Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1762 (2006) 460–467

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

http://www.elsevier.com/locate/bba

Brain-specific change in alternative splicing of Tau exon 6 in myotonic dystrophy type 1

Olivier Leroy [a], Junning Wang [b], Claude-Alain Maurage [a], Michel Parent [c], Thomas Cooper [d], Luc Buée [a], Nicolas Sergeant [a], Athena Andreadis [b], Marie-Laure Caillet-Boudin [a,*]

[a] *INSERM U422, Pl. de Verdun. 59045 Lille Cedex- France*
[b] *Shriver Center 200 Trapelo Road Waltham, MA 02452-6319, USA*
[c] *Anatomie et cytologie pathologiques, 153 quai Alsace 59500 Douai-France*
[d] *Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA*

Received 16 September 2005; received in revised form 21 November 2005; accepted 1 December 2005
Available online 29 December 2005

Abstract

Alternative splicing is altered in myotonic dystrophy of type 1 (DM1), a syndrome caused by an increase of CTG triplet repeats in the 3′ untranslated region of the *myotonic dystrophy protein kinase* gene. Previously, we reported the preferential skipping of Tau exon 2 in DM1 brains. In this study, we analyze the alternative splicing of Tau exon 6 which can be inserted in three different forms (c, p and d) depending on the 3′ splice site used. In fact, inclusion of exon 6c decreases in DM1 brains compared to control brains whereas inclusion of 6d increases. Alteration of exon 6 splicing was not observed in DM1 muscle although this exon was inserted in RNAs from normal muscle and DM1 splicing alterations were first described in this organ. In contrast, alteration of exon 2 of Tau mRNA was observed in both muscle and brain. However, co-transfections of a minigene containing exon 6 with CELF or MBNL1 cDNAs, two splicing factor families suspected to be involved in DM1, showed that they influence exon 6 splicing. Altogether, these results show the importance of determining all the exons and organs targeted by mis-splicing to determine the dysregulation mechanisms of mis-splicing in DM1.
Published by Elsevier B.V.

Keywords: Tauopathy; Myotonic dystrophy of type 1; Microtubule-associated Tau; Alternative splicing; Muscle; Brain

1. Introduction

Myotonic dystrophy of type 1 (DM1) is a multisystemic disorder characterized by myotonia, progressive weakness, wasting of skeletal muscle and extramuscular symptoms such as cataracts, insulin resistance, testicular atrophy, endocrine dysfunction and mental impairment. The brain-related symptoms include mental retardation, sleep disorders, behavioral changes, alterations in brain structure and cognitive defects [1–6]. 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*) [7]. DMPK is widely expressed, in particular in skeletal muscle and heart but also in brain [8,9]. In DM1, instead of being exported to the cytoplasm, the mutant DMPK mRNA is retained in multiple discrete foci within the nucleus [10]. The clinical features are likely caused by an RNA mechanism in which the CUG repeats alter cellular functions, including alternative splicing of various transcripts, among them cardiac troponin T (cTNT), insulin receptor (IR), muscle-specific chloride channel (ClC-1) and Tau [11–16].

Members of two protein families, CELF (CUG-BP and ETR3-like factors) and MBNL (muscleblind-like), are suspected to play a role in the misregulation of alternative splicing observed in DM1. CELF proteins are CUG-binding proteins that regulate alternative splicing [17]. MBNL proteins also regulate splicing, apparently as antagonists to the CELF proteins [18]. CUG-BP1 levels increase in DM1 muscle [13,19], whereas MBNL1 protein localizes to the DM1 foci [16,20,21]. The data are consistent with a mechanism for DM pathogenesis in which the expanded repeats cause a loss of MBNL function by sequestration and/or a gain of CELF activity, leading to the misregulation of alternative splicing of specific pre-mRNA targets [22].

* Corresponding author. Tel.: +33 3 20 62 20 73; fax: +33 3 20 62 20 79.
E-mail address: caillet@lille.inserm.fr (M.-L. Caillet-Boudin).

0925-4439/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbadis.2005.12.003

Tau alternative splicing is altered in brains of DM1 patients [15,16]. In addition to this change, DM1 brains show neurofibrillary degeneration (NFD) made of the intraneuronal aggregation of hyperphosphorylated Tau proteins [15,23]. These two pathological entities are common characteristics of a subset of neurodegenerative diseases called Tauopathies [24].

Tau expression is regulated by complex alternative splicing [25]. The adult human central nervous system mainly expresses six Tau RNAs that differ from each other by the inclusion or exclusion of exons 2, 3 and 10 [26–28]. The inclusion of exon 6, another cassette, was reported in human brain and in non-neuronal cells [29–31]. In vivo, exon 6 is preferentially included in retina, spinal cord, and the peripheral nervous system RNAs which constitutively include exons 2, 3, 4A and 10. For exon 6, three 3′ splice sites were described in humans, one canonical (c) and two sites (p and d) originally considered as cryptic [30,32]. They are used in different tissues, particularly in skeletal muscle and spinal cord [30]. The 6p and 6d variants, if translated, would result in truncated Tau isoforms that lack the microtubule-binding domain, whereas isoform 6c contains this domain. The truncated 6d Tau isoforms have been recently found in several tissues, including brain and muscle [33].

In DM1, an alteration of inclusion of Tau exon 2 and 10 has been reported in the brain tissue [15,16]. Splicing of Tau exon 2 in muscle, another tissue where Tau is expressed, have not been studied in DM1 subjects. Here, we report the pattern of Tau exon 6 splicing in brain and muscle of DM1 patients in comparison to that of Tau exon 2. This is the first report of a change in Tau exon 6 splicing associated with a human pathology and of a tissue-specific DM1 mis-splicing. Our results from co-transfections of COS cells as well as analyses of DM1 brains and glial and neuronal cell lines suggest that the three splice sites of Tau exon 6 are regulated by distinct trans-regulatory factors. The selection of the exon 6 3′ splice sites likely implies some members of MBNL and CELF splicing factor families.

2. Materials and methods

2.1. Brain and muscle tissues

DM1 brain tissue samples were obtained at autopsy of 3 patients aged from 53 to 64 years. These cases were previously described and characterized for Tau exon 2 splicing and CTG repeats [15]. Two control subjects were included. The brain tissue was obtained from Department of Neurology, Lille, France, in accordance with the local ethic committee protocol. Post-mortem delays were <48 h, and all samples were stored at −80 °C. Cortical brain regions (temporal, occipital, frontal and parietal regions) were dissected according to Brodmann's classification.

Skeletal muscles (4 controls and 3 DM1 subjects) were obtained from the Department of Pathological Anatomy, Lille, France and laboratory of Pathological Anatomy, Douai France, in accordance with the local ethics committee. The samples came from biopsies except for one sample, which corresponded to an autopsy of a DM1 patient whose brain was also analyzed.

2.2. Cell culture

NT2 cells were maintained in OptiMEM medium containing 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mmol/l glutamine. NT2 differentiation was performed as described in Ferreira et al. [34] Briefly, cells were treated with 10 μM retinoic acid (RA) (Sigma, St. Louis, MO) added in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine and penicillin–Streptomycin for 4–5 weeks. Afterwards, cells were replated at 1/4 dilution and, 2 days later, treated for two additional weeks with a mitotic inhibitor mix (10 μM 5-fluoro-2′-deoxyuridine, 10 μM uridine, 1 μM cytosine-B-D-Arabinofuranoside). Finally, after a mild trypsinization protocol, the cellular population was enriched in neuronal hNT cells.

Human neuroblastoma SKN-SH SY5Y (SY5Y), astrocytoma CCF-STTG1 (CCF) and glioblastoma T98 cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 4 mmol/l glutamine. SY5Y cells were differentiated by 1μg RA treatment for 2 weeks in serum-free medium (DMEM/Ham's F12, 2 mM glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin, (Sigma), 1× Insulin-transferrin-Selenium (Invitrogen).

Human neuroblastoma Kelly cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mmol/l glutamine as described in Caillet-Boudin et al. [35].

Monkey kidney (COS) and human epithelial (HeLa) cells were maintained in DMEM plus 10% fetal calf serum.

2.3. Constructs and transfections

Construct SV6 consists of a 900-kb insert containing Tau exon 6 placed in the *Eco*RI site of vector SVIRB [30]. The six members of the CELF family (CUG-BP1, ETR3, CELF3, CELF4, CELF5 and CELF6) were placed in pcDNA3.1/His (Invitrogen). MBNL1 isoform 3 in pCMV-SPORT6 (Invitrogen) came from the ATCC cDNA collection and was then placed into pFLAG-CMV-6b (Sigma). Protein expression was verified by using monoclonal antibodies against the FLAG or 6× His epitopes (Sigma) on westerns of crude lysates from cells transfected with each construct. Plasmid DNA was prepared by Qiagen Tip-50s and introduced into COS cells by lipofection (LT1, Mirus) 5 μg of SV6 DNA alone or with 5 μg of factor DNA were introduced into COS cells at ~30% confluence. Transfection assays were performed in COS to allow the comparison with ulterior reports on exon 2 and 3 splicing [30,36–38].

2.4. Reverse transcription and PCR assays

Total RNA was isolated with extraction kits from the following vendors: from brain and muscle, Promega; from untransfected neuronal and glial cells, Macherey-Nagel; and from transfected COS cells, by the TRIzol method (Invitrogen) 48 h post-transfection. Reverse transcription of RNA from human tissues and untransfected neuroblastoma and glioma cells was carried out according to the manufacturer's instructions (superscript one-step RT-PCR kit, Invitrogen) using 1 μg of total RNA. After reverse transcription (30 min at 55 °C), the reaction mixture was heated to 94 °C for 3 min and followed by 30–40 PCR cycles with 30 s at 94 °C, 30 s at the optimal hybridization temperature of each primer pair (see Table 1), 2 min extension at 72 °C. RT-PCR products were resolved on a 2% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide. Cloning and sequencing ascertained the identity of RT-PCR products. The ratio between isoforms was calculated by scanning the bands using a FluoroImager scanner (Claravision, France). The values were normalized to an equal quantity of 18S. For RNA from transfected COS cells, we performed RT-PCR analyses of RNA as previously described [39] for 25 cycles with primer pairs INS1/INS3 (Table 1). The isoform ratio was calculated by scanning the bands from three independent transfections using the One-Dscan program and the Scanalytics IPLab software. The statistical significance of the results was calculated by the one-way ANOVA method using Sigmastat software.

3. Results

3.1. Exon 6 splicing changes in DM1 brain but not in DM1 muscle

We first analyzed splicing of Tau exon 6 in brain from both control and DM1 subjects. Using the E4–E7 primers, we mainly

Table 1
Primer pairs used in RT-PCR analyses

Amplified region	Primers and hybridization temperature (S=sense, A=antisense, all primers written 5′ to 3′)	No. of cycles	Possible amplified products
Tau	S: TACGGGTTGGGGGACAGGAAAGAT	35	286 bp→2+3+
E1–E4	A: GGGGTGTCTCCAATGCCTGCTTCT		199 bp→2+3−
	Hybridization temperature: 65 °C		112 bp→2–3−
Tau	S: GGCTCTGAAACCTCTGATGC	40	443 bp→2+3+6c
E2–E6	A: TTGAGTTTCATCTCCTTTGC		356 bp→2+3−6c
	Hybridization temperature: 56 °C		342 bp→2+3+6p
			273 bp→2+3+6d
			255 bp→2+3−6p
			186 bp→2+3−6d
Tau	S: GAAGACGAAGCTGCTGGTCA	30	278 bp→6c
E4–E6	A: TTGAGTTTCATCTCCTTTGC		177 bp→6p
	Hybridization temperature: 56 °C		108 bp→6d
Tau	S: GAAGACGAAGCTGCTGGTCA	35	411 bp→6c
E4–E7	A: TCTTTGGAGCGGGCGGGGTTTTTG		310 bp→6p
	Hybridization temperature: 56 °C		241 bp→6d
			213 bp→6−
18S	S: AAACGGCTACCACATCCAAG	35	250 bp
	A: CGCTCCCAAGATCCAACTAC		
	Hybridization temperature: 58 °C		
IR	S: CCAAAGACAGACTCTCAGAT	35	167 bp→11+
E10–E12	A: AACATCGCCAAGGGACCTGC		131 bp→11−
	Hybridization temperature: 58 °C		
SV6/exon 6	S: CAGCTACAGTCGGAAACCATCAGCAAGCAG	25	494 bp→6c
INS1–INS3	A: CACCTCCAGTGCCAAGGTCTGAAGGTCACC		393 bp→6p
	Hybridization temperature: 65 °C		324 bp→6d
			296 bp→6−

20 nM of primers were used except for 18S primers for which we used only 2 nM for linear amplification at 35 cycles.

found transcripts without exon 6 in both control and DM1 brains. However, low levels of isoform 6c were observed in control but not in DM1 brains (Fig. 1A). Because Tau 6c, the main form of exon 6 insertion, was poorly expressed in brain (about 8% of total Tau transcripts as seen on the histogram of Fig. 1A), an internal primer was used to detect and quantify the minor forms 6p and 6d together the 6c form. By using primers E4–E6, we confirmed the decrease of exon 6c in frontal (F), temporal (T), parietal (P) and occipital (O) lobes of DM1 brains (Fig. 1B). This decrease was counterbalanced by an increase of 6d inclusion. The parallel analysis of exons 2, 3 and 6 using Tau E2–E6 primer pair clearly showed a decrease in both exon 2 and exon 6c, reflecting the decrease observed by a separate amplification of each corresponding region (Fig. 1B, C, D). Modification of the exon 6 splicing pattern in DM1 brains was not due to a difference in RNA quantity, as shown by the amplification of 18S RNA.

When we analyzed splicing of Tau exon 6 in skeletal muscle, no decrease in exon 6c or increase in 6d was observed in DM1 muscle compared to control whatever the primer pair used (Fig. 2A). This implies that changes in exon 6 splicing which occur in DM1 is brain specific. In contrast to exon 6, exon 2 inclusion decreases in DM1 skeletal muscle (Fig. 2B). Indeed, the forms without exons 2 and 3 are hardly detected in control muscle but are clearly visible in DM1 samples. As a control of splicing alterations in DM1 muscle samples, we amplified IR exon 11 (Fig. 2C). As reported elsewhere, insertion of IR exon 11 is much rarer in DM1 than in control muscle.

Therefore, the DM1 pathological change of Tau alternative splicing affects exons 2 and 6 in brain but only exon 2 in skeletal muscle.

3.2. Analysis of exon 6 splicing in neuronal and glial cells

The default in alternative splicing observed for several transcripts in DM1 results either in nonfunctional truncated proteins due to a frame shift, as described for ClC-1 [14] or in a preferential synthesis of the fetal form when the exon splicing is developmentally regulated, as described for cTNT, IR and Tau [11,13,15,16]. Interestingly, Tau exon 6 is a particular case. Indeed, Tau exon 6 is present in both fetal and adult brain and spliced isoforms resulted from the selection of three 3′ splice sites, two of which lead to a frame shift. To dissect the cellular events that regulate the splicing of Tau exon 6, we investigated the influence of cellular type and neuronal differentiation on the selection of the three splice sites of exon 6.

Since glial cells are the most abundant cell type in brain and neurons are the cells where nuclear foci mainly accumulate in DM1, we analyzed cell lines of glial (CCF-STTG1 and T98) and neuronal (neuroblastoma Kelly and SK-N-SH SY5Y, teratocarcinoma NT2) origin. HeLa cells were used as a cell line that do not originate from the nervous system. For the second part, we analyzed pro-neuronal cell lines (SY5Y and NT2) before and after differentiation with RA.

Primer pair E4–E7 detects two main bands in glial and HeLa cells, which correspond to products 6c and 6- (Fig. 3A). As shown by the histogram (Fig. 3A), the 6c form is less abundant

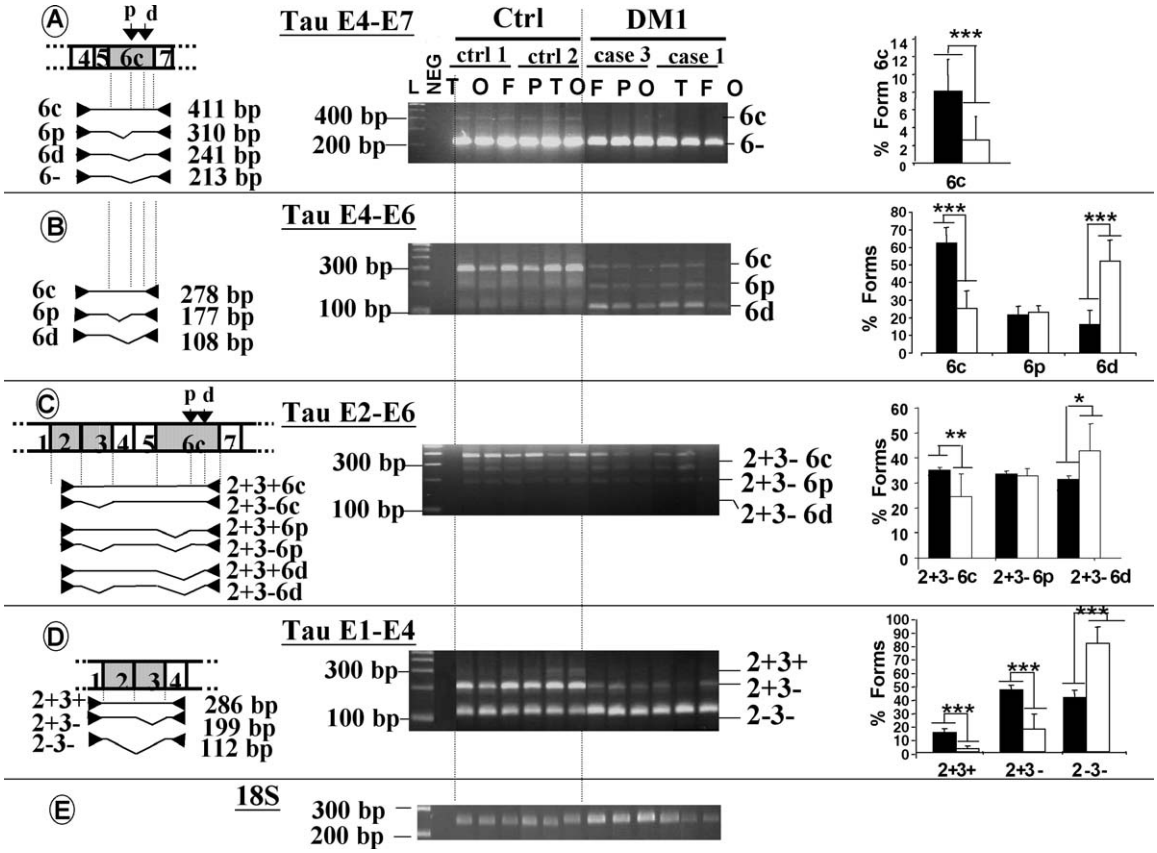

Fig. 1. Inclusion of Tau alternative exons 2 and 6 decreases in DM1 brain. The inclusion levels of the two exons were compared in control (Ctrl) and DM1 brain samples using Tau primer pairs (A) E4–E7, (B) E4–E6, (C) E2–E6 and (D) E1–E4. (E) Amplified 18S, as control for RNA quantity. At the left of each panel, a diagram shows the identity of the bands amplified by the primer pair indicated. We show the analysis of several areas (T: temporal, F: frontal, O: occipital, P: parietal) from two control (N1 and N2) and two DM1 patients (case 1 and case 3 correspond to the same patients yet described in Sergeant et al. [14], case 2 analyses (not shown) gave similar results). Tau mRNA without exon 6 is the predominant isoform in brain as seen in panel A. However, species 6c, 6p and 6d are clearly seen in panels B and C. Inclusion of 6c decreases in DM1 brains for which the ratio shifts from 6c toward 6d. Exon 2 is also decreased in DM1 (panels C and D) as first reported in Sergeant et al. [14]. At the right of each panel, an histogram illustrates the ratio of the different amplified bands, calculated for the pool of brain areas from the 3 patients and 2 controls. The values were normalized to an equal quantity of 18S. The statistical significance of the results is indicated by $*P<0.05$, $**P<0.01$; $***P<0.001$. Neg: RT-PCR in absence of RNAs. L: DNA Ladder.

in neuronal cells compared to non-neuronal cells, the 6- form being the main amplified band. We also searched for use of splice sites 6p and 6d with primer pair E4–E6. 6p and 6d are hardly detected in glial and HeLa cells. These forms were better amplified in neuronal cells, after differentiation with RA (Fig. 3B). In RA-treated cells, the decrease in 6c levels correlates with a concurrent increase of the 6p and 6d forms. Hence, neuronal differentiation strengthens the differences observed in Tau exon 6 splicing between neuronal cells and non-neuronal cells (glial and HeLa cells): a decrease of 6c form, an increase of 6p and 6d forms.

3.3. Trans-regulatory factors that are implicated in DM1 regulate the splicing of Tau exon 6

Some members of the MBNL1 and CELF protein families regulate the splicing of several exons such as cTNT exon 5, IR exon 11 whose splicing is altered in DM1 [22,25]. It is not known whether those trans-regulatory factors regulate the splicing of Tau exon 6. We addressed this question by co-transfecting COS cells with MBNL1 or CELF family members and with an exon 6 minigene (Fig. 4). CELF5 and CELF6 modestly but significantly increase 6c and 6- at the expense of 6p, the latter more so than the former. The other five factors (CUG-BP1, ETR3, CELF3, CELF4 and MBNL1) leave the ratio between exon 6 isoforms practically unchanged.

4. Discussion

4.1. Splicing of Tau exon 6 is altered in DM1 brains and not in muscles

A deregulation in splicing occurs in DM1 resulting in splicing shifts in several transcripts. In particular, mis-splicing of Tau exon 2 and 10 have been reported in DM1 brains [15,16]. Up to date, no report has been published about Tau exon 6 although it is the fourth exon possibly inserted in cerebral Tau mRNAs. Here, we report Tau exon 6 as a new target of splicing deregulation in DM1 brain. This is the first report about a change in splicing of Tau exon 6 in a human pathology. Exon 6c, the main form of the exon 6 in normal

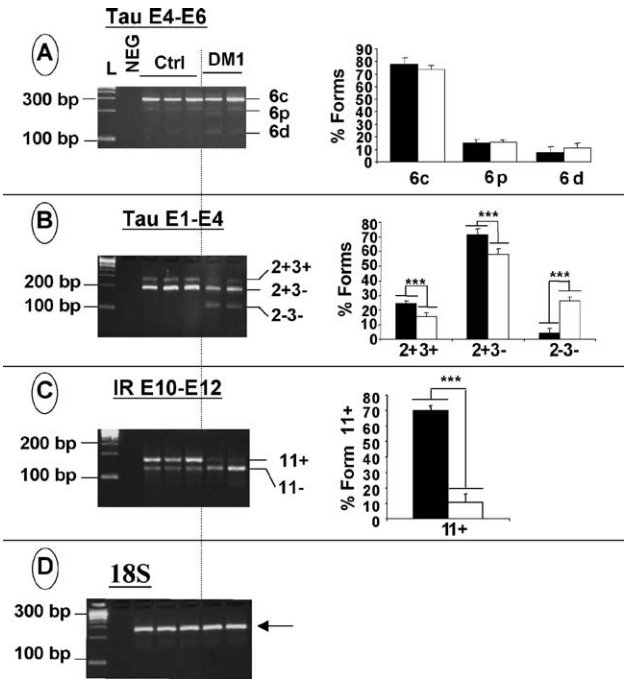

Fig. 2. Analysis of Tau splicing in control and DM1 muscles. The inclusion levels of Tau exons 2 and 6c were analyzed in 4 control and 3 DM1 muscle samples using primer pairs (A) Tau E4–E6, (B) Tau E1–E4 and compared to IR exon 11 splicing using (C) IR E10–E12. The gels show the results from 3 control and 2 DM but histograms were calculated for the 4 control and 3 DM1 samples.(A) No change is seen in relative ratios of Tau exon 6 splicing products. (B, C) Both Tau exon 2 and IR exon 11 are suppressed in DM1 muscle. (D) Amplified 18S, as control for RNA quantity. The values were normalized to an equal quantity of 18S. The statistical significance of the results is indicated by $*P<0.05$, $**P<0.01$; $***P<0.001$. Neg: RT-PCR in absence of RNAs. L: DNA Ladder.

brain [30], is preferentially skipped in DM1 brains compared to control ones. In contrast, the 6d form increases.

Tau is expressed in several tissues, especially in brain and skeletal muscle, two organs affected by splicing deregulation in DM1. Therefore, Tau RNAs were good candidates to determine if the mis-splicing is tissue specific or similar in both tissues. In fact, the tissue-specificity was dependent on the exon analyzed. The shift in exon 6 splicing that we observed in DM1 brain does not occur in DM1 muscle. Thus, the DM1 alteration of exon 6 splicing is likely brain-specific. In contrast, the preferential exclusion of Tau exon 2 is clearly detected in both DM1 brain and muscle [15,16, this paper]. Note that this splicing alteration of exon 2 in DM1 muscle is intriguing, since Tau aggregates resembling tangles are found in inclusion body myositis [40,41].

Our results agree with the fact that Tau regulated exons are known to be independently regulated in terms of both their expression profile and the details of their regulation [25]. For instance, Tau exon 2 is absent from fetal brain but constitutes ~50% of Tau protein in adult brain and is constitutively included in skeletal muscle. Tau exon 6 is present in fetal brain, whereas it is a minor isoform in adult brain and becomes prevalent in muscle. In contrast to exon 2, the splicing of Tau exon 6 is influenced by the flanking exons [30,42, this work]. Finally, exon 2 is regulated primarily by exonic and intronic silencers and their associated trans-regulatory inhibitors [37,39] but exon 6 may be primarily regulated by branch point selection and factor PTB [32]. Therefore, exons 2 and 6 are regulated by a different set of cis- and trans-regulatory elements. Altogether, these results suggest that several trans-regulatory factors are likely altered in DM1.

4.2. Splicing regulation of Tau exon 6

Several non-exclusive hypotheses could explain the tissue specificity of mis-splicing exon 6c as compared to exon 2. Indeed, the splicing factors involved could be exon-specific and/or tissue/cell-specific. The endogenous splicing pattern of Tau exon 2 and 6, and the effect of overexpression of trans-regulatory factors on Tau exon 6 splicing were analyzed in several cell lines.

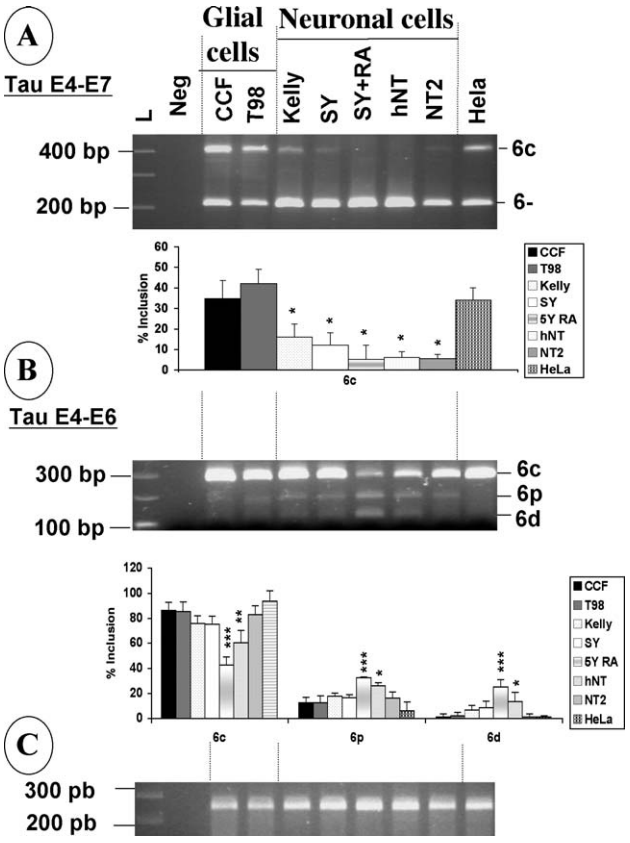

Fig. 3. Tau exon 6 splicing from endogenous transcripts differs between cell lines of non-neuronal and neuronal origin. RT-PCR analysis of cellular RNAs from two glial, three neuronal and HeLa cell lines, using primer pairs (A) E4–E7 and (B) E4–E6. (A) All neuronal cells express a significantly lower level of form 6c versus form 6- compared to glial and HeLa cells. (B) There is a significant decrease in 6c and an increase in 6p and 6d forms in differentiated neuronal cells (SY 5Y+RA and hNT) compared to other cell lines. (C) Amplified 18S, as control for RNA quantity. Histograms illustrate the ratio of the different amplified bands for each cell line, calculated by scanning the bands from at least three independent experiments. Notes statistically significant change with $*P<0.05$; $***P<0.001$.

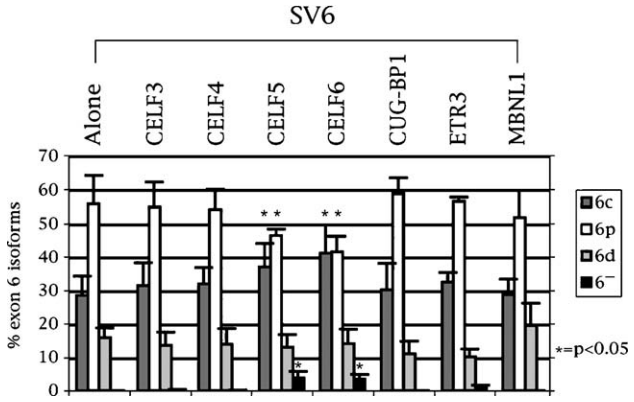

Fig. 4. CELF and MBNL1 proteins influence splicing of exon 6. The RT-PCR products come from 1:1 co-transfections of construct SV6 with the indicated factors in COS cells. The identities of the spliced species are indicated as the % of exon inclusion that was calculated by scanning the bands from three independent transfection experiments and measuring their areas using the OneDscan analysis program. Primer pair: INS1/INS3 (Table 1). Significant differences are indicated.

Our data support the hypothesis of exon-specific mechanism. Indeed, the insertion of the exon 2 and exon 6 in cellular model may be decreased by the overexpression of distinct CELF or MBNL proteins. For instance, CELF-4 favors the insertion of Tau exon 2 [37, Andreadis, unpublished results) whereas CELF -5 and -6 act on exon 6 splicing [this paper]. Then, our data suggest that DM1 splicing change of several exons (exons 2/3 and 6) on a same transcript could result from different altered processes. However, none of the CELF factors or MBNL1 seems to induce a decrease of exon 6c inclusion or an increase of 6d, the major splicing changes observed in DM1 brains. In contrast, the two most effective factors, CELF5 and CELF6, favor the complete inclusion (form 6c) or exclusion of exon 6 (form 6-) at the expense of 6p. The antagonistic effect of the CELF proteins versus MBNL1 for exon 6c splicing was not observed, as described for cTNT and IR transcripts, two other RNA targets of splicing alteration in DM1 [18].

The hypothesis of tissue-specific factor(s) agrees with a spatially and temporally controlled expression of various splicing factors [43]. The major mechanism supposed to underlie the overall splicing alterations in DM1 supposes a loss of function of MBNL that is recruited within the nuclear foci. Although differences in MBNL expression and loss-of-function due to ribonucleoprotein sequestration do not explain the distinct results observed for exon 6 in brain and muscle, MBNL may still play an important role in the control of exon 6 alternative splicing. One can imagine that additional tissue-specific trans-acting factors are also likely to be involved in the regulation of exon 6 alternative splicing in the brain and skeletal muscle, thereby explaining the differences detected.

CELF family is the second splicing factor family suspected to play a role in DM1 splicing alteration. CELF-4 factor, active on exon 2 splicing, is expressed in both muscle and brain whereas CELF-5 and -6, active on exon 6 splicing, are only expressed in brain [17,21,44]. Furthermore, each of these factors exists under various alternative splice forms and the spatial distribution and specific role of each isoform must be considered.

At last, tissues are composed by an heterogenous cell population and the splicing analysis of tissues then correspond to a global analysis of splicing from of all cell types. The analysis of homogenous cell cultures show that different cellular types have their proper splicing pattern of exon 6 inclusion. Neuronal cells express a lower level of 6c RNA compared to 6- form than in non-neuronal cells. Neuronal cells also show higher ratios of 6p and 6d inclusion compared to glial cells. Interestingly, inclusion of 6p and 6d at the expense of 6c expression becomes more pronounced after neuronal differentiation with RA. This shift could have repercussions on Tau function, since the use of 6p or 6d splice sites creates frame shifts that produce truncated Tau proteins which lack the microtubule-binding domain [30,33]. Furthermore, the simultaneous expression of the three possible forms of exon 6 mainly in the differentiated neuronal cells as compared to the non-neuronal cells also agrees with an in vivo exon 6 splicing deregulation in neuronal cells, cells mainly affected in DM1 brain [15,16,23]. These data also support the hypothesis of a connection between expression of exon 6 and neuronal differentiation [33,45]. According to Luo et al. [45], exon 6c inhibits neurite extension in RA-differentiated SY5Y cells, whereas exon 6d is compatible with neuronal differentiation and neurite extension [33]. A low level of the 6c isoforms may be needed during the neuronal differentiation process, but higher levels of 6c may be needed after the final differentiation has been reached. This is consistent with the fact that isoform 6c is the most abundant exon 6 product in control adult brain. We are now investigating if the decrease of 6c in DM1 brain corresponds to an attempt at neuronal regeneration. Such a recapitulation of a differentiation program has been described for DM1 muscle. Indeed, DM1 myoblasts fail to permanently withdraw from the cell cycle when stimulated to differentiate [46].

5. Conclusion

In conclusion, these data support that tau splicing is tissue and cell-specific. It is highly regulated during development and cell differentiation. Any modification towards this highly regulated mechanism is likely to be deleterious to several neurological disorders, the so-called tauopathies. In DM1, both exons 2 and 6 are mis-regulated although the latter is observed only in the brain tissue. Overall our data, associated with recently published results, suggest that several trans-regulatory elements are likely misregulated in DM1 and a better fundamental knowledge of the mechanisms regulating the tau exon 6 splicing could enable the identification of such trans-regulatory elements.

Acknowledgements

This work was supported by the Université de Lille II, by the INSERM and CNRS and AFM (French association against

myopathies) to Nicolas Sergeant, by NIH grants PO1 HD05515 to Athena Andreadis and RO1 AR45653 to Tom Cooper. We thank MJ Dupire for technical assistance, P. Vermersch and B. Sablonnière for helpful discussions. OL is a recipient of a scholarship from the French Research Ministry.

References

[1] A. Di Costanzo, F. Di Salle, L. Santoro, A. Tessitore, V. Bonavita, G. Tedeschi, Pattern and significance of white matter abnormalities in myotonic dystrophy type 1: an MRI study, J. Neurol. 249 (2002) 1175–1182.

[2] L. Chang, T. Anderson, O.A. Migneco, K. Boone, C.M. Mehringer, J. Villanueva-Meyer, N. Berman, I. Mena, Cerebral abnormalities in myotonic dystrophy. Cerebral blood flow, magnetic resonance imaging, and neuropsychological tests, Arch. Neurol. 50 (1993) 917–923.

[3] S. Ishii, T. Nishio, N. Sunohara, T. Yoshihara, K. Takemura, K. Hikiji, S. Tsujino, N. Sakuragawa, Small increase in triplet repeat length of cerebellum from patients with myotonic dystrophy, Hum. Genet. 98 (1996) 138–140.

[4] G. Meola, V. Sansone, D. Perani, A. Colleluori, S. Cappa, M. Cotelli, F. Fazio, C.A. Thornton, R.T. Moxley, Reduced cerebral blood flow and impaired visual-spatial function in proximal myotonic myopathy, Neurology 53 (1999) 1042–1050.

[5] G. Meola, V. Sansone, D. Perani, S. Scarone, S. Cappa, C. Dragoni, E. Cattaneo, M. Cotelli, C. Gobbo, F. Fazio, G. Siciliano, M. Mancuso, E. Vitelli, S. Zhang, R. Krahe, R.T. Moxley, Executive dysfunction and avoidant personality trait in myotonic dystrophy type 1 (DM-1) and in proximal myotonic myopathy (PROMM/DM-2), Neuromuscul. Disord. 13 (2003) 813–821.

[6] A. Modoni, G. Silvestri, M.G. Pomponi, F. Mangiola, P.A. Tonali, C. Marra, Characterization of the pattern of cognitive impairment in myotonic dystrophy type 1, Arch. Neurol. 61 (2004) 1943–1947.

[7] J.D. Brook, M.E. McCurrach, H.G. Harley, A.J. Buckler, D. Church, H. Aburatani, K. Hunter, V.P. Stanton, J.P. Thirion, T. Hudson, R. Sohn, B. Zemelman, R.G. Snell, S.A. Rundle, S. Crow, J. Davies, P. Shelbourne, J. Buxton, C. Jones, V. Juvonen, K. Johnson, P.S. Harper, D.J. Shaw, D.E. Housman, Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member, Cell 68 (1992) 799–808.

[8] P.S. Sarkar, J. Han, S. Reddy, In situ hybridization analysis of Dmpk mRNA in adult mouse tissues, Neuromuscul. Disord. 14 (2004) 497–506.

[9] L.T. Lam, Y.C. Pham, T.M. Nguyen, G.E. Morris, Characterization of a monoclonal antibody panel shows that the myotonic dystrophy protein kinase, DMPK, is expressed almost exclusively in muscle and heart, Hum. Mol. Genet. 9 (2000) 2167–2173.

[10] K.L. Taneja, M. McCurrach, M. Schalling, D. Housman, R.H. Singer, Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues, J. Cell. Biol. 128 (1995) 995–1002.

[11] A.V. Philips, L.T. Timchenko, T.A. Cooper, Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy, Science 280 (1998) 737–741.

[12] A. Mankodi, M.P. Takahashi, H. Jiang, C.L. Beck, W.J. Bowers, R.T. Moxley, S.C. Cannon, C.A. Thornton, Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy, Mol. Cell 10 (2002) 35–44.

[13] R.S. Savkur, A.V. Philips, T.A. Cooper, Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy, Nat. Genet. 29 (2001) 40–47.

[14] N. Charlet-B, R.S. Savkur, G. Singh, A.V. Philips, E.A. Grice, T.A. Cooper, Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing, Mol. Cell 10 (2002) 45–53.

[15] N. Sergeant, B. Sablonniere, S. Schraen-Maschke, A. Ghestem, C.A. Maurage, A. Wattez, P. Vermersch, A. Delacourte, Dysregulation of human brain microtubule-associated tau mRNA maturation in myotonic dystrophy type 1, Hum. Mol. Genet. 10 (2001) 2143–2155.

[16] H. Jiang, A. Mankodi, M.S. Swanson, R.T. Moxley, C.A. Thornton, Myotonic dystrophy type 1 is associated with nuclear foci of mutant RNA, sequestration of muscleblind proteins and deregulated alternative splicing in neurons, Hum. Mol. Genet. 13 (2004) 3079–3088.

[17] A.N. Ladd, N. Charlet-B, T.A. Cooper, The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing, Mol. Cell. Biol. 21 (2001) 1285–1296.

[18] T.H. Ho, N. Charlet-B, M.G. Poulos, G. Singh, M.S. Swanson, T.A. Cooper, Muscleblind proteins regulate alternative splicing, EMBO J. 23 (2004) 3103–3112.

[19] N.A. Timchenko, Z.J. Cai, A.L. Welm, S. Reddy, L.T. Aschenko, RNA CUG repeats sequester CUGBP1 and alter protein levels and activity of CUGBP1, J. Biol. Chem. 276 (2001) 7820–7826.

[20] M. Fardaei, K. Larkin, J.D. Brook, M.G. Hamshere, In vivo co-localisation of MBNL protein with DMPK expanded-repeat transcripts, Nucleic Acids Res. 29 (2001) 2766–2771.

[21] M. Fardaei, M.T. Rogers, H.M. Thorpe, M.G. Hamshere, P.S. Harper, J.D. Brook, Three proteins, MBNL, MBLL and MBXL, co-localize in vivo with nuclear foci of expanded-repeat transcripts in DM1 and DM2 cells, Hum. Mol. Genet. 11 (2002) 805–814.

[22] J.W. Day, L.P. Ranum, RNA pathogenesis of the myotonic dystrophies, Neuromuscul. Disord. 15 (2005) 5–16.

[23] P. Vermersch, N. Sergeant, M.M. Ruchoux, H. Hofmann-Radvanyi, A. Wattez, H. Petit, P. Dewailly, A. Delacourte, Specific tau variants in the brains of patients with myotonic dystrophy, Neurology 47 (1996) 711–7117.

[24] N. Sergeant, A. Delacourte, L. Buee, Tau protein as a differential biomarker of tauopathies, Biochim. Biophys. Acta 1739 (2005) 179–197.

[25] A. Andreadis, Tau gene alternative splicing: expression patterns, regulation and modulation of function in normal brain and neurodegenerative diseases, Biochim. Biophys. Acta 1739 (2005) 91–103.

[26] M. Goedert, C.M. Wischik, R.A. Crowther, J.E. Walker, A. Klug, Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated Tau protein, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 4051–4055.

[27] M. Goedert, M.G. Spillantini, R. Jakes, D. Rutherford, R.A. Crowther, Multiple isoforms of human microtubule-associated protein Tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease, Neuron 3 (1989) 519–526.

[28] M. Goedert, M.G. Spillantini, M.C. Potier, J. Ulrich, R.A. Crowther, Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein Tau containing four tandem repeats: differential expression of Tau protein mRNAs in human brain, EMBO J. 8 (1989) 393–399.

[29] C. Mavilia, D. Couchie, J. Nunez, Diversity of high-molecular-weight tau proteins in different regions of the nervous system, J. Neurochem. 63 (1994) 2300–2306.

[30] M.L. Wei, A. Andreadis, Splicing of regulated exon reveals additional complexity in the axonal microtubule-associated protein Tau, J. Neurochem. 70 (1998) 1346–1356.

[31] M.T. Vanier, P. Neuville, L. Michalik, J.F. Launay, Expression of specific tau exons in normal and tumoral pancreatic acinar cells, J. Cell. Sci. 111 (1998) 1419–1432.

[32] M.L. Wei, J. Memmott, G. Screaton, A. Andreadis, The splicing determinants of a regulated exon in the axonal MAP tau reside within the exon and in its upstream intron, Mol. Brain Res. 80 (2000) 207–218.

[33] M.H. Luo, S.W. Tse, J. Memmott, A. Andreadis, Novel isoforms of tau that lack the microtubule-binding domain, J. Neurochem. 90 (2004) 340–351.

[34] S. Ferreira, M.J. Dupire, J. Najib, A. Delacourte, M.L. Caillet-Boudin, Synthesis and regulation of Apolipoprotein E during the differentiation of human neuronal precursor NT2/D1 cells into post-mitotic neurons, Exp. Neurol. 166 (2000) 415–421.

[35] M.L. Caillet-Boudin, L. Dupont-Wallois, C. Soulie, A. Delacourte, Apolipoprotein E and Tau phosphorylation in human neuroblastoma cells, Neurosci. Lett. 250 (1998) 83–86.

[36] M.C. Arikan, J. Memmott, J.A. Broderick, R. Lafyatis, G. Screaton, S.

Stamm, A. Andreadis, Modulation of the membrane-binding projection domain of tau protein: splicing regulation of exon 3, Brain Res. Mol. Brain Res. 101 (2002) 109–121.
[37] K. Li, M.C. Arikan, A. Andreadis, Modulation of the membrane-binding domain of tau protein: splicing regulation of exon 2, Mol. Brain Res. 116 (2003) 94–105.
[38] J. Wang, Q.S. Gao, Y. Wang, R. Lafyatis, S. Stamm, A. Andreadis, Tau exon 10, whose missplicing causes frontotemporal dementia, is regulated by an intricate interplay of cis elements and trans factors, J. Neurochem. 88 (2004) 1078–1090.
[39] Y. Wang, J. Wang, L. Gao, R. Lafyatis, S. Stamm, A. Andreadis, Tau exons 2 and 10, which are misregulated in neurodegenerative diseases, are partly regulated by silencers which bind a complex comprised of SRp30c and SRp55 that either recruits or antagonizes htra2beta1, J. Biol. Chem. 280 (2005) 14230–14239.
[40] V. Askanas, W.K. Engel, Inclusion-body myositis and myopathies: different etiologies, possibly similar pathogenic mechanisms, Curr. Opin. Neurol. 15 (2002) 525–531.
[41] C.A. Maurage, T. Bussiere, N. Sergeant, A. Ghesteem, D. Figarella-Branger, M.M. Ruchoux, J.F. Pellissier, A. Delacourte, Tau aggregates are abnormally phosphorylated in inclusion body myositis and have an immunoelectrophoretic profile distinct from other tauopathies, Neuropathol. Appl. Neurobiol. 30 (2004) 624–634.
[42] A. Andreadis, J.A. Broderick, K.S. Kosik, Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons, Nucleic Acids Res. 23 (1995) 3585–3593.
[43] D.L. Black, Mechanisms of alternative pre-mRNAsplicing, Annu. Rev. Biochem. 72 (2003) 291–336.
[44] A.N. Ladd, N.H. Nguyen, K. Malhotra, T.A. Cooper, CELF6, a member of the CELF family of RNA-binding proteins, regulates muscle-specific splicing enhancer-dependent alternative splicing, J. Biol. Chem. 279 (2004) 17756–17764.
[45] M.H. Luo, M.L. Leski, A. Andreadis, Tau isoforms which contain the domain encoded by exon 6 and their role in neurite elongation, J. Cell. Biochem. 91 (2004) 880–895.
[46] N.A. Timchenko, P. Iakova, Z.J. Cai, J.R. Smith, L.T. Timchenko, Molecular basis for impaired muscle differentiation in myotonic dystrophy, Mol. Cell. Biol. 21 (2001) 6927–6938.