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Gene Deletions in X-linked Muscular Dystrophy

Mikael Lindlöf,*,‖ Anne Kiuru,** Helena Kääriäinen,* Hannu Kalimo,†† Heikki Lang,‡‡ Helena Pihko,§ Juhani Rapola,† Hannu Somer,‡ Mirja Somer,# Marja-Liisa Savontaus,** and Albert de la Chapelle*

Departments of *Medical Genetics, †Pathology, and ‡Neurology and §Children's Hospital, University of Helsinki; ‖Folkhälsan Institute of Genetics; #Department of Medical Genetics, Väestöliitto, Helsinki; and Departments of **Medical Genetics, ††Pathology, and ‡‡Clinical Neurophysiology, University of Turku, Turku, Finland

Summary

Of the approximately 170 families with X-linked muscular dystrophy of the Duchenne (DMD) and Becker (BMD) type in Finland, we have studied 90 unrelated patients for intragenic deletions by using the cDNA probes described by Koenig et al. Forty-five patients (50%) had molecular deletions of one or several of the 65 exon-containing *Hin*dIII fragments. In six deletion cases junction fragments of altered size were seen. Thirty-eight (84%) of the 45 deletions were detected using only two (1–2a and 8) of the six cDNA subclones. Using a wheelchair age of 12 years to distinguish between DMD and BMD, we found that the proportions of patients with deletions were similar. Deletions were equally common in familial and sporadic disease. BMD was more commonly caused by deletions in the 5′ end of the gene than was DMD. In at least three instances deletions of similar type resulted in diseases of similar severity. Of 14 patients with mental retardation seven had deletions; six of these comprised exons contained in probe 8. We conclude that cDNA hybridization studies provide a powerful diagnostic tool in DMD and BMD and that they promise to produce better insights into molecular-clinical correlations.

Introduction

X-linked muscular dystrophy is a degenerative disorder of the skeletal muscle comprising both severe forms leading to death in the early twenties (Duchenne muscular dystrophy [DMD]) and milder forms with a much slower course (Becker muscular dystrophy [BMD]). In addition, there are two other X-linked myopathies of dystrophic type, Emery-Dreifuss muscular dystrophy (Emery and Dreifuss 1966) and myopathy with excessive autophagy (Kalimo et al. 1988). However, these diseases are very rare and are not allelic to DMD and BMD (Thomas et al. 1986*b*; Yates et al. 1986; Saviranta et al. 1988).

The gene, located at Xp21, consists of a minimum of 60 exons distributed over a region spanning at least 1,800 kb (Koenig et al. 1987; van Ommen et al. 1987). The average size of each exon is 200 bp, and the mean size of introns is 35 kb. The gene encodes a 14-kb mRNA. The corresponding cDNA has been cloned in its entirety by Koenig et al. (1987). Parts of the cDNA have also been cloned by others (Burghes et al. 1987).

The protein product of the DMD gene, dystrophin (Hoffman et al. 1987), is a rod-shaped cytoskeletal protein (Koenig et al. 1988) with a relative mass of about 400 kD and is predominantly localized to the surface membrane of striated muscle cells (Aratha et al. 1988; Watkins et al. 1988). It is postulated that dystrophin assists the muscle membrane in resisting the stresses associated with contraction. In its absence tearing would damage the muscle and result in fiber necrosis (Beam 1988).

Deletions appear to be the most common gene defect leading to DMD and BMD. In addition, duplications have been reported (Hu et al. 1988). A considerable number of genomic DNA markers from the DMD region are available. When these markers are used, the total proportion of deletions that can be found is max-

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Address for correspondence and reprints: Dr. Mikael Lindlöf, Department of Medical Genetics, University of Helsinki, Haartmaninkatu 3, SF-00290 Helsinki, Finland.

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imally of the order of 40% (Davies et al. 1987). When the cDNA have been used as probe or when hybridizing genomic probes with long DNA molecules separated with pulsed field electrophoresis techniques, the frequency of deletions has risen to more than 50% (den Dunnen et al. 1987; Koenig et al. 1987). Deletion frequencies as high as 67% have recently been reported (Forrest et al. 1988). Phenotypic differences between DMD and BMD have been thought to depend on whether the translational open reading frame is preserved or shifted (Forrest et al. 1988; Monaco et al. 1988).

In the present study, we analyzed 90 unrelated DMD and BMD patients for intragenic deletions by using the cDNA probes described by Koenig et al. (1987). We mapped the breakpoints of the deletions relative to exon-containing *Hin*dIII restriction fragments. We sought correlations between the location and extent of the deletions, on the one hand, and clinical features of the patients, on the other. We discuss the use of the cDNA in disease diagnosis, carrier detection, and prenatal diagnosis of the disorder.

Subjects and Methods

Subjects

We studied 90 unrelated Finnish DMD and BMD patients. The total number of families with DMD and BMD in Finland (population about 5 million) is approximately 170. The patients were classified into three groups according to their clinical features. These groups are (1) DMD (patients wheelchair bound before the age of 12 years) (Emery 1987), (2) BMD (patients wheelchair bound at a later stage or not at all), and (3) patients too young to be classified. The patients were classified as DMD in 46 families and as BMD in 24 families, and in 20 families the patients were too young to be classified. In 27 families the disease was familial, as there were at least two affected males; and in 63 families only one affected male was known. Of all 90 patients 14 were classified as mentally retarded, as they had not been able to attend normal school owing to mental subnormality.

DNA Extraction

DNA was isolated from whole-blood leukocytes anticoagulated with heparin or EDTA or from fibroblasts according to methods described elsewhere (Kunkel et al. 1977). The DNA was digested with *Hin*dIII (Promega Biotech, USA) according to the manufacturer's recommendations.

Southern Blotting and Hybridization

For maximal separation of cDNA exon fragments ranging in length from 20 kb to less than 1 kb, the same digested DNA samples were loaded both on 0.7% and on 0.9% agarose gels (Sigma, USA). The gels were blotted onto nitrocellulose membranes (Schleicher & Schuell, West Germany) or Hybond® N (Amersham, UK) according to standard procedures (Southern 1975). The blots were hybridized with nick-translated plasmids or oligo-labeled cDNA inserts according to a method described elsewhere (Feinberg and Vogelstein 1984; Page and de la Chapelle 1984).

cDNA Probes

The 14-kb cDNA was subdivided into fragments numbered 1–14 starting from the 5′ end of the gene and was made available as six subclones (Koenig et al. 1987). The size of each subfragment is approximately 1 kb. Five of the six cDNA subclones were used unmodified. The sixth subclone (9–14) was subdivided by *Eco*RI-*Hin*cII double digestion into four fragments: probes 9–10, probe 11, probe 12a, and probes 12b–14. Fragments 11 and 12a were pooled before use. Fragments 12b–14 were not used, as they detect the untranslated section of the last exon (M. Koenig, personal commu-

Table 1

List of DMD cDNA Subfragments Used

Probe Name	Sizes of *Hin*dIII Genomic Fragments Detected (kb)
1-2a	3.2; 3.25; 4.2; 8.5; 3.1; 8.0; 4.6; 7.5; and 10.5
2b-3	10.5; 4.2; 6.6; 2.7; 6.0; 1.7; 12.0; 3.0; and 7.3
4-5a	7.3; 11.0; 20.0; 5.2; 4.7; 12.0; and 18.0
5b-7	18.0; 1.8, 0.4; 1.3; 1.5; 6.1; 6.2; 4.2; 11.0; 4.1[a]; 0.5[a]; 1.5[a]; and 10.0[a]
8	10.0; 1.25; 3.8; 1.6; 3.7; 3.1; and 7.0
9-10	7.8; 1.0; 8.3; 2.3; 8.8; 1.0; 6.0; 3.5; (2.8; 12.0); 6.6; and (2.55; 2.4)
11-12a	(1.45; 1.5, 2.1; 5.2; 6.8); (1.9; 2.4); 10.0; (3.4; 1.8); and 6.0

NOTE.—Parentheses indicate that the order is not known.
SOURCE.—Koenig et al. (1987) and M. Koenig (personal communication).
[a] Fragments detected with probe 7 only.

Table 2

Deletions in DMD and BMD Patients Relative to the Exon Containing *Hin*dIII Fragments Detected with cDNA Probes

Probes (fragments): 1–2a (3.2, 3.25, 4.2, 8.5, 3.1, 8.0, 4.6, 7.5, 10.5); 2b–3 (4.2, 6.6, 2.7, 6.0, 1.7, 12.0, 3.0, 7.3); 4–5a (11.0, 20.0, 5.2, 4.7, 12.0, 18.0); 5b–7 (1.8, .4, 1.3, 1.5, 6.1, 6.2, 4.2, 11.0, 4.1, 0.5, 1.5, 10.0); 8 (1.25, 3.8, 1.6, 3.7, 3.1, 7.0); 9–10 (7.8, 1.0, 8.3, 2.3, 8.8, 1.0, 6.0, 3.5, 2.8, 12.0, 6.6, 2.55, 2.4)

Family	Clinical Classification	Age in Wheelchair	Present Age of Oldest Surviving Patient	Mental Retardation	Junction fragment
1	DMD-F	9	15	no	
2	BMD-S		29	no	
3	BMD-S	18	27	no	
4	U-S		4	no	
5	U-S		8	no	
6	DMD-S	7	10	no	
7	BMD-F		9	no	(J)
8	DMD-F	12	12	no	
9	DMD-S	11	16	no	
10 ...	U-S		6	no	
11 ...	BMD-F	15	30	no	(J)
12 ...	BMD-S	16	22	yes	
13 ...	BMD-F	16	24	no	
14 ...	DMD-F	9	17	no	
15 ...	DMD-F		5	no	
16 ...	DMD-F		4	no	
17 ...	U-S		4	no	
18 ...	DMD-F	12	23	no	
19 ...	DMD-S	10	14	no	
20 ...	BMD-S		18	no	
21 ...	DMD-S	10	17	no	
22 ...	U-S		7	yes	
23 ...	BMD-S	14	22	no	
24 ...	DMD-F	10	10	no	
25 ...	BMD-F	30	69	no	(J)
26 ...	DMD-S	7	20	no	
27 ...	U-S		9	no	
28 ...	U-S		6	no	
29 ...	DMD-F	12	25	no	
30 ...	U-S		10	no	(J)
31 ...	DMD-F	10	14	no	
32 ...	DMD-S	10	22	yes	
33 ...	BMD-S	13	14	yes	(J)
34 ...	DMD-F	8	13	yes	
35 ...	DMD-S	11	16	yes	
36 ...	DMD-S	12	16	no	
37 ...	BMD-S		14	no	
38 ...	DMD-F	10	10	no	
39 ...	DMD-F	8		yes	(J)
40 ...	DMD-S	9	14	no	
41 ...	DMD-S	10	22	no	
42 ...	U-S		8	no	
43 ...	DMD-S	12	13	no	
44 ...	U-S		10	no	
45 ...	DMD-S	9	13	no	

NOTE.—The probes and their corresponding fragments (see also table 1) are shown at the top of the table. Each deletion is marked with a line. "J" indicates that a junction fragment of abnormal size was observed (an example is shown in fig. 2). Clinical data are summarized to the left of each deletion line. The exon fragments of probe 11–12a were left out of the table, as no deletions were found in this region. Several of the patients have been previously studied using genomic DNA probes (Lindlöf et al. 1986, 1987, 1988). Deletions for genomic probes were previously observed in eight patients. These can be identified as follows (first number = number in present paper; second number = number in Lindlöf et al. [1988]): 1 = 3; 6 = 2; 7 = 8; 11 = 4; 12 = 5; 13 = 6; 18 = 1; 23 = 7. F = familial; S = sporadic; U = too young to be classified.

Table 3

Proportion of Deletions Detected with the Different cDNA Probes in Patients with DMD, Patients with BMD, and Patients too Young to Be Classified (U).

Clinical Classification	No. (% of Total) for Probe					
	1-2a	2b-3	4-5a	5b-7	8	9-10
DMD	4 (31)	7 (58)	6 (60)	9 (45)	14 (56)	2 (50)
BMD	6 (46)	3 (25)	2 (20)	5 (25)	5 (20)	1 (25)
U	3 (23)	2 (17)	2 (20)	6 (30)	6 (24)	1 (25)
Total	13	12	10	20	25	4

Note.—No deletions were detected with probe 11-12a.

nication). In addition all patients with a probe 8 deletion extending in the proximal direction were separately studied with subfragment 7, excised from probe 5b–7 as a *Hin*cII fragment. A list of the probes, as well as their size and the proposed order of detectable exon-containing fragments generated after digestion with restriction enzyme *Hin*dIII and hybridization, is shown in table 1.

Results

The 90 patients were analyzed for deletions by using all the cDNA probes except 12b–14. Deletions removing portions of the cDNA were found in 45 unrelated patients. The deletion frequency was thus 50%. The extent and localization of the deletions, relative to the exon-containing *Hin*dIII fragments and cDNA probes, are shown in table 2. The number of deletions detected with the different cDNA probes is summarized in table 3. The band pattern in patients with a deletion breakpoint in the region of probe 7 is shown in figure 1.

The disease was classified as DMD in 24 of the deletion families and as BMD in 11 families. In 10 families no classification could yet be done.

Clinical data on the 45 patients with molecular deletions are shown in table 2. The proportion of deletions in the three groups is summarized in table 4. Of the 27 families with familial disease, cDNA deletions were found in 16 (59%), and of the 63 families with sporadic disease, deletions were detected in 29 (46%). Patients from 14 families showed mental retardation. A deletion was found in seven (50%) of these. The proportion of deletions in mentally normal and retarded patients is shown in table 5.

In eight familial deletion cases, we additionally studied other affected males from the same families. In each case the deletion was identical in all affected males from the same family. More than one affected male were also studied in eight families in which no deletion was found.

All the independent mutational events were characterized by the absence of at least one exon-containing fragment. In six of these deletions junction fragments of altered size were observed. One was found with probe 1–2a (patient 7), another with probe 2b–3 (patient 11), and four with probe 8 (patients 25, 30, 33, and 39). The junction fragment occurring in patient 7 and its use in carrier detection are illustrated in figure 2.

Discussion

Deletions in DMD and BMD Patients

When genomic markers are used, the frequency of deletions in DMD and BMD families varies depending on the number of markers, and on the methods used (Kunkel et al. 1986; Thomas et al. 1986*a;* Davies et al. 1987; Hart et al. 1987; Lindlöf et al. 1988). Of the 90 patients included in this study, 49 had previously been studied with the genomic markers J66-H1, J-Bir, pERT87–1, pERT87–8, pERT87–15, pERT87–30, XJ2.3, XJ5.1, XJ10.1, and pERT84–10. Deletions had been found in eight (16%) unrelated patients (Lindlöf et al. 1988). When these 49 patients were tested with the cDNA probes, deletions were found in 20 patients. Thus, among these 49 patients, the frequency of deletions increased from 16% to 41%. In the entire series, deletions were found in 45 patients (50%), which is in agreement with previously published results of studies using the same cDNA probes (Koenig et al. 1987; Witkowski 1988). Using a battery of probes, Forrest et al. (1988) have predicted a total deletion frequency as high as 67%.

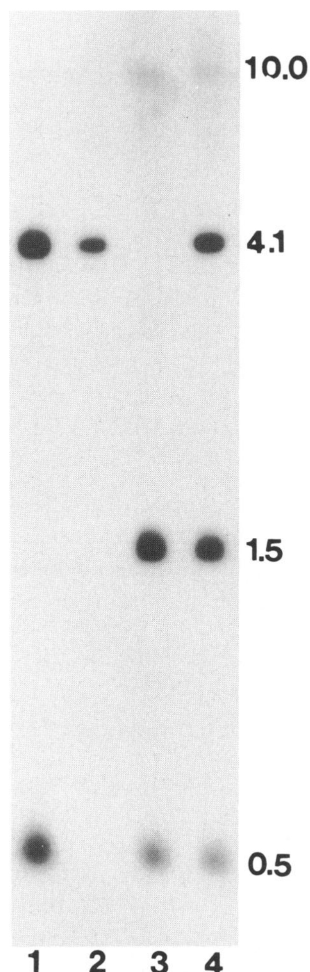

Figure 1 Hybridization of DNA from patients with a deletion breakpoint in the region of probe 7. The sizes of the fragments detected after digestion with restriction enzyme *Hin*dIII and hybridization with probe 7 are shown on the right (see also table 1). Lane 1: Patient 28 (the same banding pattern was also observed in patients 24–27). In these patients the deletions have their proximal breakpoints in the intron region between the 0.5- and 1.5-kb fragments. Lane 2: Patient 20 (the same banding pattern was also observed in patients 21–23). The deletions have their proximal breakpoints in the intron region between the 4.1- and 0.5-kb fragments. Lane 3: Patient 17. This deletion has its distal breakpoint between the 4.1- and 0.5-kb fragments. Lane 4: A control having no deletions in this region.

Table 4

Proportion of cDNA Deletions in Patients with DMD, Patients with BMD, and Patients too Young to Be Classified (U)

Clinical Classification	No. of Patients	No. (%) of cDNA Deletions
DMD	46	24 (52)
BMD	24	11 (46)
U	20	10 (50)
Total	90	45 (50)

Note.—Patients have been classified as BMD if they were able to walk after 12 years of age (Emery 1987).

The fact that deletions occur in more than half of the patients now makes it possible to confirm the diagnosis of suspected DMD or suspected BMD by finding a deletion. This will apparently be of use in the differential diagnosis of muscular dystrophies, of which there are many types (reviewed, e.g., by Gardner-Medwin 1980). To rapidly screen for DMD/BMD one may even envisage using only the two cDNA probes that together detect at least 80% of all deletions (see below). However, a major difficulty is that the absence of deletion does not rule out the disease. Immunochemical analysis of the gene product dystrophin may in the future offer the most practical way of diagnosing X-linked muscular dystrophy (Hoffman et al. 1988).

Distribution of Deletions

Of the 45 different mutations detected in the present study, 25 (patients 20–44; tables 2, 3) formed a uniform group involving exon fragments belonging to probe 8. This represents a frequency of 56% of all deletions and correlates well with previously reported data (Koenig et al. 1987; Witkowski 1988). Nine of these deletions (patients 20–28) start in the region of probe 7 between the 4.1- and 1.5-kb *Hin*dIII fragments. Band patterns characteristic of the deletion breakpoints in this region are shown in figure 1. The 3.7- or 3.1-kb fragments of probe 8, or both, are deleted in the majority (20 cases) of the deletions. Probe 8 is located approximately 7 kb from the 5′ end of the cDNA and is thus

Table 5

Proportion of cDNA Deletions in Mentally Normal and Retarded Patients

Mental Retardation Status	No. of Patients	No. (%) of Patients with cDNA Deletions
No	71	38 (54)
Yes	14	7 (50)
Total	85	45 (53)

Note.—Data on intelligence were not available from five patients.

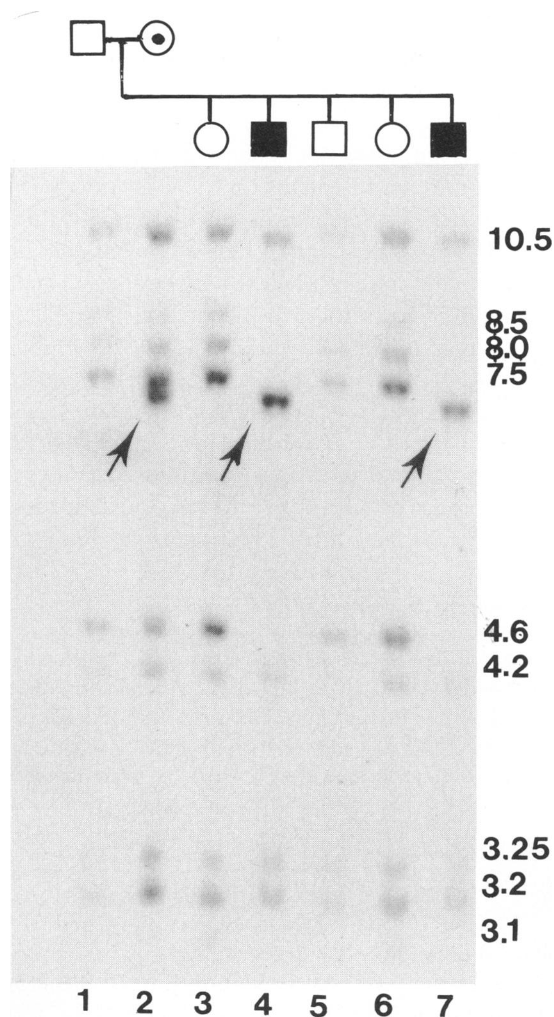

Figure 2 Use of a deletion with a junction fragment in carrier detection. DNA from patient 7 (lane 4), his father (lane 1), his mother (lane 2), his two sisters (lanes 3 and 6), his normal brother (lane 5), and his affected brother (lane 7) was digested with restriction enzyme *Hin*dIII and hybridized with cDNA probe 1–2a. The sizes of the normal fragments are shown on the right (see also table 1). The 8.5-, 8.0-, 7.5-, 4.6-, and 3.1-kb bands are missing in the DNA from the affected brothers. A junction fragment (marked with an arrow) is observed in the DNA from the affected brothers and in the DNA from their obligate carrier mother. As the sisters do not have the junction fragment, their carriership re DMD can be excluded.

near the middle of the gene. Similar results have been obtained by Koenig et al. (1987).

Another region in which deletions appear relatively frequently is near the 5′ end of the gene in the region corresponding to probes 1–2a and 2b–3. Thirteen (29%) of the deletions occurred in the region of probe 1–2a and 12 (27%) of them were in the region of probe 2b–3. However, only three of the 2b–3 deletions had their proximal breakpoint in the region of this probe. Only four (9%) of the deletions (see patients 22, 23, 36, and 45; tables 2, 3) were observed in the region of probe 9–10, and no deletions were found in the region of probe 11–12a. From these results we conclude that 38 (84%) of 45 deletions were detectable when only two cDNA probes (1–2a and 8) were used. No deletions extended over both of these probes. The unequal distribution of deletions might reflect the occurrence of regional hot spots. Another explanation is that introns in this region span extraordinarily long genomic DNA regions (Koenig et al. 1987; Wapenaar et al. 1988).

Correlation between Deletions and Clinical Severity of Muscle Disease

When analyzing our data, we used the somewhat arbitrary criterion of becoming wheelchair bound at age 12 years or less (Emery 1987) to distinguish between DMD and BMD. The use of this classification is justified because it facilitates comparisons between results from different centers. That the classification is probably outdated is illustrated by the data in table 2 that show wheelchair age for all our deletion patients. The data were similar in nondeletion patients. Instead of providing evidence for two distinct subgroups, our patients seem to form a continuous spectrum of severity. For the purpose of analysis we therefore chose to subdivide our patients just into DMD and BMD rather than to attempt to delineate a third group of intermediate severity. According to our results the presence or absence of deletions does not show any clear correlation with the severity and type of the disease (table 4).

It is notable (tables 2, 3) that BMD deletions occur more often in the region of probe 1–2a than do DMD deletions (46% vs. 31%). In all other regions DMD deletions are more frequent. How this might correlate with the quantity and structure of dystrophin is not clear. Immunochemical studies on dystrophin have demonstrated that very low levels or absence of dystrophin mainly occur in severe forms of the disease while dystrophin of abnormal size is frequently encountered in milder forms (Hoffman et al. 1988). According to one hypothesis, a major factor determining the clinical consequences might be the effect of the mutation on the translational open reading frame of the gene. Sequencing evidence in favor of this hypothesis has recently been produced (Monaco et al. 1988).

Our study does provide several examples of patients in whom the severity of the disease, on the one hand, and the extent of the deletion, on the other, are similar.

Such is the case with BMD patients 2 and 3, DMD patients 34 and 35, and the three patients, 40, 41, and 43 (table 2). We did not have any patients who evidenced identical deletions but a clear-cut difference in severity of the muscle disease. Nevertheless, before these matters can be fully understood, more-detailed studies, correlating both the DNA defect and the dystrophin abnormality with clinical features, will be necessary.

The proportion of deletions in familial disease (59%) is similar to that in sporadic disease (46%). This could be interpreted as evidence against any major negative effect on either gamete production or gamete reproduction in women with these deletions.

Mental Retardation

Mental retardation occurred in 14 (16%) of the 85 patients in whom this information was available. The proportion of patients with a deletion was almost the same in families with mentally normal probands (54%) as in families with retardation (50%) (table 5). However, close scrutiny of table 2 shows that of the seven deletion families in which mental retardation occurred, in six of them the deletion occurred in exons contained in probe 8. While the deletions in these six families are of quite variable length, it may be noted that all six show deletion of the 3.7-kb *Hin*dIII fragment. These data might suggest that DNA of importance for normal mental development in males may occur in this region. However, other interpretations are possible. Notably, probe 8 is the one most frequently deleted (in our series comprising 56% of all deletions), and several patients have deletions comprising the same region but are not mentally retarded. However, in studying the causes of mental retardation in muscular dystrophy, we are focusing on the region in which many mentally retarded patients show a deletion.

Genetic Counseling Based on cDNA Studies

By means of the cDNA probes, it is now possible to detect the mutation itself in half of all DMD and BMD patients. Only a few years ago, the mutation, in most cases a deletion, was detected in only about 10% of all cases, and genetic counseling was based on the analysis of segregation of one or several gene-specific or flanking RFLP markers. Thus the diagnostic usefulness of DNA studies is greatly improved.

The finding of a deletion by using genomic probes often makes genetic counseling of the female relatives more accurate, as carriership may be confirmed or ruled out. The same is true of those cDNA deletions in which abnormally sized hybridizing fragments are produced. An example of carrier detection using a junction fragment is given in figure 2. Unfortunately, junction fragments occur only rarely (in our series 6/45 families, corresponding to 13% of all the deletions). When a junction fragment is not generated by a deletion, carrier determination depends on the researcher's ability to observe the presence or absence of the 50% reduction in hybridization signal intensity that should occur in those bands that show deletion in the affected male(s). This method is useful, at least in some families, but it remains to be determined how generally applicable it will be. If RFLPs with favorable allele frequencies can be developed for the cDNA probes, these would be helpful in carrier detection. However, in all deletion families prenatal diagnosis of male fetuses can be based on the absence or presence of the bands that are deleted in the affected male(s).

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