

Mice transgenic for the human myotonic dystrophy region with expanded CTG repeats display muscular and brain abnormalities

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The autosomal dominant mutation causing myotonic dystrophy (DM1) is a CTG repeat expansion in the 3'-UTR of the DM protein kinase (DMPK) gene. This multisystemic disorder includes myotonia, progressive weakness and wasting of skeletal muscle and extramuscular symptoms such as cataracts, testicular atrophy, endocrine and cognitive dysfunction. The mechanisms underlying its pathogenesis are complex. Recent reports have revealed that DMPK gene haploinsufficiency may account for cardiac conduction defects whereas cataracts may be due to haploinsufficiency of the neighboring gene, the DM-associated homeobox protein (DMAHP or SIX5) gene. Furthermore, mice expressing the CUG expansion in an unrelated mRNA develop myotonia and myopathy, consistent with an RNA gain of function. We demonstrated that transgenic mice carrying the CTG expansion in its human DM1 context (>45 kb) and producing abnormal DMPK mRNA with at least 300 CUG repeats, displayed clinical, histological, molecular and electrophysiological abnormalities in skeletal muscle consistent with those observed in DM1 patients. Like DM1 patients, these transgenic mice show abnormal tau expression in the brain. These results provide further evidence for the RNA trans-dominant effect of the CUG expansion, not only in muscle, but also in brain.

INTRODUCTION

Myotonic dystrophy (DM1) is the most common form of muscular dystrophy. It involves various multisystemic clinical manifestations including myotonia, progressive weakness and

wasting of skeletal muscle, cardiac conduction defects and extramuscular symptoms such as cataracts, testicular atrophy, endocrine and cognitive dysfunction (1). Myotonia is one of the key distinguishing features of DM1, along with progressive atrophy and weakness of certain muscles of the face and jaw, the sternomastoids and the distal limb muscles. A CTG expansion in the 3'-UTR of the DMPK gene is responsible for DM1 (2–4). The size of the CTG repeat, which increases from generation to generation with sometimes very large expansions, is generally correlated with clinical severity and age at onset, providing a molecular basis for the anticipation phenomenon observed in DM1 families (5). High levels of somatic mosaicism are also observed in patients (6). The CTG repeat expansion appears to have several molecular consequences that may account, independently, for the multisystemic aspects of this disorder (7,8). Knock-out mouse models have proved useful for identification of the genes involved in some aspects of the physiopathology of DM1. Haploinsufficiency of DMPK, due to the retention of mutant DMPK mRNA in the nucleus in DM1 patients, has been shown to be involved in cardiac conduction abnormalities in mouse knock-out mutants for the homologous gene, *Dm15* (9). Furthermore, changes in the relative levels of DMPK isoforms, due to retention of CUG repeat-containing RNA but not of the isoform lacking the CUG repeat, may also contribute to the DM1 phenotype (10). Haploinsufficiency of the neighboring *Six5* (or DM-associated homeobox protein, *DMAHP*) gene in mice results in cataract formation (11,12). In DM1 patients, the *SIX5* haploinsufficiency is probably caused by change in chromatin structure, close to the *SIX5* promoter (13,14). Other genes may also be affected by a long-range cis effect or 'chromosome painting' (15,16) and/or by a trans-dominant effect of mutated DMPK mRNAs, which have been shown to be sequestered in nuclear foci (17–22). The retention of mutated DMPK mRNAs may alter RNA metabolism possibly by titration of CUG-binding proteins or by RNA interference (22,23). Transgenic

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mice expressing expanded CUG repeats developed myotonia and myopathy, showing that a trans-dominant effect of the mutation can account for DM1 features (24). In this study, we report an analysis of transgenic mice carrying the human genomic DM1 region with expanded repeats of approximately 350 CTG or with normal 20 CTG repeats. We found that homozygous mice producing sufficient quantities of mutated mRNA presented myotonia and myopathy, consistent with the RNA trans-dominant model in muscle. We also observed, as recently described in DM1 patients (25), changes in the production of microtubule-associated tau protein in the brain, which may be involved in the neurological defect in DM1 patients. These results provide further evidence for the RNA trans-dominant effect of the mutation causing features of DM1 not only in muscle, but also in the brain.

RESULTS

Transgenic mice (DM300) carrying the human DM1 region with greater than 300 CTG repeats display various phenotypes

We investigated the consequences of an accumulation of abnormal DMPK RNA carrying a large CUG expansion by analyzing transgenic mice carrying a 45 kb fragment of human genomic DNA with the CTG expansion, cloned from a DM1 patient (26,27). This DNA fragment was injected into fertilized B6D2/F1 mouse eggs and transgenic animals were crossed with C57BL/6 mice. The founders of three different lines carried human DMPK transgenes containing 304 CTG (line DM300–328), 362/184/147CTG (three-copy line DM300–1112) and 362 CTG (line DM300–1177). As previously reported, these lines showed a high level of intergenerational and somatic instability, with a strong bias towards expansion (27). Mean litter size was close to normal in the DM300–328 and DM300–1112 lines [6.5 pups per litter for 42 litters and 5.8 pups per litter for 22 litters, respectively, versus 6.2 per litter in C57BL/6 (28)] but was lower in DM300–1177, with a mean litter size of four per litter for 41 litters ($P < 0.001$). Hemizygous mice generally looked normal but a few (5% in line DM300–328 and 2% in line DM300–1177) were smaller. These mice were already smaller at 2 days of age, their weight at weaning was 50–70% that of their littermates, in litters of three to eight pups (mean of 4.6) (Fig. 1). After weaning, most of these mice developed abnormal teeth (crossed teeth, Fig. 1B). Teeth were cut at least once per week and mice were fed with pellets crushed to powder. No abnormal mice were observed in the DM300–1112 line. Mice homozygous for the transgene were obtained in each DM300 line but with a lower frequency than expected for the DM300–328 line ($P < 0.05$). In this line, homozygous mice were smaller (Figs 1 and 2), 45% (over 32) developed abnormal teeth and 52% presented an anal prolapse (Fig. 1D). In the DM300–1177 line, the phenotype of homozygous mice was more variable. Some mice had a normal body weight whereas others had low body weight, and these mice rarely had crossed teeth. In the DM300–1112 line, homozygous mice could not be distinguished from hemizygous and non-transgenic mice. The rate of mortality before the age of 1 year was higher for homozygous mice of the DM300–328 line than for those of other lines and for

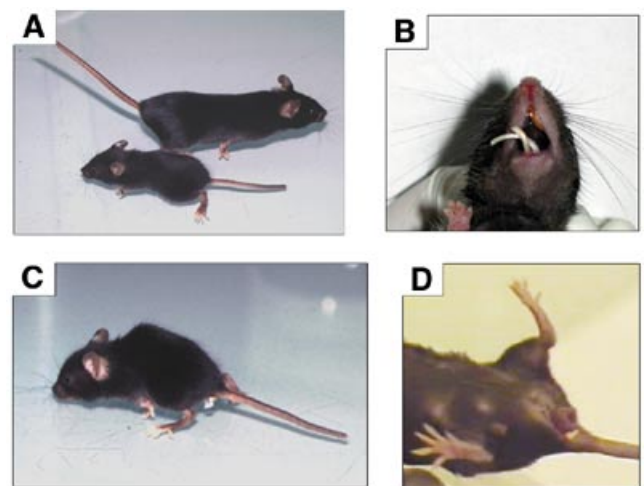


Figure 1. Phenotypic abnormalities in DM300 transgenic mice. (A) Growth retardation of a 2-week-old heterozygous DM300–328 mouse with respect to its normal littermate. (B–D) Crossed teeth, abnormal posture and anal prolapse in DM300–328 homozygous mice.

non-transgenic mice (20% versus <2% for the other lines and for the non-transgenic mice).

DM300 transgenic mice display histological muscle abnormalities

Histological and immunohistochemical analyses were carried out on the three DM300 lines, on hemizygous mice (22 mice for DM300–328, eight mice for DM300–1112 and seven mice for DM300–1177), homozygous mice (five mice) and on control littermates (40 mice). As an internal control for this study, we also examined five heterozygous transgenic mice from two lines (DM20–949, DM20–954) carrying the same human DNA fragment but with a normal repeat of 20 CTG (27). We observed no histological abnormalities in the control littermates, DM20 and DM300–1112 mice (Fig. 3A and C). Muscle fibers were homogeneous in shape and size and nuclei were generally situated at the periphery of the muscle fibers. In these mice, we observed no more than 1% centronucleated muscle fibers in the soleus and 0.5% in the gastrocnemius. However, in the DM300–328 and DM300–1177 transgenic mice, we observed heterogeneity in the diameter of the muscle fibers, fibrosis and infiltration of fatty tissue, along with large numbers of centronucleated muscle fibers in the soleus and sternomastoid muscles (Fig. 3B, D and J–L). The percentage of fibers displaying centronucleation was 1–16% in the soleus and 0.5–9% in the gastrocnemius, according to the mouse and line investigated. We also observed focal muscle degeneration and regeneration, which was confirmed by the presence of immature, newly regenerated muscle fibers expressing the neonatal MyHC (Fig. 3F). In contrast, all muscle fibers in the control mice, expressed adult MyHC isoforms. In some of the transgenic mice, we also observed atrophy of the slow muscle fibers and an increase in the number of these fibers, as shown by the ATPase reaction (preincubation pH 4.35; Fig. 3D). Ultrastructural studies in the DM300–328 line also revealed abnormalities in mitochondrial morphology, involving

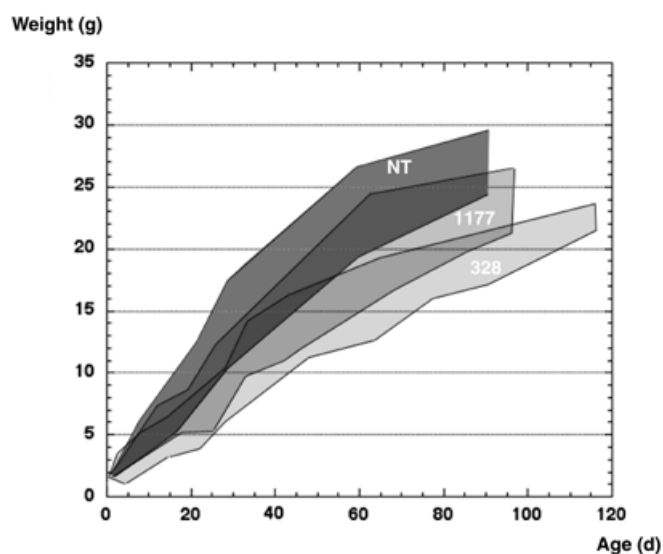


Figure 2. Mean weight for age in the various DM300 lines and non-transgenic control. Five to seven litters (20–40 mice) were weighed from birth to adult life (age is in days). Graphs have been drawn taking into account the mean weight (in grams) and SD. The DM300–328 and DM300–1177 mice tended to be smaller. The DM300–1112 mice could not be distinguished from the non-transgenic controls.

increases in both size and number, as shown in Figure 3H for the soleus.

Electromyography revealed myotonia in DM300 mice

We tested for myotonic discharges by subjecting the muscles of the fore leg (extensors) and hind leg [extensor digitorus longus (EDL), soleus] to electromyography (EMG) at room temperature, under resting conditions. A total of 29 animals were investigated, including eight homozygous and nine hemizygous mice from the DM300 lines, three hemizygous mice from the DM20–954 line and nine non-transgenic controls (Table 1). We checked for myotonic activity on insertion of the exploratory needle electrode into the muscles and following percussion of the muscle close to the area in which the electrode was inserted. Myotonic discharges were mainly detected in the DM300–328 and DM300–1177 lines (four of six homozygous mice and one hemizygous DM300–328 mouse). Such activity was preferentially observed in the extensor muscles of the fore legs. Under similar conditions, none of the transgenic mice of the DM300–1112 and DM20–954 lines and none of the non-transgenic controls displayed myotonic discharges. The frequency of the myotonic potentials largely exceeded that usually observed in humans (200–400 Hz versus a mean of 150 Hz in humans) (Fig. 4). Furthermore, the pinch-evoked muscle activity in the transgenic mice was similar to that in control mice, with no sign of myopathic alterations (Fig. 4). The amplitude of motor-unit action potentials was between 50 and 2500 μ V and the firing rate increased normally with contraction force, reaching up to 200 Hz in both normal and transgenic mice.

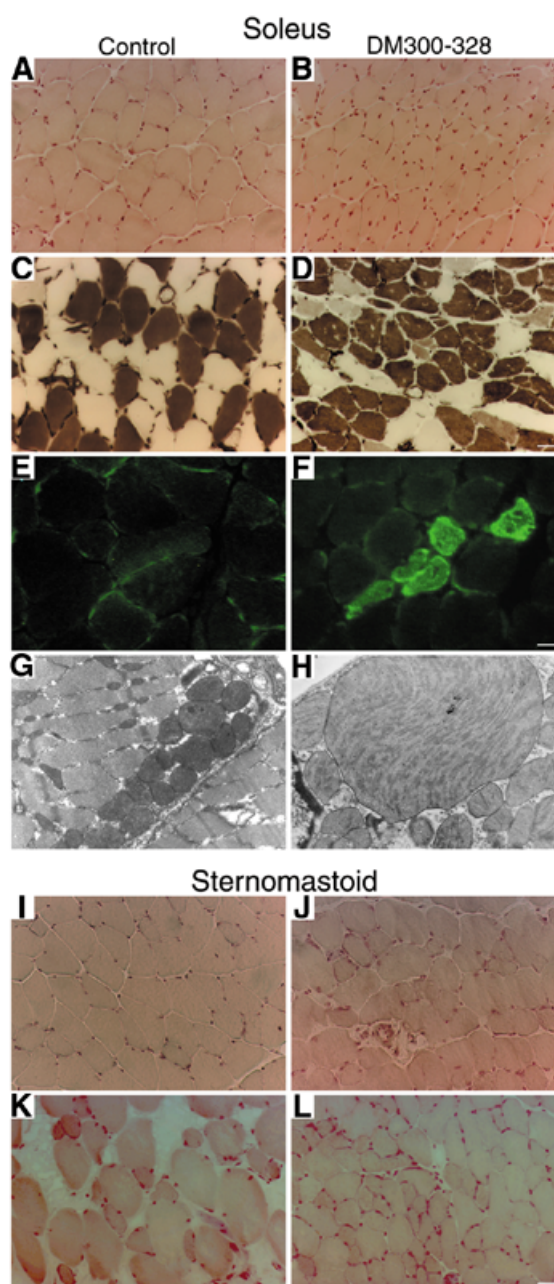


Figure 3. Histological abnormalities in muscles from DM300–328 and DM300–1177 mice. (A–H) Frozen sections of the soleus muscle of 18-month-old (A–D) and 10-month-old (E and F) controls (A, C and E) and DM300–328 transgenic hemizygous (B, D and F) mice, stained with hematoxylin (A and B), ATPase pH 4.35 (C and D) and anti-neonatal MyHC antibody (E and F). Note the presence of centronucleated muscle fibers and variability in fiber diameter in (B and D) and the presence of small newly regenerated muscle fibers stained with anti-neonatal MyHC antibody in (F). Electron microscopy of soleus muscle from 10-month-old control (G) and DM300–328 transgenic hemizygous (H) mice soleus muscle showing mitochondria with various morphological modifications. (I–L) Frozen sections of the sternomastoid muscle of 13-month-old (I and J) and 10-month-old (K and L) controls (I), DM300–328 homozygous (J) and DM300–1177 homozygous (K and L) mice, stained with hematoxylin. Note the variability in fiber diameter in (K and L) and the greater space between the muscle fibers in (K). Bar: (A–D, I–L) 75 μ m, (E and F) 37.5 μ m, magnification for electron microscopy 10 800 \times .

Table 1. Characteristics of the mice analyzed by EMG

Line	Age (months)	Genotype	Sex	CTG repeat	Myotonic discharge
DM300-328	9	+/+	f	387/362	Yes
	11	+/+	f	412/342	Yes
	4.5	+/+	m	397/377	No
	13.5	-/+	f	324	Yes
	8	-/+	m	385	No
	8.5	-/+	m	380	No
	11	-/+	f	397	No
DM300-1177	18	-/+	f	316	No
	4.5	+/+	m	359/369	Yes
	7	+/+	m	395/365	Yes
	4	+/+	m	449/415	No
DM300-1112	7.5	-/+	m	440	No
	9	-/+	f	414	No
	9	+/+	f	400/379	No
DM300-1112	9	+/+	f	400/400	No
	8	-/+	f	399	No
	8	-/+	m	420	No
	8.5-9	-/+	m, f	20	No
<i>n</i> = 3					
Controls	7.5-11	-/-	m, f	-	No
<i>n</i> = 9					

f, female; m, male; *n*, number of mice. In the three-copy line DM300-1112 (with 362, 184 and 147 CTG repeats in the founder) only the larger CTG repeat size is indicated. Control non-transgenic mice were littermates of transgenic mice that were analyzed.

The human DMPK gene is expressed in DM300 transgenic mice and the mRNAs form nuclear foci in myoblasts

Levels of the human DMPK mRNAs were analyzed by ribonuclease protection assay (RPA), in the three DM300 lines and in the DM20-954 line, using 18S RNA as an internal standard. Using a DMPK riboprobe spanning part of exons 3 and 4, we observed that the human DMPK gene was expressed in all lines, and RT-PCR experiments showed that messengers carrying the CUG expansion were produced in muscle (Fig. 5A and B). In the DM300-1177 line, we previously reported that the integrated human DNA fragment showed a deletion of ~16 kb at the 5' end (27). This deletion eliminated the promoter and the first two exons of the DMPK gene. However, with a riboprobe spanning part of exons 3 and 4, we detected expression of these exons in several tissues including brain, muscle, heart, liver and kidney (data not shown). These data suggest that the fragment was integrated close to a promoter, probably leading to the expression of a chimeric gene containing the DMPK gene sequence from exon 3 to the 3'-UTR with the repeat (Fig. 5A). Levels of total free DMPK mRNA in the gastrocnemius of 3-month-old mice (Fig. 5A) were similar in the DM300-328 and DM300-1177 lines. DMPK mRNA levels were twice that level in the three-copy DM300-1112 line and

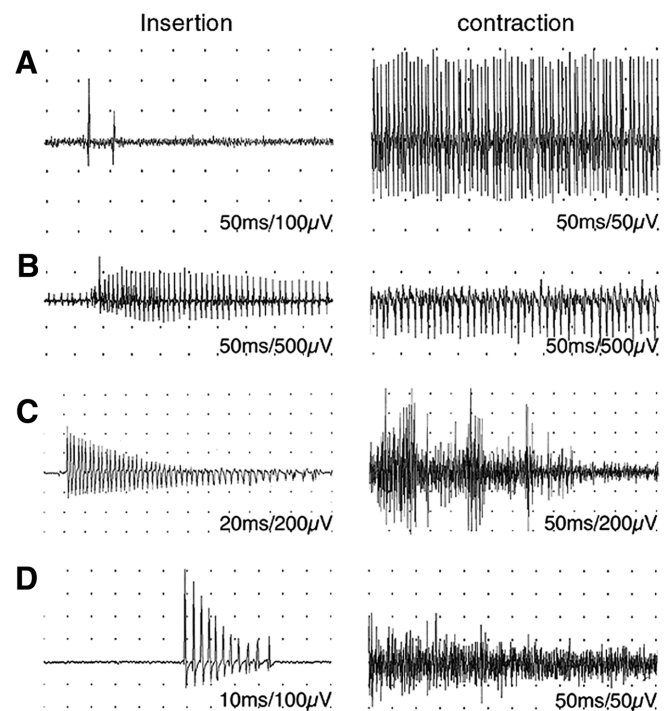


Figure 4. Myotonia in DM300 transgenic mice. Electrical activity of the extensor muscles of the right fore leg on insertion of the needle electrode (left column) or following a pinch-evoked contraction (right column): (A) non-transgenic control; (B) DM300-1177 homozygous mouse; (C) DM300-328 homozygous mouse; (D) DM300-328 heterozygous mouse. Note the shorter duration of insertion potentials in the control (A) and the myotonic discharges lasting from 40 to 400 ms or more in the transgenic animals (B-D). The motor unit action potentials recorded during muscle contraction displayed normal amplitude and frequency in the transgenic animals (B-D) with respect to the non-transgenic control (A). In all cases, the firing pattern normally followed the contraction force. Calibrations correspond to horizontal (time) and vertical (amplitude) intervals between two sampling points.

were six times higher in the three-copy DM20-954 line carrying normal 20 CTG repeats. The level of expression of the endogenous *Dm15* gene (DMPK mouse homologous gene) was higher than that of the DMPK transgene (for example 1.8 times higher in the DM20-954 line). A novel exon has been described in the 3' region of the DMPK gene, resulting in a mRNA lacking the repeats (10). This new isoform is not retained in the nucleus of DM1 cells and an imbalance between the various DMPK isoforms may be involved in the dominant effect of the DM1 mutation. We investigated whether the production of this isoform, lacking the CUG repeat, differed in the transgenic mice carrying large CTG expansion by performing RT-PCR with RNA extracted from adult DM20 and DM300 mice (Fig. 5C). Using primers respectively located in exon 12 and 16, we detected two major bands in DM20 mice of sizes consistent with these bands being the full-length isoform (with exons 12, 13, 14, full 3'-UTR including CUG) and the isoform lacking only exons 13 and 14. A third, fainter band was also observed, possibly corresponding to an isoform lacking only exon 14. In mice carrying large CTG repeats, we observed, in addition to the isoforms retaining the CUG (with or without exons 13 and 14), a smaller band, 224 nt in size.

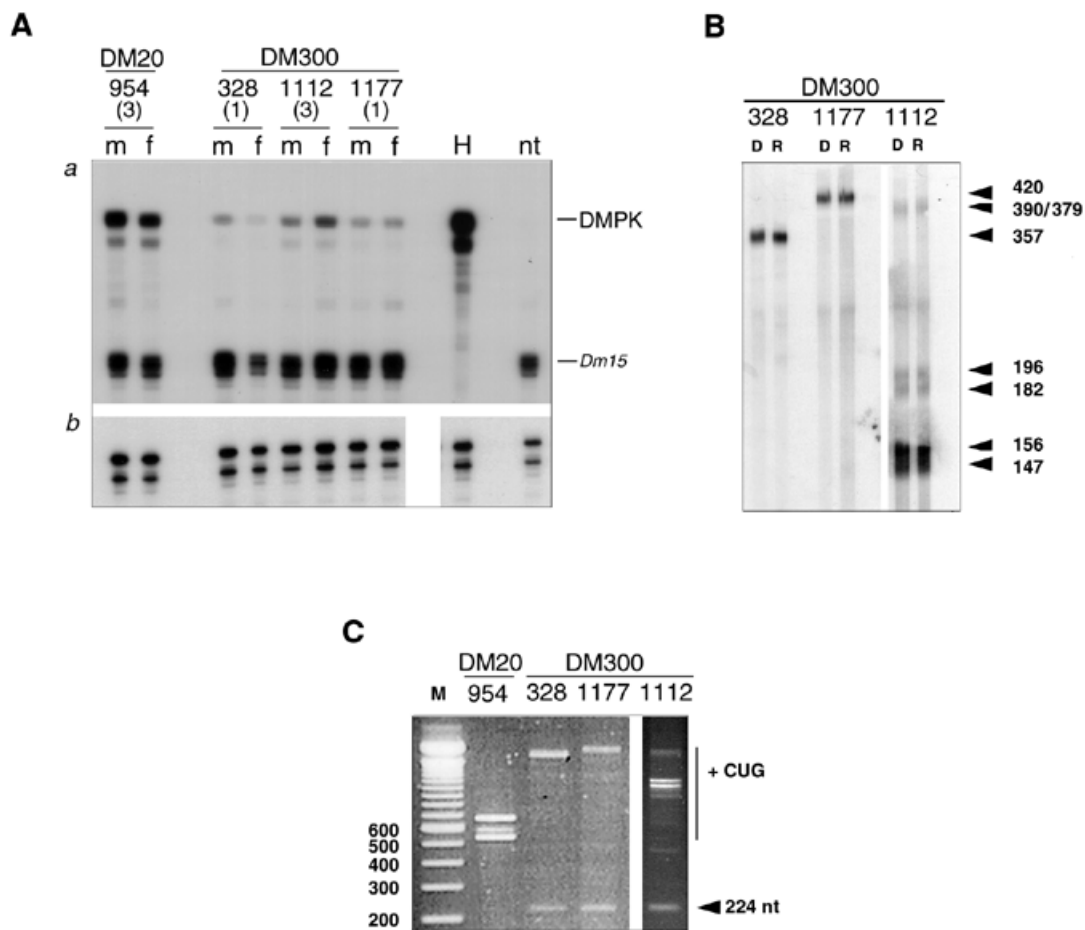


Figure 5. Expression of the DMPK transgene in transgenic mice. (A) RPA performed using RNA extracted from the gastrocnemius of 3-month-old mice. Transgenic lines analyzed by RPA and their transgene copy numbers are indicated at the top of the figure. (a) RPA using the probe that recognized the DMPK transcripts (DMPK) and the *Dm15* transcripts (*Dm15*); m, RNA from male; f, RNA from female; H, RNA from fetal human DM1 muscle; nt, RNA from a non-transgenic mouse. (b) RPA with the same RNA but using a probe specific for 18S RNA, for standardization. (B) PCR on tail DNA (D) and RT-PCR (R) on RNA extracted from muscle (gastrocnemius) were performed with primers flanking the trinucleotide repeat. The 3-month-old DM300-328 and DM300-1177 mice were hemizygous whereas the DM300-1112 mouse (10 months old) was homozygous. RNAs carrying the expansion were detected in all lines and their sizes corresponding to the size of the repeat in DNA. The number of triplets in the repeat is indicated on the right. The level of amplification of the larger repeat in the DM300-1112 line is lower in RNA as well as in DNA. This reflects the amplification bias for the smaller bands during PCR. (C) RT-PCR with primers located in exons 12 and 16. The RNA from muscle used in this experiment was extracted from a 3-month-old DM20-954 mouse and from the same mice as in (B). Bands including the repeat are indicated by + CUG in all lanes. The band corresponding to the isoform lacking the repeat (224 nt) is detected only for DM300 mice. M is a 100 bp DNA size ladder.

Sequencing experiments showed that this band corresponded to the full-length isoform (exons 12, 13, 14, 16) lacking exon 15 and the CUG repeat. This band was not detected for the DM20 line but was present in the mice of all three DM300 lines.

We investigated whether abnormal human DMPK mRNAs with greater than 300 CTG aggregated into distinct nuclear foci in the transgenic mice by isolating primary myoblasts from non-transgenic mice and from DM20-954 and DM300 transgenic mice. For the DM300-328 and DM300-1177 lines, we used homozygous and hemizygous mice that did or did not display myotonic discharges in EMG analysis. Nuclear foci were observed (Fig. 6) by RNA-FISH, using a fluorescent antisense (CAG)₃₀ oligonucleotide probe, in myoblasts obtained from DM300-328, DM300-1177 and DM300-1112

mice. In contrast, no such foci were observed in control cell cultures (derived from non-transgenic and DM20 mice).

Change in tau protein isoform production in the brains of DM300 mice

The tau protein is thought to play a role in maintaining the stability of the neuronal cytoskeleton by interacting with microtubules, and is known to be involved in the pathogenesis of neurodegenerative disorders (29). In the brain of DM1 patients, abnormally hyperphosphorylated tau proteins aggregate to give rise to the typical neurofibrillary degeneration and the production of normal tau protein is altered (25,30). Tau proteins comprise six protein isoforms in the human central nervous system (31). The brain tissue homogenates of DM1

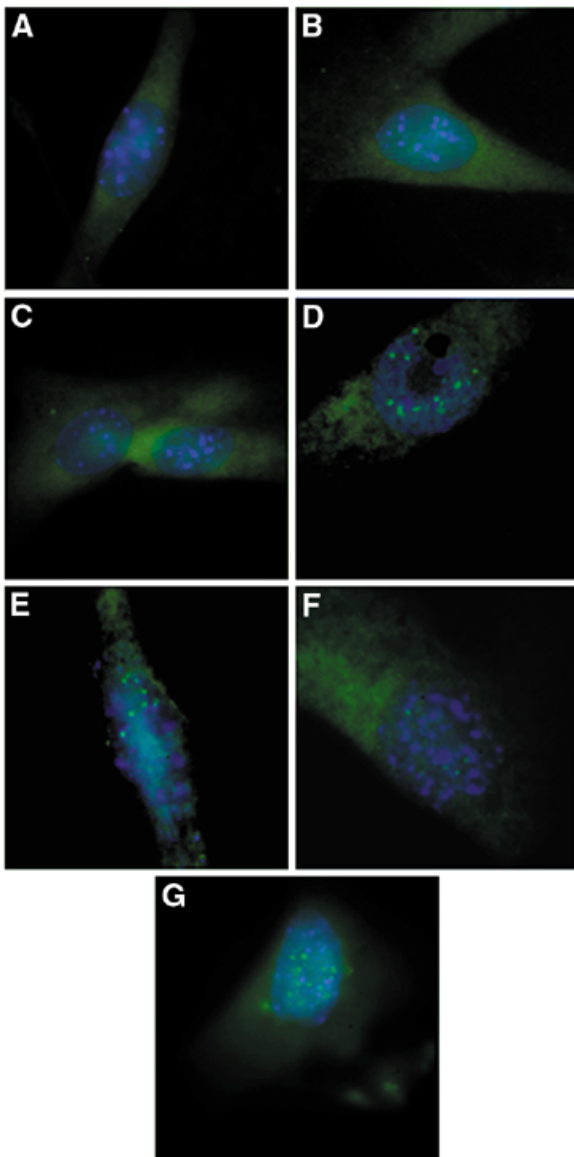


Figure 6. DMPK mRNAs aggregated into nuclear foci in DM300 myoblasts. RNA-FISH with a 5' FITC-2'-*o*-methyl (CAG)₃₀ oligonucleotide probe did not detect nuclear foci in myoblasts from (A) a control non-transgenic mouse and (B) a DM20-954 heterozygous mouse. Nuclear foci were observed in myoblasts from (D) a DM300-328 heterozygous mouse, (E) a DM300-328 homozygous mouse, (F) a DM300-1117 mouse and (G) a DM300-1112 mouse. (C) No foci were observed in myoblasts from these mice hybridized with a 5' FITC-2'-*o*-methyl (CTG)₃₀ oligonucleotide as shown for the DM300-328 heterozygous mouse.

patients display an abnormal distribution of tau protein isoforms, with two major isoforms detected instead of the six observed in control brain tissue homogenates (Fig. 7) (25). This suggests that there is a defect in the splicing of the tau RNA messenger in patients. Therefore, we investigated the levels of production of the various tau protein isoforms in controls and DM300 transgenic mice. Mice produce three tau protein isoforms (32). On western blots with mouse brain

homogenates, stained with a polyclonal antibody directed against the last 15 amino acids of tau, we detected two major tau isoforms in control mice along with tau proteins in different phosphorylation states (Fig. 7). Similar electrophoretic profiles were obtained for hemizygous DM20-954 mice and the *Dm15* knock-out mouse (33), suggesting that neither the overexpression of a normal human DMPK gene nor the loss of the endogenous *Dm15* gene are involved in regulating tau isoform production. In contrast, in homozygous transgenic DM300-328 and DM300-1177 mice, the higher molecular weight tau isoform was barely detectable (Fig. 7). Like the control, the homozygous DM300-1112 mouse showed a normal tau isoform profile. However, the results suggest that the distribution of tau isoform is altered in homozygous DM300-328 and DM300-1177 transgenic mice like in the brain tissue of DM1 patients.

DISCUSSION

We observed phenotypic abnormalities associated with histological abnormalities in skeletal muscle and myotonia only in mice transgenic for a human DMPK gene carrying an expanded CTG repeat of over 300 CTG. These results, consistent with those obtained by Mankodi *et al.* (24), provide evidence that the production of abnormal DMPK mRNAs, aggregated into nuclear foci, rather than overexpression of the three genes carried by our integrated fragments (for which the highest levels of expression were observed in the phenotypically normal DM20 lines), was responsible for the observed muscle disease and myotonia. The dental abnormalities observed in our mice may result from orofacial muscle weaknesses, as suggested for DM1 patients (34). Smooth muscle weaknesses may also account for anal prolapse in homozygous DM300 mice. This trans-dominant effect of the mutation clearly depended on the level of production of abnormal DMPK mRNA as homozygous DM328 and DM1177 mice were much more affected than the hemizygous mice. Phenotype differed also greatly both between individual mice of the same line and between mice of different lines. Therefore, differences in the amounts of expanded DMPK mRNAs produced may account for differences between lines. However, although levels of DMPK gene expression in the muscles of 3-month-old mice were similar between the DM300-328 and DM300-1177 lines, the phenotype was more pronounced in the DM300-328 line. This may be due to the mRNA in the DM300-1177 line being produced from a chimeric gene, which may display a different pattern of expression during development. Alternatively, the chimeric mRNA may be less stable. DM300-1112 mice, with three copies of the transgene, displayed no phenotype, although mRNA carrying large CUG repeats were produced and aggregated into nuclear foci. However, in DM300-1112 mice, in which the three copies are integrated into the X chromosome, the level of expression in homozygous mice was not significantly higher than that in hemizygous mice (in contrast DM300-328 and DM300-1177 mice, in which the level of expression in the homozygous mice was twice that in hemizygous mice, data not shown). The level of expression in DM300-1112 homozygous (with 2 × 3 copies) was equivalent to the level of expression in DM300-328 and DM300-1177 homozygous mice (with 2 × 1 copy). Therefore, although the level of expression was higher

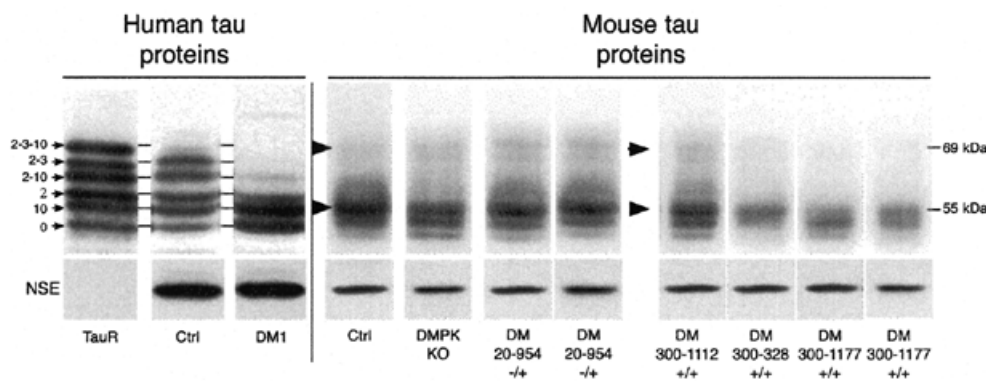


Figure 7. Tau protein expression in DM1 patients, DM transgenic mice and *Dm15* knock-out mouse (DMPK KO). A similar amount of total protein from brain tissue (100 μ g) from a human control (Ctrl) and a DM1 patient, a control mouse (Ctrl), a *Dm15* knock-out mouse and DM transgenic mice were loaded on SDS-PAGE gradient gels. Recombinant tau isoforms (TauR) were loaded to determine the distribution of each of the tau isoforms by SDS-PAGE. The presence or absence of the sequences encoded by exons 2, 3 and 10 is indicated on the left. Tau proteins were stained with a polyclonal antibody directed against the last 15 amino acids of tau, which are identical in mice and humans. As an internal standard, staining for a neuron-specific enolase (γ -enolase) was performed. The human brain tau proteins comprise six isoforms, indicated by arrows (human tau proteins panel). Two main phosphorylated tau protein isoforms were detected in mouse brain tissue (indicated by arrowheads in the mouse tau proteins panel). They correspond to the tau isoform with the exon 10 sequence and the tau isoform with the exon 2 and 10 sequences, respectively. Note that the tau protein isoform with exon 2 and 10 sequences was not detected in homozygous DM300–328 and DM300–1177 mice.

in hemizygous DM300–1112 mice (twice that in the other DM300 hemizygous mice) and nuclear foci are observed, the level of mRNA carrying the larger repeat is probably not sufficient to induce a phenotype in hemizygous and homozygous mice. Furthermore, the repeat in the mRNA with the smaller CUG repeats (approximately 184 and 147) may not be large enough to participate in the phenotype. Differences in the length of the CTG repeat in the affected tissues (resulting from somatic instability) may account for variability between mice of the same line. The genetic background should also be taken into account. Although the hemizygous mice were mated with C57BL/6 for at least five generations before homozygous mice were produced, the founders of each line had a C57Bl6xDBA background. Thus, mice of the same line may display genetic differences according to what remains of the DBA background.

Tiscornia and Mahadevan (10) described a novel DMPK exon (E16) lacking the trinucleotide repeat, in human DMPK mRNA. In cells of DM1 patients, expansions have a deleterious effect on the production of mRNA molecules including E16 and changes in the balance between the various isoforms in the cytoplasm may be involved in the physiopathology of the disease. Surprisingly, we detected the mRNA isoform with E16 only in mice carrying the DMPK gene with the expanded repeat, and not in mice carrying the normal 20 CTG repeat. This difference in the results obtained by the two studies may result from differences in the constructs (tester gene/human DMPK gene) and environments used (human/mouse; fibroblast/muscle). However, we introduced into transgenic mice an imbalance of human mRNA isoforms. The isoform with E16 (normally absent in the mouse) may have also had a dominant effect, for example by altering the composition of DMPK complexes. All three DM300 lines produced the isoforms, but it is unclear whether differences in amount of this isoform produced may account for the DM300–1112 mice showing no signs of illness. In addition, this pathological effect may not be observed in transgenic mice which express their own *Dm15*

gene. Further experiments are required to determine the levels of the isoform containing E16 in the various lines.

Tau protein is a microtubule-associated protein produced in neurons, in which it regulates the microtubule network (35,36). Sergeant *et al.* (25) recently demonstrated that the stoichiometry of the tau protein isoforms was dramatically altered in DM1 patients. We also observed changes in the levels of the various mouse tau protein isoforms in the DM300–328 and DM300–1177 transgenic mice but not in the control mice (non-transgenic, DM20 or *Dm15* KO). The human DMPK transgene was expressed in the brains of all our transgenic mice (data not shown). This suggests that the presence of mRNA carrying the CUG expansion may alter tau splicing in the brain and could participate in the brain pathology in DM1 patients. Splicing abnormalities due to the CUG expansion have already been reported for the cardiac troponin T gene (20) and probably underlies the trans-dominant effect of the mutation via the proteins binding the CUG repeat (10,37–41). No abnormalities were observed in the DM300–1112 line, providing further support for the hypothesis that the level of pathogenic mRNA is too low in this line to induce the disease phenotype.

In conclusion, our transgenic mouse model (with myotonia, muscle atrophy, morphological abnormalities and changes in the distribution of tau protein isoforms), together with the transgenic mouse model consisting of mice carrying the human skeletal actin gene with a CTG expansion (showing myotonia and muscle abnormalities) (24), the *Dm15* knock-out model (with cardiac conduction defect and mild myopathy) (33,42) and the *Six5* knock-out model (with cataracts) (11,12), suggest that DM1 is a multigenic, multimechanism disease (7,23,43). A single mutation, a CTG expansion, causes, by a cis effect, the haploinsufficiency of at least two genes involved in the disease and acts, via an RNA trans-dominant effect, on the expression of other unknown genes in the various tissues affected. Very recently, Liquori *et al.* (44) found that a CCTG

repeat expansion, in an intron of a gene unrelated to DMPK, was causing the myotonic dystrophy type 2 (DM2 or proximal myotonic myopathy). The clinical similarities between the two myotonic dystrophies underline the extensive role of abnormal RNA carrying CUG or CCUG expansion in these pathologies.

Overall, transgenic mice homozygous for the human DM1 allele are a good model for myotonic dystrophy, mirroring, in many aspects, what is observed in human patients. Our results also show that the level of mRNA carrying the CUG expansion and the size of this expansion are critical for reproduction of the symptoms linked to the trans-dominant effect of the mutation. Thus, these transgenic mice are a useful model to help us elucidate the basic mechanism of this disease and for investigating other changes in protein expression in many tissues in which the DMPK gene is expressed, as shown for tau protein in the brain.

MATERIALS AND METHODS

Transgenic mice and DNA analysis

Transgenic mice were produced and screened as previously described (27). The transgenic mice were reared in an air-conditioned, ventilated animal facility and the experiments were authorized by the head of the Veterinary Department according to French law. To determine the length of the CTG repeats in the tail, PCR analyses were performed with primers 101 and 102, and PCR products were separated by electrophoresis in a 4% denaturing acrylamide gel. Homozygous and heterozygous animals were identified by Southern blot hybridization with probe P59Pst2 (2 kb *Pst*I fragment of the DMPK gene), the EPO probe (1 kb *Pst*I fragment from the mouse erythropoietin gene) being used as an internal control (26).

Histology and immunohistochemistry

The gastrocnemius, soleus and sternomastoid muscles were removed from mice and frozen in isopentane for cryostat sectioning. We prepared 10 μ m frozen transverse sections from all these muscles for histological analysis. Frozen sections were stained with hematoxylin as described by Dubowitz (45) and ATPase enzyme histochemistry was carried out as described by Brooke and Kaiser (46). For immunohistochemistry, 5 μ m frozen sections were fixed in 1% paraformaldehyde and incubated with primary antibodies directed against neonatal MyHC (47) (diluted 1/50) and against slow MyHC (Novocastra) (diluted 1/5). The sections were then washed three times in phosphate-buffered saline (PBS) and incubated with a biotin-labeled secondary antibody, which was detected as a fluorescent signal using the biotin streptavidin-FITC system supplied by Amersham. Slides were mounted in Mowiol (Calbiochem).

Electron microscopy

Mouse muscles were fixed by incubation in 4% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) overnight at 4°C. Muscles were post-fixed for 1 h in 1% osmium tetroxide, dehydrated and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in an electron microscope (Zeiss EM 900).

Electromyography

We used a classical electromyographic approach to assess the electrical activity of skeletal muscle in transgenic and control mice. The muscles of the fore leg (extensors) and hind leg (EDL, soleus) were explored at rest, and following a gentle pinch to provoke muscle contraction. Recordings were taken from the muscle, using a disposable concentric 25 \times 0.3 mm needle; the ground electrode was a thick metal wire placed around the animal's chest. Signals were collected using a digital processing system for EMG (Viking II; Nicolet). All experiments were carried out double blind on mice aged 4–18 months, without anesthesia. The animals were handled with care and none of the animals investigated displayed signs of discomfort.

Analysis of microtubule-associated tau proteins

Mouse brain tissues were homogenized in Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 20 mM DTT, 20% glycerol), with one part brain to 10 parts buffer and boiled for 10 min. Human brain tissue from control and DM1 cases was processed as previously described (25). Western blots were performed as described elsewhere (25). Mice brain tissues were processed at the same time. The same amount of proteins was loaded on a single gel and the experiment was reproduced at least three times. Human and mouse microtubule-associated tau proteins were analyzed with an antiserum specific for the C-terminus TauCter (48). Neuron-specific enolase was used as an internal standard and was stained with the 1C1 monoclonal antibody, developed in the laboratory.

Cell culture

Primary myoblast cultures were generated from the soleus and tibialis anterior muscles (49). Myoblasts were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco BRL), supplemented with 20% (v/v) fetal calf serum (Eurobio) and kanamycin (80 μ g/ml; Gibco BRL).

Immunocytochemistry and RNA-FISH

Cells grown on glass coverslips were washed with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4 , 137 mM NaCl, 8 mM Na_2HPO_4 , pH 7) + 5 mM MgCl_2 and fixed for 15 min at room temperature in 4% paraformaldehyde. Cells were washed and stored in 70% ethanol at 4°C. They were rehydrated by incubation in PBS for 10 min at room temperature, and were then incubated with the primary antibody, directed against desmin (monoclonal mouse IgG1 isotype; Sigma) in PBS + 1% BSA for 30 min at 37°C, to check for the myogenicity of the culture. Cells were washed three times in PBS, for 5 min each, and were then incubated with the secondary antibody, a FITC-conjugated anti-mouse antibody (Sigma) at 37°C for 30 min. The slides were mounted in Mowiol and stored in the dark at 4°C.

For RNA-FISH, cells were incubated in 40% formamide, 2 \times SSC for 10 min and hybridized for 2 h at 37°C with a 5' FITC-2'-o-methyl (CAG)₃₀ oligonucleotide probe (10 ng) in a final volume of 20 μ l of 40% formamide, 2 \times SSC, 0.2% BSA, 2 mM vanadyl ribonucleoside complex and 1 mg/ml *Escherichia coli* tRNA. The coverslips were washed and mounted on slides in P-phenylenediamine (PPD, an antibleaching

agent) in PBS + 90% glycerol and stained with 4,6-diaminido-2-phenylindole (DAPI).

Slides were examined under a Leica fluorescence microscope equipped with fluorescein and double-pass band filters; images were captured with a cooled camera (Leitz, DMRB). Images from two wavelengths (DAPI, FITC) were assembled and colored using Adobe Photoshop software.

Ribonuclease protection assays (RPA)

RPA were performed with the RPAII kit (Ambion). Total RNA was extracted from tissues (gastrocnemius) according to the Chomczynski method with Rnazol (Bioprobes). With this method, it is possible to extract total free RNA (i.e. RNA not attached to the nuclear matrix). A 117 bp fragment of the human DMPK sequence (exons 3–4, 1773–1890 bp) was amplified by PCR with primers: 5'-TGAAGATGAAGCA-GACGGGC-3' (upstream) and 5'-AGGGGTAAGT-GGTTGTG-3' (downstream). This fragment was cloned into *Sma*I-digested pGEM3Zf vector (Promega) and used as a template to make an antisense RNA probe by *in vitro* transcription from the SP6 promoter (MaxiScript; Ambion), using ³²P-labeled CTP. An 18S antisense probe was used as an internal standard (Ambion). The probe (200 000 c.p.m.) was hybridized with 10 µg of total RNA in 20 µl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4, 1 mM EDTA), overnight at 60°C. The next day, samples were digested with 200 µl of digestion buffer (Ambion), ethanol-precipitated and size-fractionated on 6% polyacrylamide gels. Quantification was performed with a Molecular Dynamics PhosphorImager and ImageQuant software.

RT-PCR

RT-PCR analysis was performed as described by the kit manufacturer (Promega). DNaseI-treated total RNA was reverse transcribed with MMLV reverse transcriptase (Promega) and random hexanucleotide primers (Pharmacia). Production of the expanded repeat was detected using primers DMA (5'-CAG TTC ACA ACC GCT CCG AGC-3') and DMBR (5'-CGT GGAGGA TGG AAC ACG GAC-3'). The length of the repeat was determined by PCR as previously described (27). The various alternatively spliced forms of the DMPK 3'-UTR were amplified using primers DMASO6 (3' end exon 12: 5'-GTT GCA GGA GCG GAT GGA GTT-3') and 101 (5'-CTT CCC AGG CCT GCA GTT TGC CCA TC-3') in the same PCR conditions. Amplified product (10 µl) was mixed with 2 µl of loading buffer and run on an agarose gel (2.5%).

RT-PCR product sequencing

PCR products have been extracted from a 2% agarose gel using the QIAquick Gel extraction kit (Qiagen). Using the purified band as a template, a PCR-sequence reaction has been performed using the Thermosequenase Radiolabeled Terminator Cycle Sequencing kit (USB) and subjected to electrophoresis in a 6% denaturing acrylamide gel at 55 W for 1 h 30 min and 3 h. After electrophoresis, the radioactive sequence was detected by autoradiography after overnight exposure on Biomax-MR film (Kodak).

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