Becker muscular dystrophy patient with a large intragenic dystrophin deletion: implications for functional minigenes and gene therapy

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Abstract
The genetic defects responsible for the allelic disorders of BMD and the more severe DMD have been shown to be mutations within the dystrophin gene, which encodes a 14 kb transcript. We describe here a BMD patient who belongs to a small class of subjects with large in frame deletions of the dystrophin gene that remove apparently dispensable coding sequence, thereby producing functional truncated dystrophin. The in vitro reconstruction of these deletion derivatives of full length dystrophin transcripts should enable higher efficiency transfection of human muscle or murine germline cells using retroviral based vectors, compared with the full length transcript. This capability offers a means of examining retroviral mediated transfer as a potential therapeutic strategy in severely affected DMD patients.

The majority of mutations (~65%) in Duchenne and Becker muscular dystrophies (DMD, BMD) are intragenic deletions; approximately 10% of patients have duplications.[1 2] The severity of the clinical phenotype does not correlate with the location and extent of deletion, but does appear to correlate with the effect that the mutation has on the translational reading frame of the dystrophin transcript.[1 3 4] In the case of BMD, the reading frame is maintained and,

in those patients for whom dystrophin has been analysed by Western blotting, an altered size dystrophin is produced. These naturally occurring deletion derivatives of dystrophin in BMD patients provide a resource for mapping functionally important domains of this large (427 kd)[5] muscle protein and for permitting the construction of functional minigenes.

Analysis of the nucleotide sequence of the dystrophin transcript[5 6] together with immunohistochemistry of muscle sections[7 9] and electronmicrographic studies of purified dystrophin[10] have led to a model of dystrophin structure and function. The protein is proposed to consist of four domains: an N-terminal domain (240 amino acids) with putative actin binding function[11]; a rod shaped domain formed by 24 repeat sequences that are predicted to adopt a triple helical conformation with hinge regions[12 13]; a cysteine rich domain homologous to the entire C-terminal of *Dictyostelium discoideum* α-actinin[6]; and a 420 amino acid carboxyl-terminal region that is highly conserved in man and chicken[14] and is homologous to another high molecular weight muscle protein of unknown function.[15] The latter domain is thought to mediate interaction with a membrane protein complex.[16] Dystrophin has been shown to self associate in a manner similar to spectrin and α-actinin such that it may form a flexible subsarcolemmal network for maintaining membrane integrity, or the spatial configuration of integral membrane proteins during normal contraction induced stress.[7 13 16 17]

Here we present Southern hybridisation analysis of a BMD patient with a large deletion of the dystrophin gene within the rod shaped domain. A comparison of this patient's deletion together with those of other BMD patients reported in the literature is discussed with a view to elucidating the minimal length of coding region necessary to give rise to a functional, albeit truncated, dystrophin. The implications for using in vitro constructed minigenes in therapeutic strategies for DMD patients is discussed.

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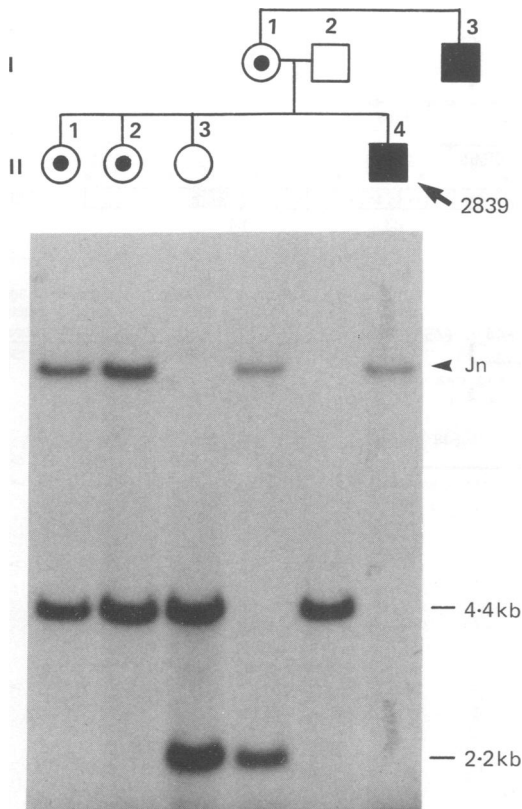S A Genet, H R Middleton-Price

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Patients and methods

The proband (patient 2389) presented at 21 years of age with proximal weakness of both legs that had become evident three years previously. This weakness was most obvious on climbing stairs and rising from a squat position. EMG investigations suggested an active myopathy and, together with a raised serum creatine kinase level (no data recorded), a diagnosis of BMD was made. The proband is now 26 years of age and continues to work as a panel beater; however, his maternal uncle, aged 44 years, is mainly confined to a wheelchair. The patient's sisters (see pedigree, fig 1) had serum creatine kinase levels of 1566 U/l (II·1), 204 U/l (II·2), and 112 U/l (II·3) (normal range 25 to 170 U/l). The sister with the highest level of serum creatine kinase is phenotypically normal but is only 20 years of age.

DNA was isolated from blood lymphocytes by the method described by Jeanpierre[18] and digested with restriction endonucleases according to the manufacturers' instructions. Digested DNA was electrophoresed in 0·8% agarose gels, capillary blotted to Hybond N or Hybond N+ membranes (Amersham), and hybridised with randomly primed ^{32}P labelled probes as described previously.[19] The DNA probes used were genomic probe pERT87-8 (*DXS164*, supplied by L M Kunkel), which detects a *Bst*XI polymorphism,[20] genomic probe P20 (*DXS169*, supplied by G van Ommen),[21] and dystrophin cDNAs[22 24] (shown in fig 2).

Oligonucleotides flanking dystrophin exon 45[1 25] were used to amplify genomic sequence by PCR from the proband's DNA following the protocol of Chamberlain *et al.*[25]

Results

The family pedigree of patient 2839 is shown in fig 1, together with the hybridisation analysis of *Bst*XI digested DNA using the dystrophin genomic probe pERT87-8 (*DXS164*). The data indicate that the BMD mutation is segregating with an altered size *Bst*XI fragment. Preliminary hybridisation analysis of this patient's DNA indicated that he carried a deletion within the dystrophin gene. The location and extent of the deletion were determined by hybridising cDNAs covering nucleotides 1418 to 7880 with *Hind*III and *Pst*I digested 2839 DNA. The hybridisation analysis was compared with that of patient 324 who has been reported previously with a large intragenic deletion of 5106 bp from exon 17 to 48, inclusive.[26]

The 5' deletion endpoint of patient 2839 occurs between exons 13 and 14 (fig 3A). This suggests that the altered size *Bst*XI fragment detected by pERT87-8 is a junction fragment because Cf16 maps within the *DXS164* locus (fig 2) The 3' deletion endpoint is located within the P20 intron, which is bounded by exons 44 and 45. The hybridisation of *Hind*III and *Pst*I digested 2839 DNA with Cf23a and Cf56b (wholly contained within Cf23a), respectively, indicated the absence of exons upstream of exon 45; PCR analysis of 2839 genomic DNA using oligonucleotides flanking exon 45 showed that this exon is intact (data not shown). The precise location of the 3' endpoint within the P20 intron is not known; however, this endpoint is 5' to the genomic region containing the P20 cosmid because both *Hind*III fragments detected by P20 are present in patient 2839 (fig 3B). The full deletion of this patient with respect to the map of the dystrophin gene is shown in fig 2.

Figure 1   Pedigree and Southern analysis of patient 2839 and family members. Upper panel shows pedigree: ⊙ = carrier female, ■ = affected male. Lower panel shows pERT87-8 hybridisation with BstXI digested DNA in which the lanes contain DNA of patient 2839 and family members as indicated in the upper panel. The lengths of the alleles detected by pERT87-8, together with the junction fragment (Jn) detected in carrier females and the affected male (patient 2839), are shown.

Discussion

Patient 2839 has an intragenic deletion of 44% of the coding region of dystrophin that is predicted not to affect the translational reading frame, and his phenotype is mild. These data suggest that a large portion of the rod shaped domain of dystrophin is dispensable without adversely affecting the patient's muscle function. In this regard, patient 2389 is similar to our earlier description of BMD patient 324 who has a deletion of 46% of dystrophin.[26] The latter patient's muscle shows a truncated dystrophin of 220 kd by Western analysis and continuous membrane staining for dystrophin by immunohistochemistry.

Few BMD patients have been described with large intragenic deletions[1 26 27] and, of these, only one has been analysed to determine dystrophin size and localisation.[26] The data (fig 2) indicate that 43% to

63% of the rod shaped domain appears dispensable although there is variation of clinical severity within this deletion range, at least in terms of the age at which patients become wheelchair bound (fig 2). In particular, patient 324, who has the largest intragenic deletion reported so far, was able to walk with the aid of a stick at 61 years of age, and his affected nephew (1353) is a weight trainer at 25 years of age.[26] The deletion range shown in fig 2 has implications for gene therapy of DMD patients based on retrovirus mediated transfection of muscle cells. The cloning of the coding region of a deleted dystrophin of 5·9 kb in length (patient 324) would permit relatively high efficiency infection of cells by retroviral recombinants[28] compared with the cloning of a full length transcript (minimum length of 11 kb). Retrovirus minigene recombinants offer a means of examining in vivo complementation of mutations that
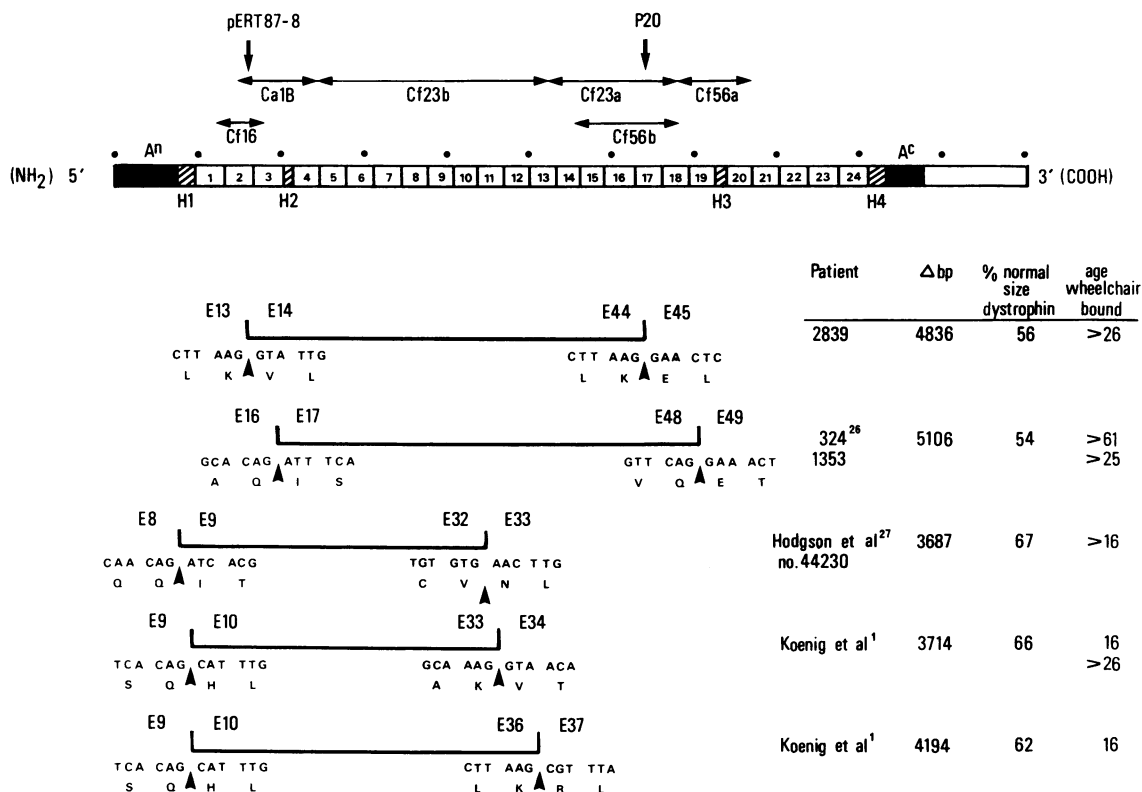

Figure 2   Location and extent of large intragenic deletions in the dystrophin gene of BMD patients. The four domains of dystrophin are represented by: filled boxes, designated A^n and A^C (regions of homology with amino- and carboxyl-terminal domains of α-actinin, respectively); open box is the putative membrane binding domain; the rod shaped domain is divided into 24 repeats with the hatched boxes indicating putative hinge regions (H1-H4).[12] The location of dystrophin cDNAs (horizontal arrows) and genomic probes (vertical arrows) used in the Southern hybridisation analysis of patients 2839 and 324 are indicated above the domain map and the dots indicate spacings of 1 kb. The patient deletions are indicated below the domain map. Exon boundaries (indicated by arrow heads) defining these deletions are shown by exon number and dystrophin nucleotide and amino acid sequences. Patients still ambulatory are indicated by ' > (age at last clinical examination)'.
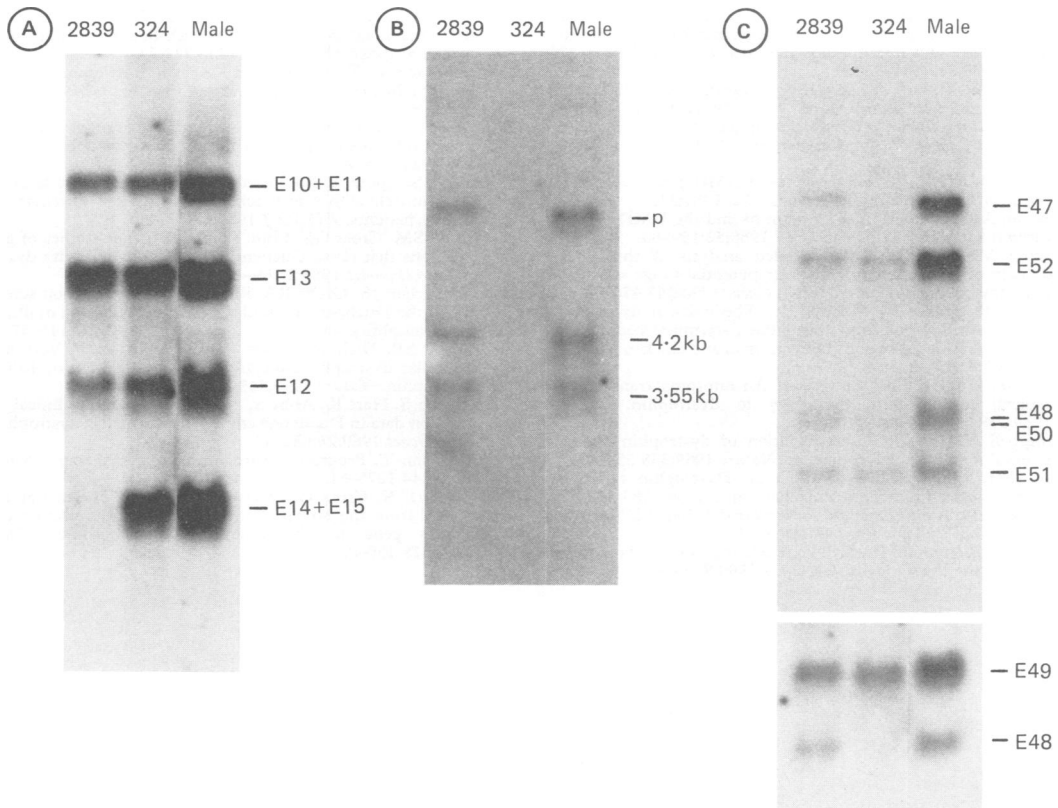
Figure 3   Southern analysis of BMD patients 2839 and 324 using dystrophin gene probes. DNA was digested with HindIII and probed with dystrophin cDNAs Cf16 (A) and Cf56a (C), and the genomic probe P20 (B). Dystrophin exon numbers are indicated alongside appropriate panels and p denotes partial digestion products.

affect translation of endogenous dystrophin genes, for example, in DMD patients and the *mdx* mouse, which is a rodent model of DMD.[29]

At a functional level, the model described by Koenig and Kunkel[13] that proposes a flexible network of dystrophin molecules dependent on hinges within the rod domain may require modification. Fig 2 shows that all the BMD patient deletions remove hinge 2, but retain the region of the rod domain downstream of hinge 3. Electronmicrographic analysis of truncated dystrophin molecules in vitro and sarcolemmal distribution in vivo should aid in determining those structural domains that are important in intermolecular associations. These studies should also provide means of evaluating dystrophin deletion derivatives constructed in vitro with regard to their suitability for gene therapy.

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