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Altered splicing of Tau in DM1 is different from the foetal splicing process

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ABSTRACT

Among the different mechanisms underlying the etiopathogenesis of myotonic dystrophy type 1 (DM1), a backward reprogramming to a foetal splicing machinery is an interesting hypothesis. To address this possibility, Tau splicing, which is regulated during development and modified in DM1, was analyzed. Indeed, a preferential expression of the foetal Tau isoform, instead of the six normally found, is observed in adult DM1 brains. By using two cell lines, we show here that the cis-regulating elements necessary to generate the unique foetal Tau isoform are dispensable to reproduce the trans-dominant effect induced by DM1 mutation on Tau exon 2 inclusion. Our results suggest that the mis-splicing of Tau in DM1 is resulting from a disease-associated mechanism.

1. Introduction

Myotonic dystrophy of type 1 (DM1) is a multisystemic disorder characterized by myotonia, progressive muscle weakness, wasting of skeletal muscle and extramuscular symptoms including brain dysfunctions (for review see [1,2]). Neurological symptoms include mental retardation, sleep disorders, behavioural changes, alterations in brain structure and cognitive deficits (for review see [3,4]).

DM1 is caused by a pathological expansion of an unstable CTG repeat ($n > 50$) located in the 3′ untranslated region of *myotonic dystrophy protein kinase* gene (*DMPK*) [5]. Clinical expression of DM1 is likely the consequence of a pathogenic RNA gain of function mechanism by which the CUG repeats alter the function of splicing factors such as MBNL and CELF families [6]. As a consequence, alternative splicing of cardiac Troponin T (hcTNT), Insulin Receptor (IR), Tau and muscle-specific Chloride Channel-1 (ClC-1) transcripts is altered in several organs (heart, skeletal muscle, brain, etc.) (for review see [7]). The modified splicing of these transcripts is paralleled by the clinical symptoms.

Noteworthy, the splicing defect consists of the preferential expression of the foetal isoforms of numerous transcripts targeted by the *trans* effect of the mutation (reviewed in [7]). This observation could suggest that CUG repeats induce an adult to foetal reprogramming of the splicing machinery in DM1. Alternatively, the DM1 foetal-type pattern could result from a disease-associated mechanism. In the brain and muscles of DM1 patients, we and others reported a modified splicing of Tau that clearly favourites the expression of the unique foetal human brain isoform [8–11]. In adult human central nervous system, six major isoforms of Tau are expressed that differ from each other by the inclusion or exclusion of exons 2, 3 and 10 (For review see [12]). In the human foetal brain, one Tau isoform lacking exons 2, 3 and 10 is expressed. Thus, as described for other transcripts such as hcTNT and IR, the DM1 altered splicing leads to an increased expression of the unique foetal isoform of Tau.

In the present study, we evaluated whether the splicing mechanism leading to a foetal-type splicing of Tau results from an adult to foetal reprogramming of the splicing machinery or from DM1 specific mechanism. Because Tau exon 2 is largely included in human adult brain and preferentially excluded in DM1 brain, we focused our study on exon 2 splicing mechanism. We compared the splicing effect of CTG repeats on exon 2 splicing from endogenous

Abbreviations: DM, myotonic dystrophy; DMPK, dystrophia myotonia protein kinase; cTNT, cardiac troponin T; ClC-1, chloride channel-1; IR, insulin receptor

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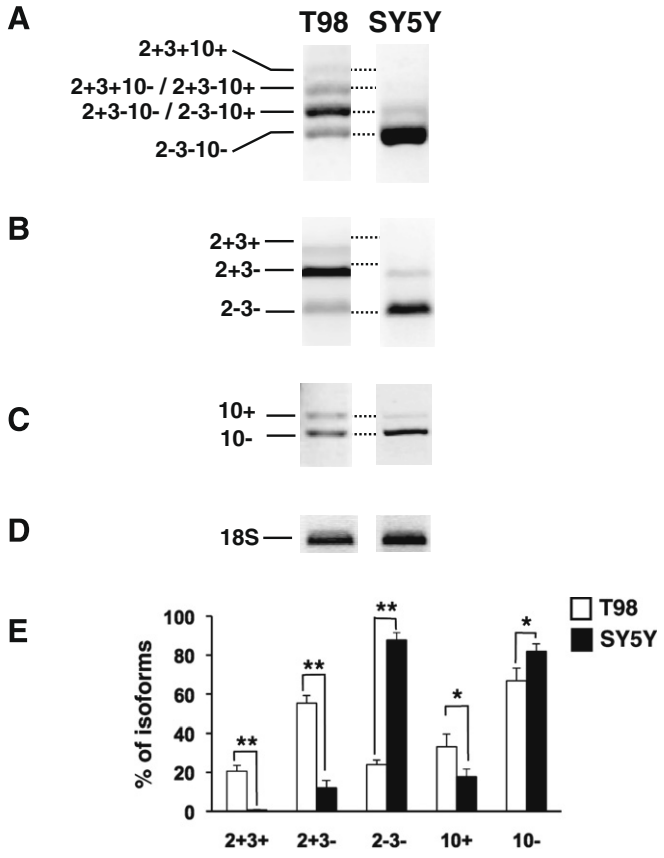

Fig. 1. Alternative splicing of endogenous Tau in human Glioblastoma T98 and Neuroblastoma SY5Y cell lines. Splicing profile is analyzed by RT-PCR using Tau E1/E13 primer pair in (A), Tau E1/E4 primer pair in (B) and Tau E9/E13 (C) (see Table 1 for the localization of Tau primers). 18S amplification is used as control of the RNA quantity amplified in (D). Note the preferential inclusion of exon 2 of Tau in T98 cell line and its preferential exclusion in SY5Y cell line. The quantitative analysis of Tau isoform expression is represented in (E). Significant differences in the isoforms expression between the two cell lines are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

or minigene tau transcripts in two human cell lines characterized by a different Tau splicing pattern.

2. Materials and methods

2.1. Cell culture and transfection

Human glioblastoma T98G (T98) and neuroblastoma SK-N-SH SY5Y (SY5Y) cell lines were maintained in DMEM medium supplemented with 10% foetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 4 mmol/L glutamine. Cell-transfections were performed using Fugene HD transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

2.2. Plasmids

Human cells were transfected with pcDNA3 plasmids containing 5, 200 or 960 CTG triplets [11] or co-transfected with human Tau 2/3 or 2Δ3 minigenes as described [13]. Tau 2/3 and 2Δ3 minigenes differ from the endogenous *MAPT* gene by a truncation of intron 1–2: the sequence of the intron upstream the exon 2 was limited to the 878 nucleotides located in the 5′ region before exon 2 (instead of the 9388 nt) (Fig. 2A). The length of intron 2–3 is identical to *MAPT* gene in Tau 2/3 minigene whereas the 3′ intron 2–3 region is shorter in the Tau 2Δ3 minigene (2092nt instead of 2439 nt in *MAPT* gene) (Fig. 2A).

Plasmids containing the 3′UTR of DMPK with 5 or 200 CTG repeats were derived from previously described constructs under a rodent ROSA promoter [14]. Briefly, the plasmids contained 5 or 200 CTG triplets flanked by the 3′UTR sequences neighboring (about 800 and 600 bp upstream and downstream) the CTG triplets. The constructs were subcloned into pcDNA 3.1 under the control of the CMV promoter [11]. The plasmid containing the 3′UTR of DMPK with 960 interrupted CTGs was a minigene construct also under the control of the CMV promoter. It contained the last 5 exons of DMPK followed by the full length DMPK 3′UTR, with 960 CTG repeats interrupted at every 20th repeat by CTCGA motif [15].

Plasmids DNA were purified using Nucleobond AX (Macherey-Nagel).

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells 48 h post-transfection using a total RNA extraction kit (Nucleospin RNA II- Macherey-Nagel). Total RNA (1.5 μg) was reverse transcribed using $pd(N)_6$ random hexamer (Amersham 27-2166-01) and Superscript First strand synthesis system for RT-PCR, according to the supplier's instructions (Invitrogen). After reverse transcription, the reaction mixture was diluted four times. Five microliters of the diluted cDNAs were amplified using Platinium PCR super mix (Invitrogen, California, USA). Briefly, the PCR mixture was heated at 94 °C for 3 min and followed by 25–35 cycles with 30 s at 94 °C, 30–60 s at the correct annealing temperature, depending on the primer pair used (see Table 1), 1 min extension at 72 °C and a final extension step at 72 °C for 7 min. RT-PCR products were analyzed by 3% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide. RT-PCR experiment was at least performed three times with total RNA extracted from three different passages of cell culture. 18S mRNA amplification was used as a control of RNA quantity [9]. Quantification of ethidium bromide luminescence was measured using a

Table 1
List of primers used to analyze the expression of Tau isoforms and the alternative splicing of Tau exons 2 and 3. The first column indicates the sequences that were amplified (Exon: E). The second contains the sequence of the forward and reverse primers (Sense/Antisense). PCR conditions and size of the fragments are indicated in the last two columns.

Sequence amplified	Sense/antisense primers	PCR conditions: $T°$ Hybridization, cycle number	Fragment size (bp)
Tau E1 > E13	5′-GTTGGGGGACAGGAAAGATC-3′ 5′-GTCTACCATGTCGATGCTGC-3′	65 °C, 35 cycles	933, 1020, 1026, 1107, 1113, 1200
Tau E1 > E4	5′-TACGGGTTGGGGGACAGGAAAGAT-3′ 5′-GGGGTGTCTCCAATGCCTGCTTCT-3′	65 °C, 35 cycles	112, 199, 286
Tau exon 2/3, Ins1 > Ins3	5′-CAGCTACAGTCGGAAACCATCAGCAAGCA-3′ 5′-CACCTCCAGTGCCAAGGTCTGAAGGTCACC-3′	65 °C, 25 cycles	290, 380, 470
Tau exon 2Δ3, Ins1 > Ins3	5′-CAGCTACAGTCGGAAACCATCAGCAAGCA-3′ 5′-CACCTCCAGTGCCAAGGTCTGAAGGTCACC-3′	65 °C, 25 cycles	290, 380
TauE9 > E13	5′-CATGGCAGACCTGAAGAATGTCAAG-3′ 5′-TCACAAACCCTGCTTGGCCA-3′	65 °C, 35 cycles	580, 487
ARN18S	5′-AAACGGCTACCACATCCAAG-3′ 5′-CGCTCCCAAGATCCAACTAC-3′	58 °C, 15 cycles	250

FluoroImager scanner (Claravision, France) and quantified using MultiGauge software (Fujifilm). Statistic analyses of a minimum of three individual experiments were calculated using Prism Software and a Mann–Whitney *U*-Test.

3. Results

Several human cell lines were analyzed in order to identify two distinct splicing patterns of Tau: one corresponding to the adult pattern and the second corresponding to the foetal profile of Tau splicing. The endogenous Tau splicing pattern was analyzed in nervous system derived human cells lines T98 and SY5Y cells. Using the Tau E1/E13 primer pair (Table 1), we observed four bands in T98 cell RNAs with a size corresponding to the four expected bands, if all 6 adult isoforms of Tau are expressed (1200 bp, ±1100 bp, ±1000 bp, and 933 bp) (Fig. 1A). These bands were cloned and sequenced to confirm the identity of the isoforms of Tau (not shown). In SY5Y cells, a major band corresponding to the foetal isoform of Tau, without exons 2, 3 and 10, was mainly observed. A minor band, corresponding to the isoform of Tau with only one exon (exon 2 or 10), was also faintly detected. RT-PCR analysis with Tau E2/E3 primers confirmed that exon 2 of Tau was preferentially included in T98 cells and excluded in SY5Y cells (Fig. 1B). Using Tau E9/E13 primer pair to analyze the splicing of exon 10, we detected a higher inclusion of exon 10 in T98 (33.2 ± 6.5%) than in SY5Y (17.9 ± 3.8%) cells (Fig. 1C and E). Thus, sequencing of the bands amplified with Tau E1/E13 primer pair as well as the separated analysis of exons 2/3 (Tau E1/E4 primer pair) and 10 (Tau E9/E13 primer pair) are in favour of a splicing pattern of Tau in T98 cells similar to an adult brain pattern, whereas in SY5Y, Tau splicing pattern is similar to a foetal one, as quantified on Fig. 1E.

In order to determine whether the RNA cis-regulating element required to reproduce the foetal pattern of Tau splicing is similar or distinct from the one implicated in the trans-dominant effect of CUG repeats, we used Tau minigenes (Fig. 2B). T98 and SY5Y cells were transfected with minigenes Tau 2/3 or Tau 2Δ3 alone. The dominant pattern was the inclusion of Tau exon 2 in both cell lines (Fig. 2C). In T98 cells, the splicing pattern of Tau minigenes is comparable to the endogenous one. In sharp contrast, we observed an alternative splicing of Tau exons 2 or 2/3 from minigenes in SY5Y cells. Indeed, Tau exon 2 is mainly included in SY5Y using Tau 2/3 and Tau 2Δ3 minigenes (Fig. 2C: 12.2 ± 0.4% and 32.2 ± 3.0% of Tau exon 2 exclusion, respectively) that is strikingly different from endogenous repression of Tau exon 2 inclusion (more than 90% exclusion). Therefore, the cis-regulating elements of Tau minigenes are sufficient to reproduce the adult pattern of Tau splicing but intronic splicing silencers necessary to reproduce the foetal splicing pattern of Tau are likely missing since the inclusion of Tau 2 is not completely repressed in SY5Y. If the cis-regulating element for the trans-dominant effect of CUG repeats and foetal pattern of exon 2 splicing is the same, then Tau minigenes should not respond to CUG repeats. Therefore, we took advantage of the differences between T98 and SY5Y cell lines to analyze the effects of CUG repeats (n = 5, 200 or 960) on Tau exon 2 splicing from both endogenous or minigene transcripts.

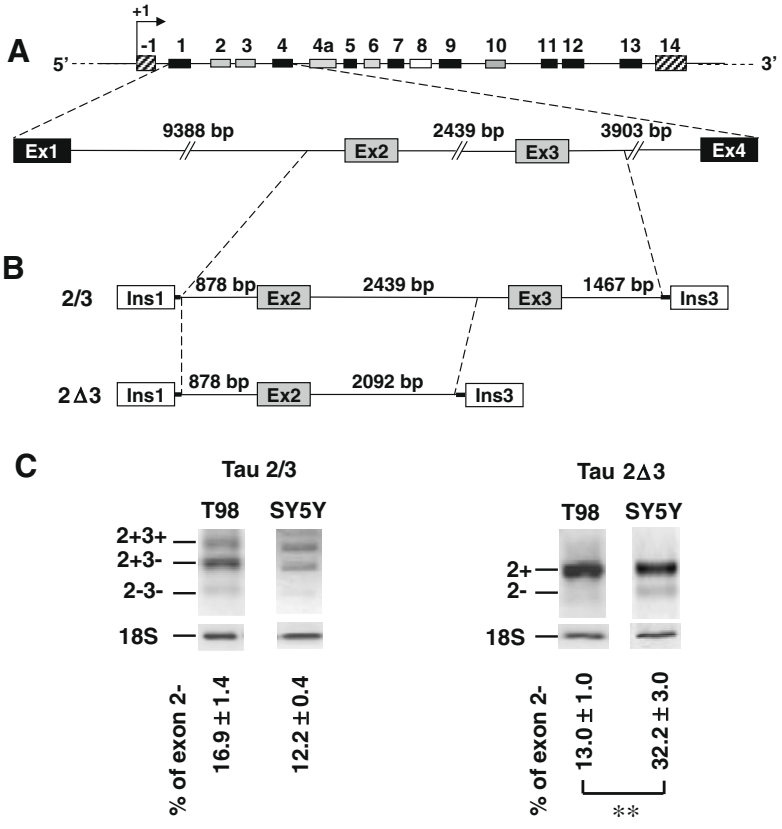

Fig. 2. Structure of the human *MAPT* gene and minigenes. (A) Schematic representation of the human *MAPT* gene with the untranslated exons in hacked squares, the constitutive ones in black squares, the alternatively spliced ones in grey and the exon 8 which is always skipped is in white. Lines represent intronic regions. Note that the scale of the *MAPT* exons and introns is artificial. *MAPT* gene is constituted of 16 exons among which 5 are being alternatively spliced. Exons 2, 3 and 10 are adult-specific exons in the human central nervous system. In order to compare the *MAPT* sequence to that of Tau minigenes, we have represented an enlargement of the *MAPT* exons 1–4 and their surrounding introns. (B) Schematic representation of Tau minigenes: Tau 2/3 and Tau 2Δ3. Rat insulin exonic and intronic sequences of the parental vector are, respectively, represented with white squares and bold lines. The length of the intronic sequences surrounding exons 2 and 3 in the human gene and minigenes are indicated above. (C) Splicing of Tau 2/3 and Tau 2Δ3 minigenes in T98 and SY5Y cell lines. Identity of the bands amplified by RT-PCR using Ins1 and Ins3 primer pair are indicated (**, $P < 0.01$).

As observed in Fig. 3A, transfection with 960 CTG repeats induced a significant increase of the exclusion of the endogenous Tau exon 2 (from 26.1 ± 2.3% to 40 ± 5.2%, $P < 0.01$) in T98 cells. Noticeably, 200 CTG moderately modified the inclusion of Tau exon 2 when compared to the normal CTG allele bearing 5 CTG repeats (Fig. 3A). In SY5Y, endogenous exon 2 exclusion was not further enhanced by CTG repeats, suggesting that the repression of exon 2 inclusion was maximum in this cell line. In contrast, the CTG 960 repeats induced a clear and significantly increased exon 2 exclusion in both T98 and SY5Y when these cell lines were co-transfected with Tau 2Δ3 or Tau 2/3 minigenes (Fig. 3B and C). Noticeably, 200 CTG repeats lightly but significantly modified the exclusion of Tau exon 2 from 2Δ3 and 2/3 minigene transcripts in SY5Y (Fig. 3B and C). Our results demonstrated that inclusion of exon 2 in minigene transcripts could be repressed by long CUG tracks in both cell lines, and contrast with the endogenous splicing pattern of Tau. We also verified that 3′UTR of *DMPK* constructs bearing the CTG repeats were overexpressed at the same level by performing a RT-PCR of the region just downstream the repeats (data not shown). Altogether, our results obtained using Tau minigenes show that the *cis*-regulating element(s) necessary to produce the foetal Tau isoform are dispensable for the *trans*-dominant effect of CUG repeats.

4. Discussion

In this study, the question addressed, as far as Tau splicing is concerned, was to determine whether the modified splicing of

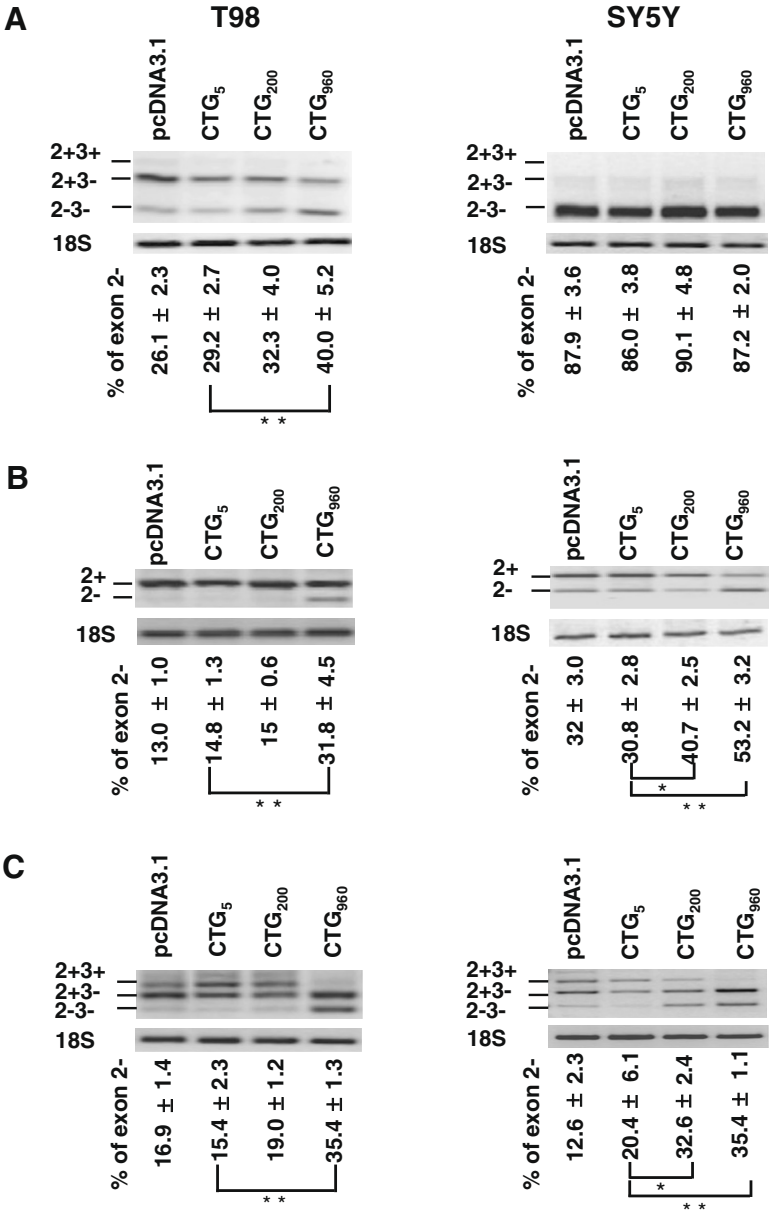

Fig. 3. Effect of CUG repeats expression on the alternative splicing of exon 2 or exons 2/3 of Tau. (A) Analysis of endogenous Tau splicing in both T98 and SY5Y cell lines transfected with pcDNA3.1 empty vector or pcDNA containing 5, 200 or 960 CTG repeats as indicated above the lanes. PCR were performed using Tau E1/E4 primers. (B) and (C) Analysis of minigene splicing after co-transfection of the CTG plasmids with Tau 2Δ3 or Tau 2/3 minigenes, respectively. The plasmids were transfected with a 1:1 ratio. Minigene splicing was analyzed by RT-PCR using Ins1/Ins3 primers. CTG 960 repeats favoured the exclusion of exon 2 from both minigenes in both cells. PCR products were quantified as detailed in materials and methods. The number underneath each lane indicates the percentage of Tau exon 2 exclusion (averaged ± S.E.M. for at least three independent experiment). Significant differences are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$.

Tau observed in DM1 implies a developmental or a disease-associated mechanism. The congenital form of DM1, which is the most severe expression of the disease, is certainly resulting from a defective development of several organs, including the brain. In the adult form, the development is "normal" and the onset and severity of the disease depend upon the length of CUG repeats in RNAs translated by the mutated *DMPK* gene. The dynamic instability and mosaicism of the mutation should also be considered as important promoting factors. Nevertheless, long CUG repeats induce a modified splicing of Tau leading to the greatly increased expression of the foetal isoform [10,11]. The defective splicing of Tau affects the three adult cassettes consisting of exons 2, 3 and 10 [8,10–11]. Herein, we focused on Tau exons 2 and 3 as we previously showed that the modified splicing of those exons was reproduced in presence of long CUG tracks [11].

Alternative splicing implies cis- (RNA) and trans-regulating (proteins) elements. In regard to Tau, several repressing factors of the inclusion of Tau exon 2 have been characterized, such as ETR-3 and MBNL1 [9,11,16]. However, less is known about the cis-regulating elements and more particularly about the intronic cis-regulating sequences. The Tau exon 2 default pattern of splicing is the inclusion [13]. Therefore, the skipping of this exon is probably due to a trans-repressing factor that recognizes an intronic splicing silencer element. Our results are in favour of such a hypothesis. In SY5Y cells, the endogenous exon 2 and exon 3 are mainly excluded whereas they are mainly included when using Tau minigenes. In contrast, exon 2 and exon 3 were mainly included in endogenous Tau transcripts of T98 cells. There is one weak exonic splicing silencer in exon 2 and one intronic splicing silencer in the 3′ region of intron 2–3 [17]. As these two elements are present in both endogenous *MAPT* and Tau minigenes, the difference of Tau splicing pattern is likely not mediated via those regulatory elements. Another possibility would be that an intronic repressing element, yet unidentified, is present in the intron 1–2 sequence. The intron 1–2 is shorter in the minigene than in *MAPT*. The missing sequence may contain one or several intronic splicing silencers that are essential to produce the foetal isoform of Tau expressed in SY5Y cells. In regards to DM1 pathophysiology, if the modified splicing pattern of Tau results from an adult to foetal reprogramming of the splicing machinery, the intronic splicing silencer necessary to produce the foetal Tau isoform in SY5Y may also be the one targeted by the trans-dominant effect of CUG repeats. To determine whether those sequences were also necessary to mediate the trans-dominant effect of CUG repeats, the minigenes Tau 2Δ3 and 2/3 were co-expressed with 5, 200 or 960 CTG repeats in both T98 and SY5Y. Inclusion of the exon 2 of Tau from the minigenes was strongly repressed by CTG 960 demonstrating that there is a cis-repressing element in Tau minigenes. Moreover, this repressing sequence implies an inducible mechanism that is not functional by default. Thus, the repression of Tau exon 2 inclusion is only activated following transfection of CTG 960 and not observed using minigenes alone. Our results demonstrate that the sequence targeted by the *trans*-splicing effect of CTG repeats is necessarily located in the minigenes. A foetal Tau isoform is expressed in SY5Y. However, Tau minigenes are lacking the cis-regulating elements necessary to reproduce this foetal splicing. In contrast, Tau minigenes have a cis-repressing element sensitive to CTG repeats, which together suggest that the latter splicing mechanism is a disease-associated mechanism. This mechanism is likely independent of the foetal splicing process, as far as Tau exon 2/3 splicing is concerned.

In conclusion, our data strongly suggest that the mechanism leading to a defective inclusion of exons 2 and 3 in DM1 is likely different from the one taking place during development. Therefore, we propose that the modified splicing of Tau in DM1 may not be the consequence of an adult to foetal reprogramming of the splicing machinery. A disease-associated mechanism would be induced by CUG repeats in mutated DMPK RNAs. Our results are important in a therapeutic point of view since molecules that would restore the normal splicing of Tau may not interfere with the mechanisms implicated to generate the Tau adult isoforms. Our data strongly suggest that both mechanisms are independent.

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