Original Paper

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Physical Mapping Evidence for a Duplicated Region on Chromosome 10qter Showing High Homology with the Facioscapulohumeral Muscular Dystrophy Locus on Chromosome 4qter

Abstract

p13E-11, a probe (D4F104S1 locus) derived from chromosome 4q35, detects *Eco*RI-rearranged fragments less than 28 kb in both sporadic and familial cases of facioscapulohumeral muscular dystrophy (FSHD). These fragments are smaller than those observed in healthy individuals. The interpretation of Southern blots is complicated by the fact that p13E-11 reveals two pairs of polymorphic alleles, one 4q35-specific and the other unlinked to 4q35, that sometimes overlap each other. We cloned a non-4q35 13-kb fragment not related to the disease from a sporadic FSHD patient of Italian origin. Haplotype analysis and in situ hybridization experiments showed that this fragment was located on the 10qter region. Restriction mapping of the 10qter clone, when compared with the 4q35 fragment, indicates a similar arrangement of *Kpn*I tandemly repeated units and flanking sequences. However 4q35 and 10q26 *Eco*RI clones can be distinguished by restriction analysis with *Sfi*I and *Sty*I. This observation could be exploited for future applications in the field of molecular diagnosis and genetic counseling. In addition the isolation of two 10q26 cosmid clones (D10S1484 and D10S1485) from a human genomic library and the construction of a detailed physical map, spanning about 40 kb, showed that the structural homology extended upstream of the *Eco*RI sites, suggesting that a duplicated FSHD locus resided in the subtelomeric region of the long arm of chromosome 10. We cannot exclude the involvement of the duplicated locus in the molecular mechanism of the disease and in the genetic heterogeneity of FSHD syndromes.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is a degenerative disease of muscles which affects specific muscle groups and displays a variety of phenotypic expression. The inheritance pattern of FSHD is autosomal dominant, but patients with a negative family history have frequently been described and are probably due to new mutations. The gene for FSHD was localized to chromosome 4q35 by linkage studies, distal to the linkage group D4S171-F11-D4S163-D4S139 [1, 2]. Recently Wijmenga et al. [3] described a probe, p13E-11 (D4F104S1), which detected polymorphic DNA fragments in normal subjects ranging between 30 and 300 kb, de novo DNA rearrangements less than 28 kb in sporadic FSHD patients and similar 'small' fragments in familial cases. The probe revealed two polymorphic loci and a 10-kb Y-specific fragment. By haplotype analysis one of the loci could be assigned to chromosome 4q35 [4] whereas the other locus segregated with 10qter microsatellite markers [5]. Physical mapping studies localized the p13E-11 probe on 4q35, close to a highly polymorphic tandem repeat unit defined by 3.3-kb *Kpn*I restriction fragments [3]. The most likely mechanism involved in the development of disease is a DNA rearrangement within the 4q35 locus resulting in deletion of a discrete number of tandem repeat *Kpn*I units, with the appearance of smaller *Eco*RI fragments of 14–28 kb in size [6]. So far, however, no cDNA clones mapping to chromosome 4q35 repeats have been identified [7, 8]. Since there is an association between deletion at the 4q35 locus and FSHD disease, other mechanisms such a long-range position effect of telomeric heterochromatin could be invoked in the pathogenesis of the disease [8].

We report here a study of an Italian sporadic FSHD family where the propositus inherited two 'small' p13E-11 DNA fragments [9]. The larger (23 kb) was derived from DNA rearrangements within the 4q35 region and was transmitted with the disease to the next generation; the smaller (13 kb) was not 4q35-specific and did not segregate with the disease. We cloned the small non-4q35 fragment and a similar-size 4q35 rearranged fragment derived from a sporadic FSHD patient in a phage vector. The results show that both 4q35 and non-4q35 human inserts contain a similar arrangement of *Kpn*I tandem repeats and flanking sequences, but are not identical, so that they can be distinguished by restriction analysis. In situ hybridization experiments have physically demonstrated that the non-4q35 p13E-11 locus (D10F104S2) is located on 10qter.

In addition the isolation and restriction mapping of two 10qter cosmid clones (D10S1484 and D10S1485) derived from a total human genomic library showed that the structural homology extended upstream of the *Eco*RI site.

Materials and Methods

Patients
4q35 haplotypes and p13E-11 fragments segregating in affected and unaffected members of family FSH21 are described in detail elsewhere [9]. Family FSH46 is a sporadic family in which the affected son carries a de novo rearrangement of 13 kb, absent in both healthy parents.

Southern Blot Analysis with 4q35 Markers
Restriction of genomic DNA and hybridization with L1LA5 (D4S163), pH30 (D4S139) and p13E-11 (D4F104S1) were as described in a previous paper [2].

Cloning of the Eco*RI* p13E-11 Fragments into λ *Phage*
Genomic DNA (200 µg) was completely digested with *Eco*RI (Biolabs) using the manufacturer's instructions. The digested fragments were separated by size on a 10–40% sucrose gradient by centrifugation in a SW41 Ti rotor at 22,000 rpm for 22 h. Fragments of

the appropriate size were selected after agarose gel electrophoresis of single gradient fractions, pooled and cloned into λ DashII/EcoRI (Stratagene). The ligation mixtures were packaged according to the Stratagene protocol and used to transform an *Escherichia coli* ER strain (p2392). The bacteriophage plaques were transferred to nitrocellulose filters (Schleicher & Schuell) and hybridized with ^{32}P-labeled p13E-11. After rescreening DNA was prepared from the lysates of positive phages following PEG precipitation and purification through CsCl density centrifugation at 38,000 rpm for 24 h using a SW50.1 Ti rotor. The positive clones isolated from the libraries constructed with the DNA of patients 21A and 46A are referred to as λ21A and λ46A.

Isolation of 10q26 Cosmids and Subclones

Cosmids C3 (D10S1484) and C5 (D10S1485) were isolated from a total DNA genomic library in pCos2EMBL, kindly provided by Poutzka et al. [10], by hybridization with a ^{32}P-labeled p13E-11 probe. Restriction mapping of the cosmids was achieved by partial digestion with EcoRI, KpnI, BglII, BamHI, PstI, SfiI, separation by agarose gel electrophoresis 0.5%, transfer to Hybond-N+ filters and hybridization with ClaI-BamHI and SalI-BamHI end fragments. The 3' region of cosmid C5 spanning from the last KpnI site to the cloning site was subcloned into pUC21 as three fragments: pEK, pPE and pECP of 1.1, 1.4 and 1.7 kb, respectively. The 3.3-kb tandem repeat was cloned as a whole or as three BamHI fragments of 1.4, 1.1 and 0.7 kb following complete BamHI digestion.

In situ Hybridization and Detection

Purified DNA from the whole cosmids and derived subcones was nick-translated with biotin-16-dUTP (Boehringer). Hybridization of metaphase chromosome preparations was carried out using a modification of the procedure described by Pinkel et al. [11]. The slides were treated in methanol-acetic acid (3:1) for 60 min at room temperature, air-dried and the target DNA denatured by immersing slides in 70% formamide/2 × SSC for 2 min at 65°C. Slides were quenched immediately in cold 70% ethanol and dehydrated in an ethanol series (70, 70, 90 and 90%). The hybridization mixture consisting of 100 ng of biotinylated probe, 5–10 μg of human Cot-1 DNA (BRL) in 50% deionized formamide, 2 × SSC, 10% dextran sulfate 1% SDS and 1% Denhardt's was denatured at 70°C for 10 min and incubated at 37°C for 60 min. The hybridization mixture was sealed under a coverslip and incubated at 42°C for 14–16 h. After hybridization the coverslips were removed by rinsing in 2 ×

SSC and washed twice in 50% formamide 1% SSC at 42°C for 5 min, washed twice in 2 × SSC at 42°C for 5 min and incubated in 4×TNFM (4 × SSC, 0.05% Tween 20, 5% nonfat milk) at 37°C for 20–30 min. The slides were then treated with alternating layers of fluoresceinated avidin and biotinylated goat antiavidin (Vector), both at 5 μg/ml concentration in 4 × TNFM. After the last wash the slides were rinsed twice in 2 × SSC at room temperature and mounted in 0.6 μg/ml propidium iodide and 3 μg/ml Citifluor (Citifluor, UK). Alpha satellite chromosome-specific probes (Oncor) were used to unambiguously identify chromosomes 4 and 10. The slides were analyzed by fluorescence microscopy with appropriate filters using a Confocal laser scanning microscope (Leica).

Pulse Field Gel Electrophoresis of Partial Digests of Isolated 10q26 Cosmids

Cosmid 5 DNA (300 ng) was digested with increasing amounts of BglII, KpnI and SfiI and electrophoresed at 15°C on 1% agarose gels. The run was at 400 V for 5 h in a Pharmacia LKB Gene Navigator system, with a constant switch interval of 0.5 s.

Results

Cloning and Restriction Mapping of 4q35 and Non-4q35 p13E-11 Fragments Derived from FSHD Patients

We cloned EcoRI fragments derived from 2 FSHD patients in λ DashII after sucrose density gradient centrifugation of EcoRI-digested genomic DNA. A non-4q35 fragment of 13 kb was cloned from patient FSH21A [9] and a 4q35 fragment of the same size was cloned from a sporadic case, FSH46A. The phage libraries derived from the 2 patients, about 5–20 × 10⁴ plaque-forming units, were screened using probe p13E-11. Restriction with the enzyme EcoRI showed that the selected λ-clones contained inserts of the correct size. The DNA extracted from the positive clones was submitted to total and partial digestion by using the following restriction enzymes: BglII, BamHI, KpnI, SfiI and StyI. DNA fragments derived from complete diges-
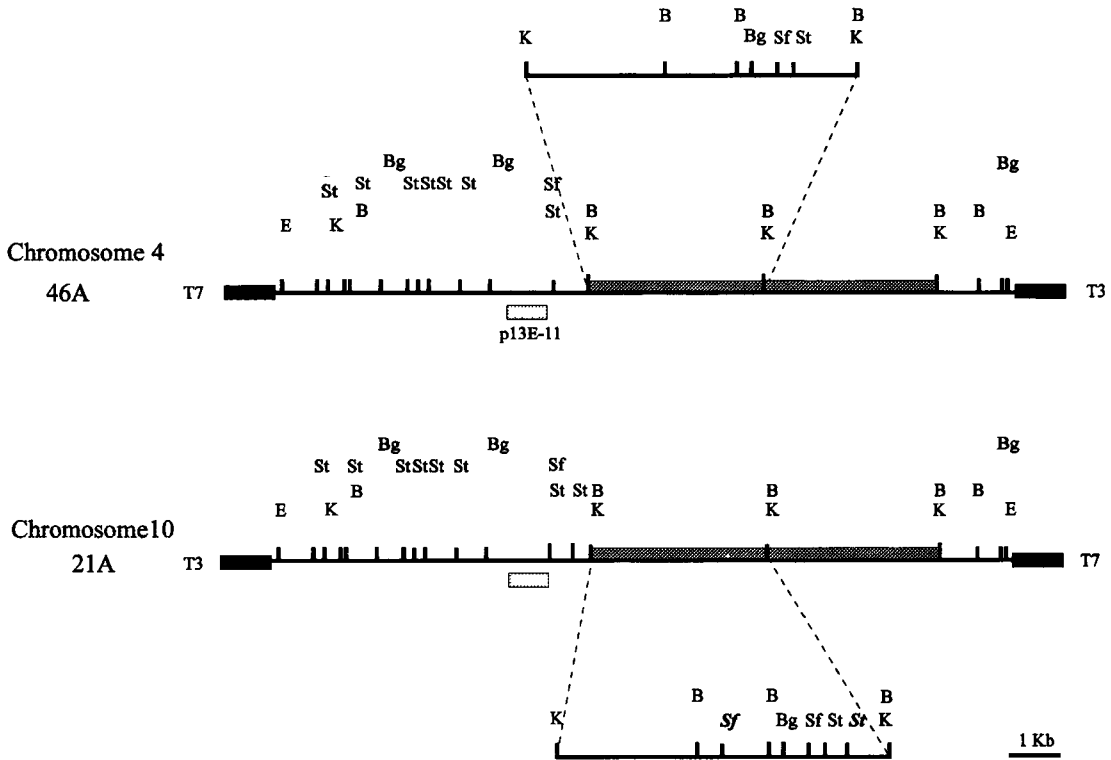
Fig. 1. Restriction maps of phage clones 46A and 21A. Restriction enzyme sites: E = EcoRI, K = KpnI, B = BamHI, Bg = BglII, Sf = SfiI, St = StyI. Each dotted bar represents the 3.3-kb repeat unit that is also expanded. The hatched box shows the position of the probe p13E-11. Partial digests of λ-clone DNAs were hybridized with γ-ATP-labeled T7 and T3 oligomers.

tion with BglII, BamHI and KpnI are identical. However some differences are detected using SfiI and StyI. Complete digestion with SfiI shows that all the 3.3-kb tandem repeat units derived from the 4q35-specific clone possess only one site for this enzyme, while the 3.3-kb repeat of the non-4q35 clone contains an additional SfiI site and is split into two fragments of 2.4 and 0.9-kb, respectively. The same is true for StyI which cut twice inside the KpnI units of the non-4q35 clones resulting in two fragments of 2.8 and 0.4-kb, respectively. A detailed map of restriction sites for BglII, KpnI, BamHI, SfiI and StyI was constructed for the different clones and is shown in figure 1. Both clones contain two 3.3-kb tandem repeats and the probe p13E-11 resides in the same 4.9-kb KpnI fragment. These results demonstrate that although a certain degree of homology exists between 4q35 and non-4q35 clones, the similarity is not complete and differences can be detected by simple restriction analysis within the KpnI repeat units of the p13E-11 EcoRI fragments.

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Homologous 4q35 and 10q26 Loci in
FSHD

*The Non-4q35 EcoRI Fragments
Segregate with 10qter Microsatellite
Markers in a Sporadic FSHD Family
(FSH 21)*

When *Eco*RI (or *Hind*III) fragments derived from healthy individuals are separated by pulse field gel electrophoresis (PFGE) and hybridized with probe p13E-11, four different fragments ranging in size from 30 to 300 kb could be distinguished [4]. Two of the fragments segregate in association with 4q35 markers (D4S139 and D4S163), while the remaining ones are transmitted in a random fashion with respect to the 4q35 markers [4]. Recently Wijmenga et al. [5] have demonstrated that the non-4q35 alleles segregate in association with 10qter microsatellite markers (D10S217, D10S212 and D10S590) in 28 CEPH families. We studied segregation of D10S212 and D10S590 markers in a sporadic FSHD family in which two *Eco*RI short fragments, 23 and 13 kb, could be detected by Southern blot after hybridization with p13E-11 (fig. 2). The 23-kb allele is a de novo rearranged fragment; it segregates with the 4q35 haplotype characterized by the allele 11.5-8.2-153 and is strictly associated with FSHD disease. The 13-kb fragment is unrelated with disease and was transmitted from the grandmother to the affected daughter with the 10qter haplotype 2-3, while the unaffected daughter not showing the short 13-kb allele inherited the 10qter 2-3 haplotype. In the third generation the granddaughter inherited from the mother the grandpaternal haplotype 3-2 and no short 13-kb fragment. It appears that the segregation of the 13-kb fragment is associated with the 10q26 haplotype 2-3.
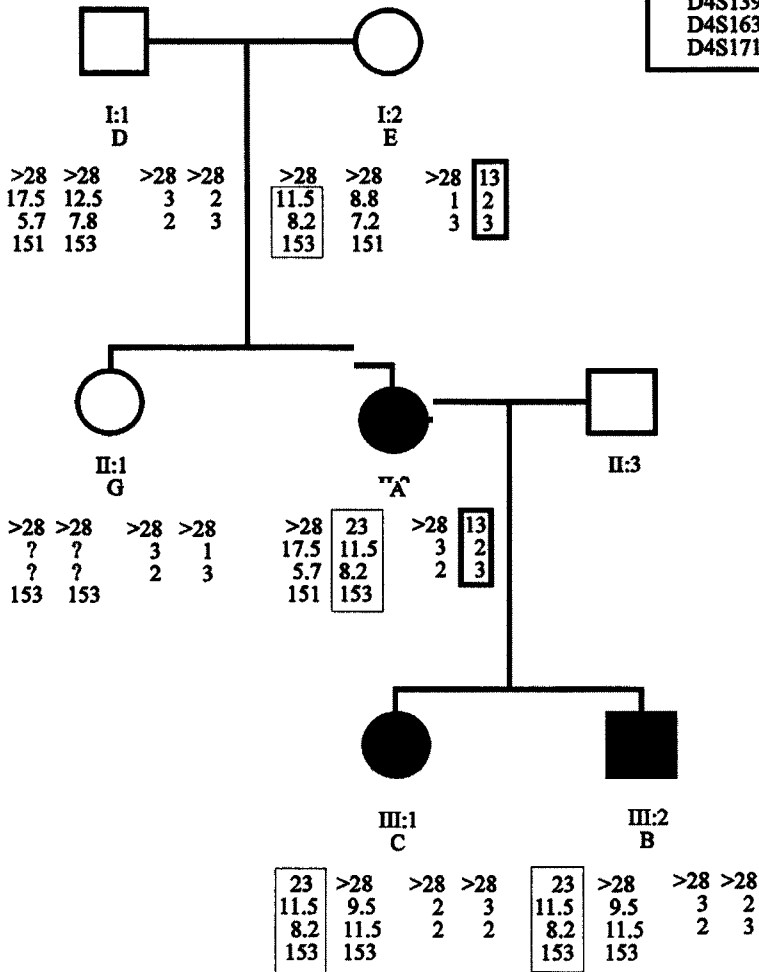
*Correlation between the Intensity of in situ
Hybridization Signals on Chromosome 10
and the Size of Non-4q35 p13E-11
Fragments Segregating in Family FSH 21*

The presence of two pairs of p13E-11 polymorphic alleles in this family, one on 4q35, the other on 10q26 was confirmed by PFGE and in situ hybridization experiments using a biotinylated probe containing 1.1 and 0.7 kb *Bam*HI fragments from 10qter 3.3-kb tandem repeat units. Hybridization was performed on metaphase chromosome spreads derived from lymphoblastoid cell lines of subjects 21E, 21A and 21C. One would expect to find no signal on chromosome 10 carrying the 13-kb fragment (containing 2 *Kpn*I repeat only), a faint signal on the deleted chromosome 4 carrying the 23-kb fragment (4 or 5 *Kpn*I repeats) and intense signals on the 4 and 10 chromosomes carrying larger alleles (more than 10 *Kpn*I repeats). As shown in figure 3 the unaffected subject 21E shows strong signals on both chromosomes 4, on one of the chromosome 10 homologues and no signal on the other; the affected subject 21A shows a reduced signal on the deleted chromosome 4 and no signal on one chromosome 10; the affected subject 21C presents a faint signal only on the deleted chromosome 4 and strong signals on the others. Therefore fluorescent in situ hybridization analysis provided indirect evidence that the non-4q35 p13E-11 locus was localized on 10qter in the FSHD family under study.

*Isolation and Characterization of Two
Non-4q35 Cosmid Clones from a Total
Human Genomic Library*

We used the probe p13E-11 to screen a total DNA human genomic library constructed in the vector pcos2EMBL [10]. As shown in figure 4, we fished out two overlapping clones: C3 containing a human genomic insert of 42 kb and C5 containing an insert of 45 kb. The restriction maps were constructed

Chromosome 4 markers	Chromosome 10 markers
D4F104S1	D10F104S2
D4S139	D10S212
D4S163	D10S590
D4S171	

I:1
D

>28 >28   >28 >28   >28 >28   >28 13
17.5 12.5   3   2   11.5 8.8   1 2
5.7 7.8   2   3   8.2 7.2   3 3
151 153   153 151

I:2
E

II:1
G

>28 >28   >28 >28   >28 23   >28 13
? ?   3   1   17.5 11.5   3 2
? ?   2   3   5.7 8.2   2 3
153 153   151 153

"A"

II:3

III:1
C

23 >28   >28 >28
11.5 9.5   2   3
8.2 11.5   2   2
153 153

III:2
B

23 >28   >28 >28
11.5 9.5   3   2
8.2 11.5   2   3
153 153

2

Fig. 2. Segregation of 4q35 and 10q26 markers in a sporadic FSHD family. The p13E-11 *Eco*RI alleles were separated by conventional agarose gel electrophoresis and the larger alleles (ranging between 30 and 300 kb) are referred to as >28 kb. The numbers 1–3 are the alleles of the microsatellite 10q26 markers. E = Grandmother; A = affected daughter; G = unaffected daughter; C = granddaughter.

Fig. 3. Fluorescent in situ hybridization on metaphase chromosome plates derived from 21E, 21A and 21C subjects, using a biotinylated probe containing cloned *Kpn*I repeats. **a-c** Chromosome 4 is indicated by arrows and chromosome 10 by boxes. On the right chromosome 4 and 10 homologues are selected and enlarged from the corresponding metaphase spread. **a, b** Chromosomes 10 are identified by using the specific pericentromeric α-satellite probe. The intensity of fluorescent signals on the tips of chromosome 4 and 10 corresponds to the different number of *Kpn*I repeats present in p13E-11 alleles segregating in FSH21 family.
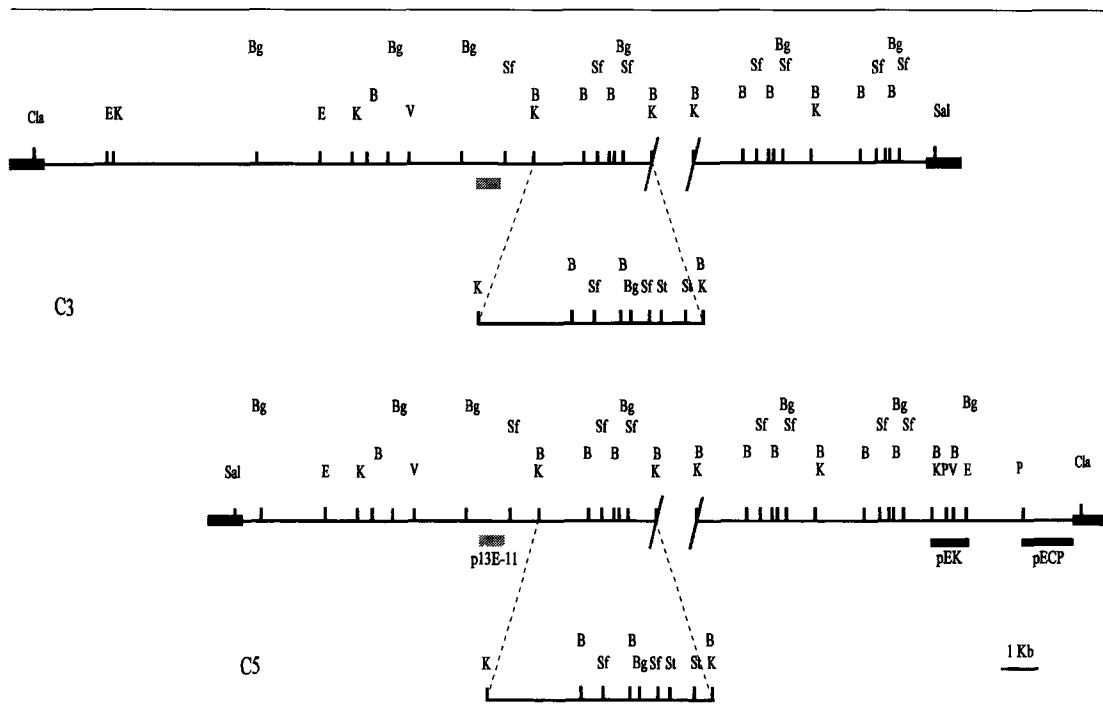
3a FSH 21E

chr 4
chr 10
FSH 21E

3b FSH 21A

chr 4
chr 10
FSH 21A

3c FSH 21C

chr 4
chr 10
FSH 21C
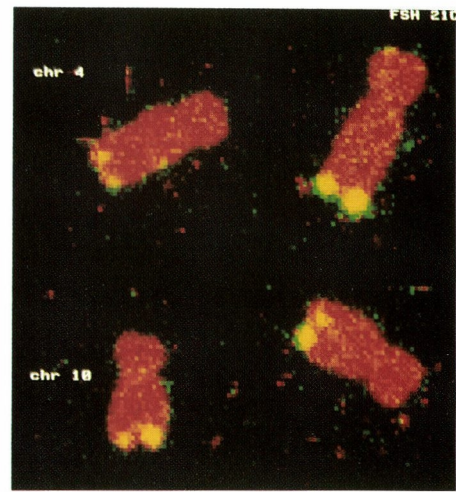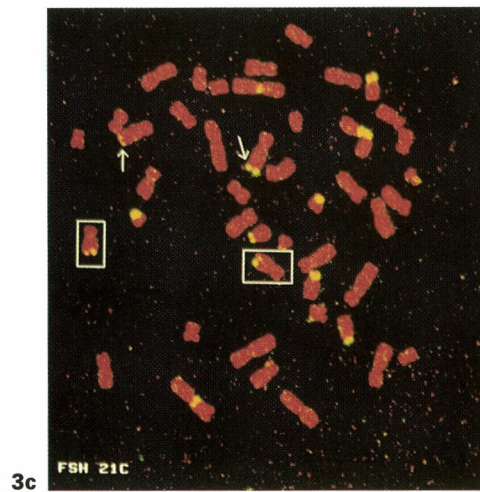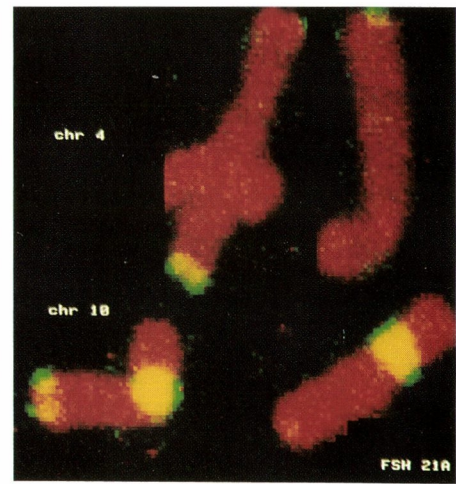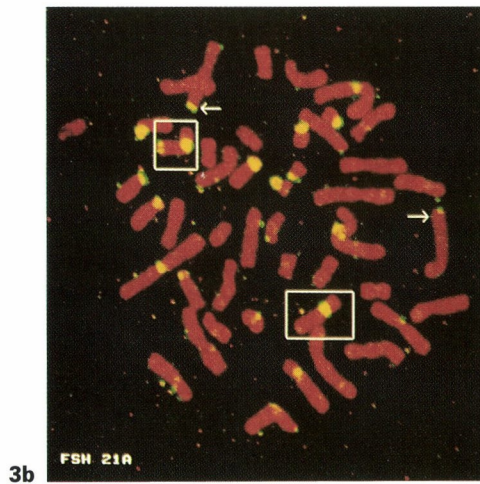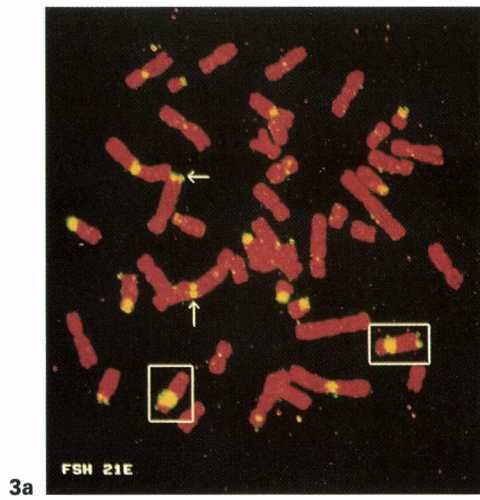
Fig. 4. Restriction maps of two 10q26 cosmid clones derived from a total human genomic library. Restriction enzyme sites: E = *Eco*RI, K = *Kpn*I, B = *Bam*HI, Bg = *Bgl*II, V = *Eco*RV, Sf = *Sfi*I, St = *Sty*I, P = *Pst*I. Only a few *Pst*I sites are reported. The expanded map corresponds to a 3.3-kb *Kpn*I repeat and shows an additional site for both *Sfi*I and *Sty*I enzymes. ▨ = Position of the probe p13E-11; ■ = subclones pEK and pECP derived from the region downstream the last *Kpn*I site. Only 3 *Kpn*I units are reported in the diagram to save space (see the gap in the baseline).

with the following enzymes: *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Kpn*I, *Pst*I and *Sfi*I. C5 contains a stretch of *Kpn*I tandem repeats of nearly 32 kb, corresponding to 10 *Kpn*I repeats, as determined by PFGE analysis of partial digests with *Bgl*II, *Kpn*I and *Sfi*I. Partial digestion with the enzyme *Sfi*I reveals a doublet for each module, since an additional *Sfi*I site cuts the repeat into two fragments. C5 extends 4 kb downstream of the *Kpn*I repeats and presents an additional *Eco*RI site. It thus contains the whole p13E-11 fragment subject to rearrangements in sporadic cases of the dis-

ease. C3 presents an interrupted p13E-11 *Eco*RI fragment containing nine 3.3-kb *Kpn*I repeats; it extends 5 kb upstream of clone C5 and stops within a truncated *Kpn*I repeat at the 3′ end. From C5 several DNA fragments derived from the region spanning from the last *Kpn*I repeat and the cloning site (fig. 4) were subcloned in pUC21. Each subclone was labeled and hybridized to genomic DNA either in the presence or in the absence of competitor DNA: no single copy sequences were detected. The sequence of the *Eco*RI end of the pEK subclone (fig. 5) shows about 90%

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Homologous 4q35 and 10q26 Loci in
FSHD

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          10        20        30        40        50
1   GAATTCTCATGTWTCTCTGCACTGATCCCCCAGGTGATGTNACTCCTGWCTAGGTTCA      pEK
    |||||||||| ||:|||||||||||||||||| |||||||||:|||||||:||||||||||
16  GAATTCTCACGTATCTCTGCACTGATCCCCAAGGTGATGTAACTCCTGTCTAGGTTCA      pLAM
          20        30        40        50        60        70


          60        70        80        90       100       110
    GCCTACAGSAGCGTTTMGRCATMTCTCTGCACTGATCACCCAGATGATGTAACACTTG
    | ||||||:|||||||| |:|||:|||||||||||||||||||||||| |||||||||||||
    GACTACAGGAGCGTTTTGACATATCTCTGCACTGATCACCCAGGTGATGTAACACTTG
             80        90       100       110       120


         120       130       140       150       160       170
    CTCTCAGCTCTGCCYACAGGGGCATTGAGACAGATCTCTCCAMTGCTCACTCAGGAGA
    ||| |||||||||:||||||||||||| |||||||||||||||:||||||||||||||||
    -TCTAAGCTCTGCCTACAGGGGCATTGTGACAGATCTCTCCAATGCTCACTCAGGAGA
    130       140       150       160       170       180


            180       190       200       210       220       230
    TGTAACAAMTTGTMCTGGGCTCNGTCTACAGGGGGGCTYTGTGATATATA-TTCCACTG
    ||||| ||:|||| ||||||| :|||||||||||||:|||||||||||| |||||||||
    TGTAA-AAATTGT-CTGGGCTTTGTCTACAGGGGGCTTTGTGATATATATTTCCACTG
      190       200       210       220       230       240


          240       250       260       270       280
    ATCAAACAGGTGATGTAAMCCTTGTCAAGGTTTGGCTCATTGGGGCTANGCGACGATA
    ||||||||||||||||||:|||||||||||||||||||| || |||||| :| || ||||
    CTCAAACAGGTGATGTAACCCTTGTCAAGGTTTGGCTTATAGGGGCTTTGTGA-GATA
          250       260       270       280       290


     290       300       310       320       330
    TCTCTGCACTGATCAGCCCAGGCAGNGAACACTTTGGCTACACT      333
    |||||||||||||||||||||||| ||:|||||| ||| |||||||
    TCTCTGCACTGATCAGCCCAGGGAGGGAACAC-TTGCCTACACT      341
    300       310       320       330       340
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Fig. 5. Sequence homology between the 3' end of 10q26 pEK subclone and 4q35 *Kpn*I-*Eco*RI subclone. Upper line: 10q26 pEK 3' end sequence; lower line: 4q35 *Kpn*I-*Eco*RI 3' end sequence from pLAM clone. This sequence is stored in GenBank, accession No. Z25282 [6]. The best alignment of the two sequences shows about 90% homology over 333 bp. The letters other than A, T, C, G correspond to the following: W = A or T, S = C or G, R = G or A, M = A or C, N = any, Y = T or C.

Fig. 6. Hybridization of pECP clone to a panel of somatic cell hybrids containing 4 and 10 human chromosomes. **a** In this panel regions of chromosome 4 present in different somatic cell hybrids are shown. Other human chromosomal material present in hybrids: HHW416 (none); HHW1372: Xqter-Xp21; HHW986: 5qter-5p15.1, 21; HHW582: 12qter-12p13; HHW848: 5pter-5p15.3, 12, 18, 20, 21, 22; HHW1499: 3, 5qter-5p22, 12, 18, 21; HHW886: 5, 6, 11, 12, 15, 16, 19, 21; HHW892: 1, 3, 5, 6, 10, 11, 12, 19, 21; HHW842: 5, 13; HHW693: 5cen-5p14.3. **b** Hybridization pattern of ³²P-labeled pECP clone on chromosome 4 hybrid DNAs digested with *Eco*RI. The first lane on the left contains human genomic DNA and the others correspond to the hybrids described in **a. c** Hybridization pattern of ³²P-labeled pECP clone to cell DNAs digested with *Pst*I (lanes A and B) and *Eco*RI (lanes C and D). CHO = Hamster cell line as a control; 109 = hybrid cell line containing chromosome 10 as the only human component.

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Homologous 4q35 and 10q26 Loci in
FSHD
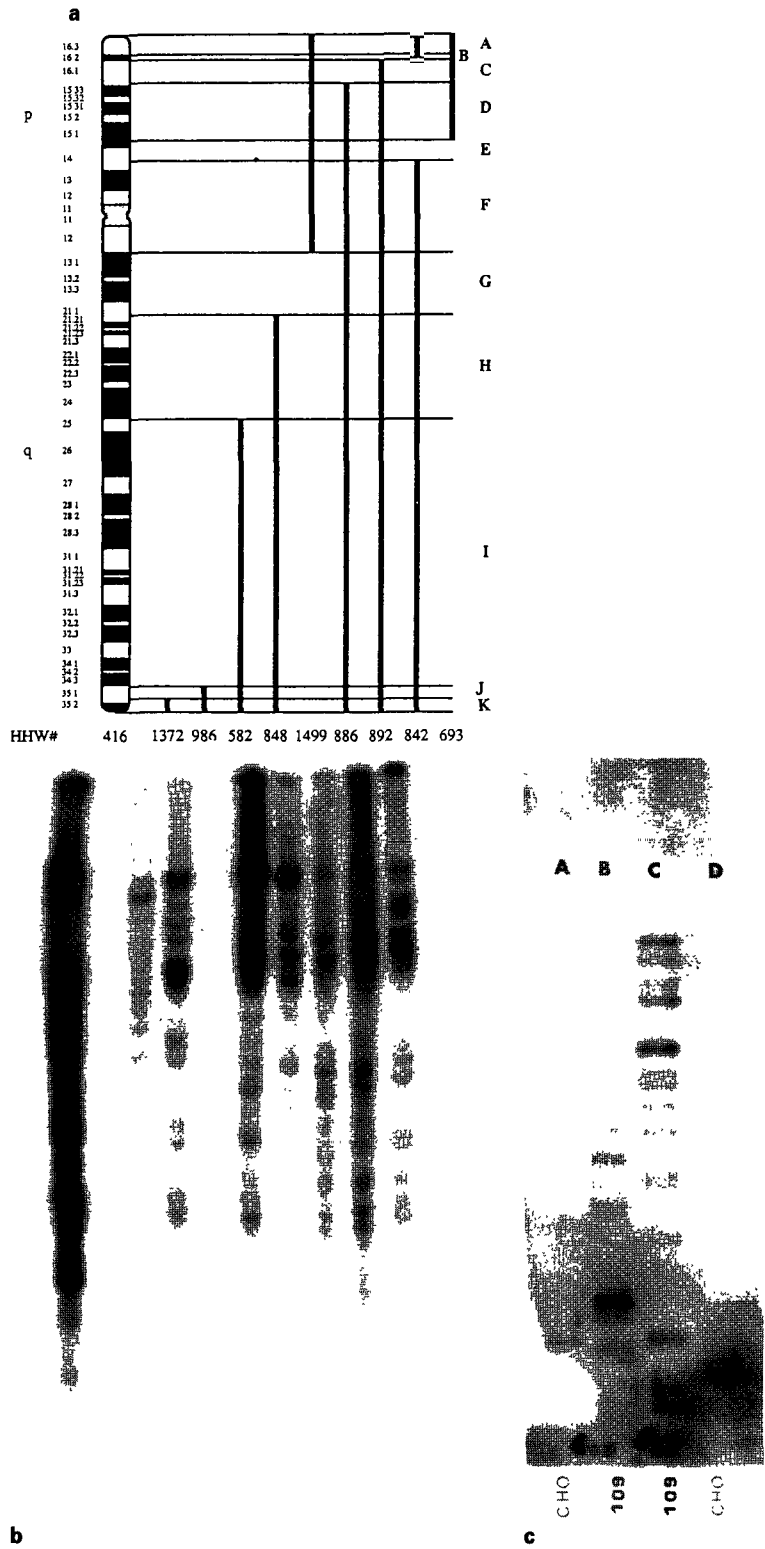
nucleotide identity over 333 bp with the corresponding 4q35 *Kpn*I-*Eco*RI fragment detected by pLAM, as described by van Deutekom et al. [6]. The pECP subclone was hybridized to a panel of somatic cell hybrids containing different portions of chromosome 4 and the whole chromosome 10. As shown in figure 6b, the pECP clone does not hybridize on the lane of the whole chromosome 4 (HHW 416) but a complex pattern of bands appears in other lanes. This result can be explained by the presence of repetitive sequences in the pECP clone that hybridize with cognate sequences derived from other chromosomes contaminating the somatic hybrids (see fig. 6a). In figure 6c, hybridization with the pECP clone shows a pattern characterized by multiple bands on the lane of the chromosome 10 hybrid cell line. In this case we cannot absolutely exclude that some fragments derive from other chromosomes. The negative results with the hybrid containing the whole chromosome 4 are meaningful, while the positive results with the chromosome 10 cell line do not definitely prove the 10qter origin of the pECP clone.

In situ hybridization with the whole cosmid C5 shows hybridization signals at the tips of chromosome 4 (4q35) and chromosome 10 (10q26), in the pericentromeric region of chromosome 1 (1q12), and in the p12 region of the 5 acrocentric chromosomes (21, 22, 13, 14 and 15). This multiple pattern is due to the abundance of repetitive sequences in the cosmid which cross-hybridize with the pericentromeric and telomeric heterochromatin of several human chromosomes [8].

In conclusion, restriction mapping and in situ hybridization experiments performed on non-4q35 cosmid clones, when compared with those published by Hewitt et al. [7] and Winokur et al. [8] for the 4q35-specific cosmid clones, show a high degree of similarity. However the presence of additional sites for *Sfi*I and *Sty*I within the *Kpn*I repeat unit and the lack of hybridization to chromosome 4 on the panel of somatic cell hybrids make it possible for us to attribute the cosmid clones to chromosome 10q26. The point must be stressed that sequence homology between 4q35 and 10q26 clones is not confined to the p13E-11 *Eco*RI fragment, cloned into phage vector, but extends 8 kb upstream of the 5′ *Eco*RI site as suggested by comparison with the 4q35 *Eco*RI-*Kpn*I restriction map published by Winokur et al. [8].

Discussion

Soon after the isolation of the p13E-11 probe, many objections were raised concerning the reliability of this marker for diagnostic and preventive purposes. The probe detects a complex pattern of bands after *Eco*RI digestion of genomic DNA and it is not easy, or even impossible, to distinguish 4q35-specific alleles from non-4q35. This is due to the observation that non-4q35 alleles are, on average, of smaller size than the specific ones (71.5 vs. 118.5 kb by PFGE) according to Wijmenga et al. [4] and in some cases overlap with the rearranged 4q-specific fragments. We observed at least two of these cases: a fragment of 13 kb not related to disease-segregating in family FSH 21 [9] together with a 23-kb 4q35 fragment; a fragment of 23 kb carried by the unaffected mother of the propositus with the same electrophoretic mobility of the rearranged 4q35 fragment transmitted from the affected father. In addition we also demonstrated, in agreement with Gilbert et al. [12], the presence of genetic heterogeneity in a sample of 21 Italian FSH families with a frequency estimated by the Homog test of 0.15 [2]. Therefore we concluded that the probe p13E-11 could not be used for the diagnosis and genetic counseling of FSHD in the ab-

sence of significant linkage of the small fragments (and possibly other 4q35 markers) to the disease locus in the family under study. The finding that 4q35 and non-4q35 clones have a high degree of homology, but can be distinguished by digestion with appropriate restriction enzymes is of great importance for the future applications of p13E-11 in the diagnostic and preventive fields. An accurate comparison of nucleotide sequences between 4q35 and non-4q35 $EcoRI$ fragments will most likely result in the identification of a certain number of restriction enzymes able to cut specifically in either one of the alleles, making simple the interpretation of the Southern blots from the affected and unaffected members of FSHD families.

We have shown that the restriction maps of the 10q26 phage and cosmid clones are similar to the ones described for the 4q35 clones and the structure homology includes the $KpnI$ tandem repeat units and flanking sequences. The sequence homology still holds in the case of the pEK subclone corresponding to the 3' end of the p13E-11 $EcoRI$ fragment. However the observation that the pECP subclone fails to hybridize to chromosome 4 suggests that the 3' limit of the homology lies between pEK and pECP. We can deduce that the homology covers at least 14 kb upstream of the $KpnI$ tandem repeats. Since the homology involves the $EcoRI$ region subject to rearrangements in the 4q35-linked FSHD cases, we postulate that a second locus with a high degree of sequence homology is present in the 10qter region and could be implicated in the 4q35-unlinked cases of disease.

We cannot exclude that unequal crossovers occur not only between homologous 4q pairs, but also between 4q and 10q subtelomeric segments, resulting in the loss of a discrete number of tandem repeats. If this is true one should find patients with a hybrid set of $KpnI$ units, recognized by restriction analysis.

Alternatively the 10q26 region might contain a duplicate copy of the gene involved in FSHD disease, but the structural organization of the subtelomeric region is such that the position variegation effect cannot be exerted. Therefore the gene would be inactivated by single point mutations occurring at a very low frequency compared to the deletion of $KpnI$ sequences. In this case it is expected that among the 4q35-unlinked families at least a few would show linkage to the 10q26 markers.

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