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ORIGINAL INVESTIGATION

Tim P. Kerr · Caroline A. Sewry · Stephanie A. Robb
Roland G. Roberts

Long mutant dystrophins and variable phenotypes: evasion of nonsense-mediated decay?

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Abstract More than 98% of Duchenne muscular dystrophy (DMD) mutations result in the premature termination of the dystrophin open reading frame at various points over its 11-kb length. Despite this wide variation in coding potential (0%–98.6% of the full-length protein), the truncating mutations are associated with a surprisingly uniform severity of phenotype. This uniformity is probably attributable to ablation of the message by nonsense-mediated decay (NMD). The rare truncating mutations that occur near the 3' end of the dystrophin gene (beyond exon 70) can however result in extremely variable phenotypes (both intra- and inter-familially). We suggest that all proteins encoded by such mutant genes are capable in principle of rescuing the DMD phenotype but that NMD abrogates the opportunity to effect this rescue. The observed variability may therefore reflect an underlying variation in the efficiency of NMD between individuals. We discuss this hypothesis with particular reference to a well-characterised Becker muscular dystrophy patient with a frameshift mutation, where expression of a truncated dystrophin rescues the muscular but not mental phenotype.

T.P. Kerr · S.A. Robb
Department of Paediatric Neurology, Guy's Hospital, London, SE1 9RT, UK

C.A. Sewry
Dubowitz Neuromuscular Centre, Department of Paediatrics and Neonatal Medicine, Imperial College School of Medicine, Hammersmith Hospital, London, W12 ONN, UK

C.A. Sewry
Department of Histopathology, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, SY10 7AG, UK

R.G. Roberts (✉)
Division of Medical and Molecular Genetics, Guy's, King's and St Thomas' School of Medicine, King's College London, 8th Floor, Guy's Tower, Guy's Hospital, London, SE1 9RT, UK
e-mail: roland.roberts@kcl.ac.uk,
Tel.: +44-7955-2510, Fax: +44-7955-4644

Introduction

The phenotypic severity of the dystrophinopathies forms a natural bimodal distribution, whereby a broad peak of diverse milder cases (Becker muscular dystrophy, BMD) is separated from a much tighter peak of more uniform severe cases (Duchenne muscular dystrophy, DMD) by a low valley of rarer "intermediate" individuals (Emery 1993). Early in the characterisation of dystrophin gene mutations (largely clustered deletions), it was noted that this distribution reflected the possibility that a deletion might either shift the open reading frame (ORF), yielding a C-terminally truncated and presumably non-functional protein, or leave it in register to encode an interstitially deleted protein (Monaco et al. 1988). The uniformity of the DMD phenotype therefore derives from the singular nature of the null state, whereas the diversity of BMD reflects the variable extent to which different interstitial deletions might compromise the function and/or abundance of the mutant protein. The "frameshift rule" is therefore predicated on the assumption that a truncated ORF will always constitute a null mutation.

During the subsequent world-wide characterisation of many thousands of dystrophin gene mutations, however, a number of apparent exceptions to the so-called "frameshift rule" have been noted. In each case, a mutation that is expected to result in the absence of full-length dystrophin is associated with a surprisingly mild phenotype. Each class of exception has been convincingly explained by a distinct mechanism: (1) disruption of the "muscle-type" promoter and/or first exon results in phenotypic rescue by alternative promoter use in all tissues except the heart, and the consequence is cardiac rather than skeletal myopathy (Muntoni et al. 1995), (2) frameshifting deletions early in the gene can allow in-frame translational re-initiation after abortive translation of the shortened cognate ORF, yielding an N-terminally truncated but otherwise full-length protein (Winnard et al. 1995), (3) ORF-terminating mutations within the gene are sometimes associated with alternative splicing of exons bearing a point mutation (or adjacent to a rearrangement); this can restore the ORF in a proportion

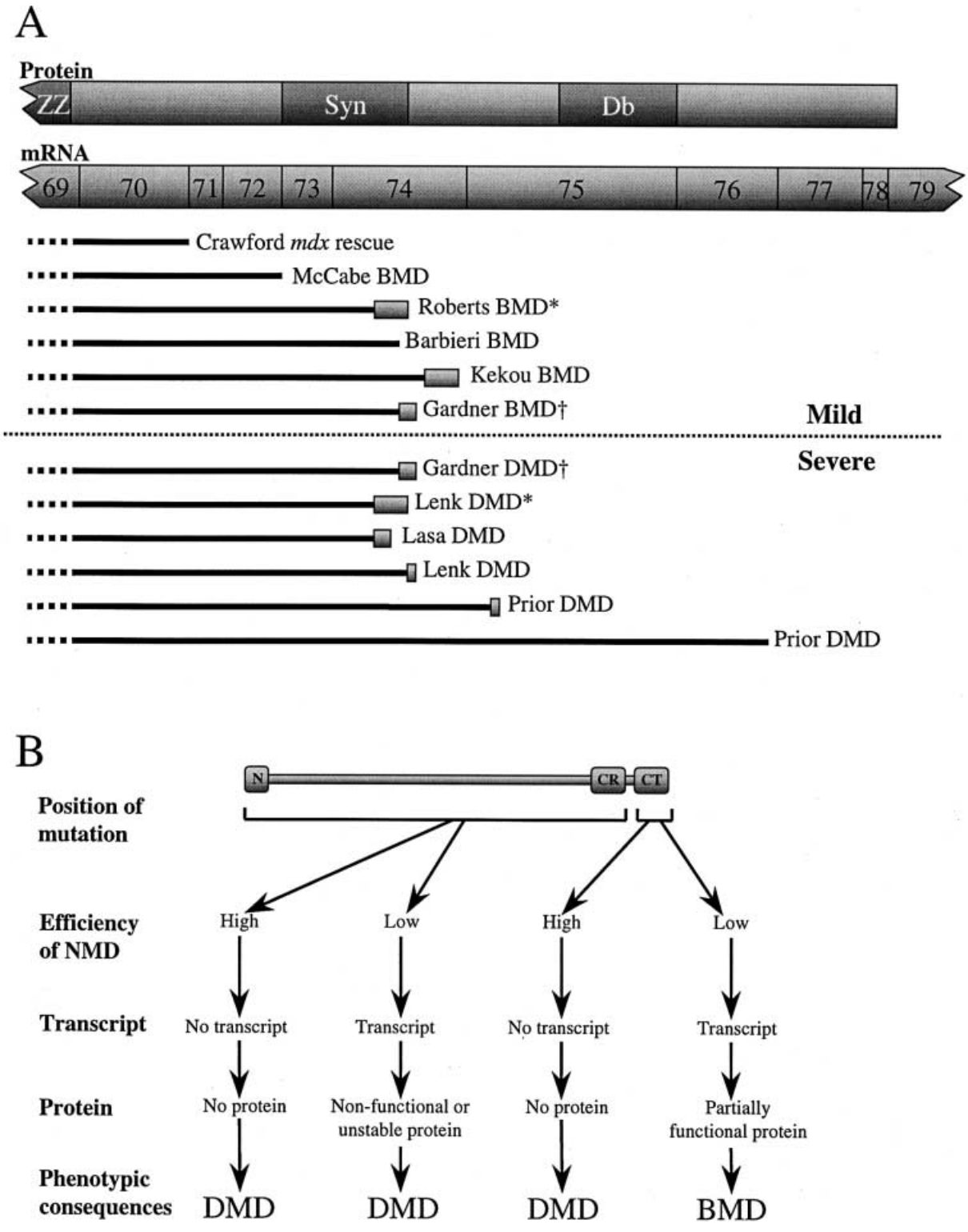

Fig. 1 **A** The 3' mutations of the dystrophin gene discussed in this paper (Barbieri et al. 1996; Crawford et al. 2000; Gardner et al. 1995; Kekou et al. 1999; Lasa et al. 1997; Lenk et al. 1993; McCabe et al. 1989; Prior et al. 1995; Roberts et al. 1992). Dystrophin ORFs of the respective patients, drawn to scale against a schematic diagram of the corresponding regions of the message (labelled with exon numbers) and protein (labelled with known syntrophin and dystrobrevin interacting regions). Nonsense mutations cause an immediate termination of the ORF (*black bars*), whereas small insertions and deletions give variable lengths of frame-shifted peptide (*grey blocks*). The mutations are divided into those that are associated with mild (*BMD*) or severe (*DMD*) phenotypes. *Asterisk* Mutation discussed in this paper and an identical defect in a DMD patient, *cross* an uncle and nephew whose mutations are identical by descent. **B** The interaction of mutation status with NMD efficiency. The diagram shows the hypothetical consequences of a variable NMD background on a common set of chain-terminating mutations (*N* N-terminal domain, *CR* cysteine-rich domain, *CT* C-terminal domain)

of transcripts, giving an interstitially deleted protein (Ginjaar et al. 2000; Roberts et al. 1992). In all three of these instances, it should be noted that the coding potential of the mRNA is still consistent with the premise of the "frameshift rule".

The original assumption that all truncating mutations are null presupposed that the C-terminal region of the dystrophin protein is in some way critical to its function (and indeed the mutations were adduced as evidence for such functional importance). One might also then expect that beyond some threshold point, C-terminal sequence might be lost with only mild functional consequences, perhaps leading to a BMD phenotype.

In the last ten years, however, it has become apparent that for the vast majority of genes, the effects of a truncating mutation act at the transcript level, rather than at that of the protein. Eukaryotic cells possess a mechanism that vets new transcripts for the integrity of the ORF, ablating those transcripts in which mutation, transcriptional error or mis-splicing has disrupted the coding region (Hentze and Kulozik 1999). In mammalian cells, this system, which is known as nonsense-mediated decay (NMD), appears to tag for destruction all transcripts in which translation is terminated before the ribosome reaches the position of the 3'-most splicing event (Hilleren and Parker 1999). In the majority of situations, in which there is autosomal inheritance, NMD should be advantageous, protecting cells from potentially harmful effects of C-terminally truncated proteins and rendering otherwise dominant mutations recessive.

Given the occurrence of NMD in most genes examined, it is likely that the dystrophin transcript is subject to this surveillance; indeed, the level of dystrophin transcripts in DMD muscle has long been known to be extremely low (Chelly et al. 1990). Thus, any truncating mutation more than 50 bp 5' of the last intron (i.e. before codon number 3665) should indeed be null, regardless of how functional the eventual protein would be, as NMD will preclude translation. This may afford an explanation for the disparity between the phenotypic consequences of a number of C-terminal dystrophin mutations (Fig. 1A). Two nonsense mutations reported by Prior et al. (1995) only remove the C-terminal 51 or 165 amino acids of the 3685-residue dystrophin protein but are associated with severe (DMD) phenotypes. Patient CM (described by McCabe et al. 1989), on the other hand, has a genomic deletion of the last seven exons (encoding the C-terminal 242 amino acids) and has a BMD phenotype. Similarly, Crawford et al. (2000) have described phenotypic rescue of an *mdx* mouse by using a cDNA transgene that encodes a dystrophin lacking the C-terminal 276 amino acids. Neither patient CM (who presumably makes use of a novel fortuitous 3' exon beyond the deletion) nor the mouse (whose transgene uses the natural dystrophin 3'UTR and will not be spliced) will suffer NMD and their truncated dystrophin proteins are therefore allowed a full opportunity to effect their rescue. The patients of Prior et al. (1995), on the other hand, have severe phenotypes largely because their NMD never gives the protein a chance.

This raises the possibility that variations in the efficiency of NMD between individuals might result in variable degrees of sensitivity to C-terminally truncating mutations (in DMD/BMD and presumably other diseases). We have examined this hypothesis (Fig. 1B) with respect to literature cases who exhibit greatly differing phenotypes in response to similar C-terminal mutations and test one aspect of the hypothesis by studying the protein of an unexpectedly mild (BMD) patient (Roberts et al. 1992) whose mutation is identical to that of a previously reported DMD patient (Lenk et al. 1993).

Materials and methods

Clinical data

The patient was first referred following concerns regarding his cognitive impairment at the age of 5 years 6 months, when he was judged to have overall cognitive functioning more characteristic of a child of 3 years 8 months and was transferred to a school for children with moderate learning difficulties. Although the issue of poor motor abilities (frequent falls and inability to run) was raised at the time, he did not attend a neuromuscular clinic until aged 9 years 6 months. He was found to have symmetrical proximal weakness in both upper and lower limbs, bilateral calf hypertrophy and marked lumbar lordosis. He walked with a wide-based waddling gait, could manage a small jump and rose from the floor with minimal Gowers' manoeuvre. Serum creatine kinase activity was markedly elevated at 15,000 U/l; no dystrophin gene deletion was detected by Southern blotting or multiplex PCR analysis. A later blood sample was analysed for dystrophin gene point mutations by chemical cleavage analysis of amplified cDNA from lymphocyte ectopic transcripts. This revealed a deletion of a single thymidine residue from exon 74 of his dystrophin gene (Roberts et al. 1992) causing a frameshift at leucine 3485. Evidence for unusual splicing was sought but not found.

Since this analysis, it has become apparent that the patient's phenotype is well within the BMD range; when last reviewed at the age of 18 years, he continued to be able to walk independently for distances up to approximately 1 km.

At the age of 10 years, formal psychological assessment by using the Wechsler Intelligence Scale for Children (WISC) had shown a verbal and performance IQ of 50 and 61, respectively, with a full-scale score of 51. These findings suggested moderate to severe learning difficulties.

A muscle biopsy taken at age 13 years showed a dystrophic appearance, with significant fibrous and fatty deposits. There were a substantial number of very small fibres, most of which stained for fetal myosin, suggesting immaturity (data not shown).

Immunohistochemistry

Unfixed cryostat sections were immunolabelled with a panel of antibodies to a variety of proteins, including β-spectrin, dystrophin (Dys 1, 2, 3), α, β, γ-sarcoglycan, β-dystroglycan, fetal myosin (all Novocastra), laminin α2, β1 and γ1 chains (all Chemicon) and utrophin (Mancho 7; the kind gift of Prof. G. Morris). These were visualised with a biotinylated-streptavidin-Texas Red method and examined by epifluorescence with a Leica Aristoplan microscope (Sewry and Lu 2001).

Results

This report was prompted by a reassessment of one of our patients in the light of his mild phenotypic progress, a sur-

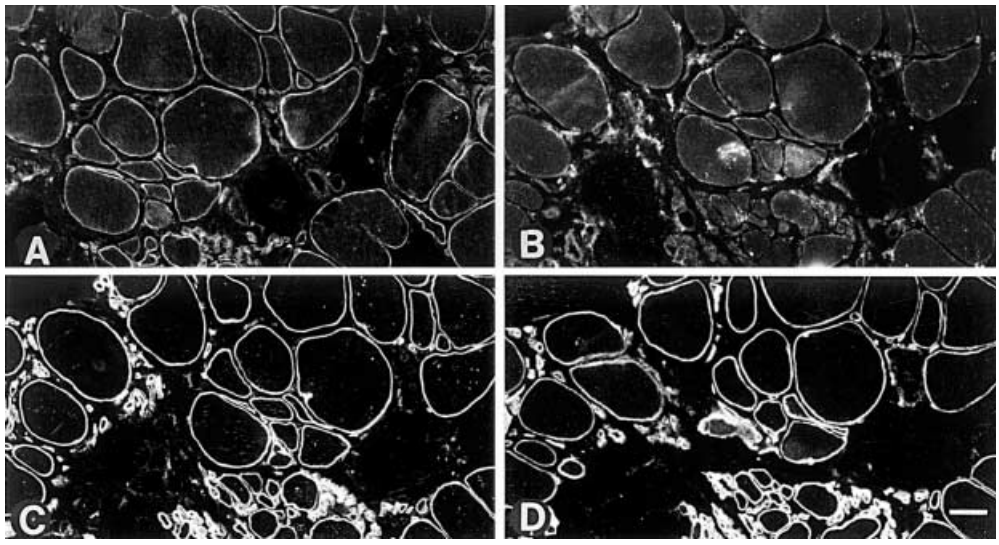

Fig. 2 Serial sections of the skeletal muscle biopsy immunochemically labelled with antibodies to (**A**) an N-terminal portion of the rod domain of dystrophin, Dys 3, (**B**) the dystrophin C-terminus, Dys 2, (**C**) β-spectrin and (**D**) α-sarcoglycan. Note the appreciable dystrophin localisation with Dys 3 but only traces with Dys 2. Expression of β-spectrin shows good preservation of the plasma membrane; expression of α-sarcoglycan also appears to be normal. *Bar* 50 μm

vey of the literature and a recent increase in our understanding of NMD. The patient concerned has been previously described as one of the first known dystrophin point mutations (patient 7; Roberts et al. 1992). His young age (11 years) then led us to categorise him cautiously as having an intermediate Duchenne phenotype but, 8 years later, it is clear that he has BMD with uncharacteristically severe cognitive impairment. His mutation is identical to that of an independently described DMD patient (Lenk et al. 1993). The availability of comprehensive immunohistochemical data provides an opportunity to address the reasons for this discrepancy.

Strikingly, the immunohistochemistry showed appreciable labelling of many fibres with Dys 3, a mouse monoclonal antibody that recognises an epitope in the region of amino acids 300–350 (Fig. 2A). This is unusual for a patient with a chain-terminating mutation. Critically, staining with the C-terminal antibody Dys 2 (raised against a peptide corresponding to the C-terminal 17 amino acids of dystrophin) revealed only occasional traces (Fig. 2B). Staining for α, β and γ sarcoglycans (Fig. 2D) and the α2, β and γ chains of laminin was normal, whereas levels of β-dystroglycan appeared to be only slightly reduced (data not shown). Immunolabelling for utrophin was pronounced on most mature fibres that lacked fetal myosin (data not shown).

Our interpretation of these data is that, despite the presence of a frameshifting mutation, a moderate amount of almost full-length dystrophin is retained. This is apparently sufficient to maintain normal levels of the sarcoglycan complex and near-normal levels of β-dystroglycan in the patient's muscle. We suggest that this is responsible for the mildness of his physical disability. We do not consider that the apparent up-regulation of utrophin could have caused the milder phenotype, as up-regulation of utrophin is common in both DMD and BMD but no correlation with clinical severity has been found (Taylor et al. 1997).

Two possible reasons for the retention of the dystrophin transcript are: (1) alternative splicing, particularly of the in-frame exon 74, which harbours the mutation, or (2) ineffective NMD. The former would result in dystrophin that retains epitopes C-terminal to the mutation, whereas the latter would bear a C-terminal truncation. A comparison of the staining with antibodies Dys 2 and Dys 3 shows that almost all of the patient's dystrophin is C-terminally truncated and is therefore unlikely to have been generated by in-frame removal of exon 74.

Discussion

NMD as a factor in phenotypic heterogeneity

We have described a patient whose chain-terminating dystrophin gene mutation is at odds with his mild muscular phenotype. A straightforward explanation for this, namely alternative splicing of the mutant exon to restore the reading frame, appears to be excluded at both the mRNA and protein level. A second possibility is that NMD, the process that causes the indiscriminate destruction of transcripts bearing prematurely terminated reading frames, may be operating inefficiently in this patient. All our data are consistent with this possibility.

Can variability in the efficacy of NMD (Frischmeyer and Dietz 1999) account for the phenotypic variability of other C-terminal chain-terminating mutations in the dystrophin gene? An examination of the genotype/phenotype relationships in relevant patients from the literature (see Fig. 1A) shows that there is no simple correlation between mutation position and severity of skeletal myopathy, e.g. the BMD patient with a deletion of exons 73–79 and the DMD patient with a nonsense mutation in exon 76. Many similar or even identical mutations between these two locations result in widely differing phenotypes.

As mentioned above, we consider the two prime candidate explanations to be alternative splicing and inefficient NMD. We know from patient CM and from transgenic mouse studies that a protein lacking material encoded by

exons 73–79 can rescue the muscular phenotype from DMD to BMD. Both alternative splicing and inefficient NMD can afford the protein the opportunity to effect this rescue. Unfortunately, most of the mutations in question (including the one described in this paper) lie in exon 74, whose loss will neatly maintain the reading frame. However, we believe that our data at both the mRNA and protein level make this unlikely in our patient. Moreover, the intrafamilial variation observed in the patients who were reported by Gardner et al. (1995) and in whom no alternative splicing was observed suggests that, as the mutations are identical by descent, the cause of variation lies outside the dystrophin gene. We therefore suggest that the variation in phenotypes shown in Fig. 1A may be explained entirely by person-to-person variation in NMD efficiency (see Fig. 1B). Although complete loss of NMD activity might result in the manifestation of certain heterozygous recessive alleles as dominant negatives (together with other constitutive side effects; Pulak and Anderson 1993), partial loss may enable the rescue of the DMD phenotype without undue activation of such cryptic alleles.

Function of the dystrophin C-terminus

A natural conclusion from these and other data, whatever the mechanism for the amelioration of the muscular phenotype, is that the C-terminal region is largely dispensable for muscle function. This is entirely consistent with findings from *mdx* mice bearing truncated dystrophin transgenes (Crawford et al. 2000). We note, however, that our patient has a severe mental phenotype, more reminiscent (both in degree and nature) of that associated with DMD, where the mutation is usually essentially null. This suggests that, although his truncated dystrophin might rescue his muscle phenotype, it is incapable of rescuing the neuronal phenotype. This in turn might suggest a particular neuronal function for the C-terminal 250–300 amino acids. Alternatively, the inter-patient variability of NMD efficiency may be tissue-dependent, with differing consequences for each of the tissue-specific dystrophin isoforms.

The only function hitherto ascribed to the C-terminal region of dystrophin is an interaction with the dystrobrevins (distant relatives of dystrophin) and the syntrophins (adaptor proteins that contain PDZ and PH domains and interact with neuronal nitric oxide synthase and voltage-gated sodium channels). However Crawford et al. (2000) have shown that dystrobrevin and syntrophin can be correctly localised in mouse skeletal muscle despite the absence of this region. It seems that, in humans, this defect is at least compatible with a mild (BMD) muscle phenotype (McCabe et al. 1989; this paper). It is possible that the C-terminus is more critical for dystrobrevin and/or syntrophin localisation in the brain or that it is required for an as-yet unrecognised brain-specific function. We note that, in non-muscle tissues, alternative splicing can remove exon 78 from the dystrophin transcript, bringing a novel ORF in-frame; this coding region is more highly conserved throughout vertebrate and invertebrate species than is the muscle-type C-terminus. The cognitive phenotype of the *mdx* mouse is at best subtle (Sesay et al. 1996; Vaillend et al. 1995, 1998) and the effects of transgene rescue have yet to be examined.

Conclusion

We have noted from the literature that truncating mutations 3' of exon 70 in the dystrophin gene result in wide-ranging and unpredictable phenotypic severity, even within the same family. On the basis of more clear-cut cases, such as patient CM (McCabe et al. 1989) and the *mdx* rescue experiments (Crawford et al. 2000), we advance the hypotheses that: (1) the C-terminal 250 amino acids of dystrophin can be lost without severe muscular phenotypic consequences, (2) in DMD cases with truncating mutations 3' to exon 70 (Prior et al. 1996), the severity of their phenotype is entirely attributable to NMD, (3) in BMD cases with such mutations, the mildness of their phenotype is attributable to a reduced efficacy of NMD. We have tested this by examining the dystrophin protein in a patient who is known to have a truncating mutation in exon 74, no unusual alternative splicing and a BMD phenotype. The presence of substantial amounts of truncated protein in his muscle rules out competing explanations for his mild phenotype. We further note that his cognitive phenotype is more akin to that of DMD, perhaps suggesting that functional requirements for the C-terminus are more critical in the brain.

Disruption of NMD might afford a route for therapy in the rare DMD individuals with C-terminal mutations (Prior et al. 1996) and in certain other recessively inherited diseases with C-terminally truncating mutations. It is likely, however, that non-specific inhibition of NMD might result in undesirable side-effects such as dominant negative activity of otherwise recessive heterozygous mutations and morphological defects as seen in nematode NMD mutants (Pulak and Anderson 1993).

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