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**DM1 CTG expansions affect insulin receptor isoforms
expression in various tissues of transgenic mice.**

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Abstract

Myotonic dystrophy (DM1) is a dominant autosomal multisystemic disorder caused by the expansion of an unstable CTG trinucleotide repeat in the 3' untranslated region of the *DMPK* gene. Nuclear accumulation of the enlarged CUG-containing *DMPK* transcripts has a deleterious effect on the regulation of alternative splicing of some RNAs and has a central role in causing the symptoms of DM1. In particular, Insulin Receptor (*IR*) mRNA splicing defects have been observed in the muscle of DM1 patients. In this study, we have investigated *IR* splicing in insulin-responsive tissues (i.e. skeletal muscles, adipose tissue, liver) and pancreas and we have studied glucose metabolism in mice carrying the human genomic DM1 region with expanded (>350 CTG) or normal (20 CTG) repeats and in wild-type mice. Mice carrying DM1 expansions displayed a tissue- and age-dependent abnormal regulation of *IR* mRNA splicing in all the tissues that we investigated. Furthermore, these mice showed a basal hyperglycemia and glucose intolerance which disappeared with age. Our findings show that deregulation of *IR* splicing due to the DM1 mutation can occur in different mouse tissues, suggesting that CTG repeat expansions might also result in *IR* misplicing not only in muscles but also in other tissues in DM1 patients.

Keywords: Myotonic dystrophy-Trinucleotide Repeats-Insulin receptor- splicing-
Transgenic mice

Introduction

Myotonic dystrophy type I (DM1) was initially described on the basis of skeletal muscle abnormalities, such as myotonia, weakness and atrophy [1]. It is a complex disorder, involving various systems, and including extramuscular symptoms. Patients often present cardiac conduction defects, cataracts, changes in smooth muscle function, neuropsychiatric disturbances and endocrine abnormalities, such as testicular failure or premature frontal pattern balding in males [1]. Previous studies also demonstrated features of metabolic syndrome, consistent with the four times higher risk of developing type II diabetes of DM1 patients [2]. Clinical investigations have provided evidence of glucose intolerance and hyperinsulinism, resulting from whole-body insulin resistance [3], an increase in fat mass [4] and dyslipidemia [5, 6]. DM1 belongs to the family of diseases caused by unstable repeat expansions. In DM1, these expansions correspond to CTG repeats in the 3' untranslated region of a serine-threonine kinase (DM protein kinase, *DMPK*) gene on chromosome 19 [7-10]. In place of the five through 37 CTG repeats observed in normal individuals [8, 9, 11, 12], CTG arrays are expanded from 50 up to 4000 CTG in DM1 patients. Expansions increase over successive generations, and their size is positively correlated with clinical severity and negatively correlated with age at onset [1]. Furthermore, CTG repeat length increases with age in several tissues, possibly reflecting disease progression. This mutation has various molecular consequences that may account for the complex and progressive multisystemic phenotype described in DM1 [13]. The CTG expansions lead to the retention of mutant *DMPK* RNA in the nucleus, resulting in *DMPK* haploinsufficiency [14, 15], and alter local chromatin structure, thereby affecting the expression of neighboring genes [16, 17]. In addition, the accumulation of large CUG-containing transcripts has a deleterious effect on the regulation of alternative splicing for some RNAs [18] and may titrate transcriptional factors, leading to potential gene expression defects [19]. However, there is growing evidence to suggest that the dominant

toxic effect of expanded *DMPK* transcripts plays a central role in the multisystemic features of DM1 [18]. Direct support for this hypothesis is provided by transgenic mouse models, in which the expression of large CUG repeat expansions accumulating as intranuclear foci leads to the development of certain symptoms characteristic of DM1 [20, 21]. The description of a similar molecular mechanism due to CCTG expansions in another gene (the zinc finger protein 9 gene, *ZNF9*) in myotonic dystrophy type 2 (DM2), which has a number of important clinical features in common with DM1, also strongly supports the hypothesis of RNA-mediated pathogenesis [18]. Nevertheless, despite descriptions of changes in the splicing and function of several genes, not all the clinical manifestations have yet been elucidated.

Previous studies suggested that insulin response defects might play a key role in the metabolic manifestations of DM1, potentially leading to type II diabetes and abnormal muscle protein metabolism [3, 22]. More recent data have suggested that early changes in insulin secretion may be the primary defect, occurring before insulin resistance and glucose intolerance [23, 24]. However, although the sequence of events leading to these metabolic disturbances remains unclear, a possible molecular mechanism for insulin resistance has been proposed. Molecular analyses have shown that the muscles of DM1 patients contain lower than normal levels of insulin receptor (IR) RNA and protein [25] and display abnormal regulation of *insulin receptor* mRNA splicing, associated with a weaker metabolic response in cultured skeletal muscle cells from DM1 patients [26]. By extending these molecular analyses to other insulin-responsive tissues and to pancreas, it may be possible to define more precisely the importance of insulin resistance in the pathogenesis of metabolic syndrome.

We studied IR splicing events and glucose metabolism in transgenic mice carrying the human genomic DM1 region with expanded repeats (>350 CTG, DM300-328) or a normal number of CTG repeats (20 CTG, DM20-954) and in wild-type mice (wt). We

have previously shown that the phenotype observed in our models depends on the level of expanded *DMPK* RNA. Both homozygous and heterozygous transgenic mice with expansions show nuclear mutant *DMPK* RNA retention in myoblasts [21]. However, only homozygous mice (from two independent lines) display muscle and brain abnormalities consistent with a dominant toxic effect of CUG repeat-containing transcripts. In this study, we observed that homozygous transgenic mice carrying >350 CTG displayed an age-dependent abnormal distribution of *IR* mRNA isoforms in various insulin-responsive tissues and in pancreas. Age-dependent abnormal glucose metabolism was also observed in the mice carrying the long repeat. Our results demonstrate that the *trans* effect of expanded *DMPK* affects *IR* expression not only in muscle, but also in other tissues involved in glucose homeostasis.

Materials and Methods

Transgenic mice

Transgenic mice were produced and genotyped as previously described [21]. For all investigations, we compared mice carrying >350 CTG repeats (DM300-328) with non transgenic mice (wt) and mice with a normal number (20) of CTG repeat (DM20-954), to confirm that the observed abnormalities were not due to overexpression of the human transgene. Mice were analyzed at the ages of 3.5 months, 5 to 6 months and 10 months. They were housed and handled according to French governmental ethics guidelines. Transgenic mice were obtained from eggs B6/D2 F1 injected eggs. They were backcrossed with C57BL/6 mice (Charles River laboratories) to ensure that the C57BL/6 background accounted for more than 98 % of the genome in both transgenic and non-transgenic control mice (over 6 backcrosses).

Glucose tolerance tests

Glucose (1 mg/g) was injected intraperitoneally, under anesthesia (pentobarbital), into mice aged 3.5, 5 to 6 or 10 months, after a 16 h fast. Blood samples were taken from the tail vein after 0, 10, 20, 30 and 60 minutes. Glucose levels were measured with a glucometer (Onetouch II; Lifescan). Plasma was recovered from each blood sample, and insulin concentration was quantified by ELISA (rat ultra-sensitive insulin ELISA kit; Christal Chem. Inc).

Insulin tolerance tests

Mice were subjected to an insulin tolerance test at the same ages. The mice were fasted for 16 h. A basal blood sample was then taken from the tail vein and the mice received an intraperitoneal injection of insulin (0.75 U/Kg of Novolin R, Novo Nordisk Pharmaceutical Industries). Blood samples were taken 15, 30 and 60 minutes after injection and glucose levels were determined as described above. Results are expressed as percentages of initial blood glucose concentration.

RNA isolation

Mice were sacrificed by cervical dislocation, according to ethical guidelines. Skeletal muscle (soleus), liver, pancreas, hypothalamus/thalamus and perigonadal white adipose tissue were frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted from frozen tissues, using RNeasy mini kits from Qiagen (Ref 74104), used according to the manufacturer's protocol. RNA was quantified by spectrophotometry and its quality was checked by electrophoresis in a 1% (w/v) agarose gel.

RT-PCR

Reverse transcription was performed with 500 ng of total RNA and SuperScript™ II Reverse Transcriptase (Ref 18064-022, Invitrogen), using hexamer primers, with control of the reaction based on PCR with oligonucleotide primers 5'-GGTGTGCACAGGAGCCAAGAGTG-3' and 5'-AGCTACTGAGCTGCTGGTGGGTC-3', which amplify the *TBP* cDNA. All amplifications were carried out in duplicate, using independent samples. We used classical RT-PCR, with oligonucleotide primers 5'-GAGGATTACCTGCACAACG-3' and 5'-CACAATGGTAGAGGAGACG-3' (Sigma), binding on either side of exon 11, for analysis of alternative splicing of the insulin receptor.

Quantitative RT-PCR

The independent duplicate RT samples were pooled and 2 µl of a 1:10 dilution was used for real-time quantitative PCR. Samples were amplified (40 standard cycles) on an ABI 7700 cycler (Applied Biosystems) in triplicate or duplicate, in two independent 25 µl reactions. The expression of the human *DMPK* transgene and murine *Dmpk* gene was quantified with a common labeled FAM-TAMRA probe (Eurogentech) 5'-AGGATGAGAACTACCTGTACCTGGTCATGGA-3' spanning the end of exon 4 and the beginning of exon 5. The human and murine gene were specifically amplified with two different pairs of oligonucleotides (Eurogentech) located in a non homologous region of the human and murine exons 4 and 5. Oligonucleotides for the human gene were 5'-GGAGAGGGACGTGTTG-3' / 5'-CTTGCTCAGCAGTGTCA-3' and those specific of the murine gene were 5'-GGAAGAAAGGGATGTATTA-3' / 5'-CTCAGCAGCGTTAGCA-3'. For quantification of the *IR* isoforms (+/- exon 11), real-time quantitative PCR was carried out with the same Absolute qPCR ROX MIX, with specific labeled FAM-TAMRA probes (Eurogentech) and oligonucleotides designed to

recognize either isoform A, *IRA* (-exon 11), or isoform B, *IRB* (+exon11). Oligonucleotides 5'-TTCGAGGATTACCTGCACAA-3' and 5'-ACATTCCCCACCTCTTCAAG-3' were used for *IRA* and oligonucleotides 5'-GGAAATCTGGAAGTGTGAGTGT-3' and 5'-TCGAGGATTACCTGCACAAC-3' for *IRB*. Both pairs of primers were located in exons 10 and 12. The corresponding probes were 5'-TTTTTGTTCCTCCAGGCCATCCCGAAAGCGAA-3' spanning the end of exon 10 and the beginning of exon 12 for the amplification of *IRA* and 5'-TCCCCAGGAAAAACCTCTTCAGGCAATGGT-3' spanning the end of exon 11 and the beginning of exon 12 for the amplification of *IRB*. The results obtained for *DMPK* or *IR* isoform quantification were normalized with a FAM-TAMRA 18S genomic control kit (RT-CKFT-18S; Eurogentech).

Immunohistochemistry

Dissected mouse pancreata were rapidly frozen in liquid nitrogen and stored at -80°C. Cryosections (10 µm) were prepared with a Leica cryostat set at -20°C. Sections were fixed by incubation for 15 minutes at room temperature with 4% (w/v) paraformaldehyde, and washed three times in 1 x PBS for two minutes each.

Sections were then permeabilized by incubation in a solution containing 0.05% (v/v) Triton X-100 and 10% (v/v) normal horse serum in 1 x PBS for 30 minutes at room temperature and washed in 1 x PBS. Double immunostaining was performed by simultaneous incubation with guinea pig anti-insulin and rabbit anti-glucagon antibodies (Dakocytomation) at 4°C overnight. Sections were washed and incubated for 1 hour at room temperature with an Alexa anti-guinea pig IgG and a fluorescein-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.). Sections were washed three times with 1 x PBS, for 5 minutes each, and were then sealed with Vectashield containing DAPI

(Vector Laboratories). Immunohistochemical signals were acquired with a Leica fluorescence microscope. Independent sections were also stained with hematoxylin-eosin.

Fluorescent in situ hybridization

Frozen sections (6 μm) of the various insulin-sensitive tissues were dried for 30 minutes, fixed by incubation in 4% (w/v) paraformaldehyde for 15 minutes at room temperature, and washed five times in 1 x PBS, for 2 minutes each. Sections were permeabilized by incubation in 2% (v/v) acetone PBS (pre-chilled at -20°C) for 5 minutes. They were then incubated in 30% (v/v) formamide and 2 x SSC for 10 minutes, hybridized with probe (1 ng/ μl) for 2 h at 37°C in buffer (30% (v/v) formamide, 2 x SSC, 0.02% (w/v) BSA, 66 $\mu\text{g}/\text{ml}$ yeast tRNA, 2 mM vanadyl complex), and washed for 30 minutes in 30% (v/v) formamide/2 x SSC at 50°C and then for 30 minutes in 1 x SSC at 20°C . Sections were then mounted in Vectashield with DAPI (Vector Laboratories). We used HPLC-purified 2-*O*-methyl RNA 20-mers (Proligo) with CAG-repeats and 5' end-labeled with fluorescein as probes.

Combined FISH/Immunofluorescence

Frozen sections (6 μm) of adipose tissue and pancreas were treated following the same FISH procedure as described before, with the exception of the permeabilization step during which we used a permeabilizing/blocking reagent (0,1% Triton X-100, 1% BSA and 3,75% goat serum in 1X PBS). After the last wash in 1X SSC, slides were incubated overnight at 4°C with 1:300 rabbit anti-HSL primary antibody (anti Hormone Sensitive Lipase, Santa Cruz) or 1:50 rat anti-insulin (R&D Systems) to label adipocytes and β cells, respectively. After three washes in 1X PBS, sections were blocked in 1% BSA and 3,75% goat serum in 1X PBS for 30 minutes at room temperature. They were then incubated with the Texas Red-conjugated secondary antibodies (Vector laboratories) for 30 minutes at

room temperature. Following incubation, slides were washed three times with 1X PBS and nuclei were stained with DAPI using VECTASHIELD (Vector laboratories).

Calculations and statistical analyses

Statistical analyses were carried out with the StatView program 5.0 (SAS Institute Inc.), using unpaired Student's t tests. For all analyses, p values <0.05 were considered significant. Results are expressed as means +/- SEM.

Results

The human DMPK transgene is expressed in various tissues in mice

In order to determine if the CTG repeat expansion could affect *IR* splicing in different tissues, we first investigated expression of the human *DMPK* transgene in several tissues from mice carrying DM1 expansions. This analysis was performed in 6- and 10-month-old mice, to follow possible age-dependent changes in the level of *DMPK* expression. We carried out real-time quantitative RT-PCR with specific probes and primers for human *DMPK* transcripts. We analyzed the quadriceps, soleus, liver, adipose tissue, pancreas and the hypothalamus/thalamus area.

We detected *DMPK* transgene expression in the various tissues with a pattern of expression very similar to the *Dmpk* endogenous gene. Interestingly, we observed that *DMPK* transgene expression (as well as endogenous *Dmpk* expression) varied with age, in a tissue-specific manner (Figure 1). In quadriceps, soleus, liver and adipose tissue, *DMPK* transcript levels increased between 6 and 10 months, whereas they decreased over the same period in the pancreas and in the hypothalamus/thalamus area. Expression was strongest in the soleus at six months of age and at 10 months of age and was the lowest in pancreas. In addition, expression of the *DMPK* transgene and endogenous *Dmpk* gene varied between

muscles types (soleus versus quadriceps in this study and unpublished data). Fluorescent *in situ* hybridization experiments showed that expanded *DMPK* transgene RNA accumulated as foci in nuclei of soleus and hypothalamus in DM300-328 mice (Figure 2). Rare foci were also observed in adipose tissue and pancreas. No RNA foci could be found in the liver of these mice.

DM300-328 mice present a muscle- and age-specific change in the splicing of insulin receptor RNA

Transcription of the *IR* gene results in the production of two alternatively spliced isoforms: *IRA* (-exon11) and *IRB* (+exon11). These two isoforms differ in function and are expressed in a tissue-specific manner [27]. *IRA* has a higher affinity for insulin and a higher rate of internalization than *IRB* [28, 29], whereas *IRB* has a higher level of kinase activity and is considered to transmit the insulin signal more efficiently than *IRA* [30]. The presence or absence of exon 11 promotes different localization of the two receptor types, which is coupled with different function [31].

Real-time quantitative RT-PCR with probes specific for the two insulin receptor isoforms, *IRA* and *IRB*, showed that the two isoforms were distributed abnormally in the soleus of 10-month-old DM300-328 mice, in which *IRB* (+exon 11) predominated. We observed a ~ 50 % increase in *IRB* RNA levels and a ~ 40 % decrease in the level of *IRA* RNA (Figure 3A). No abnormality in receptor isoform expression was observed in six-month-old mice. No isoform switch was observed in the quadriceps, suggesting that the splicing defect in the DM300-328 mice was muscle-specific (data not shown). This analysis also revealed that the levels of IR isoforms decreased with age in the soleus, both in DM300-328 and in controls.

Molecular abnormalities are also present in other insulin-responsive tissues and in the pancreas

Based on published results and the known importance of insulin in metabolic homeostasis, we extended our molecular analyses to other insulin-responsive tissues and in pancreas. In liver, at six months of age, DM300-328 mice had lower total *IR* mRNA levels than controls, with about 80% less of each isoform (Figure 3B). Strikingly, this deficiency was not maintained at 10 months of age. RT-PCR and real-time quantitative RT-PCR results at 10 months revealed an ~20% increase in *IRB* RNA levels, together with an ~20% decrease in the already low level of *IRA* RNA in DM300-328 mice ($p < 0.05$ DM300-328 compared to wt and DM20 controls). Molecular studies on adipose tissue also showed changes in the distribution of the insulin receptor mRNA isoforms, but only in 10-month-old DM300-328 mice (Figure 3C). *IRB* mRNA levels in DM300-328 mice were about 40 % higher than those in control mice, while *IRA* levels were about 50 % lower ($p < 0.001$). We next investigated the distribution of *IR* mRNA isoforms in the pancreas of DM300-328 and control wt mice. RT-PCR and real-time quantitative RT-PCR showed that the levels of both *IRA* and *IRB* isoforms were about 20% lower in 6-month-old DM300-328 mice than in wt mice ($p < 0.01$) (Figure 3D). At 10 months, DM300-328 mice had higher *IRA* and lower *IRB* levels than wt mice (figure 3D). This led to a switch in the relative distributions of the two isoforms, with *IRA* becoming more abundant than *IRB*.

Higher levels of insulin receptor expression are observed in the hypothalamus/thalamus area of DM300-328 mice

Low levels of cerebral glucose utilization have been reported in DM1 patients [32, 33], suggesting that these patients have impaired cerebral glucose metabolism. Neuronal IRs are known to have an impact on peripheral glucose metabolism [34]. We then investigated the expression of insulin receptor isoforms in the brains of DM300-328 mice.

We focused on the hypothalamus/thalamus area, in which the insulin receptor is most abundant and is involved in several types of control (food intake, body weight, energy disposal, fuel metabolism and reproduction) [35]. RT-PCR and real-time quantitative RT-PCR analyses showed no difference in the levels of expression of the *IRA* isoform (with *IRA* the most strongly expressed, and *IRB* barely detectable) between DM300-328 mice and controls, at 6 months. However, *IRA* transcript levels were higher (+40%, $p < 0.001$) in the hypothalamus/thalamus area of 10-month-old DM300-328 mice than in controls (Figure 3E). *IRB* transcript levels were too low for quantification.

DM1 transgenic mice show abnormal glucose and insulin responses

In order to investigate if the changes in IR isoforms distribution and levels observed in various tissues of the in DM300-328 mice could have some impact on glucose metabolism, glucose tolerance tests were performed after a long 16-hour fasting period, in mice aged 3.5, 5-6 and 10 months. Similar results were obtained in pilot experiments for males and females. Therefore, we chose to carry on our experiments only with males, as presented. DM300-328 males weighed 8 to 25% less than wt and DM20 controls (Figure 4D). Basal hyperglycemia and glucose intolerance were detected in DM300-328 mice at 3.5 and 6 months of age but not in controls (DM20-954) or wild-type mice ($p < 0.05$, Figures 4A). This abnormal glucose response was associated with a lack of glucose-stimulated insulin response (Figure 4B). Strikingly, no glucose intolerance was detected in the DM300-328 mice at 10 months of age, despite the persistence of the glucose-stimulated insulin response defect (Figure 4 A and B). Insulin tolerance tests in these mice showed no significant difference, at the ages of 3.5 and 6 months, between DM300-328 and control mice (Figure 4C). However a slight insulin hypersensitivity was observed in 10-month-old DM300-328 mice ($p < 0.05$). Histological analysis after the labeling of pancreatic islets with hematoxylin-eosin or the detection of immunofluorescent β cells revealed no differences in

the number or size of pancreatic islets or changes in the proportion of β cells (data not show).

Discussion

Cell culture experiments, observations in DM1 patients and mouse models have shown that expanded CUG containing mRNA are toxic and alter splicing regulation of various transcripts. In this study, we investigated the splicing regulation of *IR*, known to be affected in DM1 patients muscles and in cellular models [26, 36-40]. In our mice model, the human *DMPK* gene is controlled by its own promoter and is embedded in large human genomic sequences over 45 kb, probably containing most of the transcriptional regulatory sequences. Using quantitative RT-PCR, we observed that expression patterns of the *DMPK* transgene and the *Dmpk* endogenous gene were very similar in skeletal muscles, adipose tissue, liver and pancreas, although lower for the *DMPK* transgene (e.g. the level of expression of the endogenous *Dmpk* gene in the gastrocnemius of 3-month-old mice is ~5 times higher than that of the *DMPK* transgene [21]). We also observed that the levels of expression of both genes are tissue-specific and varied between 6 and 10 months. These data prompted us to investigate the possible splicing defects these tissues, at different ages, and the consequences that these defects could have on glucose metabolism.

At the age of six months, expression levels of both *IR* isoforms, *IRA* and *IRB*, were similar between DM300-328 in soleus, adiposes tissues and hypothalamus. However, molecular analyses clearly showed that DM300-328 mice had much lower (80% lower) levels of insulin receptor mRNA (*IRA+IRB*) in the liver than the controls. The same mice also had lower levels of *IR* mRNA in the pancreas (20% lower). At this stage, it is difficult to

explain why at 6 months of age, mice expressing the mutant *DMPK* gene showed a decrease in the levels of *IR* mRNA. Liver and pancreas are not the tissues with the highest expression of *DMPK*. *DMPK* showed a much lower expression in liver and pancreas than in muscles in which *IR* expression is not affected at 6 months. However, we can not exclude a high level of transgene expression in specific cell types in these two tissues that would affect *IR* expression in particular cells. Thus, factors involved in *IR* gene regulation at transcriptional or postranscriptional levels (such as RNA maturation and stability) could be affected by the expanded transgene. These factors may be tissue-specific and/or could be more affected by the CTG repeat in the liver and pancreas of the DM300-328 mice. It has been reported that *IR* expression at the RNA and protein levels is decreased in DM1 patients muscle [36] but there is no data on *IR* expression in the pancreas and liver of DM1 patients. Furthermore, expanded CUG-containing transcripts have been reported to sequester transcription factors such as Sp1 [19], and may have a deleterious impact on *IR* transcription. Even more surprising was the fact that the decrease of 80% in liver and of 20% in pancreas were no longer observed at 10 months of age. Instead, DM300-328 showed a shift in the *IRA* and *IRB* isoforms distribution in liver and pancreas, compared to wt and DM20 control mice of the same age. Abnormal *IRA* and *IRB* isoforms distribution was also observed in soleus, and adipose tissue. These data suggest that the expression of RNA carrying CUG expansion in transgenic mice disturbed the regulation of the *IR* gene splicing as shown in cell culture and in DM1 muscles. Furthermore, it shows that the isoforms distribution of *IRA* and *IRB* is affected in all the insulin-responsive tissues expressing the mutant *DMPK* RNA and in an age-dependent manner. It is interesting to mention here that we observed in most DM300-328 mice tissues (except pancreas and hypothalamus), a significant increase of mutant *DMPK* transcripts in the older mice. As we already know that the level of expanded CUG repeat transcripts is critical to induce phenotype and splicing abnormalities in our mice [21], it is tempting to speculate that the

IR splicing defects observed at 10 months could be linked to the increased of *DMPK* transgene expression at that age. On the other hand, this study showed that *IR* expression decreases with age in most control tissues. It is therefore possible that *IR* splicing regulation varied with age and that the mechanisms involved in aged mice are more “sensitive” to CUG toxic repeats.

Our physiological investigations in DM300-328 mice suggest that the *trans*-effect of expanded *DMPK* RNA and the observed *IR* splicing defects could contribute to the metabolic abnormalities observed in these mice. However, the disturbances observed in DM300-328 mice and DM1 patients are different. We observed no hyperinsulinemia associated with glucose intolerance and insulin resistance similar to that described in DM1 patients. Instead, DM300-328 mice showed glucose intolerance, which disappeared with age, and a defect in glucose-stimulated insulin response. Quantitative RT-PCR analyses in the insulin-responsive tissues suggested that these differences in metabolic status between the two species might arise, among other possibilities, from differences in the tissue-specific distribution of *IR* mRNA isoforms between C57BL/6 mice and humans. For example, *IRB* is the predominant isoform in human skeletal muscle, whereas *IRA* predominated in the skeletal muscles of wild-type C57BL/6 mice [27]. The different regulation of the *IR* splicing between C57BL/6 mice and human underlines that the regulation of the insulin pathways may vary between the two species. Mice on a C57BL/6 background may not fully recreate the DM1 insulin resistance features. However they remain a very useful tool to study the molecular effect of CUG repeat containing RNA on RNA splicing *in vivo*.

The mechanisms by which CUG repeats-containing RNA exert a *trans*-effect on splicing regulation remain unclear, but many studies recently carried out, have provided a

large body of information (for review see [18]). There is growing evidence to suggest that the retention of *DMPK* mRNA with CUG expansions alters RNA metabolism by titration and/or by changes in the function of CUG binding proteins such as MBNL1 and CUG-BP1 respectively members of the muscleblind-like family and the CELF family . Recent data suggest that CUG-BP1 and HnRNP H may regulate the equilibrium of splice site selection by antagonizing MBNL1 facilitation of insulin receptor exon 11 splicing in a dose-dependent manner [26, 37, 39, 41]. The predominant isoform of the insulin receptor may therefore depend on the tissue-specific or age-dependent expression profiles of these two antagonist proteins (or other members of the corresponding protein families). Thus, further molecular analyses of the expression of proteins from the CELF and muscleblind-like families in various tissues in adult transgenic mice may improve our understanding of tissue-specific and age-dependent insulin receptor mRNA processing defects. The splicing abnormalities that we observed in DM300-328 mice at 10 months of age showed a shift towards the overrepresentation of isoform B with inclusion of exon 11 in the soleus muscle. In DM1 patients, CTG expansions lead to the increase of isoform A and the exclusion of exon 11 [26]. Taken together with the observation that the repartition of isoforms A and B is also inverted in control human and mouse skeletal muscles, these results suggest that the regulation of exon 11 splicing might be different between the two species. It has been clearly demonstrated that CUG-BP1, hnRNP H and MBNL1 are able to bind the human *IR* intron 10 sequences allowing the regulation of exon 11 splicing. CUG-BP1 and hnRNP H form a suppressor required for the repression of exon 11 inclusion and MBNL1 induces exon 11 inclusion [26, 37, 39, 41]. However, these proteins can also promote an opposite splicing pattern for cardiac troponin T (cTNT): MBNL1 has been shown to favor cTNT exon 5 skipping while CUG-BP1 is involved in its inclusion [41]. Therefore, these proteins can have different roles in exon splicing, probably depending on the context and/or the number of binding sites in the splicing regulatory

sequences. Analyses of the mouse Cug-bp1, Mbnl1 and hnRnp H proteins binding to the mouse intron 10 and functional experiments using mouse *Insulin receptor* minigenes are required to determine if and how the regulation of the mouse exon 11 differs from the human *Insulin Receptor* exon 11 splicing regulation.

Conclusion

In conclusion, we showed for the first time that *DMPK* transcripts carrying CUG expansion affect *IR* splicing in transgenic mice. Furthermore, these mice provided evidence that *IRA* and *IRB* isoforms distribution can be affected not only in muscles but also in other insulin-responsive tissues and in pancreas. At this stage, it is difficult to relate the molecular abnormalities and the physiological abnormalities observed in the DM300-328 mice. Complex interactions can occur between and among insulin-responsive tissues and in pancreas, mediated by factors that promote cross-talk in insulin-driven responses. Furthermore, we observed complex age-dependent changes of the *IR A* and *B* isoforms expression in all these tissues. At 6 months of age, the lack of glucose-stimulated insulin response and glucose intolerance could be explained by the decrease of *IRA* and *IRB* expression in pancreas and liver and/or by others factors (such as defects of transcription factors involved in the regulation of the *Insulin* gene expression). At 10 months of age, despite the persistence of a glucose-stimulated insulin-response defect, the glucose tolerance appeared normal. However, multiple splicing defects were observed in all the tissues that were investigated. One possible explanation is that combined variations of *IRA* and *IRB* expression in the different tissues could induce a higher insulin sensitivity of the tissues involved in the uptake and/or storage of glucose resulting in a normal glucose tolerance.

Finally, the different regulation of *IR* exon 11 splicing between mice and human and the different physiological manifestations observed in transgenic mice compared to

DM1 patients indicate that mouse is not a good model for the study of DM1 features that involve insulin regulation. However, it remains possible that, as observed in mice, *IR* splicing regulation could be affected not only in muscles but also in other tissues in DM1 patients. The resulting *IR* splicing defects in different tissues might then participate to the metabolic syndrome observed in DM1 patients. It is also interesting to keep in mind that the splicing molecular defects could be tissue-specific and age-dependent.

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Legends

Figure 1 Age-dependent expression of the human *DMPK* transgene and the endogenous *Dmpk* gene in transgenic mice.

DMPK transgene (A) and *Dmpk* endogenous (B) transcription levels were determined by real-time quantitative PCR, with normalization according to 18S expression, in various insulin-responsive tissues and in pancreas from 6- and 10-month-old DM300-328 male mice (pool n=4-6). Expression levels are expressed in arbitrary units. *p<0.05; **p<0.01

Figure 2 Mutant *DMPK* transcripts formed nuclear RNA foci in several insulin-responsive tissues and in pancreas from transgenic mice. Nuclear foci were detected with a (CAG)₂₀ fluorescent probe in adipose tissue, soleus and pancreas from DM300-328 mice only. Foci were not detected in liver. White arrows indicate green RNA foci in the cell nuclei that are stained with DAPI. FISH experiments were combined with immunofluorescence staining for the identification of insulin-secreting cells in pancreas and hormone-sensitive lipase rich adipocytes in perigonadal White Adipose Tissue (WAT).

Figure 3 Abnormal expression of IRA and IRB in mice expressing mutant *DMPK* transcripts.

The levels of expression of the two *IR* isoforms were determined by RT-PCR (left) or real-time PCR (right), with normalization according to 18S levels in wild-type mice (wt), in control DM20-954 mice with 20 CTG repeats (DM20) and in DM300-328 mice carrying >350 CTG expansions (DM300). Isoform levels were determined in the soleus (A), liver (B), adipose tissue (C), pancreas (D) and hypothalamus/thalamus (E) of 6- and 10-month-old males (n=4-6/group). *Tbp* was used as an internal control in RT-PCR experiments. *p<0.05; **p<0.01; ***p<0.001

Figure 4 Glucose tolerance test, secretion and insulin sensitivity in transgenic mice and controls.

Blood glucose (**A**) and serum insulin (**B**) concentrations were measured at the indicated time points before and after intraperitoneal injection of glucose (1 mg/g body weight) in 16 h-fasted DM300-328 males with CTG expansions (DM 300) and in 16 h-fasted control wild-type and DM20-954 males (wt and DM 20), n=6/group. Blood glucose (**C**) was determined at the indicated time points before and after intraperitoneal injection of insulin (0.75 IU/kg), in the same 16 h-fasted mice (n=6/group). (**3.5 m**) 3.5-month-old males. (**5-6 m**) 5- to 6-month-old males. (**10 m**) 10-month-old males. Results are expressed as means \pm SEM. (**D**) Mean weight of wt, DM20 and DM300 males at 3.5, 5-6 and 10 months of age. * $p < 0.05$; ** $p < 0.01$: transgenic versus both control groups by unpaired, two-tailed Student's *t* test.







