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DIAGNOSIS OF DUCHENNE AND BECKER MUSCULAR DYSTROPHIES BY DNA POLYMORPHISM

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Summary Twenty families affected with Duchenne (DMD) or Becker (BMD) muscular dystrophy from the Japanese population were investigated using six DNA polymorphisms at the pERT87 locus. Allele frequencies for these polymorphisms were different from those found in Caucasians and 94% of unrelated women were informative for linkage analysis. DMD and BMD phenotypes cosegregated with a particular allele in each family with no obligate recombinants among 50 meiotic events. Among 20 unrelated affected males, one boy had a deletion of at least 52 kilobases (kb) surrounding the pERT87 region.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is one of the most common and serious X-linked recessive disorders with a frequency of about 1 in 3,000–5,000 newborn males (Gardner-Medwin, 1980). Becker muscular dystrophy (BMD) is clinically similar to DMD in the pattern of muscle involvement, but follows a more benign course. The underlying basic defect in these diseases and an effective treatment are unknown. Until recently, prenatal diagnosis has not been feasible and carrier detection in families at risk for DMD or BMD has depended on a somewhat equivocal test for serum creatine kinase (CK) activity (Sugita and Tyler, 1963; Harper, 1982).

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DNA restriction fragment length polymorphisms (RFLPs) have recently been used for diagnosis of various diseases including DMD. Kunkel and coworkers (1985) have isolated DNA fragments, pERT87 (DXS164), from the Xp21 region deleted in a DMD male patient. They successfully used subcloned fragments (pERT87-1, pERT87-8, and pERT87-15) from the pERT87 locus as probes to detect RFLPs diagnostic of DMD (Monaco *et al.*, 1985; Kunkel *et al.*, 1986). However, the applicability of these subclones for DMD diagnosis in populations other than American and European Caucasian populations has not yet been evaluated. Moreover, the usefulness of these probes in BMD prediction has not been fully elucidated.

In this study, we examined the feasibility of these three pERT87 subclones to detect DMD and BMD patients and carriers in affected Japanese families. The results indicate that these probes are highly useful for analyses of Japanese DMD and BMD families.

MATERIALS AND METHODS

Subjects. Unrelated healthy volunteers and members of twelve families with Duchenne or Becker muscular dystrophy from the Japanese population were investigated for DNA polymorphisms.

The diagnosis of DMD or BMD was made on the basis of clinical findings and progression of the disease, serum CK levels, muscle histology and electromyography were examined by one of the authors (J.G., I.N., M.T., or H.S.). Pedigrees with more than two affected males in one or more generations or in relatives were examined to exclude isolated cases possibly arising by new mutation.

Restriction endonuclease analysis. Genomic DNA was prepared from lymphocytes in 10–20 ml of heparinized peripheral blood by the guanidium/cesium chloride method (Maniatis *et al.*, 1982). Each DNA sample (5–10 μg) was digested to completion with 2-fold excess units of the appropriate restriction endonuclease (*Xmn*I, *Taq*I, *Bst*NI, or *Bst*XI, New England Biolabs and Takara Shuzo) in the buffer recommended by the manufacturer. The DNA samples (1.5 μg each) were separated by electrophoresis on 0.7% agarose gels and transferred to nitrocellulose filters by the method of Southern (1975). Inserts of subclones pERT87-1, pERT87-8 and pERT87-15 (provided by L. Kunkel) were labeled with [^{32}P]dCTP (New England Nuclear) using an Amersham nick translation kit. Hybridization of nitrocellulose filters was undertaken as described by Maniatis *et al.* (1982). After overnight incubation at 65°C, the excess probe was washed off in 2 × SSC (1 × SSC=NaCl 0.15 mol/liter, trisodium citrate 0.015 mol/liter), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min, followed by a wash at 55°C in 0.1 × SSC, 0.1% SDS for 50 min.

Human genomic DNA segments (OTC-d, -e, and -f) in the locus for ornithine transcarbamylase (OTC), which maps to Xp21 proximal to the DMD locus (Lindgren *et al.*, 1984; Davies *et al.*, 1985), were isolated from a human genomic library

(provided by T. Maniatis) using rat OTC complimentary DNA (Takiguchi *et al.*, 1984) as a probe. Two genomic DNA fragments (1-1 and 1-4) were isolated from a human X chromosome library (provided by Y. Sakaki). These segments were demonstrated to be unique fragments of the X chromosome by dosage analysis using a panel of DNAs prepared from normal male lymphocytes (46XY) and the lymphoblastoid cell line GM 1416, 48XXXX (Human Genetic Mutant Cell Repository, Camden, NJ).

RESULTS

Frequency of polymorphisms

The allele frequency and heterozygosity of three probes of the pERT87 (DXS164) locus in Japanese individuals including healthy volunteers and unrelated obligate carriers of DMD or BMD are shown in Table 1, together with the results from American and European Caucasian groups (Kunkel *et al.*, 1986). Racial differences were observed in the frequencies of several polymorphic sites, *e.g.* the alleles for pERT87-8 with both *Bst*XI ($\chi^2=15.39$, d.f.$=1$, $p<0.001$) and *Taq*I ($\chi^2=13.31$, d.f.$=1$, $p<0.001$) and for pERT87-15 with *Taq*I ($\chi^2=4.98$, d.f.$=1$, $p<0.05$). Although allele frequencies differed considerably between the two populations, the expected heterozygosities at all loci except the *Taq*I site for pERT87-15 were approximately 40–50% in the Japanese population, similar to those found in Americans and Europeans. The expected heterozygosities corresponded well to the observed heterozygosities, that is, the proportion of heterozygous females relative to the total females examined. These data imply that, in the Japanese population, each of three pERT87 subclones is highly informative for identifying females at risk of being DMD carriers.

Of particular interest is the proportion of DMD families in which DNA diagnoses can be made by a combination of these three pERT87 probes and four restriction enzymes. The proportion of females heterozygous at one or two of the polymorphic *Bst*NI and *Xmn*I sites detected by pERT87-1 was 14 out of 25 (56%), although the heterozygosity calculated from the allele frequencies at the two polymorphic sites was 75% (Table 2). Using the *Bst*XI site for pERT87-8 combined with the *Taq*I site provided little additional information (7 out of 18, 39%) for detecting DMD by linkage analysis. In contrast, using the *Taq*I site for pERT87-15 in combination with the *Xmn*I site improved the test applicability from 50% to 68% (13 out of 19), although the heterozygosity at the *Taq*I site was somewhat low (24%). Finally, when the two enzyme-defined sites of the pERT87-15 probe were used in combination with the polymorphic sites of pERT87-1 and pERT87-8, 30 out of the 32 unrelated Japanese women tested (94%) were informative for linkage analysis.

Nonrandom association of polymorphic sites

The above data suggest that there is a strong linkage disequilibrium between

Table 1. Allele frequency and heterozygosity of DNA polymorphisms for pERT87.

Probe	Restriction enzyme	Allele frequency		Heterozygosity (%)	
		American & European[a]	Japanese	American & European	Japanese
				Expected (Observed)	
pERT87-1	*Xmn*I	*A*: 0.66	0.53	45	50 (53)
		a: 0.34	0.47		
			(n=70)		
	*Bst*NI	*C*: 0.63	0.48	47	50 (54)
		c: 0.37	0.52		
			(n=54)		
pERT87-8	*Bst*XI	*D*: 0.60	0.26[b]	48	38 (39)
		d: 0.40	0.74		
			(n=58)		
	*Taq*I	*E*: 0.71	0.38[c]	41	47 (40)
		e: 0.29	0.62		
			(n=52)		
pERT87-15	*Taq*I	*F*: 0.67	0.85[d]	44	26 (24)
		f: 0.33	0.15		
			(n=52)		
	*Xmn*I	*B*: 0.68	0.59	44	48 (50)
		b: 0.32	0.41		
			(n=70)		

[a] Allele frequencies in American and European populations are cited from the data by Kunkel *et al.* (1986). Expected heterozygosities in both Japanese and Caucasian groups were calculated from the allele frequencies at each site. Observed heterozygosity represents the proportion of heterozygous females relative to total females examined. n, number of observed chromosomes. [b] $\chi_1^2=15.39$, $p<0.001$; [c] $\chi_1^2=13.32$, $p<0.001$; [d] $\chi_1^2=4.98$, $p<0.05$.

Table 2. Applicability of polymorphic sites for the pERT87 subclones in DMD Diagnosis.

Probe	Restriction site	Applicability (%)[a]	
		Observed	Expected
pERT87-1	*Xmn*I and *Bst*NI	56[b] (14/25)	75
pERT87-8	*Bst*XI and *Taq*I	39[c] (7/18)	67
pERT87-15	*Xmn*I and *Taq*I	68 (13/19)	62
pERT87-1, -15	*Xmn*I	81 (26/32)	74
All	*Xmn*I, *Taq*I, *Bst*XI, and/or *Bst*NI	94 (30/32)	—

[a] Applicability represents the percentage of women who are informative for one or more loci. Values in parentheses are the number of informative women/total women. Expected applicability is calculated from the observed allele frequencies at each site. [b] $\chi_1^2=4.81$, $p<0.05$; [c] $\chi_1^2=6.38$. $p<0.025$.

Table 3. Nonrandom association of the *Xmn*I and *Bst*NI polymorphic sites detected by pERT87-1 in Japanese population.

Genotype	Number observed	Frequency	
		Observed	Expected
AC/AC	5	0.20	0.06
ac/ac	6	0.24	0.06
AC/ac, Ac/aC	12	0.48	0.25
Ac/ac	1	0.04	0.13
Ac/AC	1	0.04	0.14
Others	0	0	0.36

Deduced haplotype: Common; *ac* (49%) and *AC* (45%)
Rare; *Ac* and *aC*

Haplotype frequency was deduced from the frequency of each homozygote. *A* and *a*, alleles for *Xmn*I polymorphism; *C* and *c*, alleles for *Bst*NI polymorphism. $\chi_3^2=38.96$, $p<0.001$.

the two polymorphic sites detected by pERT87-1 or by pERT87-8, whereas the two polymorphic sites for pERT87-15 are closer to equilibrium with each other. For studying linkage disequilibrium, we deduced the haplotypes from the genotypes for the two polymorphisms for pERT87-1. In theory, 10 possible genotypes (though *AC/ac* can not be distinguished from *Ac/aC*) exist as a result of random combination of four haplotypes, *AC*, *Ac*, *aC*, and *ac*. However, only four out of the 10 theoretical genotypes (*ac/ac*; *AC/ac* and *Ac/aC*; *AC/AC*) accounted for 92% of Japanese individuals (Table 3). The population frequencies for these genotypes were 0.24, 0.48 and 0.20, while their expected frequencies based on the observed allele frequencies at the two sites were 0.06, 0.25, and 0.06, respectively. The frequency of haplotype *AC* (p) or *ac* (q) calculated from the frequency of each homozygote is $p=0.45$ or $q=0.49$, respectively. The estimated frequency of genotype *AC/ac* is $2pq=0.44$, similar to the sum (0.48) of genotypes *AC/ac* and *Ac/aC*. Thus, genotype *Ac/aC* is probably rare. The frequencies for *Ac/ac* and *Ac/AC* are also very low. These data suggest that two haplotypes, *AC* and *ac*, are common and the others are rare in the Japanese population. The striking linkage between the *Xmn*I and *Bst*NI sites (allele *A* and *C* or allele *a* and *c*) for pERT87-1 probably reduces their usefulness for the diagnosis of DMD. The fact that the two *Xmn*I sites detected by pERT87-1 and pERT87-15 increased the applicability of prediction from 50% to 81% (26 of 32 tested females) suggests that the pERT87-15 region is randomly associated with the pERT87-1 region (Table 2). Further analysis of three pERT87 markers in pairwise combinations indicates that they are closer to equilibrium with one another (data not shown).

Linkage studies in DMD and BMD families

Of 16 DMD and BMD families from the Japanese population studied, DNA

diagnosis of 15 was possible by the three probes. The segregation of RFLPs demonstrated Mendelian inheritance with no obligate recombinants in 50 meiotic events in 14 DMD and 1BMD families. Representative data are shown in Figs. 1, 2 and 4. In family J (Fig. 1), since the affected boy received from his mother a 8.8 kb *Xmn*I fragment (allele *A*) hybridizing with pERT87-1 while another son with a 7.5 kb band (allele *a*) is normal, it is presumed that the maternal X chromosome carrying allele *A* is responsible for DMD. A daughter who has received allele *A* from her mother has been diagnosed as being a carrier of DMD, a prediction that is consistent with her increased CK level. Prenatal diagnosis of her male fetus will be possible by examining for the presence of 1.6 and 1.2 kb *Xmn*I fragments (allele *B*) hybridizing with pERT87-15 which also cosegregate with DMD. In family P the two affected boys have received allele *B* from their grandmother *via* their mothers, whereas the daughter has received a 2.8 kb fragment (allele *b*) from her mother and 1.6 and 1.2 kb fragments from her father. Thus, she has been diagnosed as normal with a probable accuracy of 95%. Likewise the daughter in family E is probably normal because she does not carry the DMD-X chromosome cosegregating with the 2.2 kb band (allele *d*). In families H and C, DMD cosegregates with a 2.8 kb *Xmn*I fragment (allele *b*) for pERT87-15 and a 3.3 kb *Taq*I fragment (allele *f*) for pERT87-15, respectively.

In family GK, the mother and her affected son were previously shown to have an interstitial deletion in band Xp21 by cytogenetic analysis (Saito *et al.*, 1986). Segregation of two *Xmn*I RFLPs for pERT87-1 and pERT87-15 in this pedigree shows that the DMD boy bears the deletion in the pERT87 locus and suggests that his mother is hemizygous for this locus (Fig. 2). To characterize this point more precisely, we performed a dosage analysis. The hybridization intensity of the mother in the pERT87 locus detected by two probes (pERT87-1 and pERT87-15) was similar to that seen for the single X chromosome in control males rather than that of the two X chromosomes in control females (Fig. 3). This confirms that she is heterozygous for the deletion found in her DMD son. The two pERT87 subclones span a region of 52 kb of the genomic sequence (Kunkel *et al.*, 1986), and, therefore, the lower limit of this deletion is 52 kb. However, the ornithine transcarbamylase locus (OTC-d, -e, and -f) and two other DNA segments (1-1 and 1-4) of the X chromosome were present in the DNA of the DMD boy and his mother. This is consistent with previous cytological studies. Except for this case, none of the 18 unrelated DMD and two BMD males had a deletion of the pERT87 region.

Figure 4 shows a BMD family segregating for the pERT87-8 and pERT87-15

Fig. 1. DMD families segregating for the pERT87 polymorphisms. Southern blot analysis of DNA from family members was performed as described in MATERIALS and METHODS. Sizes of the hybridizable fragments (alleles) are indicated in kilobase (kb). In family J, bands A, a and B, b were detected by pERT87-15 and pERT87-1, respectively. Symbols are as follows: □, normal male; ○, normal female; ■, affected male; ◑, obligate carrier; ⊘, manifesting carrier as detected by this experiment.

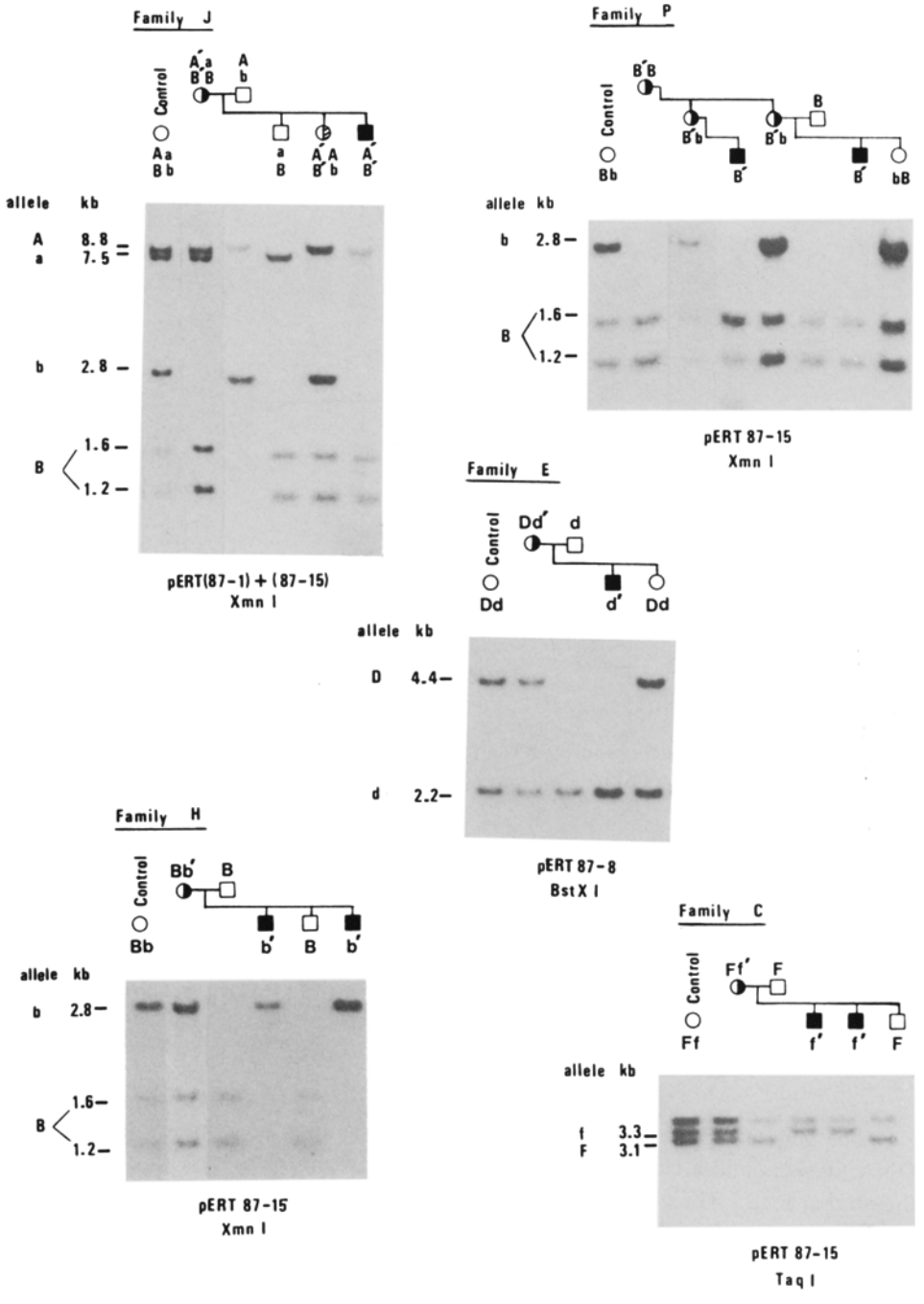
Family J
Control
Aa
Bb
A'a
B'B
A
b
a
B
A'A
B'b
A'
B'
allele kb
A 8.8
a 7.5
b 2.8
B 1.6
1.2
pERT(87-1) + (87-15)
Xmn I
Family P
Control
Bb
B'B
B'b
B
B'b
B'
B'
bB
allele kb
b 2.8
B 1.6
1.2
pERT 87-15
Xmn I
Family E
Control
Dd
Dd'
d
d'
Dd
allele kb
D 4.4
d 2.2
pERT 87-8
BstX I
Family H
Control
Bb
Bb'
B
b'
B
b'
allele kb
b 2.8
B 1.6
1.2
pERT 87-15
Xmn I
Family C
Control
Ff
Ff'
F
f'
f'
F
allele kb
f 3.3
F 3.1
pERT 87-15
Taq I

Fig. 1.

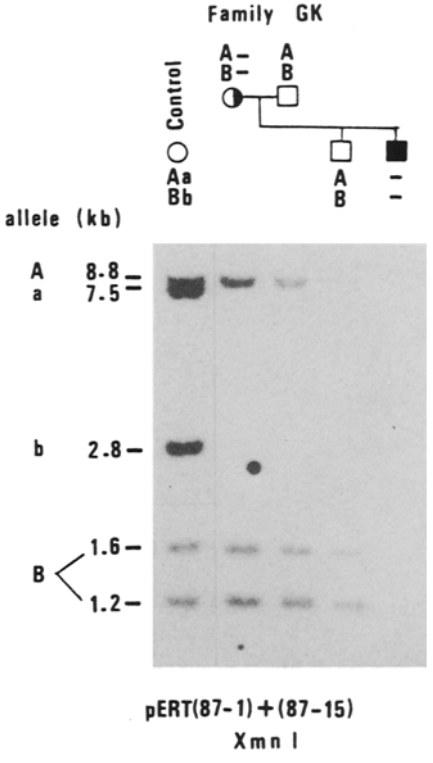

Fig. 2. Analysis of deletions for pERT87-1 and pERT87-15 in a DMD family. Symbols are the same as in Fig. 1.

polymorphisms with no obligate recombinants in eight meiotic events. In this pedigree, BMD is carried by the X chromosome with a 3.8 kb *Taq*I fragment (allele *e*) detected by pERT87-8 in the two mothers who are sisters. Therefore, three relative females who have received allele *e* from each mother are probably carriers of BMD. The linkage analysis in this pedigree indicates that the BMD gene is located near the pERT87 locus in the same region as the DMD gene.

DISCUSSION

The general applicability of DNA diagnosis requires both highly polymorphic DNA markers and a closely linked relationship between the disease in question and the marker loci. The close linkage between the DMD locus and the pERT87 probes has been previously reported (Monaco *et al.*, 1985; Kunkel *et al.*, 1986) and is confirmed by this study. In this report, we have investigated the applicability of these three DNA fragments to the diagnosis of DMD or BMD, especially in the Japanese population, because the proportion of heterozygotes often varies with race (Chak-

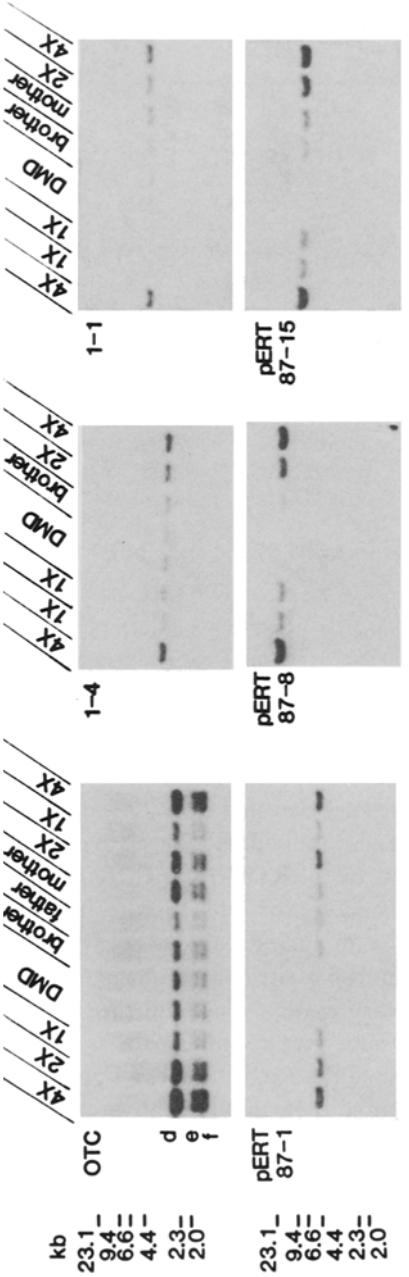

Fig. 3. Dosage analysis of DNA preparations from family members with a deletion in band Xp21. DNA (2 μg) from normal males (1X), normal females (2X), cultured lymphoblasts GX 1416 (4X) and the DMD family members were digested with *Eco*RI and applied to each lane. Southern blot analyses were performed with the probes indicated on the left of each panel.

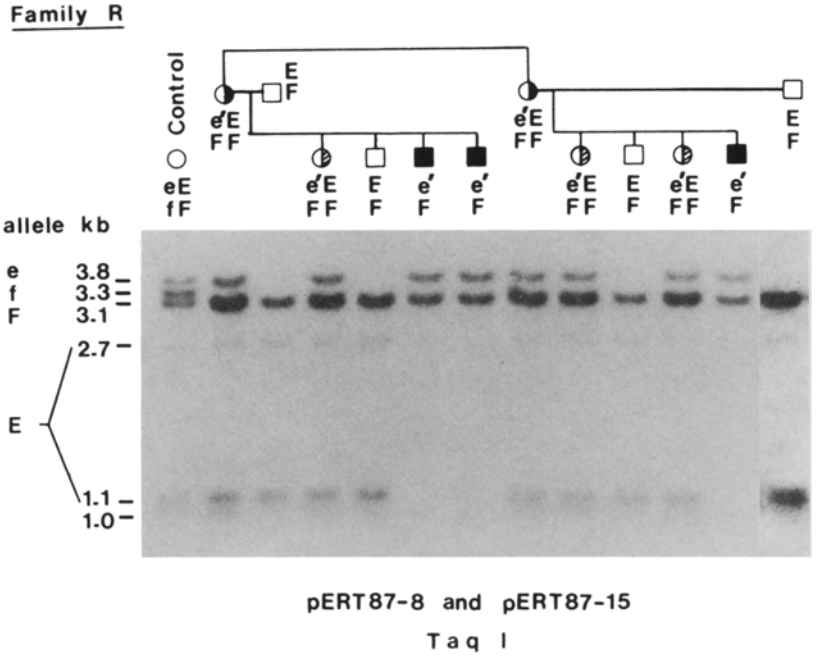

Fig. 4. BMD family segregating for pERT87-8 and pERT87-15 polymorphisms. Southern blot analysis of DNA from maternal relatives affected with BMD is shown below the pedigree. Alleles *E*, *e* and a 1.0 kb invariant band were detected by pERT87-8, and allele *F* and *f* were detected by pERT87-15.

ravarti *et al.*, 1984: Elbein *et al.*, 1986). Although, a racial difference in the frequencies of three polymorphic sites detected by *Bst*XI and *Taq*I in the pERT87 locus (Table 1) was observed, Japanese individuals are highly polymorphic for all RFLPs except the *Taq*I site detected by pERT87-15. Therefore, RFLPs in the pERT87 locus are good polymorphic markers for DMD in the Japanese population.

To obtain high efficiency in linkage analysis, several markers are necessary. However, some DNA polymorphic sites show strong linkage disequilibria (Antonarakis *et al.*, 1982; Chakravarti *et al.*, 1986), reducing their usefulness. Therefore, two or more markers sometimes give essentially the same results. Such a relationship probably exists in the case of the *Bst*NI and *Xmn*I polymorphic sites for pERT87-1 (Table 3), since only one of the 12 uninformative cases examined by the *Xmn*I RFLP for pERT87-1 was informative by the *Bst*NI RFLP. Since the RFLPs for pERT87-15 and those for pERT87-1 and pERT87-8 showed random associations, a set of *Xmn*I RFLPs detected by both pERT87-1 and pERT87-15 is the most informative and provides high efficiency with one operation within the same panel. In order to minimize the number of tests per case necessary to obtain information for DNA diagnosis, we recommend performing the screening in the following order: screening with (1) the two *Xmn*I sites for pERT87-1 and pERT87-15, (2) the two

*Taq*I sites for pERT87-8 and pERT87-15, and/or (3) the *Bst*XI site for pERT87-8.

The accuracy of diagnosis is inversely related to recombination between the disease and marker loci. In this study, we found no recombination with the six RFLPs seen by three pERT87 subclones in Japanese individuals. However, it has been reported that the pERT87 locus apparently recombines with a frequency of 5–6% in American and European populations (Fischbeck *et al.*, 1986: Kunkel *et al.*, 1986). Therefore, for a more precise diagnosis of DMD, pERT87 probes should be used in combination with other Xp21 flanking markers.

The DMD patient with a deletion in band Xp21 (family GK) is suffering from complex glycerol kinase deficiency (GKD), a syndrome characterized by congenital adrenal hypoplasia (CAH) and mental retardation, but his ornithine transcarbamylase activity is normal. Our Southern blot analysis confirmed that the OTC locus was present in this DMD patient. Francke (1984) reported a female with an Xp21 deletion who was diagnosed as being a carrier of OTC deficiency and CGD (chronic granulomatous disease). Recently, it has been shown that the X-linked CGD locus is proximal to the DMD locus and lies within the Xp21 region (Baehner *et al.*, 1986). Since the case in this report is not suffering from CGD, we speculate that one break point in this deletion is distal to the CGD and OTC loci, and the other point is distal to the DMD locus. Precise analysis of the end points of this deletion will help to determine the position of the GK locus relative to the CGD and DMD loci.

The present results together with those reported previously (Brown *et al.*, 1985; Wilcox *et al.*, 1985: Kunkel *et al.*, 1986) demonstrate a close linkage of BMD with pERT87 subclones, and suggest that BMD and DMD are caused by different mutations of the same locus or else that their loci, while distinct, are located very close to each other.

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