

Sequence Homology between 4qter and 10qter Loci Facilitates the Instability of Subtelomeric *KpnI* Repeat Units Implicated in Facioscapulohumeral Muscular Dystrophy

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Summary

Physical mapping and in situ hybridization experiments have shown that a duplicated locus with a structural organization similar to that of the 4q35 locus implicated in facioscapulohumeral muscular dystrophy is present in the subtelomeric portion of 10q. We performed sequence analysis of the p13E-11 probe and of the adjacent *KpnI* tandem-repeat unit derived from a 10qter cosmid clone and compared our results with those published, by other laboratories, for the 4q35 region. We found that the sequence homology range is 98%–100% and confirmed that the only difference that can be exploited for differentiation of the 10qter from the 4q35 alleles is the presence of an additional *BlnI* site within the 10qter *KpnI* repeat unit. In addition, we observed that the high degree of sequence homology does facilitate interchromosomal exchanges resulting in displacement of the whole set of *BlnI*-resistant or *BlnI*-sensitive *KpnI* repeats from one chromosome to the other. However, partial translocations escape detection if the latter simply relies on the hybridization pattern from double digestion with *EcoRI*/*BlnI* and with p13E-11 as a probe. We discovered that the restriction enzyme *Tru9I* cuts at both ends of the array of *KpnI* repeats of different chromosomal origins and allows the use of cloned *KpnI* sequences as a probe by eliminating other spurious fragments. This approach coupled with *BlnI* digestion permitted us to investigate the structural organization of *BlnI*-resistant and *BlnI*-sensitive units within translocated chromosomes of 4q35 and 10q26 origin. A priori, the possibility that partial translocations could play a role in the molecular mechanism of the disease cannot be excluded.

Introduction

Linkage and physical mapping strategies have identified, on human chromosome 4q35, a polymorphic *EcoRI* locus that is composed of a variable number of 3.3-kb *KpnI* tandem-repeat units (D4Z4) and that appears to be tightly linked to facioscapulohumeral disease (FSHD). In normal subjects the p13E-11 *EcoRI* fragment containing the *KpnI* repeats varies in size, in a range of 50–300 kb, whereas in sporadic and familial cases of FSHD the disease cosegregates with a fragment significantly below this—that is, in the range of 10–34 kb (Wijmenga et al. 1992; 1994; Passos Bueno et al. 1993; Upadhyaya et al. 1993; Weiffenbach et al. 1993; Cacurri et al. 1994). It has been demonstrated that the 4q35 rearrangement involves the deletion of an integral number of 3.3-kb repeat units (van Deutekom et al. 1993). Each copy of the *KpnI* repeat unit contains two homeobox sequences and two different classes of human GC-rich repetitive sequences: hhspm3, a low-copy-repeat family, and LSau, a middle-repetitive family, closely associated with beta-satellite DNA. The two homeodomains are separated by a 45-nt spacer, and the entire sequence contains an open reading frame (Hewitt et al. 1994). In the human genome the 3.3-kb repeats are present on several chromosomes other than 4q—specifically, on the short arms of acrocentric chromosomes, 1q12 and 10qter—as shown by in situ hybridization experiments (Deidda et al. 1995; Winokur et al. 1996). The spreading of *KpnI* repeat sequences on human chromosomes generates artifacts in the interpretation of DNA analysis in normal subjects and in FSHD patients, since (a) multiple *EcoRI* fragments are observed after hybridization with p13E-11 probe and (b) time-consuming linkage analysis with 4q35 and 10qter markers is required to assign the chromosomal origin of the alleles (Weiffenbach et al. 1993; Deidda et al. 1994). We found that the 10qter locus shows a high degree of homology with the 4q35 locus, as shown by restriction mapping and in situ hybridization experiments (Deidda et al. 1995). However, restriction-site differences between the

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two loci can be exploited for cleaving the interfering 10qter alleles: double digestion of genomic DNA with *EcoRI* and *BlnI* (hereafter denoted “*EcoRI/BlnI*”) resulted in a marked improvement of molecular diagnosis of and genetic counseling for the disease (Deidda et al. 1996). In this paper we report a complete sequence analysis of the p13E-11 region and the *KpnI* repeat unit derived from 10qter, a total of 4,000 bp. When the 10qter sequences are compared with the homologous 4q35 sequences published by other laboratories (Hewitt et al. 1994; Lee et al. 1995), the degree of homology is 98%–100%; this facilitates interchromosomal exchanges, resulting in the appearance of hybrid chromosomes (van Deutekom et al. 1996). Therefore, the variations in the canonical number of *BlnI*-resistant fragments separated by pulsed-field gel electrophoresis (PFGE) has to be coupled with the study of segregation of other 4q35 and 10qter markers, in order to identify the origin of atypical *BlnI* fragments. In addition, the combined use of *BlnI* and a novel restriction enzyme named “*Tru9I*,” which cuts at both ends of the array of tandemly repeated *KpnI* units of different chromosomal origin, allows the use of cloned *KpnI* sequences as a probe; this probe is able to detect cases of partial translocations, even if the p13E-11 single-copy sequence is deleted.

Patients and Methods

Patients

Diagnostic criteria for FSHD followed the guidelines proposed by the European Expert Group on FMD (Padberg et al. 1991). In each kindred, at least one patient had a muscle biopsy and electromyography to confirm the diagnosis of FSHD.

DNA Extraction

In order to isolate high-molecular-weight DNA, lymphocytes derived from either freshly collected blood or Epstein-Barr virus-transformed cells were included in 0.5% Low Melt agarose (Biorad) and were incubated with sarcosyl-proteinase K, according to the procedure described by Pharmacia. DNA samples for digestion with *Tru9I* were extracted according to the salting-out procedure (Miller et al. 1988).

Southern Blot Analysis with 4q35 Markers

[³²P] labeling and hybridization, with L1LA5 (D4S163), pH30 (D4S139), and p13E-11 (D4F104S1), of digested genomic DNAs were performed as described in a previously published paper (Cacurri et al. 1994). For the *KpnI* probe, we used the 1,179-bp fragment

derived from *BamHI* digestion of the 3.3-kb *KpnI* repeat unit.

Restriction of Genomic DNA

Digestion of genomic DNA, not included in agarose blocks, with *Tru9I* enzyme (Boehringer) was performed by incubation of 5 mg of genomic DNA with 20 U of enzyme, at 65°C overnight. Restricted samples were separated by PFGE agarose, as described below.

BlnI (Amersham) and *EcoRI* (Biolabs) digestions of DNAs included in agarose blocks were performed, respectively, in 200 ml with 60 U of enzyme and in 400 ml with 150 U of enzyme. Blocks were first rinsed three times in sterile water and then were equilibrated, for 30 min at room temperature, in the appropriate restriction-enzyme buffer. The enzymes were added in three equal portions, at 1-h intervals. After digestion, the blocks were rinsed three times in sterile water and then were equilibrated, for 30 min at room temperature, in the electrophoresis buffer (0.5 × Tris-borate EDTA).

PFGE

Electrophoresis was performed at 12°C in a Pulsaphor electrophoresis unit with an HEX electrode (Pharmacia LKB); agarose gel (Biorad) was 1.2% in 0.5 × Tris-borate EDTA. The running procedure was performed at 300 V, in 4 steps: the first step was 1 h 50 min, with pulses of 0.3 s; the second was 3 h 50 min, with pulses of 0.5 s; the third was 1 h 50 min, with pulses of 1 s; and the fourth was 2 h 50 min, with pulses of 5 s. After electrophoresis, the gels were stained with ethidium bromide and were photographed; DNA was then transferred to Hybond N+ (Amersham) for hybridization with [³²P]-labeled p13E-11.

Cloning and Sequencing

Both the single-copy p13E-11 probe and the *KpnI* repeat unit were obtained from the C3 10qter cosmid isolated from a total DNA genomic library, as described elsewhere (Deidda et al. 1995). A 4.9-kb *KpnI* fragment was subcloned in pUCBM21, and primers were designed on the basis of the sequence of the p13E-11 probe and of the D4Z4 tandem repeat, as published by Hewitt et al. (1994). The 3.3-kb *KpnI* repeat units were digested with *BamHI*, and the restricted fragments (1,387, 737, and 1,179 nt) were subcloned in pUCBM 21 and were sequenced by use of several primers designed on the basis of both the vector and the 4qter sequences, as published by Lee et al. (1995). The nucleotide sequence of double-stranded DNA was determined by the dideoxy chain-termination method with T7 DNA polymerase (Pharmacia), according to the manufacturer's protocol, and with [³⁵S]-dATP as the labeled deoxyribonucleotide.

Results

Sequence Analysis of the p13E-11 Single-Copy Probe and 3.3-kb KpnI Repeats Derived from 10qter

We performed sequence analysis of the components of the 10qter locus that were homologous to the 4q35 region. Figure 1 shows ~1,000 nucleotides of chromosome 10 sequence containing the p13E-11 probe and the initial part of the *KpnI* repeat unit (*lower sequence*), compared with the homologous tract from chromosome 4 (*upper sequence*) published by Hewitt et al. (1994). Only a few changes were observed, which were due to 4 base insertions/deletions and 13 base substitutions, which do not alter the restriction pattern of the fragment. The homology reaches values of 97.8%, in agreement with the equal intensity that the different-origin p13E-11 alleles show on Southern blot, a pattern that cannot be modified by low-ionic-strength repeated washings. Figure 2 shows ~3,300 nt of the *KpnI* repeat unit from chromosome 10 (*upper sequence*), compared with the homologous stretch from chromosome 4 (*middle se-*

quence). The average homology is 96%, with a number of discrepancies that are due to base insertions/deletions (82 total) and base substitutions (52 total). When the degree of homology is analyzed for the single components of the *KpnI* repeat units (fig. 3), the LSau family displays 94% homology, the hhsmp3 family displays 97.7% homology, the pool of other repeated sequences displays 95.2% homology, and the homeodomain regions appear to be almost identical (100% homology).

Interchromosomal Exchanges between the Homologous 4qter and 10qter Loci, Resulting in Variations in the Number of BlnI-Resistant p13E-11 Fragments (Hybrid Chromosomes)

When *EcoRI* p13E-11 alleles are separated by PFGE, which allows a better resolution of the high-molecular-weight (>50 kb) alleles, four autoradiographic bands are observed: two derived from 4q35 and two from 10qter. After *EcoRI/BlnI* digestion of genomic DNA, the two alleles of 10qter origin that contain *BlnI*-sensitive *KpnI* repeats are converted into 2.8-kb fragments that run out

P13CH4	1	GATCTATACAGCACTGATCAAACTAGCAATTTAAAGACTGGCTCAGTAAAGGGGGATACCG	P13CH4	671	CTGTCTTATGCTTATTCTACTCTGCAATCCCTTAAGGCTTTTTCTCTCCCTCCCAAAATCT
P13CH10COM	1	GATCTATACAGCACTGATCAAACTAGCAATTTAAAGACTGGCTCAGTAAAGGGGGATACCG	P13CH10COM	669	CTGTCTTATGCTTATTCTACTCTGCAATCCCTTAAGGCTTTTTCTCTCCCTCCCAAAATCT
P13CH4	62	ACAGCAATAGTCCAGSCTATAGATTTCTAGGTGCTTCACAACACATTTCCAAATATATTTT	P13CH4	732	TAAAGTGCAATTCGAACCTCAGAGGCAAAATCTCCCAAGAACTCTTGAGAACATAAATGATC
P13CH10COM	62	ACAGCAATAGTCCAGSCTATAGATTTCTAGGTGCTTCACAACACATTTCCAAATATATTTT	P13CH10COM	730	TAAAGTGCAATTCGAACCTCAGAGGCAAAATCTCCCAAGAACTCTTGAGAACATAAATGATC
P13CH4	123	CTCTGGACTTcGcTGTTTCCAAAGTAAAGAGAATTTTTCTCAATGATATTTAGATTTCT	P13CH4	793	TGACTAGTTTGGCATTGCTTTTGGGGATctgggAAAATCTGTGCACACTTCTGGAGCCCT
P13CH10COM	123	CTCTGGACTTgGgTGTTTCCAAAGTAAAGAGAATTTTTCTCAATGATATTTAGATTTCT	P13CH10COM	791	TGACTAGTTTGGCATTGCTTTTGGGGATcGggAAAATCTGTGCACACTTCTGGAGCCCT
P13CH4	184	ATTGCTATTTTCTTCCCAAgTTGGCTGTCTGTGGTATTCAGTTTCTACTAGTGTGTAGC	P13CH4	854	TGTCAAGCCATTaTTTATAAATCTATTGTGCCTCAAGTCAaAGTatAtGatgatttGGAG
P13CH10COM	184	ATTGCTATTTTCTTCCCA TTGGCTGTCTGTGGTATTCAGTTTCTACTAGTGTGTAGC	P13CH10COM	852	TGTCAAGCCATTtTTTATAAATCTATTGTGCCTCAAGTCAgAAGT GtgtgaggGGAG
P13CH4	245	AAACACTCATCTTTCTTCTCAGCAGaCACAAACTgTCATCTATATGACTCCATCATGTCTCT	P13CH4	915	ATGGGGAGACATTGGGATGCGCGcGCTGGGGCTCT CCACAGGGGGCTTCTGTGAGCCCA
P13CH10COM	244	AAACACTCATCTTTCTTCTCAGCAGgCACAAACTcTCATCTATATGACTCCATCATGTCTCT	P13CH10COM	909	ATGGGGAGACATTGGGATGCGCG CGCTGGGGCTCTcCACAGGGGGCTTCTGTGAGCCCA
P13CH4	306	TCAGCACTCCACATCAGGAGAGAAGAATCTAGTCATTAGACAATTTATCAAAAAGCCAAA	P13CH4	975	GGCAGCGAGGGCGCCCCGCGcTGTCAGCCAGCCAGCCGCGcGgCAGAGGGGATCTCC
P13CH10COM	305	TCAGCACTCCACATCAGGAGAGAAGAATCTAGTCATTAGACAATTTATCAAAAAGCCAAA	P13CH10COM	969	GGCAGCGAGGGCGCCCCGCGnTGTCAGCCAGCCAGCCGCGC GgCAGAGGGGATCTCC
P13CH4	367	CATTTCACACATATTTCTACTGTTTTAATTTCTCTCTGAAGGAGATACTGGGAGTTGGGCA	P13CH4	1036	AACTGCCCCGGCGcGCGATTTCGCCTACGcGcCCCCGcTCTCTCCGGAC GgGcCTCT
P13CH10COM	366	CATTTCACACATATTTCTACTGTTTTAATTTCTCTCTGAAGGAGATACTGGGAGTTGGGCA	P13CH10COM	1027	AACTGCCCCGGCnCGGATTTCGCCTACnCCnCCCCGcTCTCTCCGGACnGnGnCTCT
P13CH4	428	TTTTCTcATTAGCCCAgTACTGTTCTGGGTGAAAAAAaAAACTGCAGTGAGCA GCTGTA	P13CH4	1096	CCCACCTCAGGCTCCTCGGtGGCTCgCACC GgcAAAGCGG aGACCGGAacc
P13CH10COM	427	TTTTCTgATTAGCCCAgTACTGTTCTGGGTGAAAAAAgAAACTGCAGTGAGCAgGCTGTA	P13CH10COM	1088	CCCACCTCAGGCTCCTCGGgGGCTCgGCACCcggGcaAAAGCGGgagcGACCGGA
P13CH4	488	AGCCAGACTTCATCAAAATTTCTGCAcCAATGAAAAAAATTTACAAGAGAAAAcAAAA	P13CH4	1151	gcagcagcagcgcctgagcctgagcctgcaoggtgggagagcctggggcagcctcaagcgg
P13CH10COM	488	AGCCAGACTTCATCAAAATTTCTGCAgCAATGAAAAAAATTTACAAGAGAAAAc AAAA	P13CH4	1212	gccccatggccaaagtgtgcttggacaacagagctccagggggagctcgtgtgggactggg
P13CH4	549	AACCCATTAAACGTCACGGCAAGGCCAGAGTTGAATATACTGTGGTCTCTcGCTCC	P13CH4	1273	gccccgactccaggtgccccggggggg
P13CH10COM	548	AACCCATTAAACG CACGGCAAGGCCAGAGTTGAATATACTGTGGTCTCTcGCTCC			
P13CH4	610	AGTGCAAACTGTTTTTCAGAAAGCCTaCTTcTATTTTCTCTGCTGAACAGAGGAACATTTT			
P13CH10COM	608	AGTGCAAACTGTTTTTCAGAAAGCCTgCTTtTATTTTCTCTGCTGAACAGAGGAACATTTT			

Figure 1 Sequence of p13E-11 single-copy probe derived from 10qter: comparison with the homologous 4q35 sequence. *Upper sequence*, Chromosome 4 sequence published by Hewitt et al. (1994). *Bottom sequence*, 840 nt of the p13E-11 single-copy probe and 200 nt of the *KpnI* tandem-repeat unit from the 10qter *KpnI* fragment.

Ch10 1-ATCT.....-C.
Ch4 1CG.....GTCTC.....CG..G.
consensus GGGGGCTGGGGCTCTCCACAGGGGGTTTGTGAGCCAGGAGAGGGGCGCCCGGGCTGAGCCAGCCAGGCGGGCCgCAGAGGGGg----CAACTCTCCCGGGCgCgGgG

Ch10 117G.....G..AC.
Ch4 123GC.....C..GA.
consensus GATTTTGGCTAGCGCCCGGCTCTCCGAGcCGGGGGTCTCCACCTCAGCTCCTGG-GGCTC-GCACCCGGGCAAAAGC-GG--GGACCGGACCCGACGGCGAGGCCTG

Ch10 237-A.....GGC.....-
Ch4 245CAC.....CCG.....C
consensus CCGGGCCCTGGGGTGGCACAGCTGGCCCTCAAGCGGGCGCAGGCAAGGGGTGCTTGGCCa-CCACGTCCAGGGAGTCCGTGTGG--TGGGCGGGGTCCCAAGTc

Ch10 356C.....-A.....-
Ch4 367T.....GACGCCTCC.....G.
consensus GCGGGGGCGTGGGAACCCCAAGCCGGGGCAG-TCCACTCTCCAGCCCGGGCCCGGGgagcctc-GCTCCGGGGGACGGGCGAGTGAAGGCATCCCGTCCAGGGCC

Ch10 469C..G.....G.....
Ch4 489C.....T.....
consensus TCAGGAGCCcGG-GCCCTGGTCTGACTCCCTGGGGCTGTGCT-GATGAGTCTCTGGGAGCCGGAGTTCTGAGCAGGGCGCAACTCTCTAGAAACGGAGGCCCGGGGAGCT

Ch10 591C.....
Ch4 610-
consensus GGAGGCTCGAAGAGGcCGCTCGTGAAGCACCTCAGCGAGGAATAACGGGCTCTGCTGGAGGAGCTTAGGACCGGGGTGGGACGGGTCCGGTGGTTCCGGGCAGGGCCG

Ch10 713G.....-G.....
Ch4 731A.....GGG.....
consensus TGGCTCTCTTTCCGGGGAAC-CCTGGCTGGCTAGCGAGgggCGTGTCTCCGGCCCGCCCTCCACCGGGCTGACGGGCTGGGATTCCTGCTCTAGg-C-AGGCCGGTGGAGAGT

Ch10 831A.....NN.....
Ch4 853
consensus CCACAC-GCGGAACTCCATTCTTCTCTGGGATCCCGGGATCCAGAGCCnngggcAGTACAGCAGGTGGGCGGCTACTGCGACCGCGGGTTGGGGCAGCGCTGGCT

Ch10 953-G.....-G.....
Ch4 973CG..C.....
consensus GTGGGAGCAGCCgG-CAGAGCTCTCTGCCTCTCCACAGCCACCCCGCGCTGACCGCCCTCCCAACCCCCcACCCCCACCCCGGAAACCGCTGCTCCCTGGCTGGTGG

Ch10 1072-A.....A.....
Ch4 1095CTG.....G.....
consensus AGACCCCGTCCCGGAAACCGGGCCCGGACGGTCCGGCTGACACCGCTCCGGCGCTCGCTCTCTgTGCCCCCG-GCCACCGTCCCGCCCGCCCGgggcccctgGGGGC

Ch10 1181-A.C.....
Ch4 1217GC.G.....
consensus CCCTGACCGCCCGCAGCTGCAGCAGGg-G-CTGGCGGCGAAAGCAGACCCAGCCCGGCGCACACCGGGGACGCTGAGGCTTCAGGCGGGAGGGAAAGGGCGGAGATGAGAGAG

Ch10 1302AC.....CGAA..AT..CGA.....GGAGGGAG.....A.....CG
Ch4 1339GAG.GA.....-G.CG.AAG.....G.....GC
consensus gAACCGgagA--CTAGAGGcga-G-G--GGAAGTAggagggagGAGGAGGAGGAGGAGGAGGAGGAGGAAACGGAGGA-AGACAGAGCGACCGAGGACTGGGGCGGG--

Ch10 1420G.....T.....
Ch4 1450C.....C.....
consensus -GAGGGAGCCGGGAAGGACGGGGGAGGAAGGACGGGAGAAAGCGGTC-TGGCTCCGGGAGTAGCGGACCGCCCGCTCCGGGAAACGGTCAAGGTcCGGGCGGGCTGAGGGCT

Ch10 1541-
Ch4 1572C.....
consensus GGGCCACAGCCCGCCCGCGCGGGGACACCCATTGGCCCGGTTCCGGGGCCAGGGAGTGGGGGTTTCTCCGGGACAAAGACCGGGACTCGGGTTCGGCTGGGTcTTCA

Ch10 1662G.....C.....C.....
Ch4 1694A.....A.....A.....
consensus CCCCGGGTTACAGACCCGACATCCCCAGGCTGAGCCCTGCAAGCGGGCGGAGGCCCGAGCAG-CCCGGCCACGGAGAGCCACAGCAGGACGAGAGGGGTGATTTTGGTTTCGG-G

Ch10 1784TGC.....G.....N.....
Ch4 1815CGG.....A.....A.....
consensus TGGCTTTCCTC--AAGCGGCTGTGCTCAGCTCTCTCGGGCCCGAAAGGCTGGCATGCCGACTGTTGCTCCGGAGCTCTGnCGGCACCGGAAACATGACAGG-AGGTGCA

Ch10 1906C.....G..A.....-
Ch4 1936T.....C..G.....G.....
consensus AGCCCGCA-GTGCCTCTCGCTCTCTTGGCAGTTCAAACC-GCC-CACTGACAGTCCCCACGTTGGCGACGCGgGAATCCATGTCAGGCAATCAAGCCGGGAGGATCTCCTCT

Ch10 2026G.....
Ch4 2058C.....
consensus CTGGGGTCT-GCTCTGGTCTTCACTGGAAATGAACGAGAGCCACGGCTGGGTGGCGAGACCGTCCCGGCAACCGGACGCCACAGGCATTGCCTCTTCCGGAGAGGGCGCTGG

Ch10 2148T.....-
Ch4 2180C.....C.....
consensus CACACTCAAGACTCCACGGAGGTTAG-TCACACTCCCTCCACCTCCAGGcTGGTTCCTCCCTGCTGCGAGCGGTGGGAGCCAGAGAGGGCTTCCCGTTCCCGGGATCCCTG

Ch10 2268C.....C.....
Ch4 2302-
consensus GAGAGTCCGGAGACCGGCCCGCCcGAAACGGCGCCCTCCCGCTCCCGCTCTCCCGCTTCCTTCTCTCTCCGGCCCCACCACCACCCCGCCACCGcCTCCCGCCACCCCGC

Ch10 2390ACCCACAC..A.....
Ch4 2422C.....
consensus GCGCaccacacC-CGCCCCCKCCCKCCACCCACCCCGCGCGCGCCAGCCCTCGAGCCCTGGGTCCCTTCGGGGTGGGGGGGCTGCCAGGGGGCTCACGGCAATTCATG

Ch10 2512C.....G.....
Ch4 2535T.....
consensus AAGGGGTGGAGCTTGCTGCC-GTGGGCTTTACAAAGGCGGCTGGCTGGTGGTGGTGG-CGGGACGGCTCCTGGCTGACCTGCGCAGTGCACAGTCCGGCTGAGGTGCACGGAG

Ch10 2634T.....T.....
Ch4 2657C.....
consensus CCCCGCGCTCTCTCTGCGCGCTCGTCCGTGAAATTCGGGCGGGGCTCACCGGATGGCCCTCCCGACCC-TGGACAGCACCTCCCGGGAGCCCGGGGAGAGGAGGCGCCAG

Ch10 2756
Ch4 2779
consensus GAGACTCGTTTGAGCCCGGACCAAGCGAGGCGCTGGAGGCTGTTTGGAGGAACCGTACC CGGCATCGCCACCAGAGAACGGTGGCCAGGCCATCGGCATTCCGGAGCCAGG

Ch10 2876
Ch4 2901
consensus GTCCAGATTGGTTTTCAGAAATGAGAGGTCACGGCAGCTGAGCGAGCACCGGGGAAATCTGGCCCTGGCCCGGAGACGGCCCGCCAGAAAGCGGGGAAAGGGACCGCGCTCACCG

Ch10 2998
Ch4 3023
consensus ATCCAGACCGCCCTCTCTCCAGGCTTTGAGAAGGATCGCTTTCCAGGCATCGCCCGCGGGAGGAGCTGGCCAGAGAGACGGCCCTCCCGAGTCCAGGATTAGATCTGTTTCA

Ch10 3120C.....
Ch4 3143
consensus GAATCGAAGGGCCAGGCACCCGGAGCAGGTTGG3CAGGGCGCCCGcGCAGGACGGCGGCTGTGACAGCGGGCCCGCGGGGGTCACTTCTCCCTCGTGGTTCGCTTCGCCCA

Ch10 3242
Ch4 3262
consensus CCGGGCGTGGGAAAGGGCTTCCCGCACCCACGTCCT

Figure 2 Comparison of the sequence of the 3.3-kb *KpnI* repeat unit derived from 10qter and the homologous 4q35 sequence. The consensus sequence is presented in order to show base deletions/insertions (*lowercase letters*) and base substitutions (*dots*). The *BlnI* site (CCTAGG) within the *KpnI* repeat unit, which is present in the 10qter sequence and is absent in the homologous 4q35, is boxed (Deidda et al. 1996). The regions with similarity to LSau and hsp3 are indicated by boldface underlining and boldface square dots, respectively, below the consensus sequence. The homeodomain sequences are boxed.

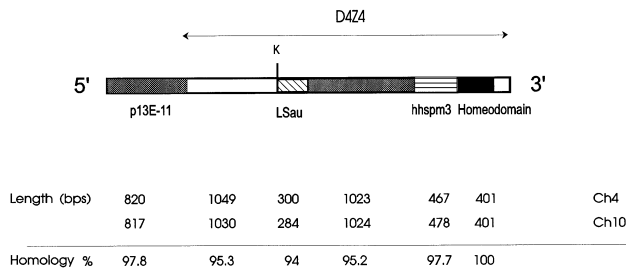


Figure 3 Sequence homology between p13E-11 and *KpnI* tandem-repeat units cloned from the 4q35 region and similar components from 10qter. The diagram represents both the nucleotide length of the p13E-11 single-copy sequence and that of the homeobox locus (D4Z4), at 4q35 (Hewitt et al. 1994; Lee et al. 1995), compared with the homologous 10qter region. The extent of homology between the different portions of 4q35 and 10qter clones is expressed in terms of percentage, ranging from a minimum value of 94%, for LSau, to a maximum of 100%, for the homeodomains.

of the gel, whereas the two alleles of 4q35 origin are preserved and reduced by ~3 kb (Deidda et al. 1996). This canonical pattern is changed in the case of chromosomal translocations that transfer the whole set of *KpnI* repeat units and, probably, other homologous sequences, including p13E-11, from one chromosome to the other. We can imagine both (1) a reduction in the number of *BlnI*-resistant fragments (monosomy) when *BlnI*-sensitive repeats of 10qter origin are moved onto a 4q chromosome and, vice versa, (2) an increase in the number of *BlnI*-resistant fragments (trisomy) when *BlnI*-resistant repeats of 4qter origin are displaced onto a 10q chromosome. Here we describe a few cases that are exceptions to the general rule of the *BlnI*-induced biallelic pattern.

Case 1.—In family FSHD 12, the disease segregates with the 4q35 haplotype characterized by the 8.7-kb (D4S139) and 10-kb (D4S163) alleles and is associated with a 25-kb *EcoRI/BlnI* fragment (fig. 4). The father, II:3, is the most severely affected individual in this family, with weakness and atrophy of facial and shoulder muscles, bilateral scapular winging, weakness of quadriceps and foot extensors, and steppage gait. He transmitted to his daughter, III:4, a 25-kb *EcoRI/BlnI* p13E-11 fragment, which is associated with the affected haplotype, and he transmitted to his son, III:5, the other 4q35 haplotype, 5.4-10, which is not associated with the disease. At 25 years of age, the daughter does not show any sign of the disease and is very likely a nonpenetrant gene carrier. The mother, II:4, is unaffected but carries three p13E-11 *BlnI*-resistant fragments, of 44, 40, and 32 kb, and has transmitted to the daughter the 40-kb allele, which is associated with the 4q35 haplotype 7.8-8.6, and she has transmitted to the son the 44-kb allele, which is associated with the 4q35 haplotype 7.4-10; and

she has transmitted an extra 32-kb fragment to both the daughter and the son. The most simple explanation is that the 32-kb additional fragment is a 10qter chromosome carrying *BlnI*-resistant *KpnI* repeats that was transmitted to both sibs.

Case 2.—A patient suffering from polymyositis with a marked involvement of facial muscles (family 85A) was submitted to our attention, for differential diagnosis. PFGE followed by Southern blot analysis showed three *EcoRI* fragments of higher molecular weight (50, 60, and 80 kb) and a smaller, 29-kb fragment (fig. 4). After *EcoRI/BlnI* digestion, the larger fragments were preserved and reduced by 3 kb, whereas the short fragment disappeared. Although this is an isolated case, it is likely that one of the larger fragments is a 10qter chromosome carrying *BlnI*-resistant *KpnI* repeats.

Case 3.—An isolated case of FSHD with severe involvement of the pelvic girdle and foot extensors had a p13E-11 small, 14-kb fragment, as shown by conventional agarose gel electrophoresis. PFGE separation of the *EcoRI* alleles showed three larger alleles, of 120, 70, and 65 kb, and one small, 14-kb fragment (fig. 4). After double digestion with *BlnI*, both the larger alleles and the small rearranged fragment were slightly reduced in size, in agreement with the 3-kb reduction usually observed for 4q35 alleles. This individual appears to carry four different sets of 4q and 10q loci, all carrying *BlnI*-resistant repeats.

A Novel Approach to Detection of Partial Translocations of KpnI Repeat Units of Different Chromosomal Origin (Hybrid Repeats)

The model proposed by van Deutekom et al. (1996) for chromosomal exchange between the 4qter and 10qter loci suggests that the whole set of *KpnI* repeats (and probably p13E-11 itself) is exchanged between the two loci. However, the crossovers (single or multiple) are more likely to occur within the *KpnI* repeat units, since they represent most ($\geq 90\%$) of the length of the p13E-11 fragments. If this is true, then the resulting hybrid *KpnI* fragments can escape detection if *BlnI*-resistant fragments of 4qter origin follow or are interspersed with the *BlnI*-sensitive repeats on a 10qter chromosome. In contrast, 4q35 p13E-11 fragments containing *BlnI*-sensitive repeats of 10qter origin would disappear if the *BlnI*-sensitive repeats precede the resistant one or would be reduced in size by more than the expected 3 kb, proportionally with the number of 10qter repeats that have been translocated distally to the *BlnI*-resistant units. Searching for restriction sites that are shared by the two loci, we found a restriction site, for the enzyme *Tru9I*, that cuts outside the array of *KpnI* repeat units of different chromosomal origins, as shown by the restriction maps of cloned fragments (fig. 5). This observation al-

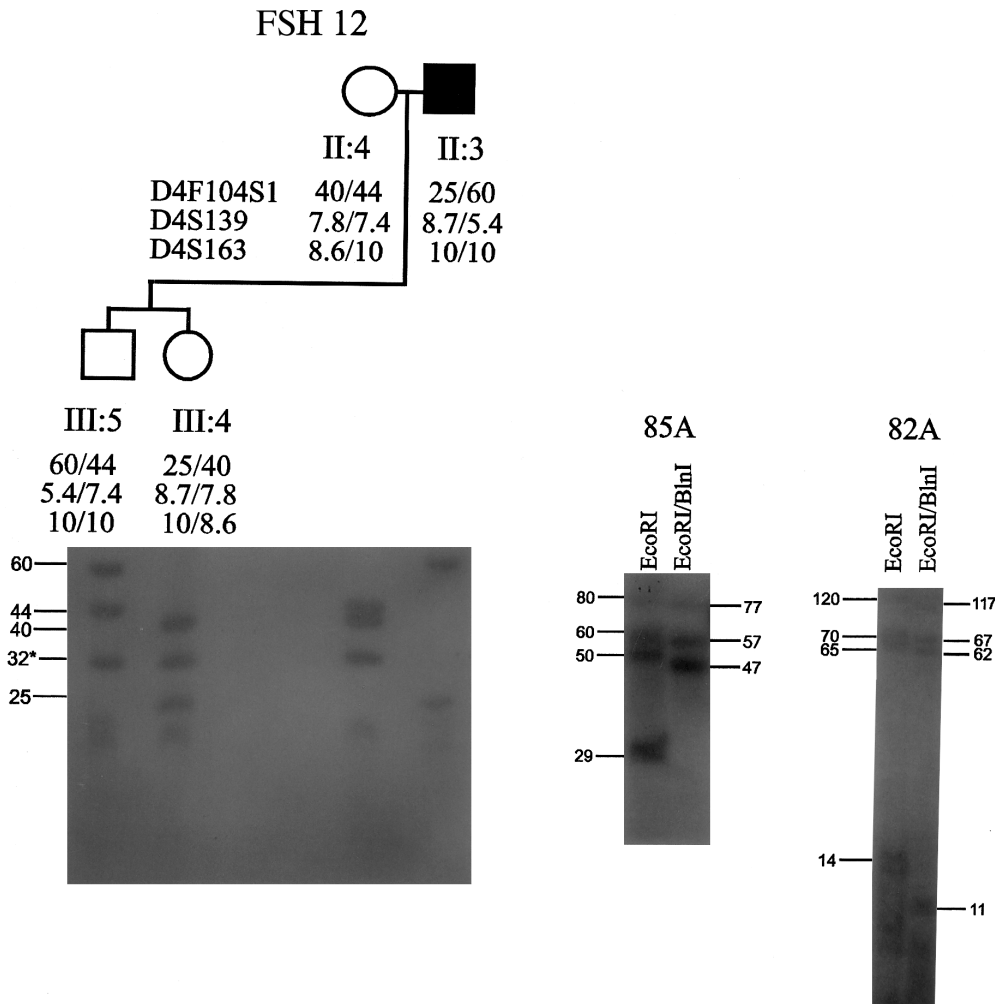


Figure 4 Variations, in the number of *BlnI*-resistant fragments, that are due to interchromosomal exchanges between 4q35 and 10qter loci. In family FSH 12, genomic DNA was restriction digested with *EcoRI/BlnI*, and the p13E-11 alleles were separated by PFGE, as described in the Patients and Methods section. Therefore, the autoradiographic bands correspond to p13E-11 alleles resistant to *BlnI* digestion. The 25-kb band is the small 4q35-specific fragment associated with the disease in the father. The extra 32-kb band (indicated by an asterisk [*]), present in the unaffected mother and in both sibs, does not segregate with the 4q35 markers, although it is not cleaved by *BlnI*. In family 85A, after *EcoRI/BlnI* digestion, three high-molecular-weight fragments appear to be *BlnI* resistant and are reduced by 3 kb, suggesting the translocation of *BlnI*-resistant *KpnI* units on one of the pair of 10qter chromosomes. Since linkage analysis with 4q35 and 10qter markers could not be performed, the size assignment of the 10q translocated chromosome was not possible. In family 82A, the subject shows four *BlnI*-resistant fragments. The 14-kb fragment is the 4q35 small fragment associated with the disease, whereas two of the larger fragments correspond to 10q translocated chromosomes.

allows us to separate out the whole set of *KpnI* repeats, whatever their chromosomal derivation, removing the p13E-11 sequence, which is cut into multiple fragments by *Tru9I* digestion. Digestion of genomic DNA with *Tru9I*, in conjunction with hybridization with cloned *KpnI* sequences in place of p13E-11, will produce fragments that are 6 kb shorter than the *EcoRI* p13E-11 fragments, and double digestion with *Tru9I* and *BlnI* (hereafter "*Tru9I/BlnI*") will measure the exact size of *BlnI*-resistant repeats, even if the latter interspersed with *BlnI*-sensitive repeats. In addition, the advantage of

Tru9I is that it cuts very frequently within the rest of genomic DNA and that it clears up the hybridization pattern of the *KpnI* repeats specific for the 4qter and 10qter loci. Figure 6 (66N [left panel]) shows the hybridization pattern obtained with *EcoRI* and with *EcoRI/BlnI*, with p13E-11 used as the probe, compared with that obtained with *Tru9I* and with *Tru9I/BlnI* digests, with cloned *KpnI* sequences used as the probe. It is worth noting that, after *BlnI* digestion, the 4q35 alleles are reduced 3 kb in size when p13E-11 is used, whereas the same alleles maintain their size when the *KpnI* probe

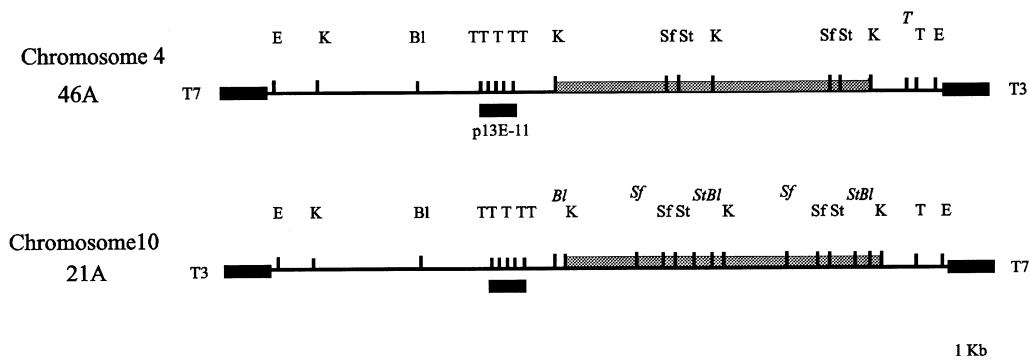


Figure 5 *Tru9I* restriction maps of 4q35 and 10qter phage clones derived from FSHD patients. The variant sites are indicated by italic letters. Only the *Tru9I* sites adjacent to the *KpnI* repeats are shown. E = *EcoRI*; K = *KpnI*; Bl = *BlnI*; T = *Tru9I*; Sf = *SfiI*; and St = *StyI*.

is used. The 10qter alleles are *BlnI* sensitive and disappear in both conditions. In conclusion, this type of hybridization detects any type of rearrangement of *BlnI*-resistant subunits, and the following examples illustrate alleles with atypical structural organization.

Family 106A

The patient in family 106A was a 39-year-old man with a mild form of FSHD, affecting mainly facial muscles and scapular fixators. PFGE analysis of p13E-11 alleles in the proband (fig. 6, lanes A [*right panel*]) shows the presence of the canonical four alleles, of 51, 47, 37, and 26 kb. After *BlnI* restriction digestion, the 47- and 26-kb alleles disappear (i.e., are 10qter like), whereas the 51- and 37-kb alleles are reduced ~3 kb in size (i.e., are 4q35 like). After *Tru9I* digestion and after *Tru9I/BlnI* digestion (fig. 6, lanes B [*right panel*]), the hybridization pattern produced when *KpnI* sequences are used as a probe shows that the 45- and 31-kb *KpnI* fragments are preserved and do not change in size, whereas the 41- and 20-kb *KpnI* fragments are cleaved, leaving an extra *BlnI*-resistant 13-kb fragment. It appears that one of the *BlnI*-sensitive alleles contains a stretch of *BlnI*-resistant repeats, but we were unable to identify which one carries the translocation. It is likely that the 37-kb *BlnI*-resistant fragment is the one causing the disease, in agreement with the mild clinical course; otherwise, several crossovers would have to be postulated, leading to a small *BlnI*-sensitive fragment of 4q35 origin and to a large *BlnI*-resistant 10qter allele.

Family FSH70

The pedigree of family FSH70 is shown in figure 7. It is a three-generation family, but only the patient, II:2, displays the typical clinical features of FSHD. Her

mother, I:1, shows only minimal signs of weakness of facial muscles, and her sister, II:3, is normal on the basis of neurological examination. Southern blot analysis with the 4q35-specific probes (not shown) indicated that the disease segregates with the 4.9-kb (D4S139) and 7.2-kb (D4S163) alleles and that it is associated with a small, 28-kb *EcoRI/BlnI* fragment. Subject II:3 has inherited the same 4q35 haplotype as is seen in the affected relatives, but she does not show any sign of the disease (i.e., she is a nonpenetrant gene carrier). The daughter, III:1, is not affected, since she inherited from the mother the other 4q35 haplotype, characterized by the 14-kb (D4S139) and 7.2-kb (D4S163) alleles and p13E-11 fragments of normal size. When the segregation of p13E-11 alleles is studied by PFGE (fig. 7A), the precise size of 4q35 alleles and of 10qter alleles can be determined: II:2 and II:3 inherited from their mother, I:1, the same 4q chromosome, carrying a small p13E-11 fragment, that is reduced to 25 kb after *BlnI* digestion but have two different 4q-chromosome fragments from the deceased father: II:2 has an atypical 4q fragment 150 kb in size, which disappears after *BlnI* digestion, whereas II:3 has a normal, 48-kb 4q band. The unaffected daughter III:1 did not receive the 4q chromosome carrying the deletion; instead, she showed the atypical 4q chromosome. When *Tru9I* and *Tru9I/BlnI* are used to detect *KpnI* alleles of different chromosomal origins (fig. 7B), the rearranged 25-kb 4q35 fragment is present in I:1, II:2, and II:3 and is absent in III:1, as expected, but, surprisingly, two extra *BlnI*-resistant fragments—a larger, 82-kb fragment and a shorter, 22-kb fragment—are detected in II:2 and in her unaffected daughter III:1. Since both II:2 and II:3 share with the mother, I:1, the same 45-kb 10qter fragment that carries the 195-nt D10S212 allele, it can be inferred that the two extra

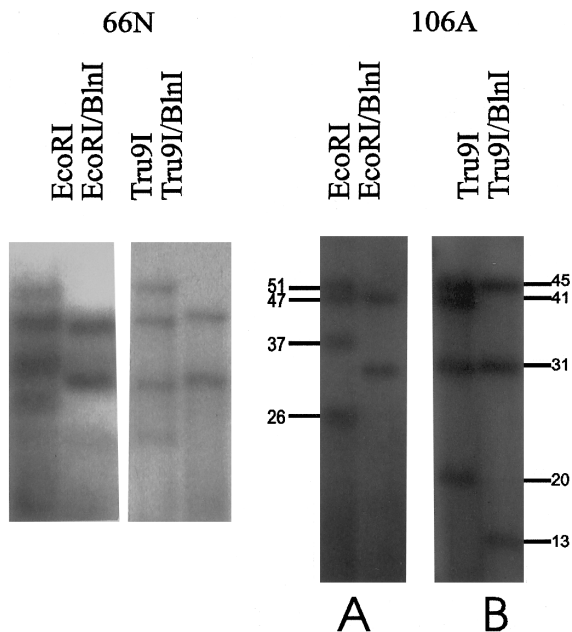


Figure 6 Effect of *BlnI* digestion on the size of p13E-11 and *KpnI* alleles in the absence or presence of partial translocations. *Left*, Southern blot analysis showing that a normal subject carries four types of p13E-11 alleles. Two of these alleles are cleaved by *BlnI* digestion and derive from the 10qter pair of chromosomes, and two resist *BlnI* digestion; the sizes of the latter are reduced by 3 kb, and they derive from the 4qter pair of chromosomes. After *Tru9I* digestion, the same pattern of four *KpnI* alleles is observed, which are 6 kb shorter than the *EcoRI* alleles: two alleles disappear after *BlnI* digestion and are very likely of 10qter origin, and two alleles maintain their size and correspond to the pair of 4q chromosomes. *Right*, Southern blot analysis in a patient with a mild form of FSHD. Lanes A show that the 47- and 26-kb fragments disappear after *BlnI* digestion (i.e., are 10qter-like), whereas the 51- and 37-kb fragments are reduced by 3 kb (i.e., are 4q35-like). Lanes B show that, after *Tru9I* digestion, four types of *KpnI* alleles are observed: the pair of *BlnI*-resistant alleles maintain exactly the same size, and the pair of *BlnI*-sensitive alleles disappear, giving rise to an extra *BlnI*-resistant 13-kb fragment.

BlnI-resistant fragments necessarily derive from the atypical 4q *BlnI*-sensitive allele. This chromosome displays a peculiar structure, with *BlnI*-sensitive repeats interspersed with *BlnI*-resistant repeats that are detected only by *Tru9I/BlnI* digestion with *KpnI* sequences used as the probe. However, the spatial arrangement of these *KpnI* repeats in the subtelomeric region cannot be determined except via the cloning of fragments derived from the 150-kb atypical allele. In the translocated 4q chromosome, the *BlnI*-resistant repeats span a total of 104 kb, whereas the remaining 46 kb are composed of *BlnI*-sensitive repeats of 10qter origin.

Discussion

The evolutionary significance of a 10qter locus with a high degree of sequence homology with the 4q35 locus

implicated in FSHD is not clear. The homology is not limited to the p13E-11 *EcoRI* fragment but, rather, extends both proximally, in the direction of the centromere (Deidda et al. 1995), and distally, reaching the telomeric sequences (Hewitt 1997): the duplication involves a DNA stretch of 100–400 kb, much larger than exon-fraction transpositions described in other hereditary diseases. Since genetic heterogeneity has been claimed for FSHD, an important question arises: Does the 10qter locus contain an active copy of the FSHD gene? Our results do not answer this question one way or the other: although the translation reading frame of the homeobox motif is preserved, as shown by 100% sequence identity, any change in promoters or other regulatory sequences that occurs in the flanking regions could inactivate the gene. Two observations exclude the presence of an active copy of the gene on 10qter: (1) the disease was not found linked to 10qter in a Danish FSHD family that did not have linkage to 4q35 (Bakker et al. 1995), and (2) the reported high frequency of chromosomal exchanges between the two loci in the normal population (van Deutekom et al. 1996) suggests that the chromosomal origin of the *KpnI* repeats has no influence on the activity of the elusive FSHD gene, unless a drastic decrease in the number of repeats occurs. It appears that the crucial pathogenetic factor is the decrease in the number of *KpnI* repeats, to less than a threshold level, rather than the interruption of a cryptic translational frame, which would favor the position effect–variegation model (Karpen 1994).

In all the cases of chromosomal exchanges that we studied, there was a transfer of *BlnI*-resistant repeats on 10qter subtelomeric regions, whereas the reciprocal event was rarely found. After PFGE separation of the alleles, we mainly encountered cases of trisomy and tetrasomy, but cases of monosomy were rare and should be differentiated by selective degradation of the larger alleles by nuclease attack or shearing during DNA extraction. To avoid this, inclusion of white blood cells within agarose and proteinase digestion of agarose blocks should be the preferred protocol. Among the 120 FSHD families studied thus far, we never found in affected individuals a 4q35-specific rearranged fragment that disappears after *BlnI* digestion, except in two isolated cases in which the 10qter origin of the fragment could not be excluded.

The main advantage of the *Tru9I/BlnI* method is the possibility of detection of partial translocations and any type of chromosomal rearrangement of *KpnI* repeat units. Here we have presented a case of a 4q chromosome with a peculiar structure consisting of two stretches of *BlnI*-resistant units alternating with *BlnI*-sensitive ones. It would be interesting to know both whether the *KpnI* repeats close to the telomere are *BlnI* sensitive or *BlnI* resistant and whether gene function or dysfunction

FSH 70

Chromosome 4 markers
 D4F104S1
 D4S139
 D4S163

Chromosome 10 markers
 D10F104S2
 D10S212

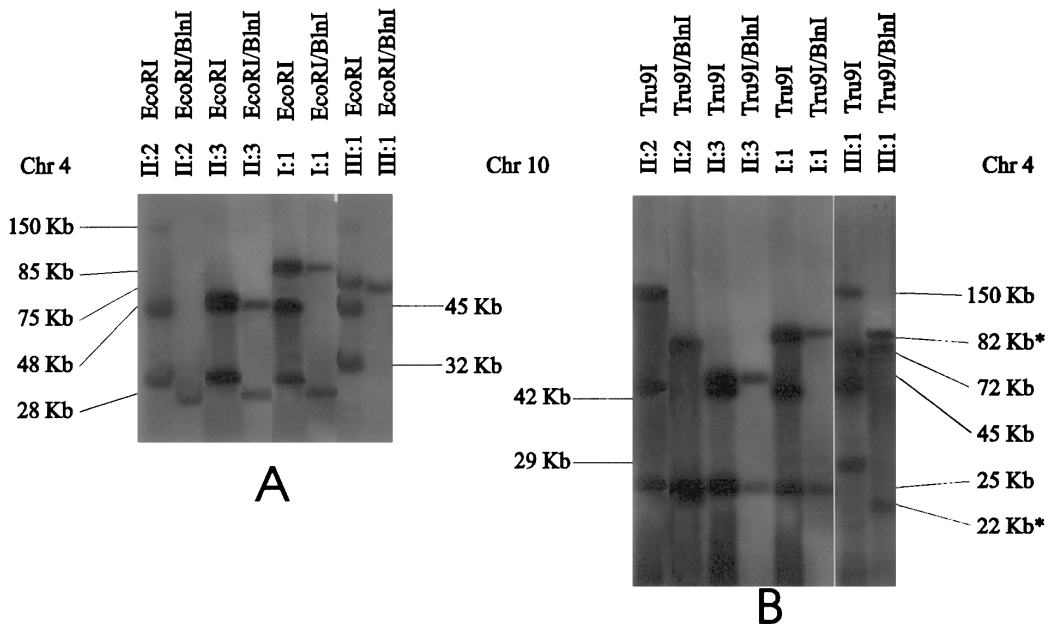
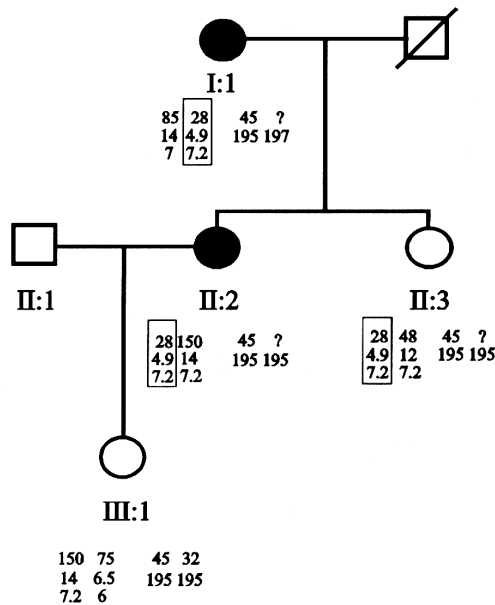


Figure 7 Segregation of a large *BlnI*-sensitive allele of 4q35 origin in a three-generation FSHD family, as shown by PFGE analysis of *EcoRI/BlnI* (A) and *Tru9I/BlnI* (B) genomic digests. The affected mother (II:2) carries two types of 4q35 chromosomes: the first is characterized by the 4.9-7.2 haplotype, of maternal origin, which is associated with a 28-kb p13E-11 *BlnI*-resistant fragment (i.e., disease related), and the other is characterized by the 14-7.2 haplotype, of paternal origin, which is associated with a p13E-11 *BlnI*-sensitive 150-kb fragment. After *Tru9I/BlnI* digestion, the 150-kb allele splits into two *BlnI*-resistant extra fragments, of 82 and 22 kb. The remaining 46 kb are *BlnI* sensitive and are interspersed within the *BlnI*-resistant fragments.

is correlated with the presence of 4q sequences in this terminal position. A priori, we cannot exclude the possibility that partial translocations of *KpnI* repeat subunits, between the subtelomeric regions of 10q and 4q chromosomes, could play a role in the molecular mechanism of the disease, either by determining a reduction in the critical number of *KpnI* repeats required for gene function or by some other unknown mechanism.

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