Am. J. Hum. Genet. 43:612–619, 1988

Normal Human Genomic Restriction-Fragment Patterns and Polymorphisms Revealed by Hybridization with the Entire Dystrophin cDNA

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Summary

Since the complete cDNA for the gene that causes X-linked recessive Duchenne/Becker muscular dystrophy (DMD/BMD) when mutated or deleted has recently been cloned and made generally available, DNA-based diagnostic studies of affected males and their families have entered into a new era. This communication sets forth the standard patterns of restriction fragments that are detected when normal human DNA cleaved with either *Hin*dIII or *Bgl*II is hybridized with seven contiguous segments comprising the entire 14-kb cDNA. Collectively, the more than 60 restriction fragments allow visualization of approximately 350 (*Hin*dIII) to 400 (*Bgl*II) kbp. This corresponds to the exon-containing one-fifth of the total genomic length of this gene, including the 3′ untranslated region. Twelve two-allele restriction-site polymorphisms that span the entire length of the gene were detected with the cDNA probes and allele frequencies determined. A diagnostic approach is proposed that starts with deletion screening of DNA from male probands, includes carrier detection based on relative fragment intensities, and extends to RFLP detection using the same autoradiographs prepared for deletion screening. Our results on deletion analysis of 32 DMD/BMD families are presented in an accompanying paper.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive fatal myopathy affecting approximately 1 in 3,300 males (Emery 1987). The entire 14-kb cDNA of the DMD gene has recently been cloned, and its protein product, called dystrophin, has been identified (Hoffman et al. 1987; Koenig et al. 1987). Mutations involving the dystrophin gene can cause either severe Duchenne-type dystrophy or milder allelic forms, such as Becker muscular dystrophy (BMD). The majority of these mutations are intragenic deletions, clustered in a region near the center of the gene and, less frequently, near the 5′ end (Forrest et al. 1987*a*; Koenig et al. 1987). The detection of gene deletions with cDNA probes leads to direct DNA-based prenatal and carrier diagnosis (Forrest et al. 1987*b*; Darras et al. 1988*b*). However, because of the large size of the dystrophin gene the restriction-fragment patterns revealed by the cDNA probes are quite complex. Weakly hybridizing, comigrating, and very small fragments exist, as do common restriction-site polymorphisms. Thus, deletions of small or comigrating fragments can easily be missed and RFLPs can be mistaken for deletions. It is the purpose of the present communication to present the complete patterns of *Hin*dIII and *Bgl*II fragments detected with the entire 14-kb cDNA in normal individuals, as a reference for the identification of deletions in DMD/BMD patients and female carriers. The exon-containing fragments that we have examined cover approximately 400 kb, close to one-fifth of the estimated size of the dystrophin gene (van Ommen et al. 1987).

A significant number of DMD/BMD families (30%–40%) have no detectable deletions and have to be studied by RFLP linkage analysis. We have found nine new RFLPs, four of them involving *Bgl*II sites, with different segments of the dystrophin cDNA. Together with

Received May 24, 1988; revision received July 14, 1988.

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0002-9297/88/4305-0007$02.00

the five RFLPs elsewhere reported, there are now 14 intragenic RFLPs that can be detected by cDNA probes at the same time that deletion screening is carried out.

Material and Methods

The normal restriction-fragment patterns were established using DNA from 27 normal male and 30 normal female individuals of different age and ethnic origin. To demonstrate Mendelian inheritance and to determine the allele frequencies of the RFLPs detected, an additional 10–28 females and a few males were tested. These were derived from normal and DMD families.

DNA was isolated from fresh leukocytes and from seven diploid lymphoblastoid cell lines that were Epstein-Barr-virus-transformed in our laboratory, digested with *Bgl*II, *Hin*dIII, *Taq*I, or *Pst*I (New England Biolabs), transferred to Hybond® filters (Amersham), and hybridized with oligo-labeled probes as described elsewhere (Darras et al. 1987). Fragments smaller than 1.5 kb were resolved on 1.5% agarose gels instead of the 0.8% gels usually employed. Electrophoresis was carried out in long gels (≥20 cm) for optimal separation of clustered and comigrating fragments. To facilitate transfer of large fragments, the electrophoresed DNA fragments were depurinated with 0.2 N HCl for 10–20 min before denaturation. The filters were washed at moderate stringency (65 C, 1 × SSC, 1% SDS) for 5–10 min, except for probe 11–14, which hybridizes to a low-level repetitive sequence and required higher-stringency washes.

As hybridization probes we used seven contiguous segments, labeled 1–3, 4–5a, 5b–7, 8, 9, 10, and 11–14, that cover the entire 14-kb dystrophin cDNA with the exception of 50 bp at the 5′ end, (fig. 1). The probes were made available by L. M. Kunkel. More recently, probes 1–2a and 2b–3, which divide region 1–3 in half, overlap for about 100 bp, and include the complete 5′ end of the cDNA in 1–2a, were obtained from the American Type Culture Collection (ATCC). The cloned segments were released from the vectors with *Eco*RI and/or *Bam*HI. Inserts were purified from low-melting-point agarose gels with phenol-chloroform extraction. As size markers we used λ *Hin*dIII and *Bst*EII digests and, occasionally, a 1-kb ladder (Bethesda Research Laboratories). Densitometry of bands on autoradiograms was carried out by using a Joyce Loebl densitometer.

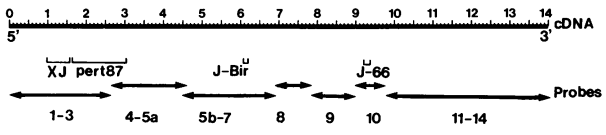

Figure 1 DMD/BMD (dystrophin) gene cDNA and fragments used as probes. The 14-kb cDNA (*top*) was divided into seven contiguous segments (*bottom*) called 1–3, 4–5a, 5b–7, 8, 9, 10 and 11–14. Relative locations of genomic probes XJ, pERT87, J-Bir, and J-66 are indicated (modified from Koenig et al. 1987).

Results

Normal Restriction-Fragment Patterns

Normal *Hin*dIII restriction patterns are shown in figure 2. The seven dystrophin cDNA probes hybridize to a total of 66 *Hin*dIII fragments, including at least two sets of comigrating fragments and at least two fragments that are recognized by adjacent cDNA probes. The X-chromosomal origin of all *Hin*dIII fragments was confirmed by dosage: with approximately the same amount of DNA loaded in each lane, male DNA (odd-numbered lanes) demonstrated single-copy intensity and female DNA (even-numbered lanes) demonstrated two-copy intensity for all fragments (fig. 2). No RFLPs were detected in *Hin*dIII-digested DNA from more than 60 individuals.

Of the 17 *Hin*dIII fragments revealed by probe 1–3, nine (10.5, 8.5, 8.0, 7.5, 4.6, 4.2, 3.25, 3.2, and 3.1 kb) hybridized with probe 1–2a and nine (12.0, 10.5, 7.3, 6.6, 6.0, 4.0, 3.0, 2.7, and 1.7 kb) with probe 2b–3. The shared 10.5-kb fragment involves the exons in which the two probes overlap. The 7.5- and 7.3-kb fragments comigrate but can be separated by using the two smaller probes. With probe 1–3 we have seen reduced intensity of this fragment in a patient deleted for the 7.5- but not for the 7.3-kb fragment (not shown). Probe 4–5a detects six fragments. The 11- and 12-kb doublet often appears fused. Probes 5b–7 and 8 hybridize to 13 and seven *Hin*dIII fragments, respectively, with the 10-kb fragment seen by both probes. With 5b–7 (fig. 2, lanes 5 and 6) the 10-kb fragment is the fainter component of the 11/10-kb doublet. Fragments ≤1.5 kb revealed by probe 5b–7 were partially resolved on long 1.5% agarose gels. The DNA in lane 5 of figure 2 is from a DMD patient with a deletion of the 1.8-, 1.5-, 1.3-, and 0.45-kb fragments. This sample was included to show that the 1.5-kb band is a doublet (unresolved even on 1.5% agarose gels), with the upper band deleted in this patient's DNA (fig. 2, lane 5). Probes 9 and 10 reveal seven *Hin*dIII fragments each. That the 1.0-kb band seen by probe 9 (fig. 2, lanes 9 and 10) is a doublet was suggested by reduced signal intensity

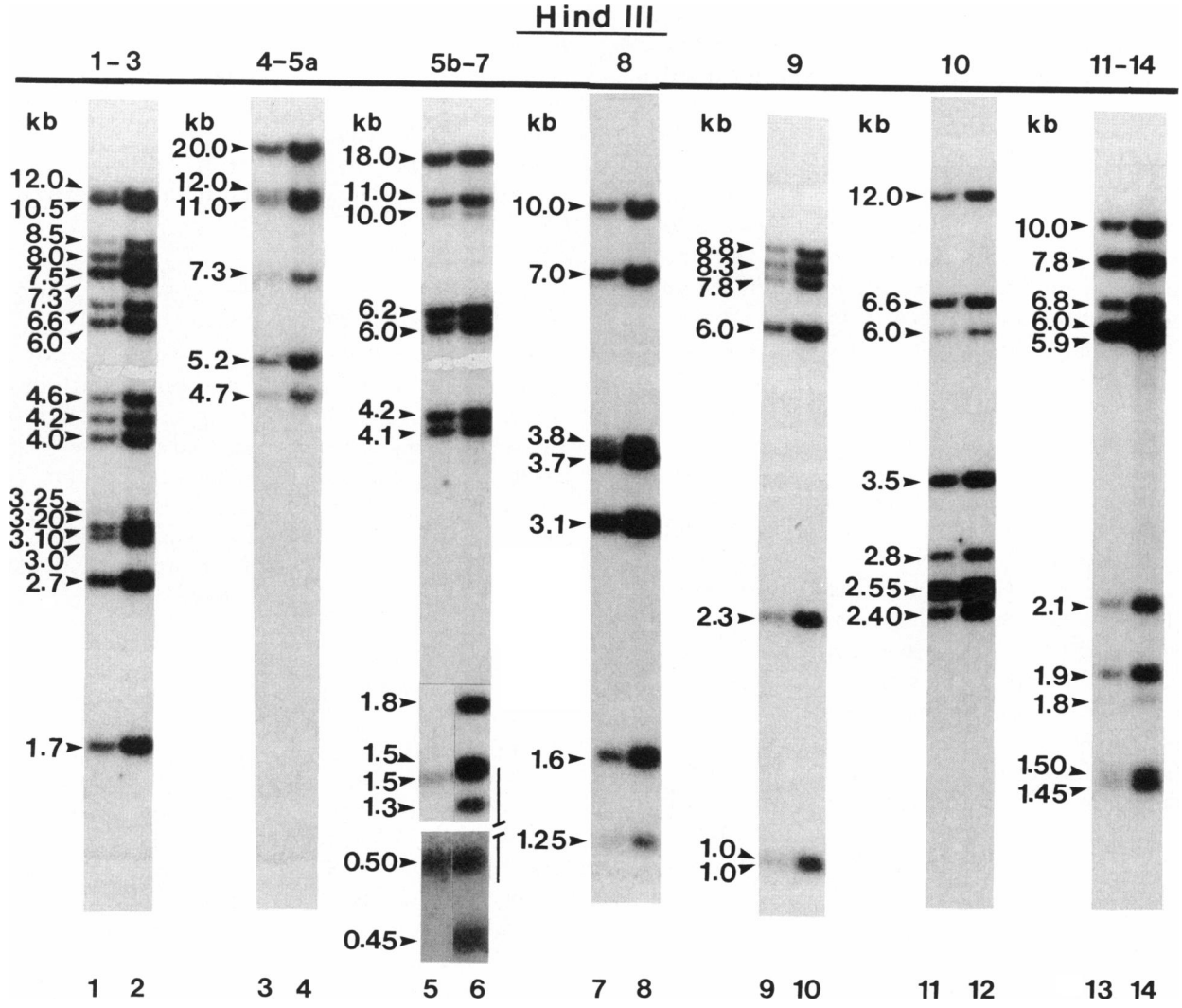

Figure 2 Normal human *Hin*dIII restriction patterns revealed by the seven adjacent segments of the dystrophin cDNA. X-chromosomal origin is demonstrated by dosage, with female DNAs (even-numbered lanes) showing two-copy intensity and male DNAs (odd-numbered lanes) showing single copy intensity, for all fragments. Closely migrating fragments appear either as clusters (3.0/3.1/3.2- and 3.25-kb bands (in lanes 1 and 2) or as doublets (i.e., 11.0/10.0-kb doublet in lanes 5 and 6). Comigrating fragments are detected by probes 5b–7 (1.5-kb band in lanes 5 and 6) and 9 (1.0-kb band in lanes 9 and 10). That the 1.5-kb band is a doublet is demonstrated in lane 5, which contains DNA from a DMD patient deleted for the 1.8-kb, one of the two 1.5-, the 1.3-, and the 0.45-kb fragments, resolved on a 1.5% agarose gel. The 10.0-kb fragment (lanes 5 and 6) is seen by the adjacent probes 5b–7 and 8. The weak 5.4-kb fragment with 5b-7 present on a previously published figure (Darras et al. 1988*b*) turned out to be a contaminant.

in a DMD individual with a deletion in this region of the gene (data not shown). The two 1.0-kb comigrating fragments fail to resolve on 1.5% agarose gels. In addition to the 10 fragments hybridizing strongly with cDNA segment 11–14 (fig. 2, lanes 13 and 14), there is a lane background with faintly hybridizing bands that do not show X dosage and are presumably of autosomal origin. A low-frequency repetitive element appears to be present in the 3′ untranslated region of probe 11–14. Washing the filters under high-stringency conditions eliminates some of the background.

When *Bgl*II-digested DNAs from normal individuals were hybridized with the seven cDNA segments, a total of 53 constant and nine variable fragments were seen (fig. 3). RFLPs were detected with probes 1–3, 4–5a, 5b–7, and 11–14 (fig. 3), white arrows. The 15 nonpolymorphic fragments seen with probe 1–3 are nicely subdivided when probes 1–2a and 2b–3 are used:

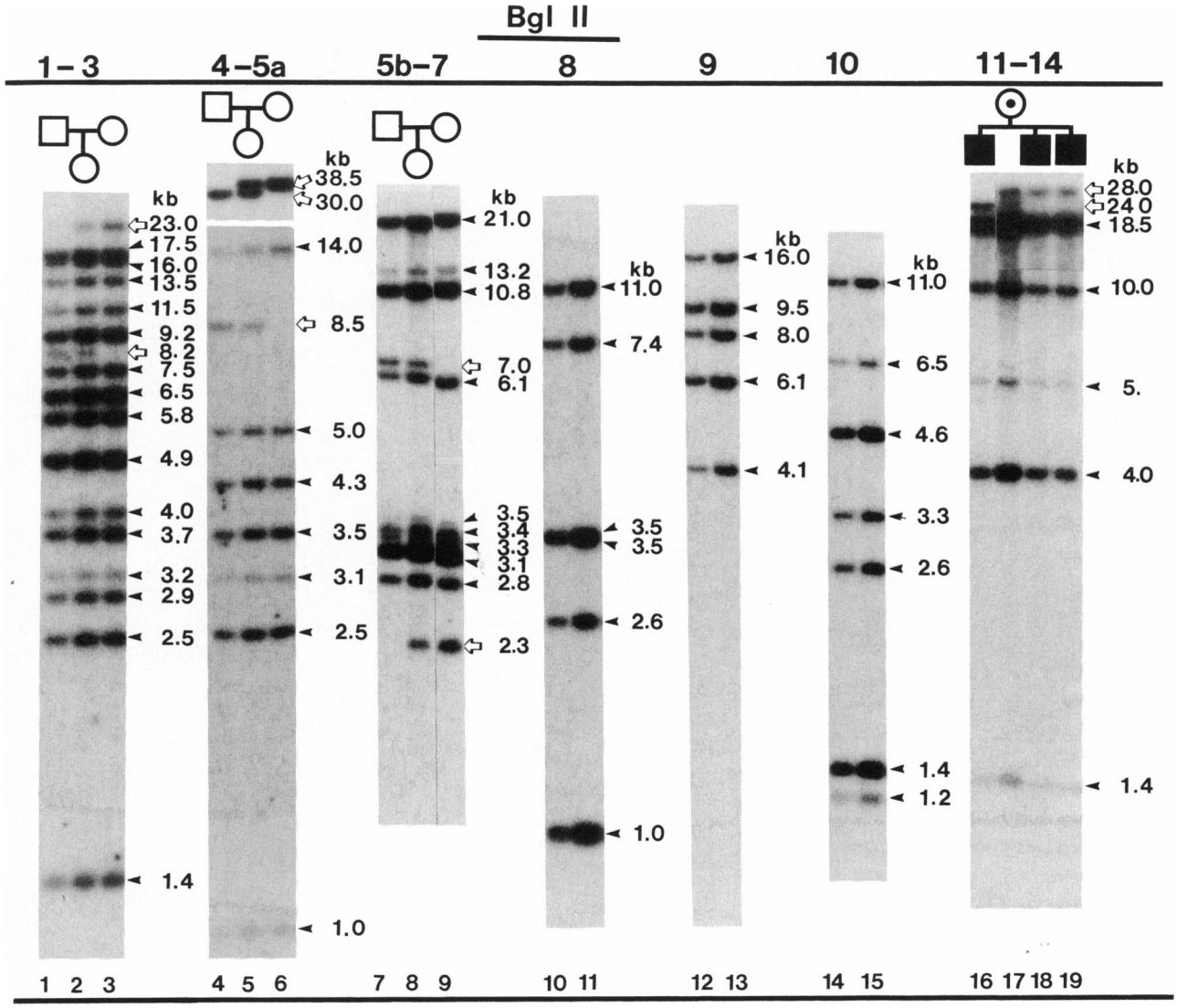

Figure 3 Normal human *Bgl*II restriction patterns and *Bgl*II-site polymorphisms revealed by contiguous segments of the dystrophin cDNA. Closed arrowheads point to constant fragments. The 3.5-kb band seen by probe 8 is a doublet, as deduced from analysis of deletion patients. Open arrows point to variant fragments caused by *Bgl*II RFLPs. Mendelian inheritance is demonstrated in the small pedigrees depicted on top of the autoradiograms for the *Bgl*II RFLPs detected with probes 1–3, 4–5a, 5b–7, and 11–14. Segregation of the 24/28-kb *Bgl*II RFLP (lanes 16–19) reveals a crossover event in lane 16 between the 11–14 polymorphic *Bgl*II site and the DMD mutation, identified in this family as a deletion of the 5b–7 and 8 region. Lane background in lanes 16–19 is caused by a low-level repetitive sequence present in cDNA probe 11–14. Weakly hybridizing autosomal bands may also be seen at lower stringency.

the 13.5-, 7.5-, 6.5-, 3.7-, 2.9, and 1.4-kb fragments hybridize uniquely with 1–2a, while the 4.9-kb band contains the overlap and hybridizes with both probes. Probe 2b–3 detects the 17.5-, 16.0, 11.5-, 9.2-, 5.8-, 4.0-, 3.2, and 2.5-kb constant fragments and the 23.0/8.2-kb RFLP alleles. Thus, for *Bgl*II-digested DNA, probe 1–3 can be used without the loss of information owing to comigrating fragments that had been seen when *Hin*dIII was used. It is unfortunate that the cluster of similar-sized *Bgl*II fragments (2.8, 3.3, 3.4, and 3.5 kb) detected with probe 5b–7 involves the region where most of the DMD and BMD deletions occur (see accompanying paper [Darras et al. 1988*a*]). For the characterization of deletions with breakpoints in this region, an alternate second enzyme such as *Pst*I or *Taq*I (Forrest et al. 1987*b*) might be more useful than

*Bgl*II. On the basis of data on a deletion patient (Darras et al. 1988*b*), we know that the 3.5-kb *Bgl*II band detected with probe 8 is a doublet, which could not be resolved on long gels. It is likely that one of these 3.5-kb fragments is also hybridizing with probe 5b–7. Since we have not used very small fragments of the DMD cDNA for hybridization experiments, the presence of similarly comigrating fragments within other single-appearing bands cannot be excluded at this time.

Restriction-site polymorphisms

When more than 50 DNA samples were digested with *Bgl*II, *Taq*I, and *Pst*I and hybridized with all cDNA probes, 12 RFLPs were detected. Mendelian inheritance was demonstrated in small families for the four *Bgl*II RFLPs with probes 1–3, 4–5a, 5b–7, and 11–14 (fig. 3; and Darras et al. 1988*b*). The size of the large (A1) allele (38.5 kb) in the 4–5a RFLP (fig. 3, lanes 5 and 6) has been inferred by adding the sizes of the A2 allele fragments (30 and 8.5 kb). Lanes 16, 18, and 19 of figure 3 contain DNAs from three affected brothers whose mother (fig. 3, lane 17) is heterozygous for the 24-kb/28-kb RFLP. Most likely, the patient whose DNA is run in lane 16 of figure 3 represents a recombinant resulting from an intragenic crossover event between the 11–14 RFLP and the DMD mutation that has been positively identified in this family as a deletion detectable with segments 5b–7 and 8 (Darras et al. 1988*a*).

Segregation of the *Taq*I and *Pst*I polymorphisms detected with probe 8 is demonstrated in two families (fig. 4). On the left, the mother is heterozygous for the 6.5-kb/5.6-kb alleles and has transmitted the 6.5-kb allele to her son and one daughter and the 5.6-kb allele to the other daughter. The family on the right contains an affected male (black symbol) who has a deletion of the 5.1 and 5.3-kb *Pst*I fragments. Since his unaffected brother has received the same 10.8-kb allele, the affected male represents a new mutation.

The *Taq*I RFLP detected by probe 5b–7 consists of a 3.2-kb allele (A1) and a second allele (A2) of 1.6 kb, which is comigrating with a 1.6-kb invariant fragment. The interpretation is difficult and may be assisted by densitometer scanning (fig. 5). On the basis of the relative intensities of the 3.2- and 2.9-kb fragments, as well as the 1.8- and 1.6-kb fragments, we believe that the five female samples in figure 5 represent the following genotypes: lanes 1 and 3, A1/A1; lane 2, A1/A2; lanes 4 and 5, A2/A2.

Additional two-allele *Taq*I RFLPs were found with probes 2b–3, 10, and 11–14, and a *Pst*I RFLP was found with probe 10. They are illustrated in figure 6 and described in the figure legend. Allele sizes, allele frequencies, and heterozygote frequencies for all 12 RFLPs have been summarized in table 1.

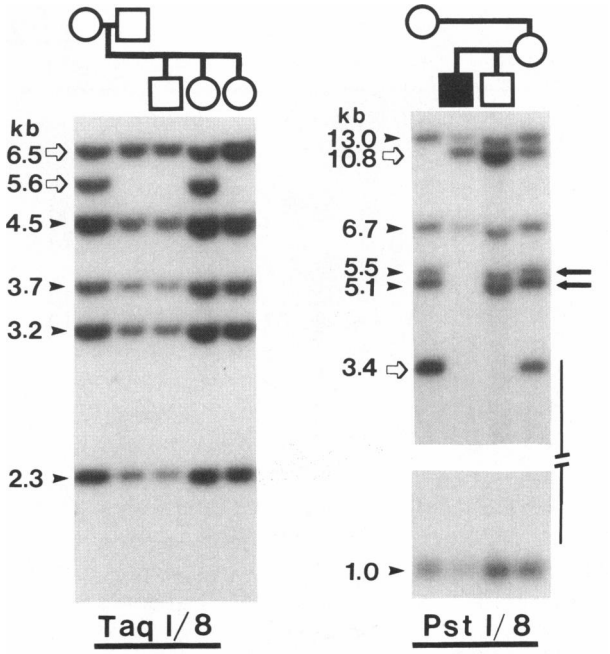

Figure 4 *Taq*I and *Pst*I RFLPs detected by the dystrophin cDNA probe 8. Open arrows point to variant allelic fragments, and closed arrowheads indicate constant fragments. Mendelian segregation is demonstrated in both pedigrees. The affected male in the family on the right is deleted for the 5.5- and 5.1-kb fragments (black arrows), which are present in the normal brother's DNA. Since both have received the same 10.8-kb *Pst*I allele from their mother and since her DNA shows double-dose intensity for the fragments deleted in her diseased son, the affected male must represent a new mutation.

Discussion

The high detection rate (60%–70%) of deletion mutations in DMD/BMD families has essentially revolutionized DNA-based diagnostic studies of this disorder. Efficient strategies can be designed by combining deletion analysis using segments of the dystrophin cDNA as probes and linkage analysis with intragenic and flanking RFLPs. Even though the cDNA is only 14 kb in length, the size of the genomic region spanned by the DMD gene is most likely on the order of 2,000 kb (van Ommen et al. 1987; Burmeister et al. 1988), which makes it by far the largest known gene. Therefore, the genomic *Hin*dIII and *Bgl*II restriction-fragment patterns are quite complex, at least with some of the cDNA segments.

In affected individuals, small deletions can be missed

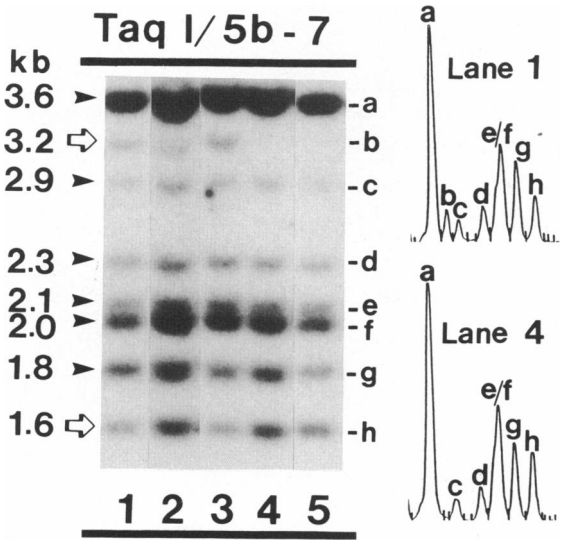

Figure 5 RFLP detected by probe 5b–7 in *Taq*I digests of DNA from five female individuals. The allelic fragment (open arrows) are 3.2 and 1.6 kb in size. However, a constant fragment also migrates to the 1.6-kb position. On the basis of relative intensities of bands, individuals 1 and 3 are considered homozygous for the A1 (3.2-kb) allele, female 2 is heterozygous (3.2/1.6 kb), and individuals 4 and 5 are homozygous for the A2 (1.6-kb) allele. Densitometer scanning of lanes 1 and 4 is shown on the right, with peaks identified with restriction fragments by small letters. With an A1/A1 genotype (lanes 1 and 3), peak b is present and taller than peak c and the g:h ratio is about 2:1. In A2/A2 individuals (lanes 4 and 5), peak b is absent and the g:h ratio is close to one. In A1/A2 heterozygotes (lane 2), peak b is smaller than peak c and the g:h ratio is about 2:3 (not shown).

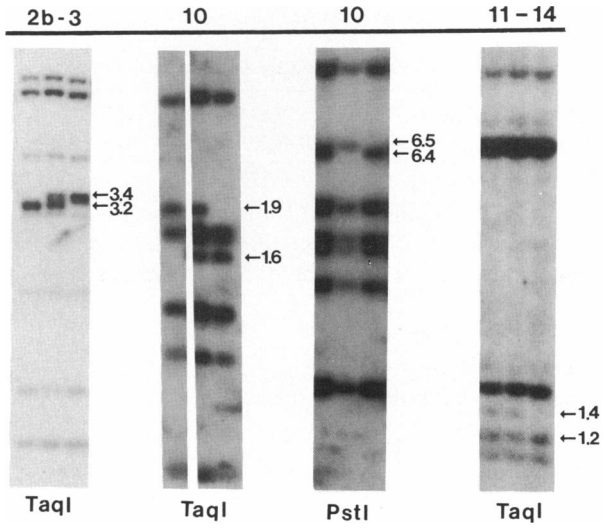

Figure 6 Four *Taq*I and *Pst*I RFLPs that span most of the dystrophin gene. The three lanes shown represent family members informative for Mendelian inheritance. Arrows point to variant allelic fragments (sizes in kb). *Taq*I/2b–3: The allelic fragments need to be distinguished from weaker constant fragments of 3.3 and 3.1 kb. Left lane, male with 3.2-kb allele; center lane, female heterozygote; right lane, male with 3.4-kb allele. *Taq*I/10: The 1.9- and 1.6-kb allelic fragments are separated by a constant doublet of 1.75 and 1.70 kb; the center lane represents a heterozygote. *Pst*I/10: Left lane (heterozygote), the 6.5- and 6.4-kb bands were clearly separated on shorter exposures; Center lane, male with 6.5-kb fragment; right lane, male with 6.4-kb fragment. *Taq*I/11–14: The 1.2-kb allelic fragment comigrates with a constant band. Of the three female samples, the right one is homozygous 1.2/1.2 kb and the other two are heterozygous. In 1.4/1.4-kb homozygotes the intensities of the 1.4- and 1.2-kb bands are about equal (not shown).

because of faintly hybridizing bands, unresolvable comigrating fragments, or clustered fragments which do not resolve well with standard electrophoretic conditions. In addition, very small fragments (≤1 kb can be missed, either because they run off the gel or are not resolved sufficiently on 0.7%–1.0% agarose gels. Many of these pitfalls can be avoided by using two restriction enzymes, such as *Hin*dIII and *Bgl*II, and by running longer or higher-percentage agarose gels. Probe 1–3 can be divided into probes 1–2a and 2b–3 as distributed by the ATCC, each of which generates a simpler fragment pattern. For *Bgl*II-digested DNA, however, probe 1–3 can be used, since all fragments are clearly separated.

Blots prepared on nylon filters have been hybridized consecutively with the seven different cDNA segments. For hybridization we have used gel-purified inserts rather than whole plasmids and have labeled them with the random-priming method of Feinberg and Vogelstein (1983) instead of with nick-translation. Labeling the whole plasmid generated a higher background and less-clean signals. Moderate-stringency washes (see Material and Methods) were used with cDNA segments 1–2a through 10 to visualize weakly hybridizing fragments. With the 11–14 segment, which contains low-repetitive sequences, more-stringent washing conditions will reduce lane background and weak autosomal bands. It may not be necessary to use probe 11–14, since this 3′ most probe is unlikely to detect deletions in DMD patients. None have been reported, and we have found deletion of this region in only one patient, who had a mild nonprogressive form of muscular dystrophy (Darras and Francke 1988). However, two RFLPs detected with 11–14 will be useful distal markers for the detection of intragenic crossovers.

Elsewhere, several intragenic RFLPs have been described with the dystrophin cDNA probes. A *Bcl*I RFLP was detected with probe 5a, and an *Xmn*I RFLP with probe 6b (Koenig et al. 1987). We also have seen the latter RFLP on *Xmn*I filters hybridized with probe 5b–7

Table 1

RFLPs Detected by DMD cDNA Probes

Probe, Enzyme, and Allele	Allele Size (kb)	Allele Frequency	Chromosomes Tested	Heterozygote Frequency	Females Tested
2b-3:					
BglII:					
A1	23	.71	51	.41	22
A2	8.2	.29			
TaqI:					
A1	3.4	.26	43	.38	23
A2	3.2	.74			
4-5a:					
BglII:					
A1	38.5	.68	25	.44	10
A2	30/8.5	.32			
5b-7:					
BglII:					
A1	7.0	.30	43	.42	18
A2	2.3	.70			
TaqI:					
A1	3.2	.36	39	.46	20
A2	1.6	.64			
PstI:					
A1	14.0	.05	40	.10	20
A2	12.0	.95			
8:					
PstI:					
A1	10.8	.12	52	.21	27
A2	3.4	.88			
TaqI:					
A1	6.5	.92	39	.15	20
A2	5.6	.08			
10:					
PstI:					
A1	6.5	.92	40	.15	20
A2	6.4	.08			
TaqI:					
A1	1.9	.05	40	.10	20
A2	1.6	.95			
11-14:					
BglII:					
A1	28	.02	57	.04	28
A2	24	.98			
TaqI:					
A1	1.4	.16	42	.27	22
A2	1.2	.84			

that includes the 6b segment (data not shown). The *TaqI* and *PstI* RFLPs that we have found with probe 8 are most likely identical to the recently described *PstI* and *TaqI* RFLPs seen with cDNA probe Cf56a (Forrest et al. 1987*b*). This implies that cDNA probes 8 and cf56a are overlapping. The *TaqI* RFLP detected by probe 5b–7 must also be identical to the one detected by probe Cf23a (Forrest et al. 1987*b*). The smaller allele, however, was measured as 1.6 kb on our blots, instead of 1.8 kb as published elsewhere. The *TaqI* RFLP detected with probe 2b–3 is likely to be the same one previously reported with genomic probe pERT 87–15 (Kunkel et al. 1986). Previously unreported *TaqI* RFLPs were found with probes 10 and 11–14. Thus, *TaqI* RFLPs are seen with each of the cDNA segments except 4–5a. Since *Hin*dIII digestion did not reveal any RFLPs and, in ad-

dition, generated numerous comigrating fragments, it may not be the most useful enzyme for diagnostic studies even though an almost complete *Hin*dIII fragment map has been established. In contrast, four *Bgl*II RFLPs were detected by segments 1–3, 4–5a, 5b–7, and 11–14 on the same blots that we used for deletion screening. A *Bgl*II RFLP reported as having allelic fragment sizes of 30 and 8 kb has been detected with genomic probe pERT 87–30 (Monaco et al. 1987). This may be the same RFLP we have seen with cDNA probes 1–3 or 2b–3. A strategy that combines deletion screening and RFLP search with cDNA probes would involve the preparation of *Bgl*II and *Hin*dIII filters that are hybridized successively with probes 8, 5b–7, 1–3, and 4–5a; and if no deletion were found, it would also involve the preparation of *Taq*I filters probed with 2b–3, 5b–7, 8, 10, and 11–14. The application of this strategy to the study of 32 DMD/BMD families is presented in an accompanying paper (Darras et al. 1988*a*).

Acknowledgments

We are indebted to L. M. Kunkel, M. Koenig, and the ATCC for cDNA probes, to B. Foellmer and L. Battat for technical assistance, to L. Bouffard for secretarial help, and to the Muscular Dystrophy Association of America for financial support.

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