DMD transcript imbalance determines dystrophin levels

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ABSTRACT Duchenne and Becker muscular dystrophies are caused by out-of-frame and in-frame mutations, respectively, in the dystrophin encoding DMD gene. Molecular therapies targeting the precursor-mRNA are in clinical trials and show promising results. These approaches will depend on the stability and expression levels of dystrophin mRNA in skeletal muscles and heart. We report that the DMD gene is more highly expressed in heart than in skeletal muscles, in mice and humans. The transcript mutated in the mdx mouse model shows a 5′ to 3′ imbalance compared with that of its wild-type counterpart and reading frame restoration via antisense-mediated exon skipping does not correct this event. We also report significant transcript instability in 22 patients with Becker dystrophy, clarifying the fact that transcript imbalance is not caused by premature nonsense mutations. Finally, we demonstrate that transcript stability, rather than transcriptional rate, is an important determinant of dystrophin protein levels in patients with Becker dystrophy. We suggest that the availability of the complete transcript is a key factor to determine protein abundance and thus will influence the outcome of mRNA-targeting therapies.—Spitali, P., van den Bergen, J. D., Verhaart, I. E. C., Wokke, B., Janson, A. A. M., van den Eijnde, R., den Dunnen, J. T., Laros, J. F. J., Verschuuren, J. G. M., ’t Hoen, P. A. C., Aartsma-Rus, A. DMD transcript imbalance determines dystrophin levels. FASEB J. 27, 000–000 (2013). www.fasebj.org

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Duchenne muscular dystrophy (DMD) is the most common and most severe inherited neuromuscular disorder (1). It is caused by reading frame-disrupting mutations in the DMD gene, which abolish dystrophin protein function (2). In Duchenne muscles, only traces of dystrophin can be found with immunofluorescent staining, and full-length dystrophin is absent by Western blotting (3). In contrast, mutations that maintain the DMD reading frame allow production of dystrophins, albeit of a different size and/or abundance (4). These mutations are found in the patients with less severe Becker muscular dystrophy (BMD; ref. 5).

In Duchenne muscles, the lack of protein epitopes encoded by the portion of the transcript upstream from the mutation has been considered to be the result of instability of the prematurely truncated dystrophin protein. However, DMD transcripts can generally be picked up in patient-derived muscles (6). Several papers report quantitative studies of the DMD transcript in patients with DMD, mostly using microarray technology (7–12), in which probes are localized at the 3′ end of the gene (see Supplemental Fig. S1 for a graphical representation of probe positions within the DMD gene), concluding that the DMD gene is less expressed in patients with DMD compared with controls. Chelly et al. (6) reported that out-of-frame but not in-frame mutations can cause a reduction in the transcript levels <10%. These results were confirmed in a recently published study (13). However, this may only hold true for a subset of DMD causing mutations, because it has also been reported that reading frame-disrupting mutations can cause a reduction of 40–75% in the transcript levels (14). Very few studies have attempted an extensive profiling of the DMD transcript in patients, mainly because of the huge size of the gene (2.2 Mb) and transcript (14 kb; 6, 13–15). What is known is that the transcription of the entire DMD gene requires around 16 h and that splicing occurs cotranscriptionally (16). Notably, an excess of nascent transcript relative to mature transcripts was detected in human control muscle tissue (15).

In the past few years, a lot of effort has been put into the development of therapeutic strategies to slow down the disease progression. Among these strategies, anti-
sense oligonucleotide (AON)-induced exon skipping (17) and read-through of stop codons (18) have shown great potential and made it to clinical trial phases III (19) and II (20), respectively. The exon-skipping approach aims to reframe the DMD transcripts to allow the production of a BMD-like dystrophin, whereas read-through uses compounds that force the cell to ignore premature stop codons, allowing the production of full-length proteins. Because these approaches target the precursor mRNA and mature mRNA, respectively, transcript amounts will be crucial for the potential success of these therapies.

Therefore, we have explored dystrophin transcript levels in the mdx mouse model, carrying a nonsense mutation in exon 23, and compared them with those in wild-type (WT) mice. We report the transcript levels in several skeletal muscles and in heart and show that there is a prominent 5′–3′ transcript imbalance in skeletal muscles of mdx mice, meaning a nonhorizontal representation of the transcript levels along the transcript with reduced transcript levels toward the 3′ end. We also show a significant transcript imbalance in 22 patients with BMD. We finally demonstrate that transcript imbalance, rather than transcript levels, determines the amounts of dystrophin protein in patients with BMD. We suggest that transcript imbalance is a key factor for determining protein abundance, and thus it may influence the outcome of mRNA targeting therapies.

MATERIALS AND METHODS

Mice

Muscles (untreated or injected) were obtained from experiments described previously (21–23). Four to 7 muscles were included per group.

Muscle biopsies

Muscle biopsy samples were obtained from the tibialis anterior muscles with standardized techniques under local anesthesia from 22 patients with BMD after informed consent was obtained. The muscle tissue was immediately frozen in liquid nitrogen-cooled isopentane and stored at −80°C before proceeding with further analyses.

Western blot analysis

Muscle samples were homogenized in 75 mM Tris-HCl (pH 6.8) and 13% (w/v) sodium dodecyl sulfate (SDS) buffer using MagNA Lyser Green Beads (Roche Diagnostics, Almere, The Netherlands) in a BBY24M Bullet Blender Storm (Next Advance, New York, NY, USA). Protein concentrations were determined using a Pierce bichinonic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer’s instructions. A 30-μg sample of (mouse) protein lysate in treatment buffer with 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.001% (w/v) bromphenol blue was heated for 5 min at 95°C and loaded on 1.0-mm-thick native polyacrylamide gel electrophoresis (PAGE) Tris acetate (polyacrylamide) gels, with a linear resolving gel gradient of 3–8% (Bio-Rad, Veenendaal, The Netherlands) and run on the Trans-Blot Turbo system for 1 h at 75 V (0.07 A) and 2.5 h at 150 V (0.12 A) in Running Buffer (XT Tricine; Bio-Rad) in an ice container (unpublished results). Proteins were blotted on a nitrocellulose membrane using ready-to-use Trans-Blot Turbo transfer packs and the Trans-Blot Turbo transfer system from Bio-Rad at 2.5 A and ~25 V for 10 min. For patients with BMD, 10 and 20 μg of protein lysates were loaded on a hand-made 4–7% gradient SDS-PAGE gel and run overnight at 4°C. Proteins were blotted onto a nitrocellulose membrane for 6 h at 4°C. Blots were first blocked in Tris-buffered saline (TBS) containing 5% (w/v) milk (Elk, Cappiina Melkunie, Zaltbommel, The Netherlands), washed in TBS plus 0.05% (v/v) Tween 20 (TBST), and incubated overnight with monoclonal NCL-DYS1 antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) or rabbit polyclonal antibody (ab15277; Abcam, Cambridge, UK) in TBS. Rabbit polyclonal antibody to sarcomeric actinin (ab72592; Abcam) was used as a loading control. Membranes were washed in TBST and incubated for 1 h with the fluorescent secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680LT donkey anti-rabbit IgG (Li-Cor, Lincoln, NE, USA) in TBS, and protein levels were quantified with the Odyssey system and software (Li-Cor). Four mice per group were included, and two technical replicates were performed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated using TriPure reagent as described previously (21, 24). The RNA concentration was measured on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and integrity was checked with a total RNA nanochip assay on the Agilent 2100 bioanalyzer (Agilent, Amstelveen, The Netherlands). Human control skeletal muscle (AM7982) and heart (AM7966) total RNAs were purchased from Ambion (Bleiswijk, The Netherlands). cDNA was synthesized using 400 ng of total RNA as input material and with random hexamer primers (20 ng), and gene expression levels were determined by SYBR Green-based real-time qPCR (95°C for 10 s, 60°C for 30 s, and 72°C for 20 s for 45 cycles followed by melting curve determination) on the LightCycler 480 (Roche Diagnostics Ltd., West Sussex, UK). All primer pairs were used at the final concentration of 0.1 μM and spanned at least 1 splice junction to avoid contaminating genomic DNA amplification; primer sequences are listed in Supplemental Table S1. To determine the relative expression levels in skeletal muscles and heart, we used Gapdh as the reference gene. Amplification curves were imported in LinReg software (version 11.3), which allows evaluation of the PCR efficiency in each well based on the SYBR Green fluorescent accumulation. This allows calculation of expression levels even when different fragments are not amplified with the same amplification efficiency or when the amplification efficiency is not 100%. The output results in LinReg are relative amounts corrected for amplification efficiency (listed in the Excel results file in the N0 column), which represent the relative amount for that specific amplified region. The relative amounts (N0 values) of DMD 1–2 exon-exon junction were then corrected for the N0 values of Gapdh expression by dividing the exon 1–2 N0 values for the Gapdh N0 values. The number obtained was set to 1 for quadriceps muscle, and graphs show the transcript levels across muscles.

To analyze the transcript imbalance, the expression N0 values of the amplified exon-exon junctions were corrected for the expression levels of the exon 1–2 junction, which was used as reference instead of Gapdh. The ratios obtained
were plotted in a scatterplot in which exons are plotted on the y-axis and the ratios between the N0 values (so the relative quantities) on the y-axis. This analysis was applied to infer the expression of each exon-exon junction relative to that of exon 1, thus relative to the transcription at the promoter site. The same analysis was applied to calculate the regression lines in mice and human samples. The exon junctions analyzed in mice were exons 1–2, exons 20–21, exons 25–26, exons 44–45, exons 62–64, and exons 63–64. We included the last primer pair to identify the possible presence of the Dp71 isoform, which was only detected in diaphragm muscles as reported in Supplemental Fig. S2A.

Exon-skipping quantification using RT-qPCR was performed using a primer pair detecting only the skipped exon-exon junction (m22/24Fsh and m24r2 listed in Supplemental Table S1) and primer pairs covering 3 splice junctions along the *mdx* dystrophin transcript. These 3 independent regions along the *mdx* dystrophin transcript were used to quantify the total transcript: exon 1–2 junction, exon 44–45 junction, and exon 62–64 junction. The N0 values resulting from the analysis of the m22/24Fsh-m24r2 amplification were divided by the N0 values obtained by amplification of either the exon 1–2 junction, exon 44–45 junction, or exon 62–64 junction used to quantify the total transcript. These ratios were transformed to a percentage by multiplying the values obtained for 100.

Transcript analysis and quantification for patients with BMD were done with the same principle, using more primer pairs covering the exon 1–2, exon 2–3, exon 3–4, exon 7–8, exon 27–28, exon 43–44, exon 55–56, exon 62–64, and exon 63–64 junctions. The Dp427m expression in Fig. 5B was determined using Gapdh as the reference gene while the slope (transcript imbalance) was built using the exon 1–2 junction as a reference (see Fig. 5A). LinReg software was used to determine the N0 values for the quantification.

**Statistical analysis**

The differences in expression levels between heart and skeletal muscles in mouse and human were tested using a 1-way analysis of variance (ANOVA) and an independent-samples t test. A Bonferroni multiple testing correction was used to assess differences between groups. A value of $P \leq 0.05$ was considered significant.

The statistical analysis of the slope was performed using a linear mixed model, in which mice or patients were considered the random effect. The differences in slope angles in mice were determined by the estimation of fixed effects considering the interaction of the covariate (exon) and group (*mdx*/WT). A value of $P \leq 0.05$ was considered significant.

The correlation analysis was performed using the Pearson correlation test, and a value of $P \leq 0.05$ was considered significant.

**RESULTS**

**Dystrophin protein and transcript levels in control skeletal and heart muscles**

To determine dystrophin protein levels in all muscles, we performed a Western blot for quadriceps, tibialis anterior, gastrocnemius, triceps, biceps, diaphragm, and heart muscles of WT mice (Fig. 1). Dystrophin levels were higher in diaphragm and heart than in tibialis anterior, quadriceps, and gastrocnemius muscles (Fig. 1B). Transcript levels, quantified at the 5′ end, were more abundant in heart than in all the skeletal muscles, including diaphragm for which transcript levels were similar compared with those of the other skeletal muscle groups (Fig. 1C). The overexpression of the dystrophin transcript in heart compared with that in skeletal muscles was confirmed in human control tissues, in which the expression of dystrophin transcript was almost 10-fold higher in heart (Fig. 1D).

**5′-3′ Dystrophin transcript imbalance**

To determine the effect of a nonsense mutation on transcript levels, we studied the DMD expression in *mdx* mice and compared the results with those obtained in WT mice. The *mdx* dystrophin transcript was more abundant in heart than in skeletal muscles in *mdx* mice.
and WT mice (Figs. 2A and 1C). Comparable levels of expression were observed for mdx and WT mice, except for the triceps for which the expression levels were higher for mdx mice (Fig. 2B).

To determine whether transcript levels were equal throughout the 14-kb dystrophin transcript, we investigated the expression at several exon-exon junctions. Expression levels of the exon-exon junctions were plotted relative to exon number, and regression lines were used to analyze the slopes obtained. We observed negative slopes in quadriceps, gastrocnemius, and diaphragm muscles of WT mice (P<1E−2). TA, tibialis anterior; Q, quadriceps; G, gastrocnemius; TR, triceps; BI, biceps; D, diaphragm; H, heart.

Figure 2. Dystrophin expression levels in mdx and WT mice. A) Dystrophin transcript is more abundant in heart than in other skeletal muscles in mdx mice (n=4−7 mice/group). Results of RT-qPCR of exon 1−2 junction in mdx mice are shown in the graph. DMD gene expression in quadriceps is set to 1. A significant difference is observed between heart and the other muscles (P < 5E−2). B) Dystrophin transcript is expressed equally between mdx and WT mice (n=4−7 mice/group). Results of the RT-qPCR of exon 1−2 junction in mdx and WT mice are shown in the graph. DMD gene expression for all muscles of mdx mice is set to 1 to facilitate the comparison between mdx and WT. A significant difference was observed in the triceps expression values between mdx and WT (P<1E−2). TA, tibialis anterior; Q, quadriceps; G, gastrocnemius; TR, triceps; BI, biceps; D, diaphragm; H, heart.

Up-regulation of the internal isoform Dp71 (promoter located in intron 62) for both WT and mdx mice. Dp71 up-regulation was not detected in any the other muscles tested (Supplemental Fig. S2A).

Therapeutic exon skipping does not recover mdx dystrophin transcript imbalance

AON-mediated exon skipping aims to restore the open reading frame of dystrophin transcripts. To assess whether transcript reframing influences transcript stability, we delivered an AON inducing exon 23 skipping locally (intramuscular injection into the gastrocnemius muscle) and subcutaneously into mdx mice. Exon 23 skipping was observed in treated mice, whereas we did not observe any spontaneous exon skipping in non-treated mice (Fig. 4A–C). As can be recognized from Fig. 4D, exon skipping per se did not correct the difference between 5’ and 3’ expression levels observed in the mdx mice compared with that in WT mice. However, this imbalance has a dramatic effect on one

Figure 3. 5′–3′ Dystrophin transcript imbalance. Representative 5′–3′ transcript imbalance in tibialis anterior, triceps, and heart of mdx and WT mice is shown. In each graph, exons are plotted on the x-axis and expression levels on the y-axis. Each dot indicates the expression levels for a specific exon-exon junction per mouse. Black dots represent WT mice, and white dots represent mdx mice. Because dots are superimposed, the dot representing exon 1−2 junction expression in mdx mice is present but not visible on the graphs. Regression lines for mdx and WT mice are shown as continuous and dashed lines, respectively. The P values shown in figure represent the difference in slope steepness between mdx and WT mice. A significant difference in the dystrophin transcript 5′–3′ imbalance is present in all muscles.

Figure 4. Exon skipping does not correct mdx transcript imbalance. A) Intramuscular injection of AON showing exon skipping in mdx mice. B) Subcutaneous injection of AON showing skipping in mdx mice. C) Exon skipping in treated mice. D) Exon skipping per se did not correct the difference between 5′ and 3′ expression levels observed in the mdx mice compared with that in WT mice.
of the most important outcome measures in the AON-mediated exon-skipping approach: the exon-skipping percentage. The exon skipping percentage is defined as the ratio between the skipped transcripts and the total transcripts (skipped and nonskipped). In a qPCR approach, we quantified the skipped transcript in the locally treated mice, and we used 3 different regions along the transcript as references to quantify the total amount of the *mdx* dystrophin transcript. Because of the transcript imbalance, the exon-skipping percentage significantly increased when distal exons were used to quantify the total transcript amount (Fig. 4E). The exon-skipping percentage reached values higher than 100% when the total *mdx* dystrophin transcript was quantified, considering the most distal exons.

5′-3′ Transcript imbalance negatively influences protein levels

To study whether transcript instability is also caused by protein-nontruncating mutations, we analyzed the dystrophin transcript in a group of 22 patients with BMD carrying heterogeneous deletions, among which a deletion of exons 45–47 was the most frequent [11 (50%) patients]. Mutations were confirmed at RNA levels for each patient. We then quantified the expression of several exon-exon junctions in all patients, highlighting various degrees of transcript imbalance. As depicted in Fig. 5A, the transcript was significantly less stable in patients with BMD than in healthy control skeletal muscle (*P*=5.2E−3).

To test whether transcript levels and/or transcript instability has an effect on dystrophin protein levels, we determined protein amounts by Western blot using 2 different antibodies (Supplemental Fig. S2B). A strong correlation was observed in the protein levels as detected by the 2 different antibodies (*r*=0.93, *P*<0.001). No correlation was found between transcript levels (exon 1–2 junction) and the protein levels for either of the antibodies (*r*=0.051 *P*=8.1E−1 for the monoclonal antibody and *r*=0.005 *P*=7.6E−1 using the polyclonal antibody; Fig. 5B). Notably, we found a strong correlation between the slope angles and the protein levels quantified with either of the 2 antibodies (*r*=0.554 *P*=7.5E−3 for the monoclonal antibody and *r*=0.570 *P*=4.5E−3 using the polyclonal antibody; Fig. 5C).

DISCUSSION

DMD is caused by out-of-frame mutations within the *DMD* gene encoding dystrophin. AON-mediated exon skipping and forced read-through of stop codons are among the most promising approaches for restoring dystrophin expression in patients with DMD because these approaches are currently being tested in phase III and II clinical trials, respectively. Both approaches have the DMD transcript as a therapeutic target, making the transcript levels an important parameter to take into account. The aims of this study were to shed light on the transcript levels in control and dystrophic muscles and to study the correlation between transcript and protein levels in mice and patients.

Thus, we studied dystrophin protein levels in several muscles, and surprisingly we observed that dystrophin levels were not equal in all muscles. Specifically, dystrophin was more abundant in diaphragm and heart than in other skeletal muscles such as tibialis anterior, quadriceps, and gastrocnemius. Notably, transcript levels were also higher in heart than in other muscles in both WT and *mdx* mice. The higher *DMD* gene expression in the heart could be due to the fact that cardiomyocytes are mononucleated cells, whereas other muscles present multinucleated fibers. Transcript levels were comparable among the other muscles for both WT and *mdx* mice, and no differences were observed in the expression levels between *mdx* and WT mice with the only...
exception of the triceps for which mdx mice showed higher expression. The observation that transcript levels were comparable between mdx and WT mice was surprising, given the evidence in the literature that the transcript is less abundant in patients with DMD than in controls (6–12). However, we realized that gene expression studies in patients with DMD found in the literature were performed using microarray probes mapping to the 3' part of the gene (see Supplemental Fig. S1 for a depiction of probes mapping to the DMD locus). Because the quantification we show in Figs. 1 and 2 was performed at the 5' end of the transcript, we decided to quantify exon-exon junctions along the transcript; this led to the discovery that mdx dystrophin transcript levels are reduced toward the 3' end of the gene. This information was missed by the previous studies because no probes mapped to the 5' end of the gene and because the analysis was performed by pooling the expression levels of all probes together. This reduction (which we named transcript imbalance due to the discrepancy in the 5' and the 3' levels) was observed in quadriceps, gastrocnemius, and diaphragm muscles of WT mice and in human control muscle. This result strongly suggests that the transcript levels are not constant across the gene, perhaps because of incomplete transcription. However, we did not observe the same level of imbalance in the tibialis, triceps, and heart of WT mice, suggesting that factors involved in the transcriptional process of the DMD gene or in the transcript turnover might be differentially represented among different muscles. Further investigation is needed to address this point in WT mice and in mdx mice, in which a protein-truncating mutation causes a significantly more pronounced 5'–3' imbalance in all muscles compared with those in WT mice. Differences between the 5' and 3' ends have been reported in 3 patients with BMD (25) and in human control muscle tissue before (15). It was suggested that this difference was caused by increased transcriptional initiation due to growth or regeneration, although it has also been hypothesized that because of the sheer size of the DMD gene, not all initiated transcripts could be completed. We exclude the possibility that regeneration causes higher transcription initiation, because we observed comparable levels of 5' ends of transcripts in mdx and WT mice and a significant increase of myosin heavy chain 3, a marker of muscle fiber regeneration, in mdx skeletal muscles compared with that in WT (see Fig. 2 for a comparison of DMD expression levels in mdx and WT mice and Supplemental Fig. S2C for a comparison of myosin heavy chain 3 and DMD qPCR in quadriceps muscle). We hypothesize that the varying degrees of transcript imbalance in different skeletal muscles could be caused by either transcriptional or post-transcriptional mechanisms. Further research is needed to elucidate the conformation and structure of the DMD gene, the manner in which the transcription machinery proceeds along this huge locus, and the factors that are differentially bound to the transcript in different muscles.

The transcript imbalance has an effect on the quantification of exon skipping by RT-qPCR. The exon-skipping percentage has been used to identify the most effective AON chemical characteristics (26) and to optimize AON sequences for clinical development (27). Our findings highlight how the exon 22–24 junction is more expressed compared with the most distal part of the gene (Fig. 4). However, it is not known which of the transcripts is complete and which is not.
when focusing on the area immediately flanking the skipped exon.

We further investigated the transcript in 22 patients with BMD carrying heterogeneous mutations within the DMD gene. We observed a significant reduction in transcript levels toward the 3' end compared with that in control muscle. This was unexpected, given the absence of a premature stop codon in these patients. Notably, a previous study showed that only 3 of 11 patients with BMD showed a reduction at the 3' end of the transcript compared with that at the 5' end (25); however, the quantification strategy in this study used only 2 primer pairs. Amplification bands of 10 dystrophic controls were resolved on a polyacrylamide gel and quantified; the ratio between the 2 amplified bands was used then to normalize the ratio in the experimental samples. We think that the normalization procedure might be responsible for missing the transcript imbalance in these patients. Notably, we observed a significant correlation between the dystrophin protein levels and the transcript imbalance rather than transcript levels. This is an unanticipated finding demonstrating that detailed studies on RNA quantification should be of interest for therapeutic approaches with the aim of converting the DMD phenotype into BMD, as well as other transcript-targeting approaches. It also raises questions about the mechanism underlying the 5'-3' transcript imbalance. We hypothesize that different mechanisms could be responsible. One possibility is that the mutated transcript may be recognized by factors triggering the mRNA instability. Alternatively, the presence of a premature nonsense mutation may have consequences for the DNA structure impairing the transcription process downstream of the mutation.

Our findings have obvious consequences for transcript-targeting therapies such as stop codon readthrough and exon skipping, because these rely on the presence of intact mRNAs. Thus, the identification of factors triggering the mRNA instability will have a significant effect on the success of RNA-based therapeutic strategies.

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