsy of three patients aged from 53 to 64 years. Two of them (DM1.1 and DM1.3) suffered from the adult form of the disease, and the other (DM1.2) had congenital DM1. The founder mutations consisted of 150 CTG repeats for both DM1.1 and DM1.3 and 1,000 CTG repeats for DM1.2. These cases were previously described and characterized for Tau exon 2 splicing and CTG repeats (Sergeant et al., 2001). Skeletal muscles were obtained from the Department of Pathological Anatomy, Lille, France, in accordance with the local ethics committee. They were biopsy derived, with the exception of one sample, which belongs to the DM1.3 patient, whose brain was also analyzed. Two control subjects were included. Post-mortem delays were always less than 48 hr, and all samples were stored at  $-80^{\circ}\text{C}$ . Four cortical brain regions were dissected according to Brodmann's classification: areas from the temporal (area 22), occipital (area 18), frontal (area 10), and parietal (area 39) regions. All tissues were analyzed and included for histogram representation, but only the results obtained for two samples are illustrated.

### Cell Culture and Transfection

Human neuroblastoma SKNSH-SY 5Y (SY 5Y) cells and glioblastoma T98 cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, 50 IU/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mmol/liter glutamine as described previously (Caillet-Boudin et al., 1998; Ferreira et al., 2000). Minigenes SV2/3 and SV2/ $\Delta$ 3 were described elsewhere (Andreadis et al., 1995). ETR-3 and CELF4 plasmids were described by Ladd et al. (2001). Plasmid DNA was purified by using Nucleobond AX (Macherey-Nagel, Düren, Germany). Transfection was performed with ethylenimine polymer

ExGen500 (Euromedex France) according to the manufacturer's instructions. Cells were harvested 48 hr after transfection.

### RT-PCR Amplification

Brain or muscle RNAs were isolated by using the RNeasy kit according to the manufacturer's instructions (Promega France). The RNA concentration was determined by absorption at 260 nm. RT-PCR was performed with 1  $\mu\text{g}$  of total RNA using M-MLV reverse transcriptase (Promega France) and random hexamers (5  $\mu\text{mol}/\text{liter}$ ). RT-PCR controls without RNAs were performed. Specific primer pairs used to study the expression of splicing factors and number of PCR cycles are detailed in Table I. Primers of Tau exon 2 and IR exon 11 were chosen in the neighboring exons. The final volume of PCR was 25  $\mu\text{l}$  and contained 15 pmol of primers, 1.5 mM  $\text{MgCl}_2$ , 1 U *Taq* polymerase (Invitrogen, La Jolla, CA), and 200 nM dNTP. PCR conditions consisted of 5 min at  $94^{\circ}\text{C}$ ; cycles of 1 min denaturation at  $94^{\circ}\text{C}$ , annealing for 2 min at  $60^{\circ}\text{C}$ , 2 min extension at  $72^{\circ}\text{C}$ ; and a final extension step at  $72^{\circ}\text{C}$  for 7 min. RT-PCR were performed in triplicate. *18S* RNAs were used as internal controls. RT-PCR products from muscle RNA amplification were analyzed by 2% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide. Brain RT-PCR products were resolved by electrophoresis on a 4% or 5% (w/v) polyacrylamide gel, and the bands were stained with Sybr Gold (Interchim France). The intensity of Sybr Gold luminescence was measured with a FluoroImager scanner (Clarus France) and was analyzed with ImageQuant software (GE Healthcare Sweden). The cDNA signals were normalized to those of *18S*. Statistical analyses were performed via one-way ANOVA in SigmaStat Software (SPSS France).

Cellular RNAs were extracted by using a total RNA extraction kit (Macherey-Nagel) according to the supplier's instructions. RT-PCRs were performed with the superscript one-step RT-PCR system from Invitrogen according to the manufacturer's instructions, using 1  $\mu\text{g}$  total RNA. After the reverse transcription step (30 min at  $55^{\circ}\text{C}$ ), the reaction mixture was heated to  $94^{\circ}\text{C}$  for 3 min; followed by 30–40 cycles of 30 sec at  $94^{\circ}\text{C}$ , 30 sec at the  $56$ – $65^{\circ}\text{C}$  hybridization temperature according to the primer pair (see Table I); 2 min extension at  $72^{\circ}\text{C}$ ; and a final extension step at  $72^{\circ}\text{C}$  for 7 min. RT-PCR products were analyzed via 2% or 4% (w/v) agarose gel electrophoresis and stained with ethidium bromide. *18S* amplification was used as internal control. RT-PCR amplification was performed at least three times with total RNA extracted from three different cell culture passages.

### Polyacrylamide Gel Electrophoresis and Western Blotting

Cellular protein extracts were obtained by resuspending the cell pellet in Laemmli's buffer and heating at  $100^{\circ}\text{C}$  as previously described (Dupont-Wallois et al., 1995). After a 10% SDS-PAGE, ETR-3 was detected by immunoblotting onto nitrocellulose membrane with the monoclonal antibody ab7656 (Abcam, Cambridge, United Kingdom). Briefly, proteins were transferred to nitrocellulose membrane (Hybond; GE Amersham Biosciences) at 2.5 mA/cm<sup>2</sup> per gel using the Semidry Novablot

TABLE I. Primers and PCR Conditions for Amplification of CELFs, MBNL, Tau, IR, and Minigene Transcripts

Sequences amplified	Sense primers	Antisense primers	PCR conditions Temp No. of cycles	Band size (bp)
CUGBP1	5'-TCCTCTGGGAACCTCAACAC-3'	5'-TACCCGAGTAGGCCTGAGTG-3'	60°C/26	382
ETR-3	5'-AAGACCAACCTCATCGTCAA-3'	5'-TTGCTGGTCTTGAAGGAGAC-3'	56°C/30	120
CELF4	5'-TGCTCAACAAGCAACAGTCC-3'	5'-AGCCATGGGGTTCAGGTAG-3'	60°C/26	394
MBNL1	5'-ATTACAACCCGTGCCAATGT-3'	5'-CAGCCTGGTTGACCTGGTAT-3'	60°C/24	440
ARN18S	5'-AAACGGCTACCACATCCAAG-3'	5'-CGTCCCAAGATCCAACACTAC-3'	58°C/15	250
GAPDH	5'-CCATGGCACCCTCAAGGCTGA-3'	5'-GCCAGTAGAGGCAGGGATGAT-3'	62°C/22	440
Tau E1-E4	5'-TACGGGTTGGGGGACAGGAAAGAT-3'	5'-GGGGTGTCTCCAATGCCTGCTTCT-3'	65°C/35	286 → 2 <sup>+</sup> 3 <sup>+</sup> 199 → 2 <sup>+</sup> 3 <sup>-</sup> 112 → 2 <sup>-</sup> 3 <sup>-</sup>
Tau E4-E7	5'-GAAGACGAAGCTGCTGGTCA-3'	5'-TCTTTGGAGCGGGCGGGGTTTTTG-3'	56°C/35	411 → 6c 310 → 6p 241 → 6d 213 → 6 <sup>-</sup>
Tau E4-E6	5'-GAAGACGAAGCTGCTGGTCA-3'	5'-TTGAGTTTCATCTCCTTTGC-3'	56°C/40	278 → 6c 177 → 6p 108 → 6c
IR E10-E12	5'-CCAAAGACAGACTCTCAGAT-3'	5'-AACATCGCCAAGGGACCTGC-3'	58°C/35	167 → 11 <sup>+</sup> 131 → 11 <sup>-</sup>
IR E10-E11	5'-CCAAAGACAGACTCTCAGAT-3'	5'-CATCTAGATCTTACTGCAAT-3'	52°C/35	118 → 11 <sup>+</sup>
INS1-INS3	5'-CAGCTACAGTCGGAAACCATCA-GCAAGCAGA-3'	5'-CACCTCCAGTGCCAAGGT-CTGAAGGTCACC-3'	65°C/35	494 → 6c 393 → 6p 324 → 6d 296 → 6 <sup>-</sup>

Transfert System (GE Amersham Biosciences) according to the manufacturer's instructions. Proteins were reversibly stained with Ponceau red to check the quality of protein transfer. Membranes were blocked in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 (v/v; TBS-T), and 5% (w/v) skimmed milk for 30 min. Membranes were incubated for 1 hr at room temperature and ETR-3 antibody at a final dilution of 1/1,000 (v/v) in TBS-T. Membranes were further incubated for 1 hr at room temperature with secondary antibody (anti-mouse immunoglobulins conjugated to horseradish peroxidase; Sigma France) at a final dilution of 1/6,000 (v/v) in TBS-T. Notably, membranes were rinsed three times for 10 min each in TBS-T between each step of the procedure. The immunoreactive complexes were revealed by using the ECL Western Blotting Kit and Hyperfilms (GE Amersham Biosciences). Western blot films were digitized with a UMax scanner (GE Amersham Biosciences).

## RESULTS

### DM1 Splicing Alterations in Human Brain and Muscle Tissues

Alternative splicing of Tau exons 2/3 and 6 and IR exon 11 was first analyzed in brain and muscle tissues of control individuals (Fig. 1A). The analysis of Tau splicing showed that exon 2 was alternatively spliced in the human brain tissue, whereas it was always included in muscle (Fig. 1A). Indeed, the isoform lacking both exons 2 and 3 was present in brain but absent in muscle from control subjects. In contrast, Tau exon 3 was alternatively spliced in both brain and muscle tissues of controls: both

isoforms 2<sup>+</sup>3<sup>-</sup> and 2<sup>+</sup>3<sup>+</sup> were detected in both tissues. Tau exon 6 and IR exon 11 were hardly detected in brain but were clearly observed in muscle from control subjects.

The analysis of Tau exons 2, 3, and 6 and IR exon 11 splicing showed that these alternative splicing events were altered in DM1 brains. This was shown for Tau exon 2/3. Reduced inclusion of Tau exon 6c and IR exon 11 was also seen in DM1 brain (Fig. 1A,B). Because this reduction was particularly low, internal primers of Tau exon 6 and IR exon 11 were used. Figure 1B showing the detailed analysis in four brain regions further supports both decrease of exon 6c and IR exon 11 inclusion in DM1 brain. In contrast, only inclusion of Tau exon 2/3 and IR exon 11 was reduced in DM1 muscle but not that of Tau exon 6 (Fig. 1A).

### Expression Pattern of CELF and MBNL RNAs in the Human Brain

The loss of function of CELF and/or MBNL is supposed to be instrumental in DM1. However, little is known about the expression of CELF and MBNL family members in human brain. We analyzed the expression of *CUG-BP1*, *ETR-3* (*CUG-BP2* or *NAPOR*), *CELF4*, and *MBNL1* in four neocortical regions of the human brain. Levels of these transcripts were determined by using RT-PCR amplification of mRNA isolated from temporal, frontal, parietal, and occipital cortex of two controls and three DM1 patients. Results are shown for transcripts amplified from the frontal cortex (Fig. 2A). A reduced expression of *CUG-BP1*, *ETR-3*, *CELF4*, and *MBNL1* was ob-

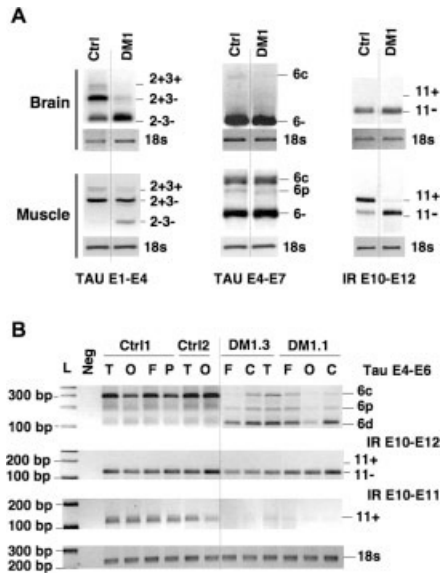


Fig. 1. Splicing patterns of Tau and IR transcripts in brain and muscle from control and DM1 subjects. **A:** Tau exons 2/3 and 6 (respective primer pairs: Tau E1–E4 and Tau E4–E7) and IR exon 11 (primer pair: IR E10–E12) alternative splicing analysis. Note that splicing patterns are tissue specific. Tau exons 2/3 alternative splicing was clearly altered in both DM1 brain and muscle. A faint band corresponding to Tau 6c isoform was detected only in control brain (Ctrl) as compared to DM1 brain. **B:** More detailed analysis of Tau exon 6 and IR exon 11 splicing in several cortical brain areas of two controls and two DM1 brains (DM1.3 and DM1.1 per Sergeant et al., 2001). Amplification with the Tau E4–E6 primer pair confirmed the variation of Tau exon 6 splicing in DM1 brains. The IR E10–E12 primer pair showed that E11 exclusion is the major event of brain IR splicing. However, the E10–E11 primer pair showed the possible low presence of exon 11<sup>+</sup> IR transcripts in control samples. T, O, F, P correspond respectively to temporal, occipital, frontal, parietal cortical areas. 18S amplification has been used as loading control. Neg, no template control of RT-PCR; L, ladder.

served (Fig. 2B) and was significant for *CUG-BP1*, *CELF4*, and *MBNL1*. These results therefore suggest an altered expression of *trans*-regulatory splicing factors in the brains of DM1 patients.

**Differential Expression of ETR-3 and CELF4 in Neuroblastoma SY5Y and Glioblastoma T98 Cells**

To analyze the potential *trans*-regulatory function of CELF and MBNL in the brain regulation of alternative splicing of Tau exons 2/3 and 6, we first analyzed the endogenous pattern of Tau splicing and compared with that of IR exon 11 in several human cell lines. As previously shown (Dupont-Wallois et al., 1995; Smith et al., 1995), neuroblastoma SY5Y cells mainly express a single *Tau* RNA isoform lacking exons 2 and 3. In contrast, Tau exons 2 and 3 were alternatively spliced in T98 glioblastoma cells (Fig. 3A). IR exon 11 was skipped in SY5Y, whereas it was alternatively spliced in T98 cells (Fig. 3A). Therefore, T98 cells expressed an adult-like pattern of Tau isoforms, whereas SY5Y were showing a fetal-

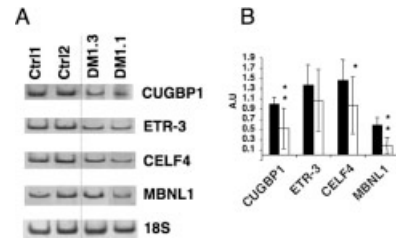


Fig. 2. RNA expression of *CELF* and *MBNL* family members in both normal and DM1 brains. **A:** RT-PCR analysis of *CELF* (*CUGBP1*, *ETR-3*, *CELF4*) and *MBNL1* transcripts in frontal brain area from two normal (Ctrl1, Ctrl2) and two DM1 patients (DM1.1, DM1.3 per Sergeant et al., 2001). 18S amplification has been used as loading control. **B:** Semiquantitative analysis of cerebral *CELF* and *MBNL* transcripts. Histograms correspond to the average  $\pm$  SD of four brain areas (temporal, occipital, frontal, parietal) from controls (solid bars) and DM1 patients (open bars). The values were normalized to an equal GAPDH amplification. Note the decreased expression of all factors analyzed in DM1 samples compared with controls. Significance of the results was determined by one-way ANOVA method in Sigmapstat software. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

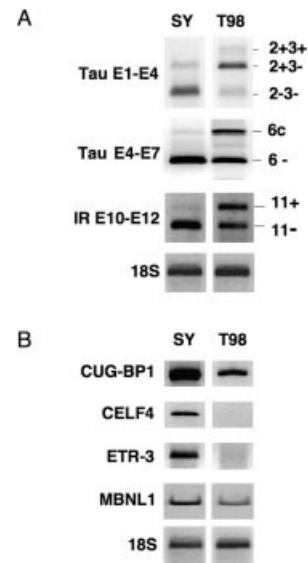


Fig. 3. Analysis of Tau and IR splicing patterns in SY 5Y and T98 cells. **A:** Splicing patterns of Tau exons 2/3 and exon 6 and IR exon 11 in neuronal SY 5Y (SY) and glial T98 cell lines. The corresponding regions were amplified by using the Tau E1–E4, Tau E4–E7, and IR E10–E12 primer pairs. Note the preferential inclusions of Tau exon 2, Tau exon 6c, and IR exon 11 in T98 compared with SY 5Y cells. **B:** Transcript analysis of *CELF* and *MBNL* family members in SY 5Y and T98 cells. *CUGBP1* and *MBNL1* mRNAs were expressed in both cell lines. In contrast, *ETR-3* and *CELF4* were expressed in SY 5Y but not in T98 cells. 18S amplification has been used as control loading in A and B.

like phenotype, similar to that observed in DM1. The two cell lines were therefore used to investigate the pattern of *CELF* and *MBNL1* expression. *CUG-BP1* and *MBNL1* transcripts were similarly expressed in SY5Y and T98 cells, whereas analyses of *CELF4* and *ETR-3* expression showed

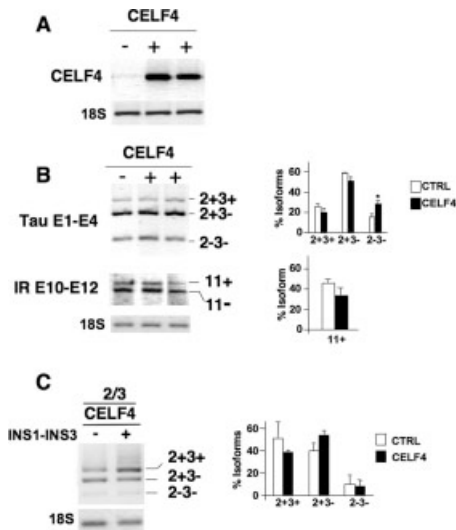


Fig. 4. CELF4 transfection of T98 cells. **A:** Efficiency of cell transfection by CELF4 was checked by RNA expression. **B:** Analysis of Tau and IR splicing before (–) and after (+) transfection did not show a change in alternative splicing of Tau exons 2/3 and IR exon 11 upon CELF4 transfection. **C:** Analysis of Tau exons 2/3 splicing in T98 cells cotransfected with CELF4 and Tau exons 2/3 minigene. Histograms represent the percentage of each Tau RNA isoform averaged from three experiments. Note the slight increase of Tau exons 2/3 exclusion, but only for endogenous transcripts. \* $P < 0.05$ . 18S amplification has been used as loading control in A–C.

striking differences between SY5Y and T98 cells (Fig. 3B). Both *CELF4* and *ETR-3* were expressed in SY5Y cells but were almost entirely absent in T98 cells. Altogether, those observations suggested that *CELF4* and/or *ETR-3* could regulate the alternative splicing of Tau exons 2 and 3.

### Effect of *ETR-3* and *CELF4* Expression on Splicing of Tau Exons 2, 3, and 6 and IR Exon 11

T98 cells were transfected with *CELF4* or *ETR-3*, and the splicing of Tau exons 2, 3, and 6 was analyzed by RT-PCR. When transfecting T98 cells with *CELF4* (Fig. 4A), we did not observe a clear change in the endogenous splicing of Tau exons 2/3 (Fig. 4B). A faint but significant increase in Tau  $2^{-}3^{-}$  isoform level was not counterbalanced by a significant decrease in Tau  $2^{+}3^{+}$ / $2^{+}3^{-}$  isoforms. No significant changes were observed when analyzing Tau minigene or endogenous *IR* transcripts (Fig. 4C). In contrast, expression of *ETR-3* in T98 (Fig. 5A) resulted in a significant decrease of *Tau* exon 2 and *Tau* exon  $2^{+}3^{+}$  isoforms and a significant increase of *Tau* isoforms lacking those alternatively spliced cassettes (Fig. 5B). A similar effect of *ETR-3* on Tau exons 2/3 skipping could be observed in cotransfection of *ETR-3* and Tau exons 2/3 minigene (Fig. 5C). *ETR-3* expression also resulted in a preferential skipping of IR exon 11 (Fig. 5B). In contrast, Tau exon 6 splicing was not modulated by *ETR-3* in T98 cells (Fig. 5B). Therefore, the present data showed that *ETR-3* is an efficient *trans*-regulatory factor of Tau

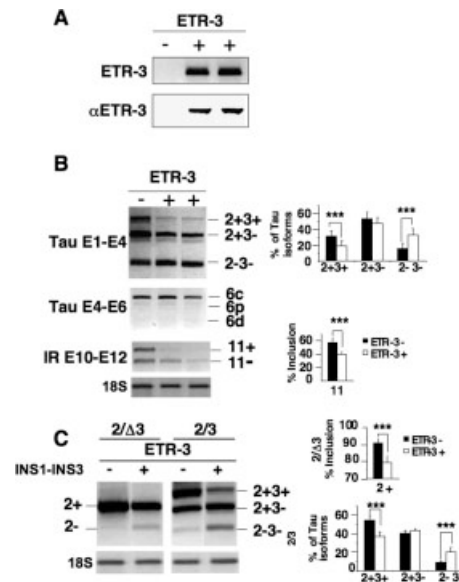


Fig. 5. *ETR-3* transfection of T98 cells. **A:** The efficiency of cell transfection by *ETR-3* was checked by RNA and protein expression. **B:** Analysis of Tau and IR splicing before (–) and after (+) transfection showed a change in alternative splicing of Tau exons 2/3 and IR exon 11 upon *ETR-3* transfection, whereas Tau exon 6 splicing did not change. Histograms represent the percentage of Tau RNA isoforms and IR exon 11 inclusion. **C:** The *ETR-3* effect on Tau exons 2/3 splicing was checked by cotransfection with minigenes containing only exon 2 and its intronic sequences ( $2/\Delta 3$ ) or both exon 2 and 3 ( $2/3$ ) and the corresponding intronic sequences. Tau exon 2 exclusion was promoted by *ETR-3* transfection. Histograms are averages of three individual experiments and correspond to the percentage of Tau exon 2 inclusion ( $2/\Delta 3$ ) and the percentage of Tau isoforms expressed ( $2/3$ ). 18S amplification has been used as loading control in B and C. Asterisks indicate a significant difference between splicing pattern as determined by a one-way ANOVA test. \*\*\* $P < 0.001$ .

exons 2/3 and IR exon 11 alternative splicing but not of Tau exon 6 in T98 cells.

## DISCUSSION

Because the splicing defects observed in DM1 are supposed to result from the intranuclear inclusion of *DMPK* mRNA bearing a CUG expansion and consequently the loss or gain of function of specific *trans*-regulatory splicing factors, we analyzed the brain expression pattern of several *trans*-regulatory splicing factors with potentially altered function in DM1. We show an overall decrease of *CUG-BP1*, *ETR-3*, *CELF4*, and *MBNL1* transcripts in DM1 brain. The brain decrease of *CUG-BP1* transcripts, previously shown in muscle, was also present in brain (Watanabe et al., 2004). These data contrasted with the increased protein level of *CUG-BP1* observed in DM1 muscle (Philips et al., 1998; Savkur et al., 2001; Charlet et al., 2002). We investigated the expression of *CUG-BP1*, *ETR-3*, and *MBNL1* proteins by using either Western blot or immunohistochemistry. However, those proteins were seldom detected in human brain tissue (data not shown). Given the complexity of the

regulation of gene expression, the quantification of mRNA levels might not correspond to the levels of final gene product. Thus, the reduced expression of those *trans*-regulatory splicing factors might also result from the loss of specific subneuronal populations, a defective differentiation of neurons during development or a dedifferentiation of neurons associated with aging in DM1 brains. Further work will be needed to have a complete overview of the expression pattern of those splicing factors in the brain of patient suffering from DM1.

The defective splicing of Tau exon 2 in DM1 is likely similar to the splicing pattern Tau of exon 2 observed in brain of human fetus, suggesting a loss of function or a lack of expression of *trans*-regulatory splicing factors necessary for Tau exon 2 alternative splicing in adult. From minigene studies, the default splicing pattern of Tau exon 2 is inclusion (Andreadis et al., 1995; Gao et al., 2000; Li et al., 2003). Tau exon 2 is mainly inserted in the peripheral nervous system as well as in nonneuronal human tissues (for review see Andreadis, 2005). In sharp contrast, exon 2 is alternatively spliced in the human adult brain. Together, these observations suggest that *trans*-regulatory repressors may regulate alternative splicing of Tau exon 2. By comparative study of Tau exon 2 splicing pattern and the transcript expression of *CELF* and *MBNL* *trans*-regulatory family members in 2 human brain cell types, we showed that *CELF4* and *ETR-3* are likely regulating the splicing of Tau exon 2. Overexpression of *CELF4* in T98 cells had a modest effect on the splicing of endogenous Tau exons 2/3. A modest effect of *CELF4* had been reported for Tau exon 2 minigene (Li et al., 2003). The difference in *CELF4* effect on endogenous or minigene transcripts could be due either to the ratio of *CELF4* /transcript levels or to the differences in the intronic sequences present in minigene and endogenous transcripts. *CELF4* was also inefficient in endogenous IR exon 11 splicing in these cells.

In contrast, *ETR-3* expressed in T98 cells was a regulatory factor of both tau exons 2/3 and IR exon 11 splicing. *ETR-3* promoted Tau exons 2/3 skipping on both endogenous and minigenes Tau transcripts. *ETR-3* seems more effective in inclusion of both exons 2 and 3 than in inclusion of only exon 2. The *ETR-3* action on IR splicing agrees with a previous report (Han and Cooper, 2005). However, the presence of *ETR-3* in normal brain suggests a more complex regulation with regard to Tau splicing regulation. Indeed, *ETR-3* synthesis in normal brain ruled out the hypothesis of a Tau splicing misregulation that might be due only to an abnormally expressed *ETR-3*. Tau missplicing appears to be associated with a statistically significant decrease in *CUG-BP1*, *CELF4*, and *MBNL1* expression, as assessed by RT-PCR. A similar pattern of splicing is also observed for *Tau* transcripts in neuronal cell lines but is absent in glial cells. However, the former express greater levels of *CUG-BP1*, *CELF4*, *MBNL1*, and even *ETR-3* transcripts than the latter, in clear contrast to what we observed in DM1 brains. Altogether, this suggests that *ETR-3* involves other partners that may modulate its activity on Tau splicing. Alternatively, it can also be hy-

pothesized that the cell type-specific and disease-associated splicing of *Tau* transcripts might be controlled by different regulatory mechanisms. However, the normal brain *ETR-3* expression could be consistent with a direct effect of *ETR-3* on brain IR exon 11 exclusion.

In contrast to Tau exon 2, *ETR-3* did not affect exon 6c insertion. This result further supports our hypothesis that Tau exons 2 and 6c are independently regulated and also suggests that all the splicing misregulations observed in DM1 probably are not due to a unique mechanism (Leroy et al., 2005). Such a hypothesis is also supported by the fact that exon 6 splicing misregulation is brain-specific, whereas exon 2 splicing misregulation occurs in both brain and muscle (Leroy et al., 2005). Both exon- and tissue-specific regulation are probably involved. Several hypotheses are possible: cerebral exons 2/3 and exon 6c splicing are regulated 1) by the same brain-specific factor but different factors in muscle; 2) by a same muscle-specific factor but different factors in brain; or 3) by two different exon-specific factors with an activity differentially regulated in these tissues.

In conclusion, we have found that the expression of *trans*-regulatory splicing factors of both *CELF* and *MBNL* family members is modified in the brain tissue of DM1 patients. Because of the complexity and heterogeneity of brain tissue, this observation would necessitate further investigation to identify the mechanisms underlying this loss of expression. We showed that *ETR-3* is likely a strong repressor of Tau exon 2 and more likely of Tau exons 2/3 inclusion, whereas *CELF4* has little effect in our cell system. Together, these results further support the hypothesis that *CELF* proteins regulate the splicing of several transcripts, now including *Tau*, using distinct protein domains (Han and Cooper, 2005). However, it remains to be determined whether the effect of *ETR-3* is mediated by a direct interaction of the protein with Tau transcripts. Moreover, regulation of *CELF* proteins by posttranslational modifications or interaction with other protein partners or cellular distribution must be investigated before we can understand the common and distinct regulatory mechanisms shared by this family of proteins.

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