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Down-regulation of mitochondrial mRNAs in the *mdx* mouse model for Duchenne muscular dystrophy

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Abstract In our search for genes up- or down-regulated genes in the *mdx* mouse model for Duchenne muscular dystrophy, we isolated a down-regulated mitochondrial DNA clone. In addition to this clone, all protein-coding mitochondrial genes tested had tissue-specific and age independent down-regulated expression. This implied mechanisms at the RNA level since no change in the mitochondrial DNA contents were detected. Cytochrome *c* oxidase activity showed the same range of down-regulated expression. These data provide a molecular basis for energetic metabolism modifications in *mdx* mice.

Key words: Dystrophin; Gene expression regulation; Mitochondrial RNA

1. Introduction

The *mdx* mouse is a puzzling model for severe Duchenne muscular dystrophy, which is reported to be caused by mutations in the dystrophin gene [16,17]. In the *mdx* mouse, a point mutation [2] created a premature stop codon [24] but, unlike humans, this animal has almost no motile alterations. In fact, between 3 and 4 weeks of age, *mdx* mice undergo a temporary major DMD-like crisis with muscle fiber degeneration and necrosis [9,10,19]. Regeneration and recovery are evident by 5 to 6 weeks and muscle fibers are somehow protected from further definitive necrosis. One exception concerns the diaphragm which shows a characteristic DMD histological pattern of necrosis and connective tissue proliferation in the aging *mdx* mouse [25].

Very little is known about the cascade of events starting with dystrophin deficiency and ending with dramatically progressive DMD, and it is not yet understood how the *mdx* mouse can largely compensate for dystrophin deficiency. We have developed a strategy based on the assumption that, regarding the drastic modifications that occur in skeletal muscles, the absence of dystrophin creates cellular signals that are important enough to interfere with transcription of a significant population of genes. To identify such up- or down-regulated genes, we constructed a cDNA *mdx* mouse muscle library and differentially screened it with probes from *mdx* mouse muscles and control muscles. A down-regulated clone was isolated in *mdx* mouse skeletal muscle. This clone contained 80% of the ATPase 6 gene from mitochondrial DNA. Further analysis showed that down-regulation affected other mitochondrial RNAs. This under-expression was found to be specific to skeletal muscles and brain, but not dependent on age, and paralleled the down-regulated activity of partially mitochondrial encoded cytochrome *c* oxidase.

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Abbreviations: DMD, Duchenne muscular dystrophy; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; ND, NADH dehydrogenase; CO, cytochrome *c* oxidase; Cyt *b*, cytochrome *b*.

2. Materials and methods

2.1. Tissues and RNA extraction

Tissues were dissected from 3-month-old C57BL/10mdx and C57BL/10ScSn control male mice, frozen in liquid nitrogen and stored at −80°C. Muscle samples (posterior legs and the bottom part of the back) were checked by histology and immunofluorescence with an anti-dystrophin monoclonal antibody [21]. Total RNAs were extracted using a guanidine isothiocyanate method [5], from a 1-to-4 g pool of tissues from 5 to 10 animals. RNA concentration and purity were assessed by measuring absorbance at 260, 270 and 280 nm [26] and gel electrophoresis.

2.2. Differential screening procedures

A Uni-Zap XR (Stratagene) phage library, derived from 3-month-old *mdx* mouse skeletal muscles, was plated at low density (4 to 5 colonies/cm^2) and transferred onto four nylon membrane replicate sets. The first and third replicates were hybridized with the *mdx* probe and the second and fourth replicates were hybridized with the control probe. ^{32}P-labeled single strand cDNA probes (1×10^8 to 5×10^8 dpm/μg) were prepared from 1 μg of poly(A$^+$) RNA from 3-month-old *mdx* and control mouse skeletal muscles and column purified according to Sambrook et al. [23]. Clones showing differential hybridization signals, on both duplicate membranes and with different X-ray film exposures, were recovered as pBluescript SK phagemids according to the supplier's protocol. Sequence determinations were performed on both strands using T3 and T7 primers and the Taq Dye Primer Cycle Sequencing Kit (Applied Systems at Généthon, Evry, France). Sequences were compared to database sequences with the Blast program.

2.3. Northern blots

Total RNAs were size-fractionated in denaturing 1% agarose gels in the presence of ethidium bromide (25 μg/ml) and transferred to nylon membranes as described by the supplier (Amersham). Double-strand cDNA probes were made from PCR-prepared DNA fragments that were [α-^{32}P]dCTP labeled by random priming. S14 inserts were T7-SK primed; other mitochondrial DNA probes were generated by PCR with the pAM1 DNA template, a plasmid coding for the complete mouse mitochondrial genome (generous gift of Dr. Patrick Lestienne) and the following primers: 5′-CGCCATAGCCTTCCTAACATT-3′/5′-ATGGTGGTACTCCCGCTGTAA -3′ (ND1); 5′-AATCGTTGATTATTCTCAACC-3′/5′-TAGGTTGGTTCCTCGAATGTG -3′ (COI); 5′-CCCATTCCAACTTGGTCTACA-3′/5′-AATTATTGAAGCAGATCAGTT -3′ (COII); 5′-AACTAGATACATCAACATGAT-3′/5′-GGGGTTTTTACTTTTATGGTT -3′ (ATPase 8); 5′-CAAACTCATGCATATCACATA-3′/5′-GGAAAAGTCAGATTACGTCTA -3′ (COIII); 5′-CCCTCCTTCCAACATACTCC-3′/5′-TTTGGTTGGTTGTCTTGGGTT -3′ (ND6); 5′-CCTCCTGCCCCATCCAACAT-3′/5′-CGAT-

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AATTCCTGAGATTGGTA -3′ (cyt *b*). GoldStar DNA polymerase (Eurogentec) was used in 30 cycle reactions (1 min/95°C, 1 min/50°C and 1 min/72°C). Ribosomal 18S RNA oligonucleotides were [γ-^{32}P]dATP kinased according to the standard procedure. All probes were purified through G50 spin-columns. The hybridizations were carried out overnight in 50% formamide, 5× SSPE, 5× Denhard't solution, 1% SDS and 100 mg/ml salmon sperm DNA, at 42°C. The membranes were washed in 0.1× SSPE, 0.2% SDS at 50°C, exposed to Amersham Hyperfilms for autoradiography and scanned with a phosphor imager analyser (Fuji Bas 1000) for quantification with the MacBas program (Fuji). Data were averaged and variance was estimated according to the standard error. The amounts of RNAs (routinely 10 to 20 μg) were normalized by re-probing the same lanes or probing half of the lanes with GAPDH and/or 18S rRNA. No significant differences were noted between these two internal standards.

2.4. Mitochondrial enzyme assays

100 mg of diaphragm slices were homogeneized in 10 volumes of 10 mM phosphate buffer (pH 7.5) on ice. Citrate synthase and cytochrome *c* oxidase activities were measured in increasing quantities of diaphragm homogenates in the presence of 1 mM lauryl-maltoside, at 25°C. Cytochrome *c* oxidase activity was measured, at 550 nm, as the oxidation rate of reduced cytochrome *c* by diaphragm homogenates [22]. Citrate synthase activity was initiated by the addition of 0.5 mM oxaloacetic acid and monitored at 412 nm for 6 min in the presence of excess substrate (0.1 mM 5,5′-dithio-bis(-2-nitrobenzoic acid)) and cofactor (0.3 mM acetyl coA).

3. Results

3.1. S14 clone selection by differential screening

Three thousand clones of a 3-month-old *mdx* mouse muscle cDNA library were differentially screened with probes prepared with muscles from 3-month-old *mdx* and control mice. Three clones showing differential hybridization signals, between *mdx* and control probes, were isolated. The investigation carried out with one clone, designated S14, is reported here. In the library screening, this clone exhibited a lower hybridization signal with the *mdx* probe than with the control probe (Fig. 1A). It contained a 560 bp insert and its expression, detected by Northern blot, in muscles of 3-month-old *mdx* mice as compared to control is shown in Fig. 1B. The size of the main hybridized RNA band was approximately 800 bp. Quantification revealed twofold lower expression in *mdx* mouse muscle (ratio control/*mdx* = 2.12 ± 0.12; n = 11). Sequencing and research in Genebank and Swissprot databases indicated that clone S14 represented about 80% of the ATPase 6 gene. This is a mitochondrial gene encoding subunit 6 of the ATPase complex, located from positions 7927 to 8606 on the 16 295 bp circular mitochondrial mouse genome [1]. S14 encompassed the region from position 8045 to position 8605; below it is referred to as the ATPase 6 probe.

3.2. Down-expression probed with ATPase 6 concerns the other mitochondrial RNAs

In the mitochondrial genome, each complete DNA strand is transcribed as one polycistronic RNA chain before further cleavages at the tRNA level [6]. This explained why the size of the band we detected on Northern blots corresponded to a mRNA encoded by the two overlapping ATPase 6 and ATPase 8 genes [1]. To determine whether transcripts down-regulated in *mdx* mouse muscle were limited to this ATPase 6/8 RNA, eight probes along the mitochondrial genome were synthesized by PCR and assayed in comparative Northern blots (Fig. 2A). The same twofold under-expression (ratio control/*mdx* = 1.95 ± 0.12; n = 29) was reproducibly observed in *mdx* mouse muscles with all mitochondrial probes for the main bands and larger bands corresponding to characteristically longer premature mitochondrial transcripts. One complete hybridization pattern is shown in Fig. 2B with the NADH dehy-

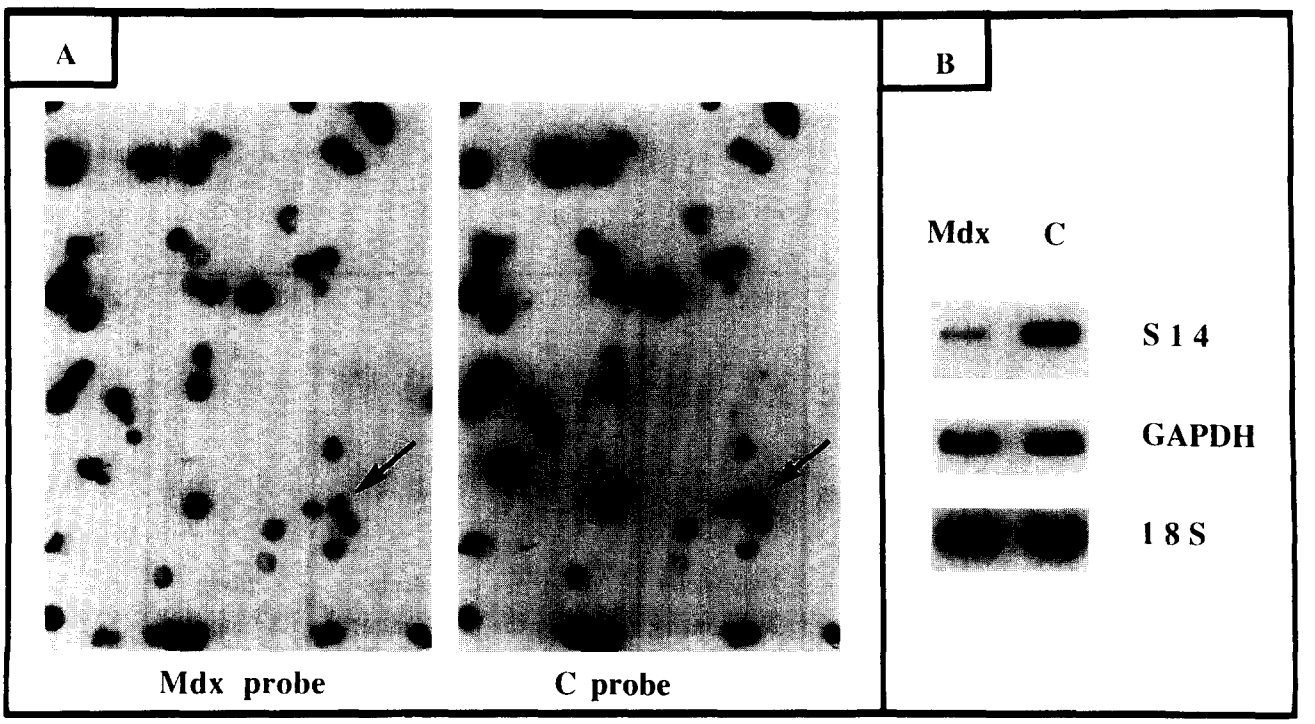

Fig. 1. S14 clone selection. Down-regulation observed in the differential screening (A) of the S14 clone (arrow) was confirmed on Northern blots (B). Control: C.

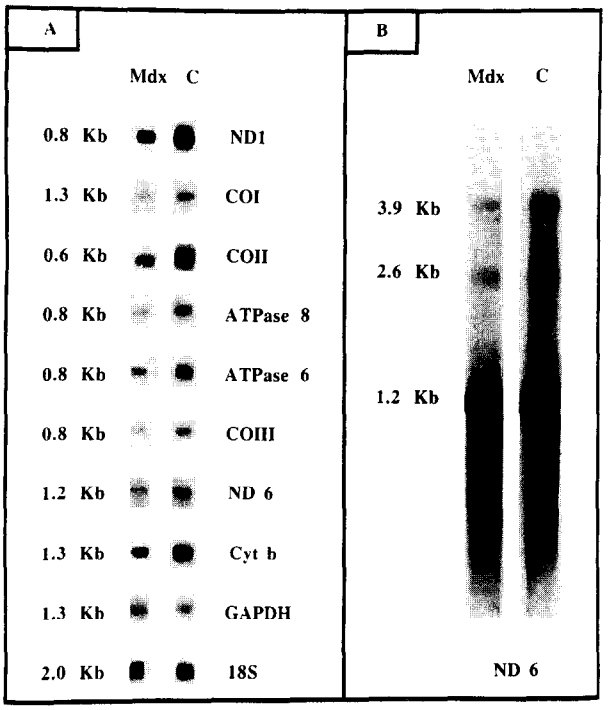

Fig. 2. Quantitative Northern blot analysis of eight mitochondrial gene transcripts from *mdx* and control muscles. (A) RNA blots were comparatively analyzed with the probes listed on the right; the apparent approximate transcript sizes are presented on the left. (B) Complete hybridization patterns shown with ND 6 probe, after over-exposition to highlight the two minor larger bands. Control: C.

drogenase 6 probe. We concluded that down-regulation, initially detected with the ATPase 6 probe, actually involved all mitochondrial RNA transcripts we detected, regardless of their maturation status.

3.3. mdx *mouse muscle does not contain less mitochondrial DNA than control mouse muscle*

One explanation for the down-regulated expression of mitochondrial RNAs in *mdx* mouse muscle could have been a reduction in the amount of mitochondrial DNA. We tested this hypothesis by comparing mitochondrial DNA contents in *mdx* and control mouse muscles. Detection was made by Southern blots of *Hin*dIII digested genomic DNA, prepared from muscles, hybridized with the ATPase 6 used as mitochondrial DNA probe, and a nuclear DNA probe to normalize the signals. The nuclear probe was a 1 kb *Xho*I–*Hin*dIII cDNA fragment coding for the mitochondrial ATPase α subunit. Quantification of ATPase 6 probe hybridization signals (not shown) did not show any significant differences between *mdx* and control mice after normalization by the nuclear probe (ratio control/*mdx* = 0.97 ± 0.10; $n = 3$), demonstrating no change in the amount of mitochondrial DNA in *mdx* mouse skeletal muscle. The same result was obtained with another mitochondrial probe, derived by PCR from cytochrome *b* DNA and two other nuclear probes, β actin and calsequestrin. Down-regulated expression of mitochondrial RNA could therefore not be the result of less mitochondrial DNA in *mdx* mouse muscles, but implies a regulatory mechanism at the RNA level.

3.4. *Down-regulation of mitochondrial RNAs is tissue specific and age independent*

Dystrophin transcripts are normally expressed in a tissue specific manner (for a review see [13]) and the impact of dystrophin deficiency is only particularly severe in the diaphragm of the aging *mdx* mouse [25]. Moreover, a very characteristic regeneration crisis affects approximately 1-month-old *mdx* mouse muscle [8,19]. It was thus of interest to test the age dependance and tissue specificity of down-regulation of mitochondrial RNAs. Fig. 3 shows Northern blots from *mdx* and control mice, probed with ATPase 6 and made from different tissues; (i) 1-, 3- and 9-month-old limb muscles, diaphragm, heart and brain, tissues where dystrophin RNAs are normally transcribed, and (ii) liver which does not contain dystrophin transcripts. In limb muscles, the same twofold down-regulation found at 3 months was measured at 1 month (ratio control/*mdx* = 2.10 ± 0.07; $n = 3$) and 9 months (ratio control/*mdx* = 1.92 ± 0.10; $n = 5$), indicating no age dependence of down-regulated expression of mitochondrial RNAs. The twofold under-expression observed in limb muscles from the *mdx* mouse was detected in diaphragm (ratio control/*mdx* = 2.20 ± 0.16; $n = 3$) and brain (ratio control/*mdx* = 1.80 ± 0.12; $n = 8$), but not found in the liver (ratio control/*mdx* = 1.03 ± 0.11; $n = 3$) or heart (ratio control/

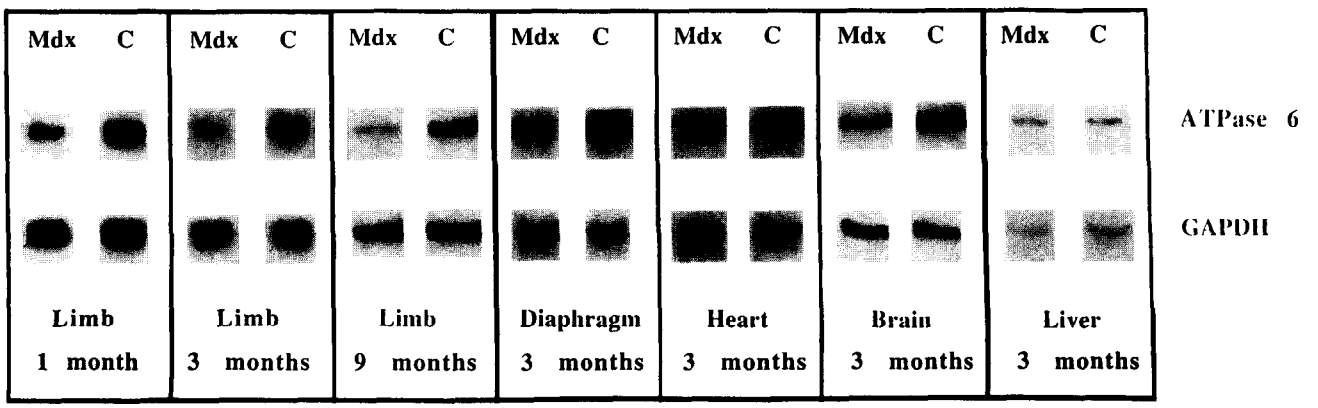

Fig. 3. Age independance and tissue specificity of the mitochondrial RNA down-regulation. The levels of expression of mitochondrial RNAs were compared in *mdx* and control mice by ATPase 6 Northern blot analysis in limb muscles (1-, 3- and 9-month-old), diaphragms, hearts, brains and liver, using 10 μg of total RNA for the three former tissues and 30 μg for the two latter tissues. Only heart and liver did not present lower ATPase 6 expression. Control: C.

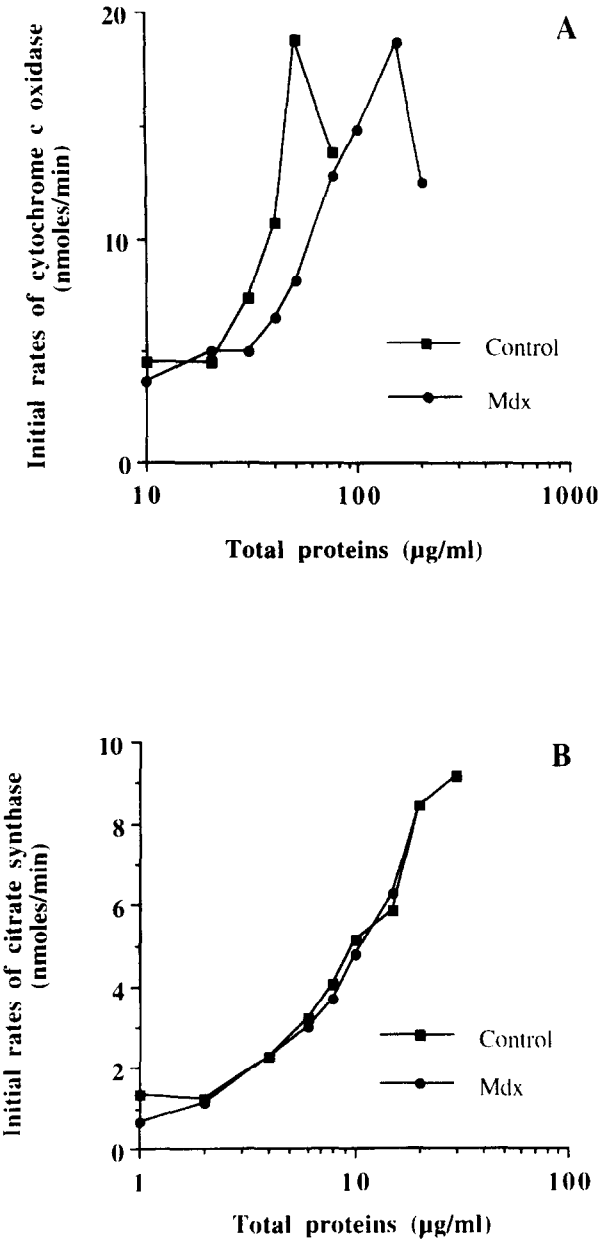

Fig. 4. Comparison of cytochrome *c* oxidase activities in *mdx* and control mouse diaphragms. Cytochrome *c* oxidase activities (A) were normalized with the citrate synthase activities (B). Assays were performed in increasing quantities of diaphragm homogenates and expressed relative to the protein concentrations of these homogenates. Three determinations (Bio-Rad reagent) were carried out for each protein concentration and the difference between estimations was less than 10%.

ndx = 1.00 ± 0.12; *n* = 3). This shows the strict tissue specificity of mitochondrial RNA down-regulation. It did not completely match the tissue specificity of dystrophin and was not modulated by the difference between diaphragm and other skeletal muscles concerning damage caused by a lack of dystrophin.

3.5. *Down-regulation of mitochondrial RNAs parallels down-regulation of a mitochondrial enzyme activity*

To determine the consequences of the twofold down-regulation of mitochondrial RNAs at the translation level, the activity of the cytochrome *c* oxidase complex containing three of the main mitochondrial DNA encoded enzymes, cytochrome oxydases I, II and III, was compared in *mdx* mouse and control muscles. Three-month-old diaphragms were chosen because these muscles present a homogeneous distribution of fast and slow fibers [16] (Pons, personal communication), thus eliminating artifactual differences from the tissue samples. As shown in Fig. 4, this activity was standardized according to the activity of citrate synthase, a nuclear DNA encoded enzyme functioning in mitochondria. The same twofold lower difference (ratio control/*mdx* = 1.80 ± 0.20; *n* = 3; calculated at 50% activity), detected in the expression of mitochondrial RNAs, was measured for cytochrome *c* oxidase activity in *mdx* mouse muscles. Down-regulation of mitochondrial RNAs could thus directly induce quantitatively similar down-regulation at the translated product level.

4. Discussion

Through differential screening, we isolated a mitochondrial clone encompassing 80% of the ATPase 6 gene and down-regulated in *mdx* mouse skeletal muscles. Extension of ATPase 6 down-regulation in *mdx* mouse skeletal muscles to all mature RNAs and their precursors is in agreement with the specificities of mitochondrial transcription: one promoter controls transcription of each mitochondrial DNA strand in one long premature polycistronic transcript [29]. Since the amount of mitochondrial DNA remained unchanged in the *mdx* mouse and the same twofold under-expression was found for all mitochondrial genes tested, the simpliest explanation would be transcription regulation affecting all transcripts in the same way.

There are at least two arguements that favor a highly significant impact of the observed down-regulation. (i) The twofold difference could be enough to modulate some aspects of the cellular activity since this down-regulation did not concern only one gene, but rather a complete set of genes cooperating in the very essential oxidative phosphorylation pathway. It is likely that such key genes for ATP production cannot undergo manyfold deregulation, especially in tissues such as brain and muscle, which rely more on mitochondrial ATP production. Variations in the expression of mitochondrial genes were thus reported to be 2- to 4-fold during myogenic differentiation [30], and 1.5- to 8-fold in more extreme situations such as mitochondrial myopathies [15] and cancer cells [28]. In addition, the general trend in *mdx* and DMD muscles of a shift towards oxidative slow-type muscle fibers [3,18,20,25], richer in mitochondrial DNA and RNA [31], partially reduces the decrease in the overall mitochondrial RNA content here. (ii) The second element is under-expression of the encoded proteins. It cannot be ruled out that the twofold lower cytochrome *c* oxidase activity in *mdx* muscles, an essential enzyme of the respiratory chain, was due to its nuclear encoded subunits. However, the fact that the same twofold difference was noted in enzymatic activity and RNA expression suggests that down-regulation of mitochondrial RNAs leads to a parallel under-expression of the encoded proteins.

Our data suggest a direct causal relationship between the lack of dystrophin and down-regulation of mitochondrial RNA transcripts, instead of a secondary effect such as non-specific consequences of regeneration or necrosis. First, this down-

regulation was found at 1-month-old, when the muscle fibers go through a major degeneration/regeneration crisis, and at 3- and 9-month-old, when fiber regeneration is complete. Second, it affected limb and diaphragm muscles in the same manner, although only the latter reproduced strong DMD-like muscle damage [25]. The altered mitochondrial genetic expression was thus in line with the lack of dystrophin in skeletal muscles, regardless of their ages or physiological status. Third, the brain, which is not known to show any degeneration or necrosis, presented the same down-regulation as muscles. In the *mdx* mouse, both brain and muscles fail to express dystrophin transcripts. Moreover, the fact that the situation in liver provided a good negative control was in full agreement with our hypothesis, since this tissue does not normally express dystrophin. However, the non-deregulation of mitochondrial transcripts in *mdx* hearts indicated a cardiac specific phenomenon. The heart, as skeletal muscles and brain, normally expresses dystrophin transcripts and is very reliable for mitochondrial energy production. However, it oversteps the 1-month-old necrosis phase and, more generally, cardiac function seems to be partly protected in DMD patients since cardiac tissue resists the lack of dystrophin much longer than skeletal muscles [14]. One explanation for this cardiac adaptation could be the existence of a short protein product of the dystrophin gene, named DP71, which has been found in heart but not in skeletal muscles [12].

So far very few modifications in mitochondria from muscles lacking dystrophin have been reported. However, Cullen and Watkins [9] have observed unexplained ultrastructural changes in DMD and *mdx* mouse mitochondria. These changes could be correlated with the down-regulated expression of mitochondrial genes. Very recently, energy metabolism measurements have highlighted an impaired oxidative metabolism in *mdx* mouse skeletal muscles [11]. Our data provide a molecular basis for the defective regulation of cellular energy production in the *mdx* mouse and could be explained by a cytoskeleton role. On one hand, one possible function of dystrophin is in anchorage of the cytoskeleton to the sarcolemma [27]; on the other hand, the mitochondrial matrix is likely associated with the cytoskeleton [4] and some of the mitochondrial mRNAs could belong to the growing known population of mRNAs cytoskeleton-dependent in terms of their transport and translation [7]. The absence of dystrophin could thus induce some reorganization of the cytoskeleton, which could deregulate the expression of mitochondrial genes.

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