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Research Article

Genomic DNA from a patient with dystrophic myopathy, glycerol kinase deficiency, and congenital adrenal hypoplasia was investigated using cDNA probes for the Duchenne muscular dystrophy (DMD) locus. Genomic probes had not detected a deletion in this patient. Southern analysis of Hind III-digested genomic DNA from this patient identified a deletion when the three distal Hinc II DMD cDNA fragments were used as probes. The deletion began in the genomic region corresponding to the 1.05-kb Hinc II cDNA fragment and extended through the 3' end of the DMD gene. This represents a centromeric breakpoint that corresponds to a position approximately 10.2-10.6 kb from the 5' end of the 14-kb DMD cDNA. These investigations demonstrate the value of the DMD cDNA probes for improved diagnoses in patients with molecular lesions involving the DMD locus. Furthermore, this novel deletion involving the coding portion of the 3' end of the DMD gene assists in the ordering of exons in this region and will provide insight into the functional role of the carboxy terminus of the DMD gene product, dystrophin.

Complementary DNA Probes for the Duchenne Muscular Dystrophy Locus Demonstrate a Previously Undetectable Deletion in a Patient with Dystrophic Myopathy, Glycerol Kinase Deficiency, and Congenital Adrenal Hypoplasia

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Abstract

Genomic DNA from a patient with dystrophic myopathy, glycerol kinase deficiency, and congenital adrenal hypoplasia was investigated using cDNA probes for the Duchenne muscular dystrophy (DMD) locus. Genomic probes had not detected a deletion in this patient. Southern analysis of Hind III-digested genomic DNA from this patient identified a deletion when the three distal Hinc II DMD cDNA fragments were used as probes. The deletion began in the genomic region corresponding to the 1.05-kb Hinc II cDNA fragment and extended through the 3′ end of the DMD gene. This represents a centromeric breakpoint that corresponds to a position ~ 10.2–10.6 kb from the 5′ end of the 14-kb DMD cDNA. These investigations demonstrate the value of the DMD cDNA probes for improved diagnoses in patients with molecular lesions involving the DMD locus. Furthermore, this novel deletion involving the coding portion of the 3′ end of the DMD gene assists in the ordering of exons in this region and will provide insight into the functional role of the carboxy terminus of the DMD gene product, dystrophin.

Introduction

The association of Duchenne muscular dystrophy (DMD),[1] glycerol kinase deficiency (GKD), and congenital adrenal hypoplasia (AHC) is recognized as a microdeletion syndrome involving contiguous loci on the short arm of the X chromosome in the Xp21 region (1–5). Patients with this complex phenotype may have classical DMD or a milder dystrophic myopathy as seen in the original two brothers described with this disorder (6, 7), the eldest of whom is the subject of these investigations. These patients also have developmental delay (3–8).

Despite the clinical phenotype involving multiple loci, not all patients have evidenced deletions using the previously available probes (4, 9). The patient reported here showed no detectable deletion (4). The current investigations using the recently cloned DMD cDNA probes (10) demonstrate that the centromeric breakpoint is in the genomic region corresponding to the 1.05-kb Hinc II cDNA fragment located between 3.1 and 4.1 kb from the 3′ end of the full-length DMD cDNA.

Methods

The patient, C.M., is the elder of two brothers with dystrophic myopathy, AHC, severe developmental delay, osteoporosis with pathological fractures, hyperglycerolemia, glyceroluria, and GKD (5–8, 11). The dystrophic myopathy shows the histological features of early DMD (7), but is clinically a mild form of Becker muscular dystrophy (BMD) with absence of muscular pseudohypertrophy at 15 yr of age. Previous investigation showed no evidence of deletion using the following Xp21 probes (ordered Xpcen → pter): DXS84 (754), DXS142 (pERT87), J-Bir, DXS68 (L1.4), DXS28 (C7), DXS67 (B24), DXS41 (99-6), and DXS43 (D2) (4).

Genomic DNA from C.M. was prepared from lymphoblastoid cells (4) according to described methods (12, 13). Genomic DNA was isolated from leukocytes of DMD controls using 10–20 ml of heparinized or EDTA-anticoagulated whole blood according to the method of Kunkel et al. (14) adapted to automated equipment (DNA Extractor, model 340A; Applied Biosystems, Foster City, CA). The purified nuclear DNA pellet was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA quantitated using a minifluorometer (Hoefer Scientific Instruments, San Francisco, CA) (15, 16). A 20-μg aliquot of genomic DNA was digested with Hind III (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 10 μg of digested DNA was electrophoresed on a horizontal agarose gel (17). DNA fragments were transferred to a nylon membrane (Zetabind; Bio-Rad Laboratories, Richmond, CA) with 10× standard saline citrate (18). Conditions for hybridization, washing, and autoradiography have been previously described (19, 20).

The following DMD cDNA probes were used, and directly corresponded to the nucleotide regions from Koenig et al. (10): 1–2a (1a, 1b, and the 5′ portion of 2, noted here as 2a); the 5′, 1.5-kb Eco RI fragment (originally released as 9–7); 4–5a, the 1.8-kb Eco RI fragment spanning the approximate region of nucleotides 2,700–4,500 of the cDNA (originally released as 30–1); and 8, the 0.9-kb Eco RI fragment spanning the approximate region of nucleotides 6,900–7,800 (originally released as 44–1). Four probes were subcloned from the 6.1-kb Eco RI fragment spanning the 3′ end of the cDNA, corresponding to regions 9–14 of Koenig et al. (10) (originally released as 63–1). This region was divided into four fragments by digestion with Hinc II. The fragments were designated by the original regional terminology of Koenig et al. (10), followed by a restriction enzyme abbreviation (E = Eco RI and Hc = Hinc II), since these probes represented modifications from the originally defined regions. Although these modifications were relatively minor they did assist in the clarification of the exon order in this

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1. *Abbreviations used in this paper:* AHC, congenital adrenal hypoplasia; BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; GKD, glycerol kinase deficiency.

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Table I. Summary of DMD cDNA Fragments Used in these Investigations

Fragment designation	Approximate size	Restriction enzyme(s)	Approximate region of DMD cDNA (nucleotides) (10)	Correspondence with previously reported probes (10)	Alternative designation (21)
	kb				
1–2a	1.5	Eco RI	1–1,500	1a; 1b; 5′ portion of 2	9–7
4–5a	1.8	Eco RI	2,700–4,500	4; 5a	30–1
8	0.9	Eco RI	6,900–7,800	8	44–1
9–10EHc	1.9	Eco RI-Hinc II	7,800–9,700	9; 10	63–1EHc1.9
11Hc	1.05	Hinc II	9,700–10,850	11a; 5′ portion of 11b	63–1Hc1.05
12Hc	0.6	Hinc II	10,850–11,500	3′ portion of 11b; 12a, which overlaps with 5′ portion of 12b	63–1Hc0.6
12b–14HcE	2.5	Hinc II-Eco RI	11,500–13,900	3′ nonoverlapping portion of 12b; 13; 14	63–1HcE2.5
14	1.0	Hind III-Eco RI	12,900–13,900	14	63–1HE1.0

portion of the cDNA (see below). These four probes were: 9–10EHc, the 1.9-kb Eco RI-Hinc II fragment spanning the approximate region of nucleotides 7,800–9,700; 11Hc (11a; 5′ portion of 11b), the 1.05-kb Hinc II fragment spanning the approximate region of nucleotides 9,700–10,850; 12Hc (3′ portion of 11b; 12a), the 0.6-kb Hinc II fragment spanning the approximate region of nucleotides 10,850–11,500; and 12b–14HcE (3′ nonoverlapping portion of 12b; 13; 14), the 2.5-kb Hinc II-Eco RI fragment spanning the approximate region of nucleotides 11,500–13,900. Probe 14 was identical to that of Koenig et al. (10), the 1.0-kb Hind III-Eco RI fragment spanning the approximate region 12,900–13,900. These DMD cDNA probes are summarized in Table I. Two genomic probes within the DMD region were also used: J66-H1 (DXS268), which was cloned from patient DL66.6 and maps between JBir (DXS270) and L1 (DXS68) (21, 22); and P20 (DXS269), which was 5′ to J66-H1, between JBir and J66-H1 (23). The cDNA and genomic probes were radiolabeled with ^{32}P-dCTP using random primers (24).

Results

The DMD cDNA probes 1–2a, 4–5a, and 8 showed intact signals with genomic DNA from the patient C.M. (Fig. 1, *A–C*). This result was anticipated since the genomic probes P20 and J66-H1, describing a region 3′ to probe 8, gave signals that were present and intact (data not shown). When C.M.'s genomic DNA was probed with the subclone from region 9–14 comprising the 3′ terminal DMD cDNA, probe 14, no signal was detectable (Fig. 1 *D*). Therefore, to further define the exact breakpoint the four restriction fragments, derived by Hinc II digestion of region 9–14, were hybridized individually with genomic Southern blots from the patient and controls. The 5′, 1.9-kb fragment, 9–10EHc, revealed the presence of each of the expected 13 Hind III fragments in the patient's DNA (data not shown). However, when the 1.05-kb fragment 11Hc was

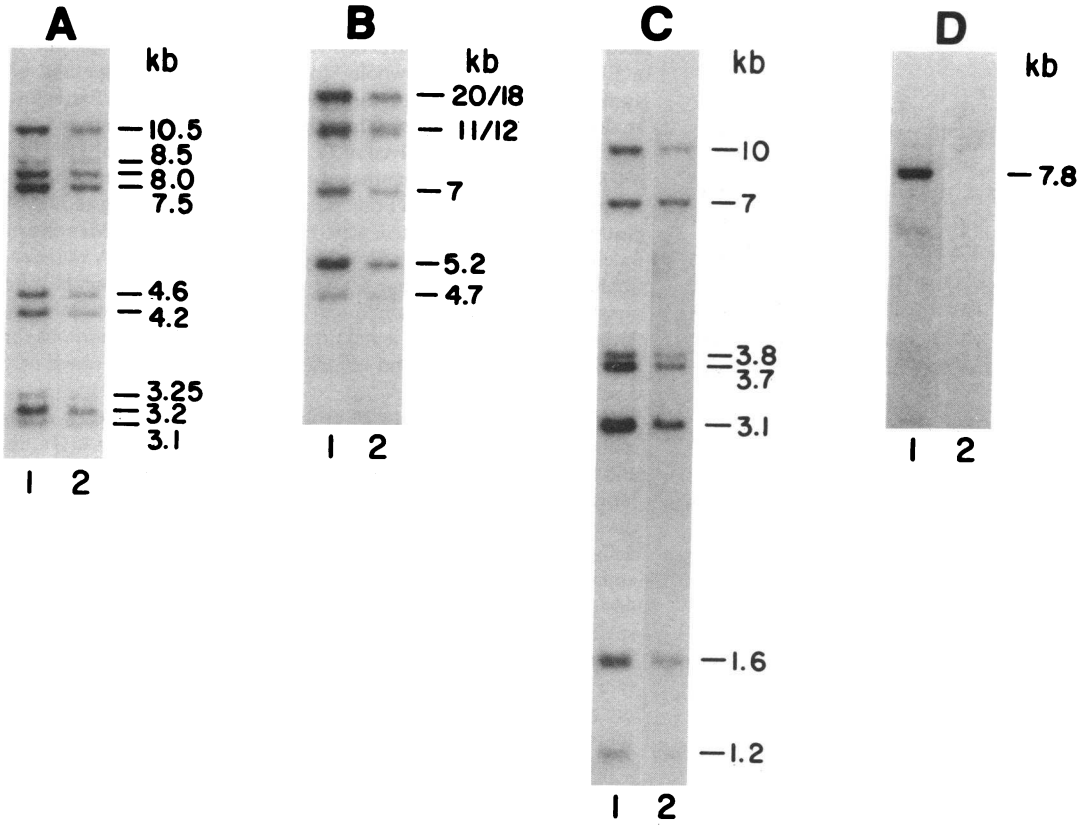

Figure 1. Autoradiograms of Southern blots from genomic DNA digested with Hind III and probed with the DMD cDNA fragments 1–2a (*A*), 4–5a (*B*), 8 (*C*), and the 3′ terminal subclone 14 (*D*) are shown. The same Southern blot was used for the hybridizations shown in *A* and *B*, and another was used for *C* and *D*. Lane *1* represents DNA from a control DMD individual without deletions involving these four cDNA regions. Lane *2* represents DNA from C.M.

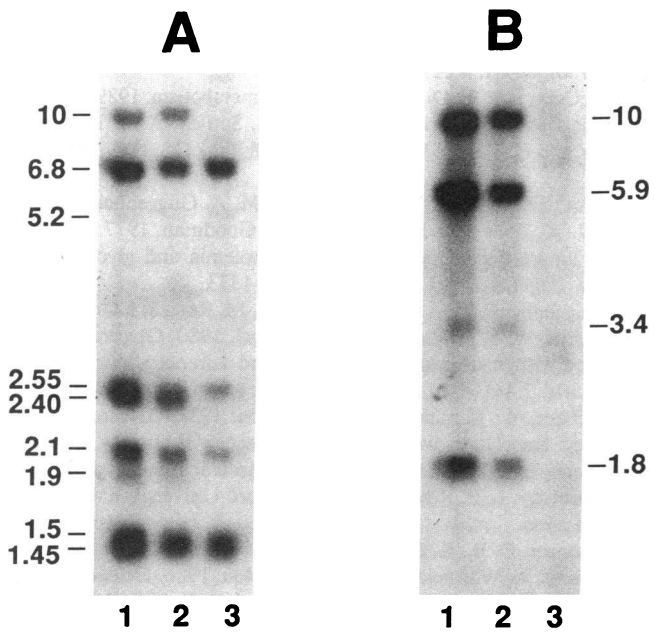

Figure 2. Autoradiograms of Southern blots from genomic DNA digested with Hind III and probed with the DMD cDNA fragments 11Hc (*A*) or 12Hc (*B*) are shown. Lanes *1* and *2* contain control DNA from two individuals not deleted for the DMD gene. Lane *3* represents DNA from C.M.

used as a probe only six of the nine control Hind III fragments were detectable in the patient (Fig. 2 *A*). This localized the centromeric breakpoint of his deletion to this region of the DMD gene. In agreement with these data the 0.6-kb probe, 12Hc (Fig. 2 *B*), and the 2.5-kb probe, 12b-14HcE, (data not shown) failed to hybridize with the patient's DNA.

Discussion

The DMD cDNA probes have demonstrated a deletion in the genomic DNA from a patient with mild BMD, GKD, AHC, and severe developmental delay. The regions probed with cDNA clones 1–2a, 4–5a, and 8, and the genomic clones P20 and J66 were intact. The centromeric deletion breakpoint was shown to be within the region probed with 11Hc and no signal was detected with the 3′ probes 12Hc, 12b–14HcE, or 14. A comparison of these Southern hybridizations with the data previously reported by Koenig et al. (10) allowed localization of the centromeric breakpoint of this patient's deletion relative to the known order and approximate sizes of hybridizable Hind III fragments, as summarized in Fig. 3. The centromeric breakpoint is localized between the sixth and seventh (5′ → 3′) Hind III fragments detected by probe 11Hc, a region ~ 10.2–10.6 kb from the 5′ end of the DMD cDNA. The deleted Hind III fragments total ~ 33 kb from the 3′ genomic terminus of the DMD gene, representing a minimal estimate of the DMD portion of the deletion; however, genomic Hind III fragments not detected by the DMD cDNA could increase this distance greatly.

The exact order of many of the Hind III fragments in the 3′ region of the DMD cDNA has not been determined because of the extreme scarcity of deletions originating or terminating in this region. One patient has demonstrated a centromeric breakpoint distal to J66 (22) and others are expected due to phenotypic involvement of the DMD along with telomeric loci (4, 9); however, cDNA characterizations of these patients' deletions have not been reported. The results presented here showing hybridization of these DMD subclones with the patient's genomic DNA allow further clarification of the gene structure in this region. Fig. 2 *A* shows the patient to be deleted for a 2.4-kb Hind III fragment within probe 11Hc, and this was confirmed on additional Southern blot hybridizations. However, other Hind III fragments previously reported to be 3′ to the 2.4-kb fragment (10) were present, including the 2.55-kb fragment. This suggested that this region might contain two 2.4-kb Hind III fragments, one of which was deleted and the second of which was present in patient C.M. Probe 12Hc detected the three Hind III fragments reported by Koenig et al. (10), as well as a faint fourth fragment 3.4-kb in size, suggesting the presence of an additional exon in this region. Furthermore, probe 14 detected the 7.8-kb fragment in the controls, rather than the 6-kb Hind III fragment anticipated from the previously reported order (10); with this change in order and apposition of the 5.9- and 6-kb fragments it became clear that these latter two fragments were identical. The proposed clarified order for this region is shown in Fig. 3.

None of the 104 patients with DMD examined by Koenig et al. (10) exhibited deletions involving the 3,500 nucleotides at the 3′ end of the DMD cDNA. An additional series of patients with isolated DMD or BMD, who were characterized using the DMD cDNA probes, has revealed no deletions involving this 3′ portion of the DMD cDNA (Baumbach, L., J. Chamberlain, P. Ward, J. Witkowski, and C. T. Caskey, unpublished observations). The deletion in this patient, C.M.,

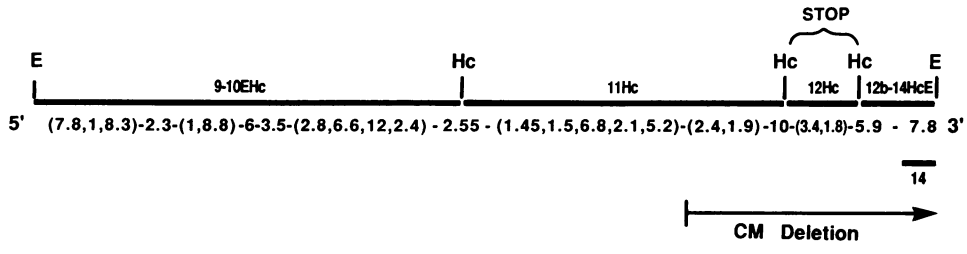

Figure 3. Summary of hybridization results for DMD cDNA regions 9–14. Shown are the size (in kilobases) and 5′ → 3′ order of the human Hind III fragments detectable by Southern analysis with five different cDNA subclones. The four fragments derived by digestion of 9–14 with Eco RI and Hinc II are represented along the top of the bar and are aligned to indicate the genomic Hind III fragments (below the bar) detectable with each. The approximate location of the DMD mRNA stop codon (Koenig, M., and L. Kunkel, unpublished observations) is also shown at the top. The genomic Hind III fragment detected with the 3′ cDNA subclone and the genomic DMD exon-containing Hind III fragments deleted in patient C.M. are shown below the bar. The order of the detectable genomic Hind III fragments is taken from Koenig et al. (10), modified as discussed in the text. Additional Hind III fragments containing exons too small for detection under the hybridization conditions used may also be present.

clearly involves this region, and similar patients with the complex DMD, GKD, and AHC microdeletion phenotype have deletions extending beyond the 3′ terminus of the DMD gene (4, 5, 8, 22). The current data suggest that if a deletion includes the 3′ portion of the DMD locus it will have a high likelihood of extending in a telomeric direction to involve additional loci. The observation that our patient, C.M., with an extremely mild and apparently nonprogressive dystrophic myopathy, has a deletion of the 3′ portion of the DMD locus may be helpful in understanding the clinical spectrum of DMD and BMD, though molecular heterogeneity is evident among patients with BMD (25, 26). The end of the coding sequence of the DMD transcript has been localized to within region 12a. Therefore, the current investigations demonstrate that the deletion in patient C.M. encompasses up to 1 kb of the 3′ coding region of the dystrophin mRNA (27). Although all previous DMD and BMD deletions have involved the 5′ and central regions of the DMD cDNA (10) leading to speculation regarding the role of the spectrin-like repeats in the pathogenesis of these disorders (28), this patient shows that truncation of dystrophin with loss of even a relatively small portion of the carboxy terminus results in a myopathic phenotype, though it may be mild. The relationship of these microdeletions to the developmental delay exhibited by these patients is also quite intriguing, particularly with the recent evidence that the DMD gene is expressed in the brain (27, 29).

The availability of the DMD cDNA probes permits detailed delineation of breakpoints in these microdeletion patients, including those with previously undetectable deletions. This will improve the diagnostic capabilities for these patients and will be particularly valuable to their families for carrier detection and prenatal diagnosis. In turn these patients provide clarification of the genomic organization of the DMD region, as well as valuable insights into the relationships between the structure and function of the gene product.

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