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Transcription of the dystrophin gene in Duchenne muscular dystrophy muscle

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Dystrophin is the recently discovered defective gene product in Duchenne and Becker muscular dystrophy (DMD and BMD). Dystrophin transcripts have been amplified and identified in diagnostic needle muscle biopsy samples using the polymerase chain reaction (PCR) procedure. Using 5′- and 3′-primers, dystrophin transcripts can be detected in both DMD and BMD muscle biopsies, on either side of defined deletions within the dystrophin gene.

Dystrophin; Duchenne muscular dystrophy; Polymerase chain reaction; RNA

1. INTRODUCTION

Duchenne and Becker muscular dystrophy (DMD and BMD) are two allelic X-linked disorders which arise from mutation in the same gene [1]. The gene has recently been identified and shown to span 2000 kilobases [2] comprising approx. 0.05% of the human genome. The corresponding human cDNA sequence has been isolated [3] and antibodies have been raised against a fusion protein constructed from a fragment of cDNA toward the 5′-end of the molecule. This has led to the identification of a protein, dystrophin, which, although present in normal muscle, is absent in skeletal muscle from boys with DMD [4,5]. Abnormal abundance or abnormal molecular mass forms of dystrophin are found in patients with the milder BMD phenotype [5,6].

Approx. one-third of all cases of DMD (which affects 1 in 3500 newborn males) are due to a new mutation in the gene. In skeletal muscle, dystrophin is encoded by a large 14 kb RNA transcript processed from more than 65 exons; as a result, less than 1% of the total gene is transcribed and the large amount of splicing that takes place during transcription probably contributes to the high levels of mutation which are observed [7]. The large dystrophin transcript is rapidly degraded [8] and this degradation, coupled with its low abundance (0.001–0.01% total muscle RNA) makes the transcript very difficult to detect on Northern blots, using diagnostic muscle biopsy samples. Expression in the 5′-end of the gene has been observed in DMD muscle however, particularly in regenerating muscle fibres, using the greater sensitivity of in situ RNA hybridization [8].

Using the polymerase chain reaction (PCR), dystrophin mRNA can be detected in many tissues [9,10]. Unlike Northern blot analysis, which requires extensively purified high molecular mass RNA, PCR does not have such stringent requirements for intact message (indeed, it only requires that the average molecular mass of template

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Abbreviations: PCR, polymerase chain reaction; DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; G6PD, glucose-6-phosphate dehydrogenase

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mRNA be slightly greater than the largest fragment amplified). Here, we show that using the PCR amplification procedure, we can readily detect dystrophin transcripts in diagnostic muscle biopsy samples. Using both 3′- and 5′-primers, we are able to detect these transcripts on either side of defined deletions within the dystrophin gene.

2. EXPERIMENTAL

2.1. *RNA extraction and cDNA synthesis*

Needle muscle biopsies were snap-frozen in isopentane cooled in liquid nitrogen and stored at −70°C or in liquid N_2. Control muscle samples were obtained during surgical procedures for non-muscle-related disorders. Excess mounting medium was removed with a razor blade and total RNA was extracted from the frozen muscle using an acid guanidinium thiocyanate (pH 4.0)/phenol/chloroform method [11]. cDNA was obtained from isolated total RNA (5 μg) using Moloney murine leukaemia virus reverse transcriptase (Pharmacia) and either reverse or pd(N)6 random primers (Pharmacia).

2.2. *Polymerase chain reaction*

cDNA was amplified with 0.3 μg synthetic dystrophin oligonucleotides (final volume 50 μl) (Oligonucleotide Service Facility, Royal Postgraduate Medical School) in a programmable temperature bath (Techne) at an annealing temperature of 58°C. If the first amplification was negative then the quantity of the template was doubled and the process repeated; if this second amplification was negative, then G6PD cDNA was co-amplified as a control. (The levels of dystrophin mRNA and G6PD mRNA are approximately equal in human muscle.) Amplified products were separated on 1.8% agarose gels and visualized with ethidium bromide. Molecular mass markers were obtained from a combined *PvuII*/*TaqI* (Boehringer) digest of pEMBL DNA (gift from Professor L. Luzzatto, Royal Postgraduate Medical School), obtaining fragments of 1443, 1008, 613, 357, 278, 193 and 103 bp.

3. RESULTS

Three pairs of primers derived from the dystrophin cDNA sequence were synthesized as forward and reverse primers to amplify 3 short segments spanning 3 regions of the dystrophin coding sequence. Segment I (nucleotides 35–237) is situated in the first exon, extending into the untranslated region. Segment II (nucleotides 1854–2070) is situated in exons 14–16 and encompasses part of the cDNA which was originally used by Kunkel and colleagues to construct a 90 kDa fusion protein [4]. Segment III (nucleotides 9727–9975) is situated 3′ to the so-called 'hot spot' region which is located between 7 and 8 kb from the 5′-end of the molecule (fig.1). The fourth segment (nucleotides 865–1348) amplified the central region of the G6PD cDNA.

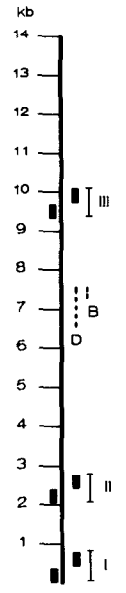

Fig.1. Location of dystrophin PCR primers. Solid line represents 14 kb dystrophin cDNA. Filled rectangles indicate forward (left) and reverse (right) primers, encompassing PCR-amplified segments; I, 204 bp; II, 218 bp; III, 248 bp. (D---; B---) Deletion in dystrophin cDNA from DMD and BMD patients, respectively, referred to in fig.3. The sequences (5′-3′) of the primers are: segment I (forward), TCTGGGAGGCAATTACCTTC, (reverse) ACAGTCCTCTACTTCTTCCC; segment II (forward), ACCGCTGGGTTCTTTTACAA, (reverse) TACAGTTTGCCCATGGATTG; segment III (forward) GCGTGGATATGTGTCTGAAC, (reverse) CCCCCAAAGGATGCAACTTCA; segment IV (forward) GTCAAGGTGTTGAAATG, (reverse) CTGGCTCCTGCAGAAGAC.

RNA was isolated from small blocks (5–15 mg) of muscle biopsy samples taken for diagnostic evaluation. The final yield was 1.0–1.5 μg RNA/mg tissue. Total RNA was reverse-transcribed into single-strand cDNA using either pd(N)6 or one of the specific forward primers, complementary to the 3′ part of the mRNA segment to be amplified. Both methods were successful but random primers were finally chosen because they gave more reproducible results than using a single tube containing a set of specific primers.

The size of the amplification products was as predicted from the sequences of dystrophin and G6PD cDNA respectively [3,12], namely

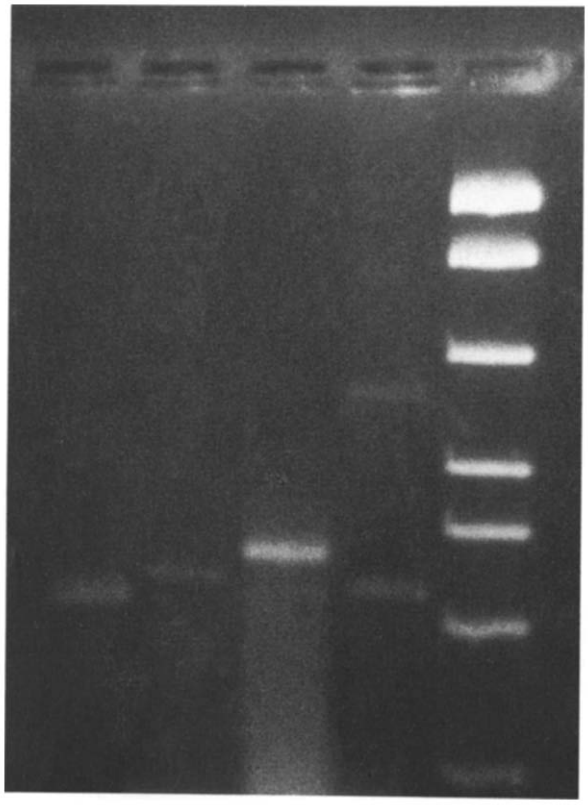

Fig.2. Agarose gel electrophoresis of amplified dystrophin RNA segments from normal muscle biopsies. Lanes: 1, segment I; 2, segment II; 3, segment III; 4, coamplification of segment I and G6PD (segment IV).

(dystrophin) segment I, 204 bp; segment II, 218 bp; segment III, 248 bp; (G6PD) segment IV, 483 bp. Fig.2 shows these amplification products from normal muscle and the co-amplification of dystrophin segment I and G6PD segment IV. The specificity of the amplified products was established by (i) correct predicted size; (ii) lack of amplification when the opposite primers were used for cDNA synthesis; (iii) lack of amplification of dystrophin segments in human myoblasts. Fig.3 shows the results of a typical experiment amplifying total RNA obtained from both DMD and BMD muscle. This particular DMD patient was deleted for the cDNA probe 44.1 (exons 34–39) while the BMD patient had a smaller deletion (exons 38–39) which was detected with the same probe, both deletions occurring in the central (7–8 kb) region of dystrophin cDNA (fig.1). Amplified RNA can therefore be detected 3′ to the identified deletion in both cases. Although

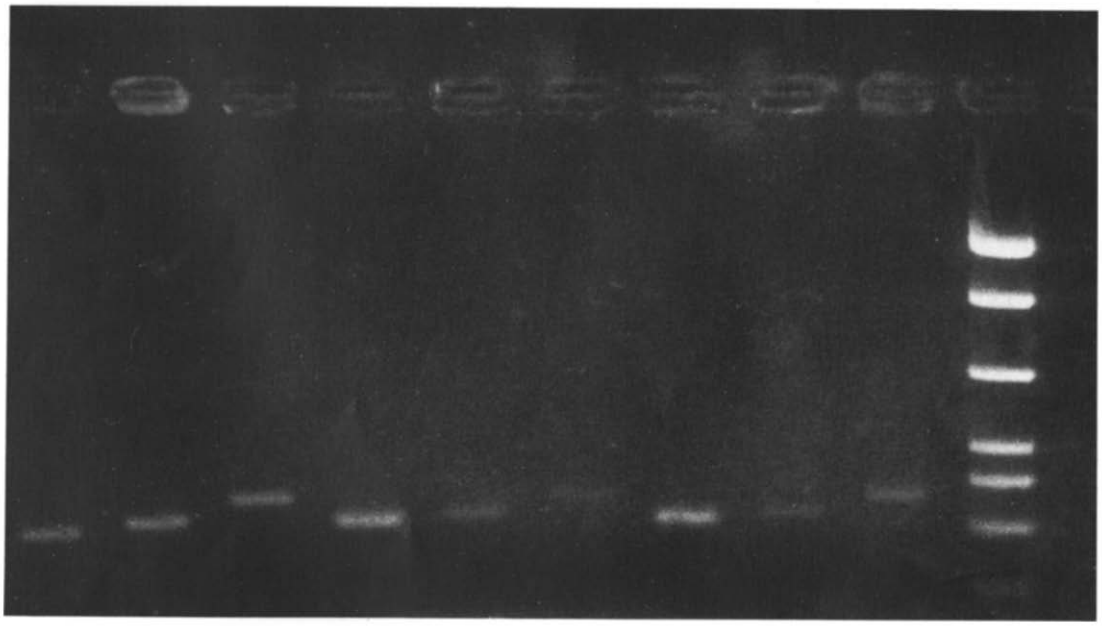

Fig.3. Agarose gel electrophoresis of amplified dystrophin RNA segments from DMD and BMD muscle. Lanes: 1–3, normal muscle (1, segment I; 2, segment II; 3, segment III); 4–6, DMD muscle (4, segment I; 5, segment II; 6, segment III); 7–9, BMD muscle (7, segment I; 8, segment II; 9, segment III).

transcriptional amplification products are obtained for all three regions, there is uneven amplification among the primers; products obtained with segment II were distinctly reduced, in both these examples and many others.

4. DISCUSSION

Dystrophin transcripts in skeletal muscle are rare, accounting for 0.001–0.01% of total RNA. Problems in detection are compounded by the instability of the large message. However, using the PCR technique, we can reproducibly detect dystrophin message in routine needle muscle biopsies. Samples have been handled without taking any specific precautions. We have overcome the problems associated with partially degraded RNA in stored tissues by only amplifying small segments (200–500 bp); PCR does not require intact message and it only requires that the molecular mass of the template be slightly greater than that of the fragment to be amplified.

The three dystrophin segments were amplified in both normal and DMD and BMD muscle. In muscle from the DMD patient shown here (as well as many others; not shown) the dystrophin message was detected in the distal region of a genomic deletion encompassing several exons, indicating that transcription was not aborted after the deletion.

The possibility of using PCR techniques on needle muscle biopsies will permit the study of dystrophin at the RNA level in virtually all patients for which this analysis is of interest. Such information will help in elucidation of many unresolved questions: for example, the mechanism of DMD gene inactivation in the 35% of patients who do not exhibit genomic deletions or duplications in the Duchenne gene. It will also be possible to study whether non-deletion mutations involve splicing of the large dystrophin mRNA [7], or whether some of the DMD patients actually have true null mutations. Finally, it should be feasible to test the hypothesis that frameshift deletions in patients with a BMD phenotype produce a semi-functional protein as a result of reinitiation of the reading frame from an internal start codon [13].

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