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Four Novel Dystrophin Point Mutations: Detection by Protein Truncation Test and Transcript Analysis in Lymphocytes from Duchenne Muscular Dystrophy Patients

Key Words
Duchenne muscular dystrophy
Point mutation
Illegitimate transcripts
Protein truncation test

Abstract
About 30% of cases of Duchenne muscular dystrophy (DMD) result from point mutations randomly distributed in the immense dystrophin gene. As already observed for the gross rearrangements, most of the DMD point mutations identified so far give rise to truncated proteins. Here, we report results of a comprehensive search for point mutations within the dystrophin gene based on illegitimate transcript analysis by using the RT-PCR technique in combination with a method capable of selectively detecting translation-termination mutations, called the protein truncation test (PTT). The RT-PCR-PTT procedure was successful in detecting mutations in 4 out of the 6 DMD patients who were investigated. These mutations, Q2972X in exon 59, 3474insC in exon 24, delT393–G394+5 in exon/intron 3, and 2436delAG in exon 18, had not been previously described. Moreover, several alternatively spliced forms of ectopic dystrophin mRNA were characterized in normal controls or in DMD patients. Most of these differentially spliced messages consisting of exon skipping or intronic sequence insertion are reported here for the first time.

Introduction

Duchenne (DMD) and Becker (BMD) muscular dystrophies are allelic neuromuscular disorders which are caused by defects of the dystrophin gene, one third of which arising from new mutations. This 2.5-Mb gene in Xp21 contains about 80 exons and includes multiple promoters (7 have been identified so far) [1]. Gross rearrangements (mostly intragenic deletions) are responsible for about 65% of DMD and BMD cases, the remaining one third of cases resulting from more subtle alterations.

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Unlike the large deletions, which are easily detected by using standard multiplex amplification procedures [2, 3], the detection of small mutations has been delayed for years due to the large size and complexity of the dystrophin gene. However, their identification is essential for diagnostic purposes, as it allows a highly accurate direct DNA testing for carrier and prenatal diagnosis.

For 3 years, most of the procedures that have been used to identify point mutations in the DMD gene have consisted in scanning only a limited number of exons for sequence variations using attractive methods such as single strand conformation analysis (SSCA) or heteroduplex analysis (HA). However, very few mutations have been detected when we consider the number of patients who have been investigated [for review, see ref. 4, 5]. We had previously applied the PCR-SSCA approach to the screening of 40 exons of the dystrophin gene (≈20% of the coding sequence) in 20 non-deleted DMD or BMD patients, and could characterize only 2 point mutations [6, 7]. Recently, Prior et al. [8] analyzed 71 exons (≈80% of the coding region) in 158 patients by using a PCR-HA strategy and 'only' found 29 disease-causing mutations. These results argue for a fundamentally different approach based on mRNA rather than classical genomic DNA studies to search for point mutations in large genes in which no prevalent mutational hot-spots are observed.

Illegitimate transcription [9] has made possible the dystrophin mRNA analysis of DMD and BMD patients when muscle biopsies were not available. The analysis of ectopic dystrophin mRNA was first used by Roberts et al. [10], who screened 10 overlapping reverse transcription (RT)-PCR products corresponding to the entire dystrophin-coding region (11 kb) by using the chemical cleavage of mismatch method.

As almost all the mutations causing DMD lead to premature termination of protein synthesis, we believed that the protein truncation test (PTT), previously developed by Roest et al. [11], could be the most suitable strategy to search for mutations in the dystrophin gene. This in vitro transcription/translation procedure reveals the presence of a translation-terminating mutation in producing a lower-molecular-weight protein instead of the full-size wild-type product. The PTT has been successfully applied to the APC [12, 13], NF1 [14] and BRCA1 [15] tumour suppressor genes, and recently to the dystrophin gene [16]. In our laboratory, we first used the RT-PCR-PTT to analyze the effects on the mRNA structure of two novel DMD mutations which we had detected by PCR-SSCA applied to genomic DNA [7].

Here, we report the use of the RT-PCR-PTT method to search for unknown point mutations in 6 other non-deleted DMD patients, which resulted in the identification of 4 previously undescribed causative mutations, 2 cases remaining unresolved. During this study, alternatively spliced forms of dystrophin mRNA could also be characterized in lymphocytes from normal individuals or from the DMD patients under investigation. Most of these splices, which involve the loss of a single exon, had not been reported before; their physiological significance is unclear.

Materials and Methods

Patients

For the 6 male patients included in this study, the diagnosis of DMD was based on clinical data, family history, grossly elevated serum creatine kinase activity, then confirmed by dystrophin analysis (immunostaining and/or Western blotting) on muscle biopsy when available (table 1). They were selected for point mutation screening, as no gross rearrangement could be detected either by Southern blotting of *Hin*dIII-digested genomic DNA hybridized with cDNA probes or by two different sets of nineplex-PCR [2, 3 17]. Moreover, a previous screening based on PCR-SSCA methodology had been performed for patients 4 and 5 (40 exons analyzed) and for patient 6 (20 exons analyzed), but had failed to detect any mutation-causing disease [6, 7].

DNA was extracted from blood samples from patients and relatives (25 subjects) by a standard salting-out procedure.

RNA Extraction and RT-PCR

RNA was extracted by standard method [18] either from peripheral blood lymphocytes (PBL) purified using Histopaque 1077 (Sigma), or from lymphoblastoid cell lines. An aliquot (3 µl) of each sample was analyzed on a 1% agarose gel in order to check concentration and quality of RNA.

The entire coding sequence was then amplified for each of the 6 patients and for each of 5 normal controls in 10 overlapping nested reactions (mean size 1.1 kb), as described by Roberts et al. [19]. Briefly, approximately 500 ng to 1 µg of RNA were used as template for the first-strand cDNA synthesis. RT was performed according to manufacturer's instructions (BRL) from a specific reverse primer Xb (X = 1–10) in a total volume of 12.5 µl. A 30-µl PCR mix containing primers Xa (forward) and Xb was then added to the RT reaction, and the first round of amplification (PCR_1) was performed, consisting of 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 5 min. A 2-µl aliquot of PCR_1 was used in the subsequent round of nested PCR (PCR_2) using inner primers (T7-Xc and Xd) in a final volume of 50 µl (30 cycles were performed as above). Primers Xa and Xb are those described by Roberts et al. [19], whereas primers T7-Xc and Xd are those reported by Gardner et al. [16]. One fifth of the nested PCR_2 reaction was analyzed on 1.5% agarose or 5% non-denaturing acrylamide gels.

Table 1. Clinical data available for the 6 DMD patients of the study

Patient No.	Current age, years	Diagnosis	Dystrophin analysis[a]	CK[b] IU/l	Familial/isolated case, other data
1	10	DMD/intermediate	negative[c] (IF + WB)	9,200	isolated
2	8	DMD	negative (IF)		isolated, memory and concentration impairment
3	9	DMD	NA	>5,000	familial, problems at school, symptomatic carrier
4	23	DMD	NA	975 (9)	familial, good intellect
5	9	DMD	negative (WB + IF)	10,450 (4)	familial
6	10	DMD	negative (WB + IF)	13,900 (9)	isolated, mental retardation (IQ 58)

[a] IF = Immunofluorescence; WB = Western blot; NA = not available.
[b] Figures in parentheses indicate age. CK = Creatine kinase.
[c] A weak signal has been detected with NCL Dys1 antibody on Western blot.

PTT Analysis

The primers T7-Xc were modified in order to contain a T7 promoter and a eukaryotic translation initiation sequence. For each of the ten overlapping reactions, 2 µl of the nested PCR (PCR_2) were used directly for an in vitro transcription/translation reaction in the presence of ^{35}S-methionine (kit TnT/T7-coupled reticulocyte lysate system – Promega). Electrophoresis in 15% SDS-PAGE to separate the resulting products and visualization of the peptides were as previously described [7].

Direct Sequencing Analysis

PCR-amplified genomic DNA or cDNA was purified with Centricon 100 (Amicon) and directly sequenced by using sequenase 2 (USB) and ^{35}S-dATP as isotope. Additional primers were used for cDNA sequencing (S3F: nucleotides 2821–2845; S3R: nucleotides 3423–3400; S9R: nucleotides 9450–9427). In order to further analyze each mutation at the genomic DNA level, new primers were designed to amplify exons 18, 24, and 59 and their intron boundaries (sequences available upon request).

Restriction Analysis of the Mutations

When a point mutation created or abolished a restriction site, the relevant exon of the dystrophin gene was specifically amplified from genomic DNA of the patient and all the available relatives. 10 µl of the PCR products were then digested with the appropriate restriction enzyme, and the resulting fragments were analyzed on 2% agarose or 8% polyacrylamide gels.

Results

The analysis of dystrophin RT-PCR fragments (11 kb) in 6 DMD patients resulted in the identification of the disease-causing mutation in 4 of them. One mutation was detected because it induced an abnormally sized RT-PCR fragment (in patient 3), and the three others because they generated truncated peptides (in patients 1, 2, 4).

Identification of a 5′ Consensus Splice-Site Mutation

In patient 3, reaction 1 (nucleotides 212–1175) displayed a shorter fragment than the one expected on acrylamide gel (fig. 1a). Direct sequencing of the altered-size band revealed a mRNA lacking exon 3 (fig. 1b). This result was most likely indicative of a splice-site mutation, as exon 3 was present in the genomic DNA standard multiplex procedure. Sequence analysis of the amplified corresponding genomic region revealed a 7-bp deletion in the exon-intron junction, delT393–G394+5 (fig. 1c, table 2). This mutation removes the donor splice site and induces the complete skipping of exon 3 from the mature mRNA transcript, causing an in-frame deletion of 93 nucleotides in the coding sequence. A peptide of about 38 kDa (nor-

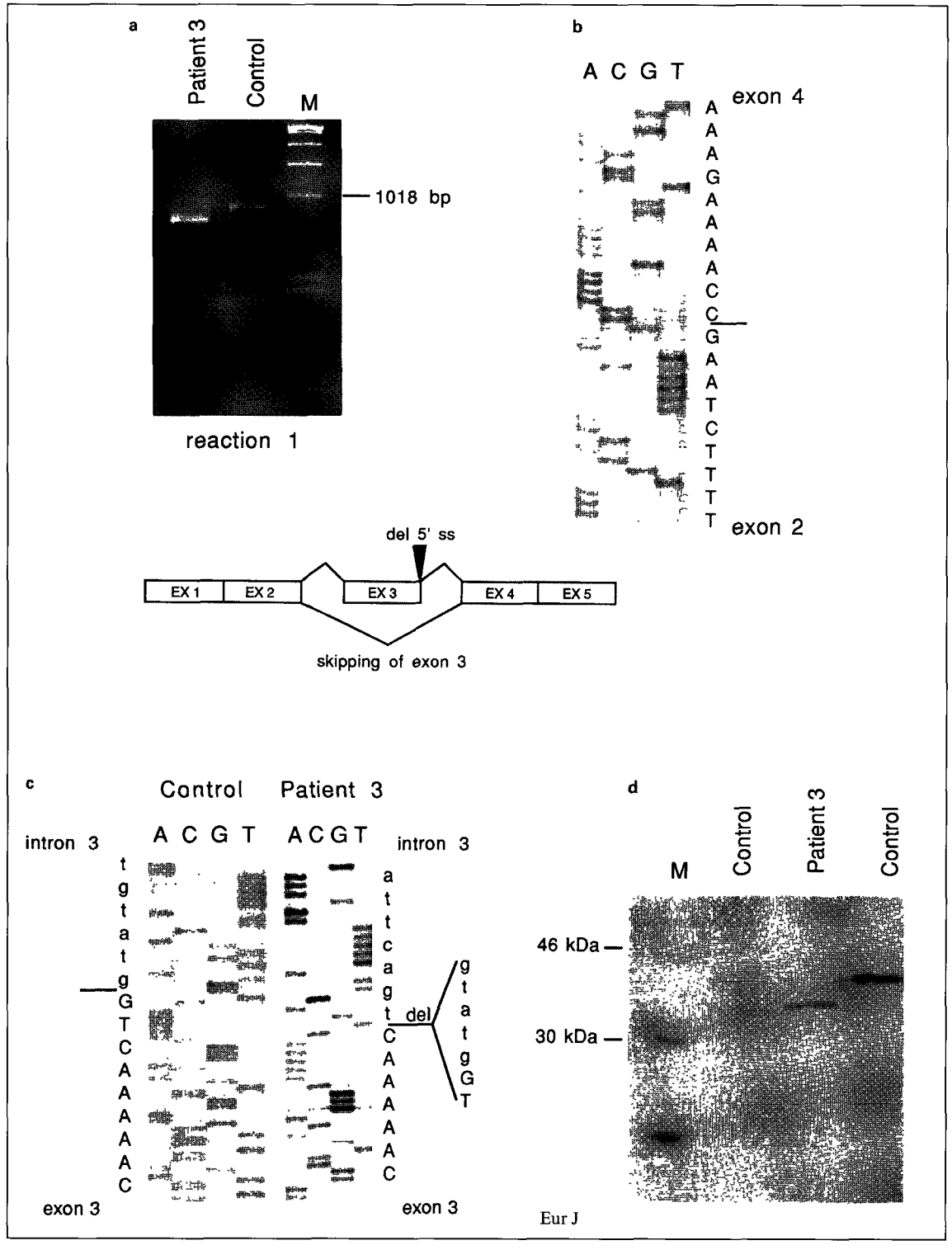


Fig. 1. Characterization of the 393delT–G394+5 mutation (patient 3). **a** Polyacrylamide gel electrophoresis of products of nested RT-PCR for reaction 1 from patient 3 and from a normal control. M = Size marker (1-kb ladder, BRL, USA). **b** Direct sequencing of the reaction 1 reduced-size product obtained for patient 3, demonstrating the loss of exon 3 from the mature mRNA. **c** Partial sequence of exon 3 and its intronic boundaries amplified from the genomic DNA of patient 3 reveals a 7-bp deletion at the junction exon 3/intron 3. **d** PTT. The calculated band of 38 kDa is observed for patient 3, as expected from the in-frame skipping of exon 3 from the mRNA.

Table 2. Identification of four previously undescribed mutations in the dystrophin gene

Patients[a]	RT-PCR reaction[b]	Nucleotide change[c]	Name[d]	Exon	Protein domain[e]	Consequence[f]	Enzyme change[g]
1	RT9	C→T at 9122	Q2972X	59	R24	Gln→Stop at 2972	(+) *Dde*I
2	RT3	insertion of C after 3474	3473insC	24	R7	frameshift	none
3	RT1	deletion of TGGTATG between 393 and 394+5	delT393-G394+5	3/intron 3	N	5′ ss deletion of 31 aa from F32 to L62	(–) *Bsr*I
4	RT3	deletion of AG from 2436 or 2438	2436delAG	18	R4	frameshift	(–) *Hinf*I

[a] Patient's number is as defined in table 1.
[b] Numbering of RT-PCR fragments is as defined by Gardner et al. [16].
[c] Numbering is according to Koenig et al. [29].
[d] Mutations are designed according to the nomenclature proposed by Beaudet and Tsui [28].
[e] N = N-terminal domain; R = spectrin-like repeat.
[f] 5′ss = 5′ splice site; aa = amino acids.
[g] Creation (+) or suppression (–) of the corresponding enzyme restriction site.

mal size 41.5 kDa) was detected by PTT (fig. 1d). Therefore, patient 3 is expected to produce an interstitially truncated dystrophin lacking 31 amino acids in the amino-terminal domain. However, no muscular biopsy was available to confirm the effect of the microdeletion on the protein structure.

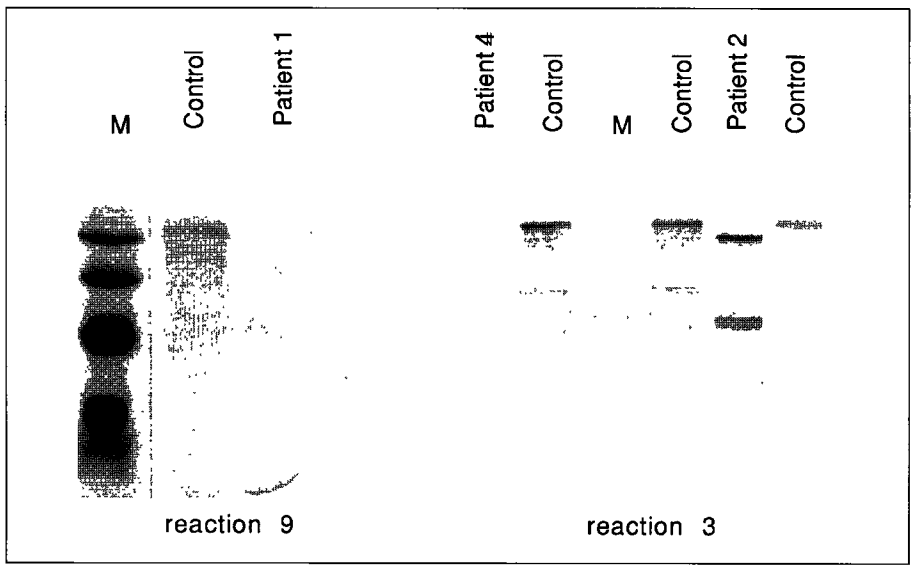
In this familial case of DMD (a maternal uncle of patient 3 died of DMD at age 23), a direct carrier testing could be performed since the deletion suppresses a *Bsr*I site (table 2). The mother, the grandmother and the grandaunt of the proband were shown to carry the mutation, as expected from haplotype analysis and clinical data [both the grandmother (who is a 45XO mosaic at an estimated rate of 20%) and the grandaunt (who presents a normal karyotype) are manifesting carriers]. Nevertheless, the origin of the mutation could not be determined in this family.

Identification of Three Truncating Mutations

All full-length RT-PCR products obtained for the 5 remaining patients were analyzed by PTT. Three patients exhibited an abnormal electrophoresis pattern for one of the 10 RT-PCR fragments studied. Patient 2 displayed a slightly reduced-sized peptide for reaction 3 (nucleotides 2342–3594), whereas no peptide could be detected either for patient 1 in reaction 9 (nucleotides 8786–10046) or for patient 4 in reaction 3 (fig. 2a). In these 3 cases, the sizing of the band (or its absence) made it possible to localize the site of the mutation and to focus the sequencing step. Indeed, the absence of peptide suggested that in patients 1 and 4 the muation was probably at the 5′ end of the RT-PCR fragment, whereas the quasi-full-length peptide detected for patient 2 helped to localize the truncating mutation at the 3′ end of reaction 3. Direct cDNA sequencing using appropriate primers led to the identification of the disease-causing mutation in each case (fig. 2b), consisting of a nonsense mutation in patient 1(Q2972X, due to a C to T substitution at nucleotide 9122), a 1-bp insertion in patient 2 (3474insC) and a 2-bp deletion in patient 3 (2436delAG) (table 2).

The premature stop codon resulting from the chain-terminating mutation lies 336 and 102 bp from the 5′ end of the T7-PCR product in patients 1 and 4, respectively, and is expected to generate truncated peptides of 12.5 and 3.4 kDa which could not be detected on PTT gels because of their very low content in ^{35}S-methionine.

In the 3 cases reported here, the relevant exon has been amplified from genomic DNA in order to confirm the



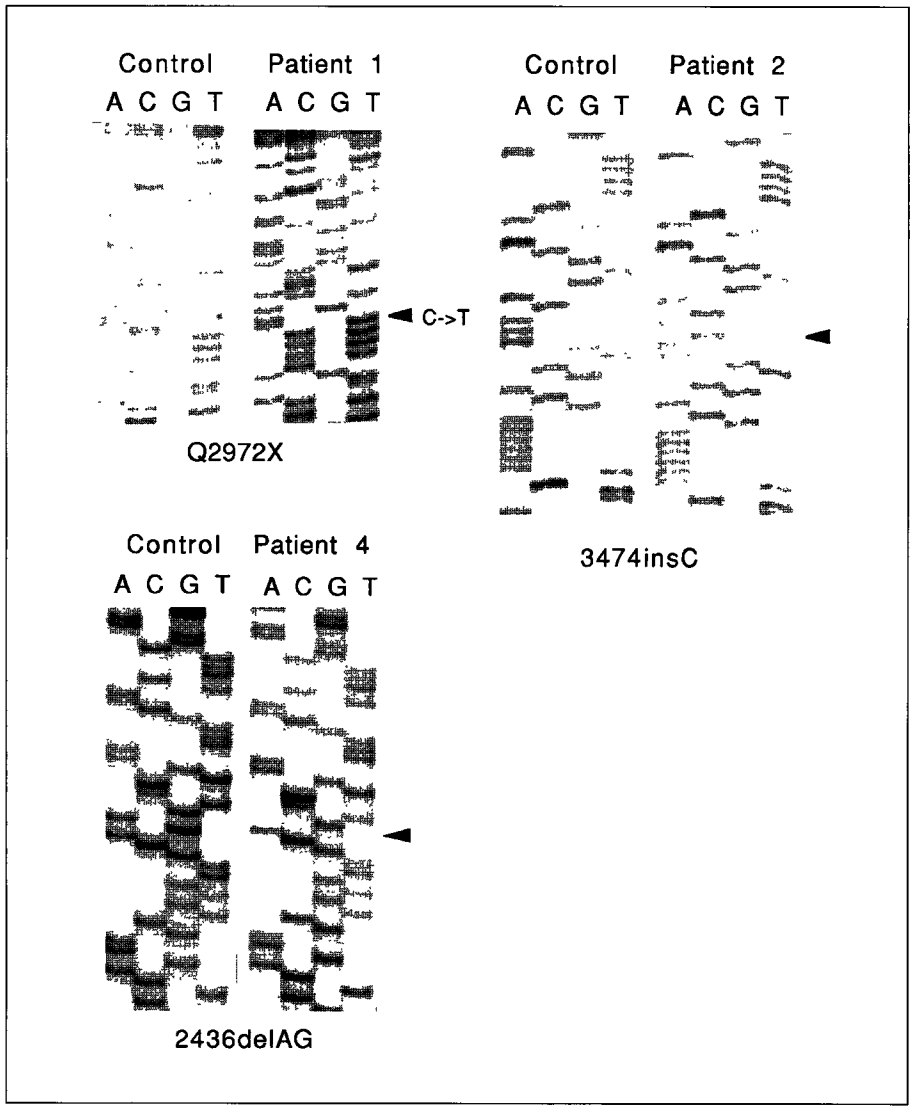


Fig. 2. Identification of three translation-terminating mutations. **a** PTT: no peptide has been detected for patient 1 in reaction 9 and for patient 4 in reaction 3. Patient 2 displayed a reduced-size band in reaction 3. M = Molecular size marker (Rainbow™ (^{14}C), Amersham, UK: 46, 30, 21.5, 14.3 kDa). **b** Characterization of the causative mutation by direct cDNA sequencing of the RT-PCR products with abnormal PTT patterns. We identified a nonsense mutation (Q29772X) in patient 1, a 1-bp insertion in patient 2 (3474insC), and a 2-bp deletion in patient 4 (2436delAG). Normal controls were sequenced in each case. The dark arrows indicate the position of the sequence variation.

molecular defect identified at the mRNA level. Direct carrier assessment was further carried out in the three families either by sequencing (family 2) or restriction enzyme digestion (families 1 and 4). Both patients 1 and 2 are isolated cases of DMD. As the mutation was absent in the maternal DNA of patient 2, it appears that the 3474insC mutation is likely to be a de novo mutation. Mothers of patients 1 and 4 were found to be carriers, and the segregation analysis allowed us to establish that in the family 1 the mutated haplotype was inherited from the maternal grandfather.

Patients with No Detectable Mutation after PTT Analysis

In patients 5 and 6, no mutation could be detected by RT-PCR-PTT. As they are known to be negative for dystrophin protein both by immunofluorescence and Western blot, the possibility that these 2 patients may constitute cases of autosomal phenocopies can be ruled out [16]. Moreover, patient 5 has 2 affected brothers in whom dystrophin analysis was also negative.

Alternatively Spliced Forms of Dystrophin mRNA in Illegitimate Transcripts

The use of the highly sensitive RT-PCR method allows the detection of differentially spliced messages even though they correspond to a very low proportion of the dystrophin transcripts. Some of these alternative splicing events are reproducibly detected and have been well characterized in specific tissues and/or in lymphocytes, such as the skipping of exons 9, 38, 68, 74, and 78 [20; Roberts, unpubl. data]. We used primers designed with the aim of avoiding the amplification of these alternatively spliced species from the PBL of DMD patients [16]. Nevertheless, we detected in the DMD patients or in normal controls under investigation some shorter or longer RT-PCR products in addition to the full-length product expected from the position of the primers (table 3). After gel purification of each extra band, direct cDNA sequencing revealed the loss of exon 39 (patient 1), exon 48 (1 normal control), exons 14 and 15 (patient 2), or exons 46 and 50 (patient 4); in patient 2, we also found an insertion of a putative 41-bp intronic sequence at the junction exon 55/exon 56.

The level of expression of these species could not be estimated from illegitimate transcription. Moreover, they could be artefactually overrepresented by the process of RT-PCR, which sometimes has a tendency to favour shorter target sequences. The frequency of these alternative spliced events in the ectopic dystrophin transcripts has not been established. Only one of the splice events detected here has been reported elsewhere. Roberts et al. [10] described the loss of exon 39 from the mature mRNA transcript in normal PBL samples and also in 1 patient who carries the Q1851X nonsense mutation in exon 39. In this last case, the skipping of exon 39 may eliminate the nonsense mutation as the reading frame is maintained.

Table 3. Alternative spliced forms of dystrophin mRNA

Patient[a]	RT-PCR reaction	Modification	Protein domain[b]	Consequence[c]
1	RT6	loss of exon 39	R14	deletion of 46 aa
2	RT2	loss of exons 14 and 15	R2-R3	deletion of 70 aa
2	RT8	insertion of 41 bp at the junction exons 55/56	R22	frameshift
4	RT7	loss of exons 46 and 50	R18 R19, H3	frameshift
Normal control	RT7	loss of exon 48	R19	deletion of 62 aa

[a] Numbering as referred to in table 1.
[b] R = Repeat domain; H = hinge domain.
[c] aa = Amino acids.

Discussion

Mutations Causative of DMD

The 4 mutations, consisting of 1 splicing mutation in exon/intron 3 (delT393–G394+5), 1 nonsense mutation (Q2972X) in exon 59, and 2 frameshifting mutations (3474insC in exon 24, 2436delAG in exon 18) have not been reported before among the 100 mutations described in DMD patients [4, 8, 15, 16]. No apparent clustering of point mutations has been demonstrated so far in the dystrophin cDNA, with the exception of the 28 CpG sites which may constitute hot-spots for nonsense mutations, since they can undergo oxydative deamination of 5-methylcytosine to thymine. To our knowledge, 2436delAG is the first mutation described in exon 18. Interestingly, 40 exons (not comprising exon 18) of the DMD gene had previously been explored for sequence variation in patient 4

who carries the 2436delAG mutation by using a PCR-SSCA-based method applied to genomic DNA. This observation confirms that the most reliable approach to search for the mutations scattered throughout this immense gene relies on the entire coding sequence screening.

The mechanisms of mutagenesis responsible for small DNA lesions appear to be highly dependent on the local DNA sequence context [21]. The sequence surrounding Q2972X contains 3 tandemly repeated dinucleotides (CT), a 4th one being created by the substitution C for T. The deletion 2436delAG occurs in a repeated sequence (AG). We noticed also the presence of 3 dinucleotide repeats TG, one of them immediately flanking the 3′ breakpoint of the 7-bp deletion delT393–G394+5. These findings are consistent with a role of the short direct repeats in the generation of deletions (or insertions), as they may promote misalignment and slipped mispairing during DNA replication [for review, see ref. 21]. Cooper and Krawzack [21] also demonstrated that small insertions may be explained by reference to neighbouring inverted repeats or symmetric elements. Considering the mutation 3474insC, we noticed that the insertion of C within a run of three A creates a perfect palindromic sequence (ACACA).

As expected from the DMD phenotype of the patients, 3 of the 4 mutations that we describe result in protein truncation and, therefore, do not allow the synthesis of any functional dystrophin. The absence of dystrophin in the muscle is not compatible with the correct structure and function of the large complex of sarcolemmal glycoproteins that form a link between the intracellular cytoskeleton proteins and the extracellular matrix component, laminin [for review, see ref. 22]. One mutation (Q2972X) would also disrupt the Dp116 transcript which is expressed in peripheral nerves in normal individuals [23].

The case of the delT393–G394+5, which results in an in-frame deletion of exon 3, has to be discussed with regard to the observed severe DMD disease that affects patient 3, since it does not follow the reading frame rule. Whereas in-frame deletions are usually responsible for a BMD phenotype, those involving the NH2-terminal of dystrophin are associated with a great phenotypic variability ranging from mild or intermediate to severe phenotypes. This can be observed in the description of 6 other deletions of the single exons 3 or 5 [reviewed in ref. 4, 24], or from the case of a DMD patient with transcripts lacking exon 5 [10]. The NH_2-terminal is known to be essential for a proper function of dystrophin as it interacts with F-actin of the subsarcolemmal cytoskeleton [25]. Nevertheless, as it has already been discussed elsewhere [10, 24, 26], it still needs to be clarified why patients with an out-of-frame deletion removing exons 3–7, 3–6, or 5–7 might have a milder phenotype than patients with single exon in-frame deletion in this area. As hypotheses, the presence of alternatively spliced transcripts or the presence of an independent mutation restoring the reading frame have been discussed [10]. It should also be admitted that an interstitial deletion in the actin-binding domain can be worse than a complete absence of this domain; a dominant negative effect such as an aggregation of proteins due to conformational/functional changes may explain a severe phenotype. Consistent with this last hypothesis are the description of the missense mutation L54R in exon 3 in a DMD patient [27] and the observation of symptomatic females carrying in-frame alterations in the actin-binding domain.

We failed to identify the causative mutation in 2 of the 6 patients analyzed here. Recently, another study using the RT-PCR-PTT strategy for 22 patients gave a similar range of efficiency, as mutations could be detected for 17 patients [16]. Contrasting with the conclusions of Prior et al. [8] from the genomic DNA analysis of 80% of the dystrophin coding sequence, the mRNA analysis reveals that at least 80% of the DMD point mutations lie in the coding region, and that the splice site mutations only represent 5–7% [4, 16]. In our 2 cases negative for mutation (patients 5 and 6), the dystrophin data and/or the familial data as well as the haplotype segregation clearly show a Xp21-linked inheritance of the disease. For these patients, the dystrophin gene should be further screened for missense mutations; however, Gardner et al. [16], using a powerful technique for detecting DNA alterations, failed to demonstrate any missense in the coding region of their 5 unidentified cases, 3 of them with no dystrophin in their muscle. Alterations in intronic or extragenic control regions will have to be investigated.

Alternative Splicing Events in Illegitimate Transcripts

Undescribed spliced forms of dystrophin transcripts have been observed during this study, whose biological significance, if any, has to be defined. Most of them maintain an open reading frame (transcripts 39^-, 48^-, and $14+15^-$) and therefore may encode proteins with interstitial deletions located in the rod domain of the dystrophin. However, the absence of previous reports on these differentially spliced forms in any tissues in which dystrophin is normally expressed renders this unlikely.

The biological significance of the spliced forms of dystrophin mRNA that result in loss of frame, as observed in

patients 2 (insertion of 41 bp at the junction exon 55/56) and 4 (transcripts 46⁻ and 50⁻), is more ambiguous. The origin of the 41-bp sequence insertion does not correspond to the 5′ end of intron 55, of which the first 40 bp have been recently reported (Genbank, accession number L37431). This insertion could be a consequence of the utilization of a cryptic splice site in intron 55, the complete sequence of which is still unknown. Interestingly, the same additional sequence has also been observed in 1 non-deleted BMD patient of our series, which has not been included in this report as the entire coding sequence has not been studied yet.

Whether these alternative transcripts represent 'noise' in the mRNA splicing machinery or artefacts of the RT-PCR process remains unresolved. Moreover, data of exhaustive studies of dystrophin transcript analysis from a specific and illegitimate source are not available to compare the frequency and the extent of these phenomena in each case, so that it is unknown whether there are more aberrantly spliced transcripts in illegitimate compared to specific tissues. The detection of such alternative splicing forms by using the RT-PCR method might complicate, in the future, the interpretation of transcript analysis of human gene diseases.

In conclusion, the RT-PCR-PTT appears at this time to be a very efficient procedure to search for truncative point mutations in large genes. The sequencing step is greatly reduced as the size of the truncated peptides pinpoints the location of the mutation (as observed for patients 1, 2 and 4), and also because silent changes are not detected.

While the linkage analysis cannot be applied when a spontaneous mutation is suspected (one third of cases), the identification of a point mutation in the non-deleted cases of DMD makes it possible to propound a highly accurate direct diagnosis for carrier testing and prenatal diagnosis, as realized for families 1 and 2.

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