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Facioscapulohumeral muscular dystrophy (FSHD) has recently been shown to be associated with deletions that are detectable using probe p13E-11 (D4F104S1). Although these deletions reside within large, highly polymorphic restriction fragments (20–300 kb), the "mutant" fragment is usually shorter than 28 kb and can routinely be detected using conventional agarose gel electrophoresis. Yet, the complete visualization of the alleles requires pulsed-field gel electrophoresis (PFGE). Family studies showed that p13E-11 detects two nonallelic loci in this size range, only one of which originates from chromosome 4q35. We have assigned the other p13E-11 locus to chromosome 10qter by linkage analysis in CEPH pedigrees. Knowing the location of both loci improves the diagnostic reliability, as the exact origin of "small" *EcoRI* fragments can be determined by haplotyping. Since FSHD shows genetic heterogeneity, this 10qter locus became an interesting candidate to be the second FSHD locus. However, analysis of a large chromosome 4–unlinked FSHD family did not provide evidence for linkage on chromosome 10qter. © 1995 John Wiley & Sons, Inc.

Key words: FSHD • Facioscapulohumeral muscular dystrophy • linkage • DNA diagnostics

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## THE FSHD-LINKED LOCUS D4F104S1 (p13E-11) ON 4q35 HAS A HOMOLOGUE ON 10qter

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**F**acioscapulohumeral muscular dystrophy (FSHD) is one of the three major forms of muscular dystrophy, along with the Duchenne/Becker and limb girdle forms. The onset of FSHD is characterized by weakness of facial, shoulder girdle, and upper-arm muscles. Where there is progression of the disease, the weakness and atrophy

spreads to the foot extensor, upper arm, and pelvic-girdle muscles.<sup>4,5</sup> Although FSHD shows a characteristic autosomal-dominant inheritance pattern, isolated patients are discovered quite frequently, suggesting a high new mutation frequency of the disease.

The gene defect involved in the majority of families is localized to chromosome 4q35, distal to the linkage group cen . . . D4F171–F11–D4S163–D4S139.<sup>7</sup> More recently, specific deletion fragments associated with the etiology of FSHD have been cloned and characterized.<sup>9</sup> These deletions occur within polymorphic *EcoRI* or *HindIII* fragments and can be detected by conventional Southern analysis using probe p13E-11 (D4F104S1).<sup>6,8,10,14</sup> In general, the deletion fragments are smaller than 28 kb. However, to reveal the complete polymorphism detected by p13E-11, separation by pulsed-field gel electrophoresis (PFGE) is required since fragments can range in size from less than 20 kb to more than 300 kb. Interestingly, PFGE reveals the presence of two nonallelic loci.<sup>13</sup> Using haplotype analysis, one locus could be assigned to chromosome 4q35. Despite the fact that FSHD patients

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exhibit p13E-11 detectable fragments that are smaller than 28 kb, sometimes healthy individuals also display "small" p13E-11 fragments that can be visualized quite easily on conventional agarose gels. These "small" fragments most likely originate from the non-4q35 locus since polymorphic fragments originating from 4q35 seem to be at least 50 kb in length in normal individuals.<sup>13</sup>

Although a "small" p13E-11 detectable fragment by itself is not diagnostic for FSHD, it becomes so when its origin is defined to be chromosome 4q35. In this study, we localize the second p13E-11 polymorphic locus to chromosome 10qter, which will facilitate the differentiation between the two p13E-11 loci and thereby improving the diagnostic power.

Since FSHD shows genetic heterogeneity,<sup>3</sup> it was attractive to consider this second p13E-11 locus to be the second FSHD locus. We investigated the potential linkage between the second p13E-11 locus and FSHD in a large Danish FSHD family that was excluded for linkage with 4q35 markers.

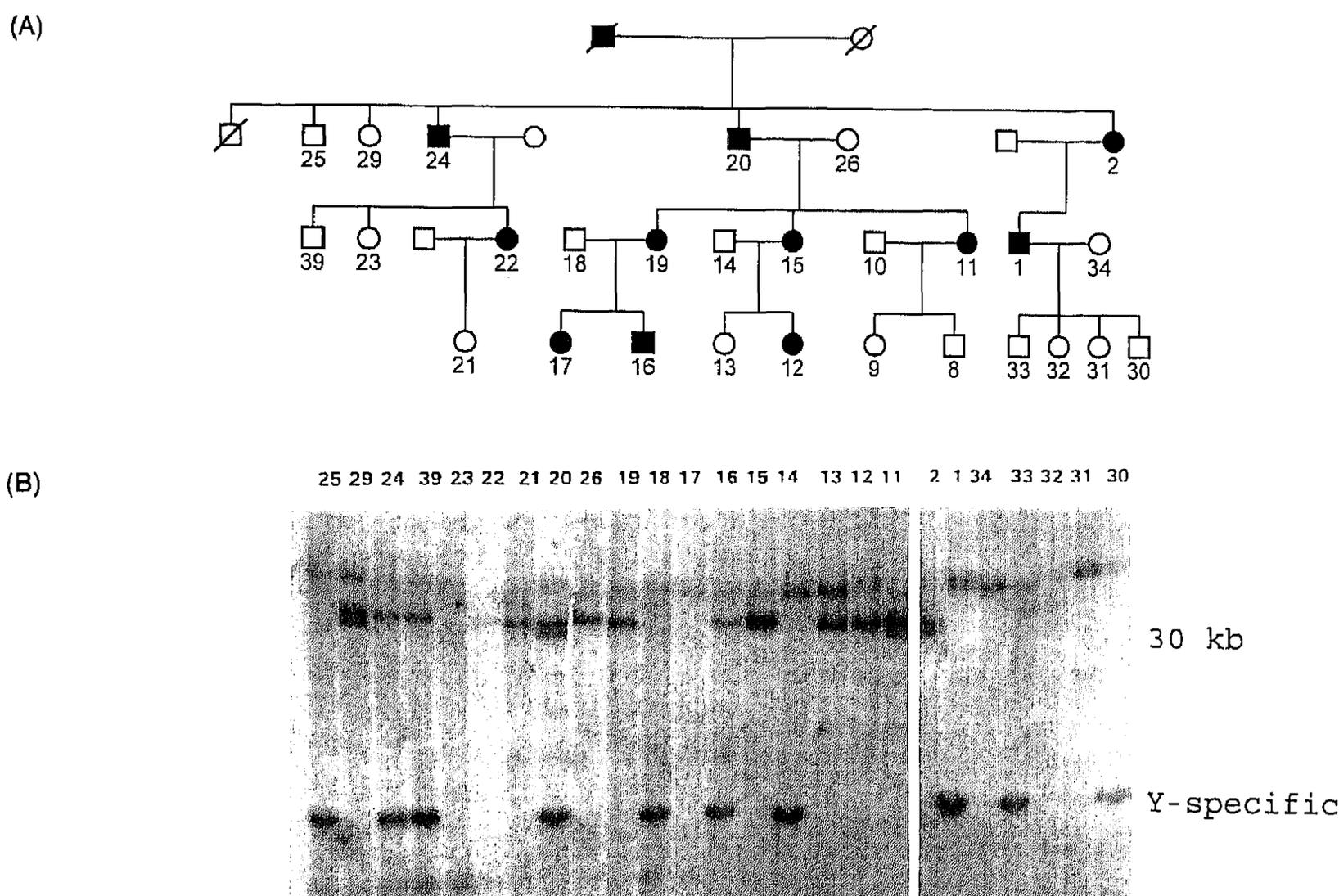
#### MATERIALS AND METHODS

**Subjects.** This study encompasses a large multi-generational Danish FSHD family consisting of 11 affected and 12 unaffected individuals (see Fig. 1A). This family shows an autosomal-dominant in-

heritance pattern. Most patients were subjected to repeated physical investigations by independent neurologists. Three cases had a muscle biopsy and electromyography to establish the diagnosis of FSHD.

**Analysis of p13E-11.** The Centre d'Etude Polymorfisme Humaine (CEPH)<sup>2</sup> provided, as a part of the EUROGEM project, ready-to-hybridize Southern blots of *Eco*RI-digested DNA samples from the CEPH pedigrees members. They also provided microtiter plates with DNA samples of members of the CEPH pedigrees. Hybridization with  $\alpha^{32}\text{P}$ -dCTP-labeled probe p13E-11 was performed overnight at 65°C according to Church and Gilbert.<sup>1</sup> Filters were washed to a stringency of  $1 \times \text{SSC}/0.1\% \text{SDS}$ , followed by autoradiography for 1–3 days using an intensifying screen.

**Analysis of Microsatellite Markers.** Amplification of microsatellites is performed in a microtiter plate. The reaction volume is 15  $\mu\text{l}$  containing 10 mmol/L Tris-HCl, pH 9.0; 50 mmol/L KCl; 0.01% gelatine; 1.5 mmol/L  $\text{MgCl}_2$ ; 0.1% Triton X-100; 0.06 U Supertaq DNA polymerase (HT Biotechnology Ltd.); 200  $\mu\text{mol/L}$  each of dATP, dGTP, and dTTP; 2.5  $\mu\text{mol/L}$  dCTP; 0.75  $\mu\text{Ci}$   $\alpha^{32}\text{P}$ -dCTP (at 3000 Ci/mmol); 30 ng of each oligonu-



**FIGURE 1.** (A) Danish FSHD pedigree, number below individuals indicate DNA sample numbers. (B) Southern blot of *Eco*RI-digested genomic DNA, hybridized with probe p13E-11. Sample numbers correspond with numbers in the pedigree (A).

cleotide primer; and 30 or 200 ng of genomic DNA template. Samples are processed in a thermocycler (MJ Research) through 27 cycles (30 ng DNA), or 20 cycles (200 ng DNA) consisting of 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C. Some of the microsatellite loci are amplified simultaneously.

After PCR, 1 volume of formamide sample buffer (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) is added to the samples. The samples are denatured (5 min at 94°C) and loaded on a DNA sequencing gel (6% acrylamide/bisacrylamide 19:1, 7 mol/L urea, 0.35% APS, 0.07% TEMED, in 1 × TBE). Electrophoresis is performed for 3 h at 40 mA and 65 W. Prior to loading and electrophoresis the upper buffer compartment is filled with 0.5 × TBE, the lower buffer compartment with 5/6 × TBE/0.5 mol/L NaAc and the gel is prerun for 0.5 h. Dideoxy sequencing reactions of phage M13mp18 are loaded on the gel as size standards. After electrophoresis the gel is fixed for 20 min in 5% methanol/5% acetic acid. The gel is dried and exposed to an X-ray film at room temperature for 2 h or overnight.

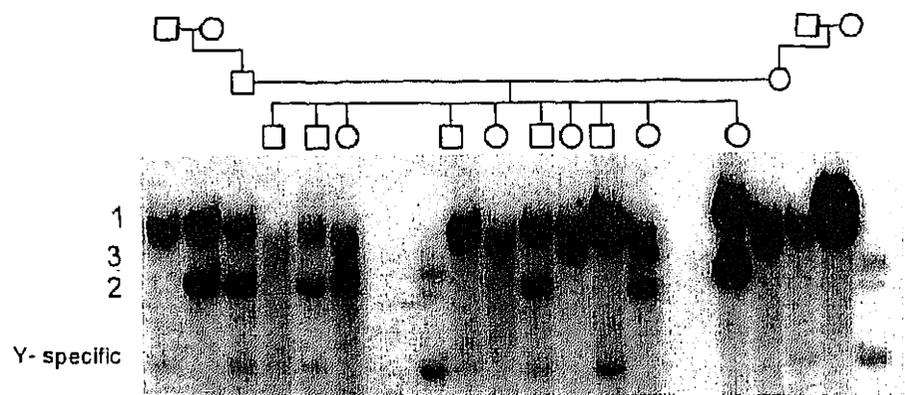
**Linkage Analysis.** Linkage analysis in the CEPH pedigree set was carried out to establish the position of the nonchromosome 4 p13E-11 locus on the genetic linkage map. Since not all *EcoRI* fragments detected by p13E-11 can be identified using conventional agarose gels, the polymorphism was scored as a two- or three-allele system. Individuals with only fragments larger than 28 kb were scored as homozygous (1,1) and individuals with a fragment smaller than 28 kb were scored as heterozygous (1,2).

Two-point linkage analysis in the Danish FSHD family was carried out using the MLINK program of the LINKAGE package, Version 5.1, assuming equal recombination fractions in males and females, a gene frequency of 0.00005, and a penetrance of 95%.<sup>12</sup>

## RESULTS

### Assignment of the Second p13E-11 Locus to 10qter.

From a total of 20 CEPH pedigrees both pairs of grandparents were genotyped using p13E-11. Among these 80 individuals (160 chromosomes), 8 showed a fragment which was smaller than 28 kb, implying a 10% chance of detecting a "small" p13E-11 fragment. Of seven CEPH pedigrees analyzed with p13E-11, four (1346, 1375, 1416, and 1423) showed segregation of the "small" band. In CEPH pedigree 1416, two different and easily distinguishable "small" p13E-11 fragments were



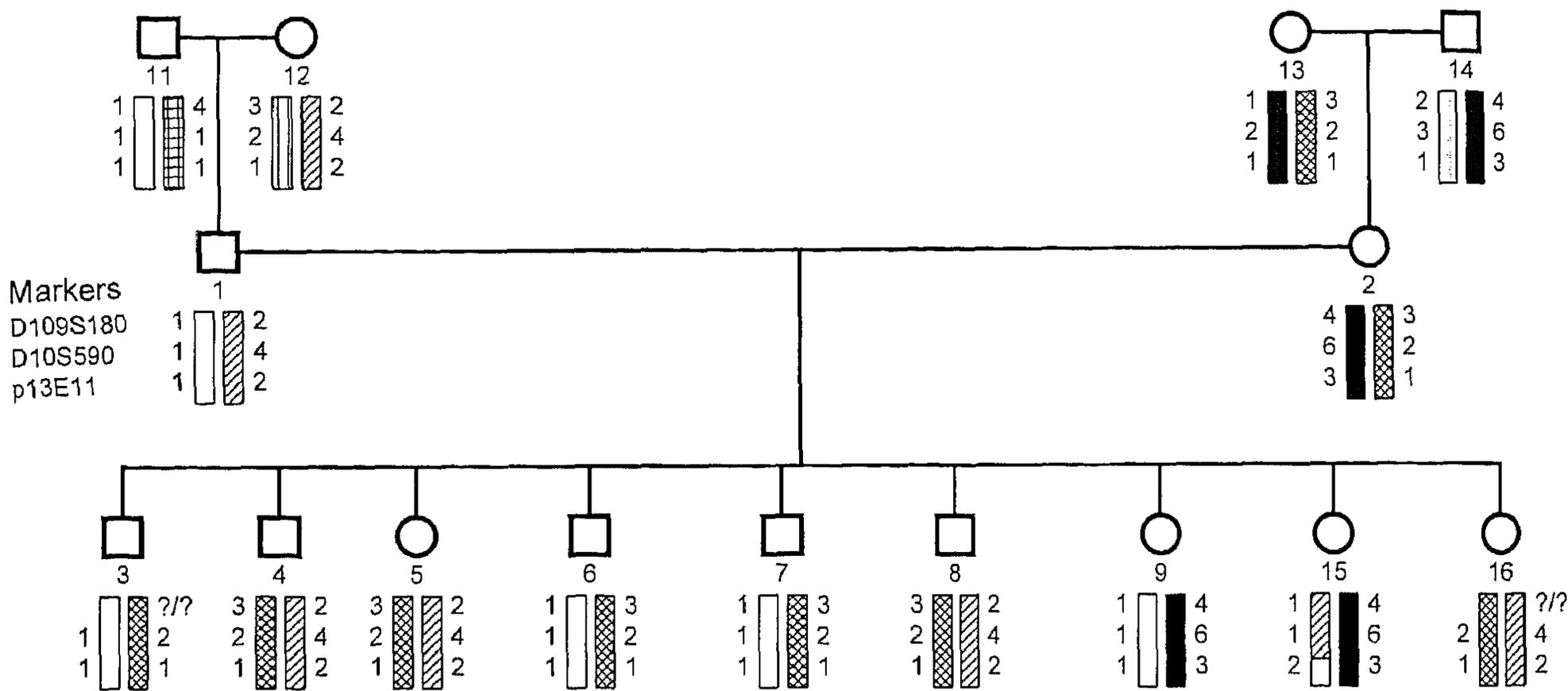
**FIGURE 2.** CEPH pedigree 1416 with Southern blot results for probe p13E-11. Alleles longer than 28 kb are indicated with a 1, two alleles shorter than 28 kb are indicated by 2 and 3, respectively.

found to segregate independently (Fig. 2), yielding a total of 18 possibly informative meioses. The other three families showed one "small" p13E-11 fragment of which the segregation could be followed through the pedigrees, yielding a total of 36 possibly informative meioses. Using the data from the Version 6.0 CEPH database, significant linkage was detected between these "small" p13E-11 fragments and different chromosome 10q markers in the four families (data shown for pedigree 1416; Figs. 2 and 3). Several additional markers (e.g., AFM198zb4, D10S212 and AFM304wh1, D10S590) mapping to chromosome 10q were tested in the four CEPH pedigrees. The results of the two-point linkage analysis are shown in Table 1. The most closely linked marker, D10S590, showed a recombination frequency of 0.05 with a lod score of 4.0. Multipoint linkage analysis suggests the following locus order to be the most likely one: D10S217-(13 cM)-{D10S212-(1 cM)-D10S180-(1 cM)-D10S590}-(6 cM)-p13E-11 ... tel. The position of p13E-11 with respect to this linkage group is distal, based on one recombination event between D10S590 and p13E-11 in CEPH family 1416 (see Fig. 3).

Two-point linkage analysis between the FSHD locus and a marker from the FSHD gene region on chromosome 4q35 (pH30; locus D4S139) was performed in the large Danish FSHD family. This family shows five (out of 19 informative) meioses where FSHD apparently recombines with D4S139 (see Fig. 4A). Exclusion of linkage for this FSHD locus to chromosome 4q35-qter is evident with a lod score of -2.2 at 10 cM.

**Table 1.** Lod scores for p13E-11 vs. chromosome 10 markers in CEPH pedigrees.

	cM	Lod
P13E-11 vs. D10S590	5.0	4.0
P13E-11 vs. D10S180	5.6	3.74
P13E-11 vs. D10S212	6.6	5.84



**FIGURE 3.** CEPH pedigree 1416 showing cosegregation of p13E-11 alleles with alleles of two chromosome 10 markers.

Analysis of this family with p13E-11 (Fig. 1B) revealed the presence of a detectable fragment of 30 kb on a conventional agarose gel. Linkage analysis between this "30-kb" p13E-11 fragment and pH30 yielded no recombinants and a lod score 3.24 confirming that this 30-kb p13E-11 allele is located on chromosome 4q35 (Fig. 4A).

To test whether chromosome 10q contains a second FSHD locus, multipoint linkage studies were performed between FSHD and the 10q markers D10S212 and D10S590, and linkage was excluded with a lod score of  $-2$  at 15 cM (in 18 informative meioses 6 recombinants were detected; see Fig. 4B).

## DISCUSSION

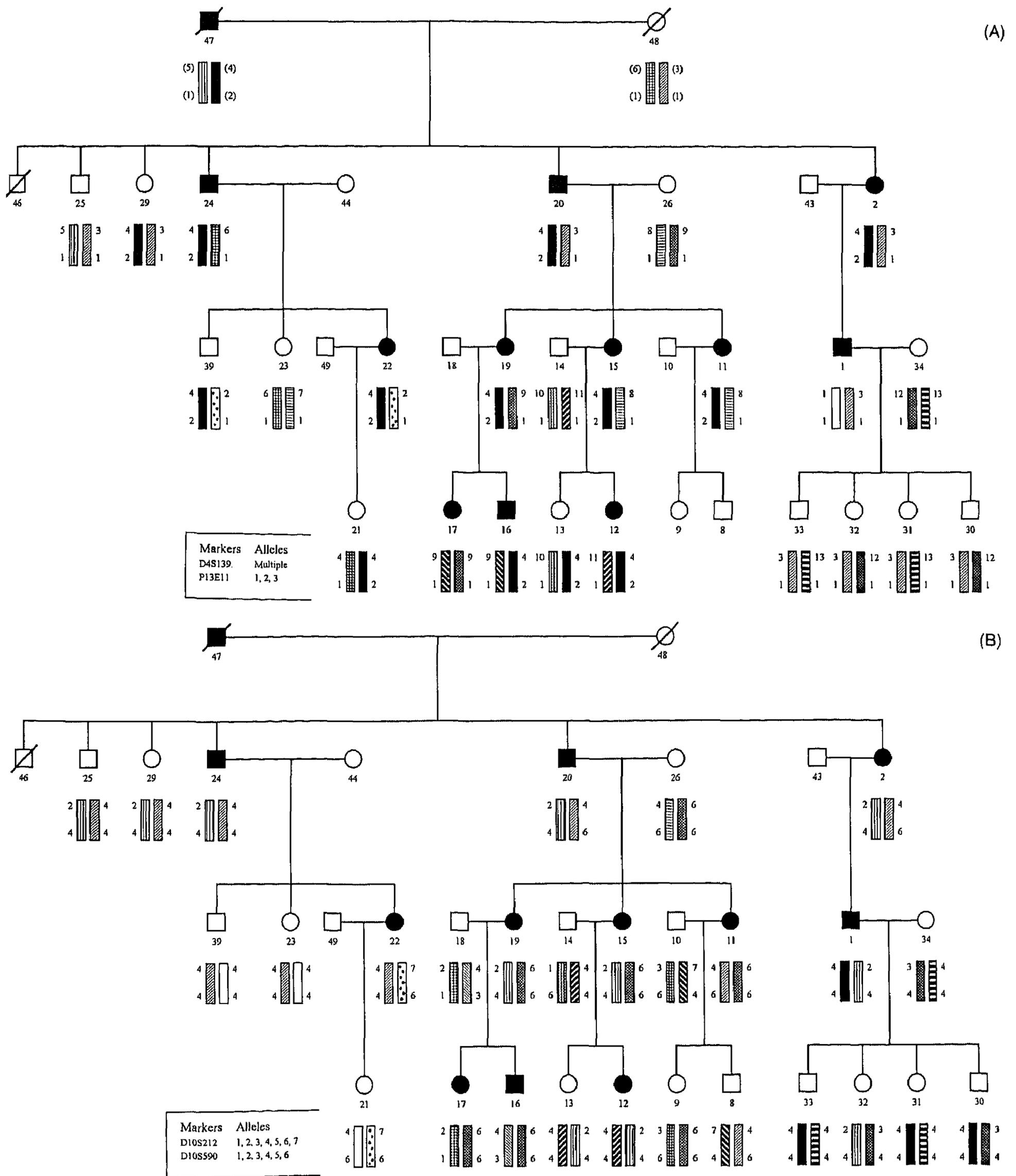
The molecular diagnosis of FSHD can be carried out successfully using probe p13E-11. However, due to the complexity of the banding pattern generated by p13E-11, a reliable diagnosis requires the detection of a *de novo* DNA rearrangement, or tight linkage of FSHD to a "small" (<28 kb) p13E-11 fragment. In 10% of 80 random individuals studied, "small" p13E-11 fragments can be found, which are not associated with FSHD. The occurrence of these nonchromosome 4 fragments in FSHD families can hamper the diagnosis. The latter fragments were mapped to chromosome 10q. Hence, adjacent markers from chromosomes 4 and 10 can be used to decide whether a given fragment originates from the FSHD locus on chromosome 4q, or from the other locus on chromosome 10q.

The mapping of a locus to chromosome 10q

which is highly homologous to the FSHD locus on chromosome 4q,<sup>13</sup> is in line with previous observations, that the p13E-11-derived cosmid clone shows cross-hybridization with chromosomes 1q12 and 10q, and the p-arm of acrocentric chromosomes using fluorescent in situ hybridization.<sup>12</sup> In addition, a CA repeat isolated from a p13E-11<sup>+</sup> chromosome 4 cosmid clone (D4F106S1)<sup>14</sup> was found to map to 10qter using a somatic cell hybrid panel (Wright TJ, Hewitt J, personal communication). On the basis of this similarity, it is possible that these regions on both chromosome 4 and 10 contain similar genes as well, including the putative FSHD gene. Therefore, FSHD families unlinked to the 4q locus might show linkage to the chromosome 10 locus. One such family was tested in this study, but appeared to be unlinked. However, this does not exclude the possibility that other FSHD families unlinked to chromosome 4 can show linkage to the markers on chromosome 10.

The striking homology between the 4q and 10q telomeres may offer an explanation for the disruption of the FSHD gene, because cryptic translocations between these two chromosomes might occur. At present this hypothesis cannot be tested due to the lack of probes distal to D4F104S1. However, the fact that sporadic patients show only one rearranged fragment and not two reciprocal products as would be expected from a translocation, strongly favors the deletion-type of mutation described by van Deutekom et al.<sup>9</sup>

The observation that a 30-kb p13E-11 fragment in the Danish FSHD family is linked to chromosome 4 but not to the FSHD is unexpected, as the so called normal p13E-11 alleles on chromo-



**FIGURE 4. (A)** Danish FSHD pedigree showing cosegregation of a 30-kb allele of p13E-11 with the chromosome 4q35 marker D4S139. The FSHD in this pedigree does not segregate with chromosome 4q35. Individuals 29, 39, 1, 21, and 17 all show a crossover. **(B)** Danish FSHD pedigree demonstrating nonlinkage of FSHD with the chromosome 10q markers D10S590 and D10S212. Individuals 22, 11, 16, 17, 13, and 32 all show a recombination.

some 4 are believed to be over 50 kb.<sup>13</sup> Therefore, one has to be careful in the interpretation of Southern blot detectable p13E-11 alleles (both smaller and larger than 28 kb) in relation to FSHD.

A clear relationship can only be proven if a de novo fragment arises with the sporadic occurrence of FSHD, or if in a family linkage to chromosome 4 is proven. Exclusion of linkage of a "small" frag-

ment to chromosome 10q markers might be an indication of a FSHD-associated p13E-11 allele on chromosome 4.

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