Direct detection of 4q35 rearrangements implicated in facioscapulohumeral muscular dystrophy (FSHD)

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Abstract

The p13E-11 probe has been shown to detect DNA rearrangements in sporadic and familial cases of FSHD. Its use, however, has been hampered by the fact that it detects at least two pairs of *Eco*RI alleles, one derived from the 4q35 region (D4F104S1), the other from 10q26 (D10F104S2). We have cloned p13E-11 *Eco*RI fragments from the 4q35 and 10q26 subtelomeric regions and shown the presence of several restriction site differences within the *Kpn*I tandem repeat units. The two loci present a different distribution of restriction sites for the enzyme *Bln*I which allows differential cleavage of the *Kpn*I units derived from 10q26, leaving intact the 4q35 pair of alleles. This method of differential restriction greatly facilitates the interpretation of Southern blots obtained from affected and unaffected subjects, with an important improvement in reliability for diagnosis and genetic counselling. In addition, this method can be used to investigate the molecular mechanism of the 4q35 rearrangement implicated in the disease and to ascertain whether the rearrangement is because of interchromosomal exchange between 4qter and 10qter *Kpn*I repeats.

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Key words: 4q35 rearrangements; facioscapulohumeral muscular dystrophy; *Bln*I restriction; DNA diagnosis.

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disorder which affects specific muscle groups and displays a variety of phenotypic expression. The gene for FSHD has been localised, by linkage studies, to chromosome 4q35 distal to the linkage group D4S171-F11-D4S163-D4S139.[1 2] Wijmenga *et al*[3] first described a new probe, p13E-11 (D4F104S1), telomeric to D4S139, which detects polymorphic DNA fragments in normal subjects ranging from 30 kb to 300 kb, de novo DNA rearrangements less than 28 kb in sporadic FSHD patients, and similar "small" fragments in familial cases. Physical mapping studies have localised 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 10 to 28 kb in size.[4] The probe shows two polymorphic loci and a 9·5 kb Y specific fragment. By haplotype analysis one of the loci could be assigned to chromosome 4q35[5] whereas the other locus segregates with 10qter microsatellite markers.[6] Unfortunately 4q35 and 10qter p13E-11 alleles segregating in the same FSHD family sometimes overlap with each other on conventional agarose gel electrophoresis and make the interpretation of Southern blots difficult. In order to analyse the structural differences between the two pairs of p13E-11 alleles, we cloned the non-4q35 13 kb fragment segregating in a FSHD Italian family and a similar sized 4q35 fragment from a sporadic case of FSHD into a phage vector.[7] Haplotype analysis with microsatellite 10qter markers and in situ hybridisation experiments with cloned *Kpn*I repeats in different members of the Italian family have shown that the non-4q35 fragment is located in the 10qter region, in agreement with the haplotype assignment by the Leiden group.[6] Restriction mapping of the 10qter clone, when compared with the 4q35 fragment, indicates a similar arrangement of *Kpn*I tandem repeat units and flanking sequences. However, 4q35 and 10q26 *Eco*RI clones can be distinguished by restriction analysis with *Sfi*I and *Sty*I. We soon realised that this observation could be exploited for future applications in the field of molecular diagnosis and genetic counselling. We assumed that an accurate comparison of nucleotide sequences between 4q35 and non-4q35 *Eco*RI fragments would probably result in the identification of restriction enzymes able to cut specifically in either one of the alleles, facilitating the interpretation of the p13E-11 hybridisation patterns from the affected and unaffected members of FSHD families. Here we report that restriction enzyme *Bln*I cleaves the variable *Kpn*I region of the 10qter p13E-11 fragments, allowing the direct identification of 4q35 alleles implicated in the disease.

Materials and methods

PATIENTS

4q35 haplotypes and p13E-11 fragments segregating in affected and unaffected members of family FSH21 are described in detail elsewhere.[8] Family FSH46 is a sporadic family in which the affected son carries a de novo rearrangement of 13 kb, absent in both healthy parents.

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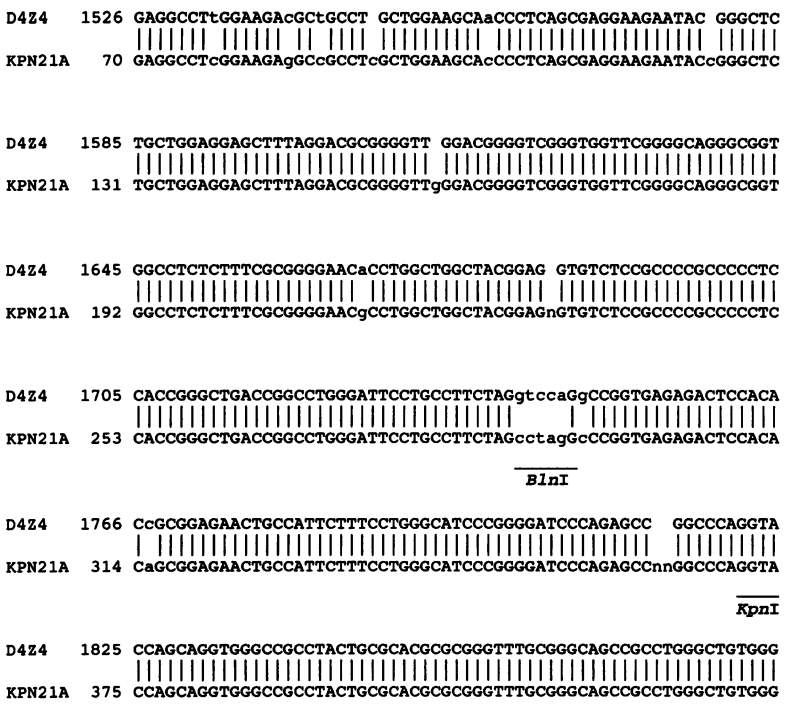

Figure 1 Comparison of 300 nucleotide sequences derived from 4q35 and 10q26 KpnI repeat units. D4Z4: 4q35 tandem repeat sequence as derived from Hewitt et al[9]; KPN21A: 10q26 tandem repeat sequence cloned from a sporadic FSHD patient. The BlnI (CCTAGG) and KpnI (GGTACC) sites are indicated.

SOUTHERN BLOT ANALYSIS WITH 4q35 MARKERS

Restriction of genomic DNA and hybridisation with L1LA5 (D4S163), pH30 (D4S139), and p13E-11 (D4F104S1) were as described in a previous paper.[2] Digestion with enzyme *Bln*I (Boehringer) was carried out by incubating 10 μg of genomic DNA with 20 U of enzymes at 37°C overnight. Digested samples were separated by conventional agarose gel electrophoresis at 2 V/cm for 48 hours.

CLONING OF THE *Eco*RI p13E-11 FRAGMENTS INTO λ PHAGE

Genomic DNA (200 μg) was completely digested with *Eco*RI (Biolabs) according to 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 hours. Fragments of the appropriate size were selected after agarose gel electrophoresis of single gradient fractions, pooled, and cloned into λ DashII/*Eco*RI (Stratagene). The ligation mixtures were packaged according to the Stratagene protocol and used to transform an *E coli* ER strain (p2392). The bacteriophage plaques were transferred to nitrocellulose filters (Schleicher & Schuell) and hybridised with ^{32}P labelled 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 hours 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.

PULSED FIELD GEL ELECTROPHORESIS

High molecular weight DNA was isolated from freshly collected blood, or from EBV transformed cells, and added to 0·5% Low Melt agarose (Biorad) according to the procedure described by Pharmacia LKB. Blocks were rinsed three times in sterile water and then equilibrated for 30 minutes at room temperature in the appropriate restriction enzyme buffer. Digestions with *Bln*I (Boehringer) and *Eco*RI (Biolabs) were carried out respectively in 200 μl with 60 U and in 400 μl with 150 U of enzyme. The enzymes were added in three equal portions at one hour intervals. After digestion, the blocks were rinsed three times in sterile water and then equilibrated for 30 minutes at room temperature in the electrophoresis buffer (0·5 × TBE).

Electrophoresis was performed at 12°C in a Pulsaphor electrophoresis unit with an HEX electrode (Pharmacia LKB); the agarose gel (Biorad) was 1·2% in 0·5 × TBE. The running procedure was carried out at 300 V, in three steps: the first one was five hours with pulses of one second, the second three hours with pulses of 20 seconds, and the third two hours with pulses of 45 seconds. After electrophoresis, gels were stained with ethidium bromide and photographed; DNA was then transferred to Hybond N$^+$ (Amersham) for hybridisation with α-^{32}ATP labelled p13E-11.

Results

SEQUENCE DIVERGENCE BETWEEN *Eco*RI 4q35 AND 10q26 CLONED FRAGMENTS IS OBSERVED WITHIN THE *Kpn*I TANDEM REPEAT UNITS

We have already shown by restriction mapping of 4q35 and 10q26 cloned fragments from FSHD patients that restriction site differences exist between the *Kpn*I tandem repeat units derived from different chromosomes: an additional site for *Sfi*I has been described within the 10q26 *Kpn*I units.[7] A more accurate sequence comparison of the *Kpn*I fragments showed that 10q26 alleles displayed new restriction sites for the enzymes *Sty*I and *Bln*I (C/CTAGG) that were absent in 4q35 homologues. In fig 1 a 300 nucleotide sequence derived from 4q35 D4Z4 units[9] is compared with the corresponding tract derived from subcloned 10q26 *Kpn*I units to show a 10qter specific *Bln*I site occurring about 80 nucleotides upstream of the *Kpn*I site.

This observation prompted us to construct a detailed restriction map for *Bln*I using the phage clones derived from the two loci, with the aim of finding a restriction enzyme that could specifically cleave either one of the p13E-11 alleles on genomic DNA. *Sty*I cannot be used for this purpose since it cuts within the *Kpn*I units of both alleles.

*Bln*I RESTRICTION MAPS OF PHAGE CLONED *Eco*RI FRAGMENTS DERIVED FROM 10q26 AND 4q35 REGIONS

Restriction maps were constructed after partial digestion with *Bln*I of cloned fragments. As reported before[7] clone 46A contains a 13 kb

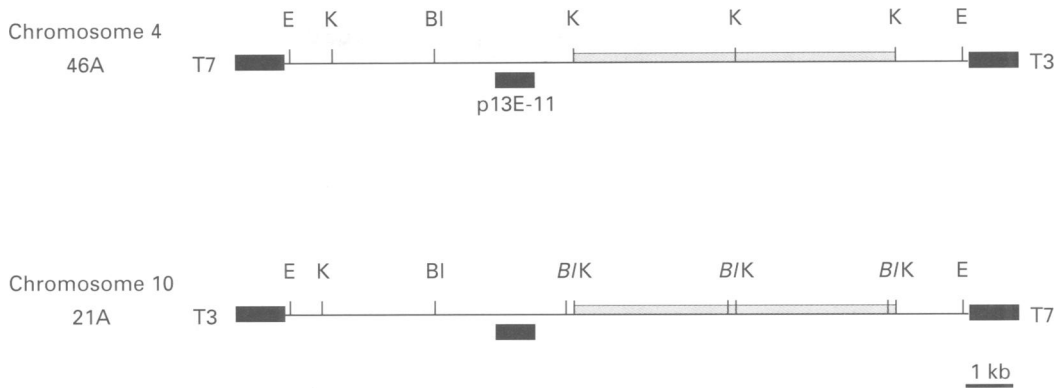

Figure 2 BlnI restriction maps of 4q35 and 10q26 phage clones derived from FSHD patients. Restriction sites: E, EcoRI; K, KpnI; Bl, BlnI. Each dotted bar represents the 3·3 kb repeat unit and the hatched box shows the position of probe p13E-11. Partial digests of λ clone DNA were hybridised with γ-ATP^{32} T7 and T3 oligomers.

*Eco*RI fragment derived from a 4q35 de novo rearrangement in a sporadic FSHD patient. Clone 21A contains a similar sized *Eco*RI fragment, unrelated to the disease, which segregates in patient 21A in association with 10qter microsatellite markers and has been mapped by in situ hybridisation to 10q26.[7] As shown in fig 2, both 4q35 and 10q26 clones contain a common *Bln*I site about 3 kb upstream of the *Kpn*I site in the first repeat unit. However, the 10q26 clone shows an additional *Bln*I site about 80 nucleotides upstream of the *Kpn*I site of each repeat. The same results were obtained with *Bln*I restriction of the 43 kb cosmid clone, which contains at least 10 *Kpn*I repeat units of the 10q26 type, indicating that the additional *Bln*I site is a constant feature of each 10q26 repeat unit. Therefore, we can assume that after double digestion of genomic DNA with the enzymes *Eco*RI and *Bln*I, the p13E-11 alleles derived from 4q35 will be shortened by 3 kb (the distance between the 5′ *Eco*RI site and the first *Bln*I site), while the 10q26 alleles will be cleaved into *Bln*I fragments that will not show any hybridisation signal with p13E-11, except for a small piece of 2·8 kb spanning the distance between the first two *Bln*I sites.

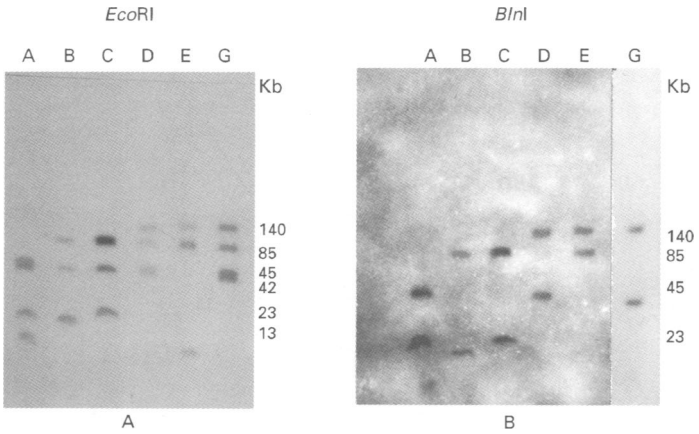

Figure 3 Pulse field gel electrophoresis of 4q35 and 10q26 p13E-11 alleles derived from family FSH21 members after EcoRI and BlnI restriction. A, B, and C refer to affected FSH subjects, D, E, and F to unaffected members of family FSH21. In panel A both 4q35 and 10q26 alleles are shown and are clearly separated by size. In panel B only the 4q35 alleles that are not cleaved by BlnI are left in the gel. The 23 kb fragment is the 4q35 rearranged allele segregating with the disease and is preserved after BlnI digestion. The differences in the migration of the 23 kb fragments (not observed by conventional agarose gel electrophoresis) are probably the result of different concentrations of sample DNA in the agarose plugs. The 13 kb fragment is a small 10q26 allele and is cleaved by BlnI restriction.

PULSED FIELD GEL ELECTROPHORESIS OF 4Q35 AND 10Q26 P13E-11 ALLELES AND EFFECT OF *Bln*I DIGESTION ON THE HYBRIDISATION PATTERN

Fig 3 (panel A) shows a clear pattern of separation of the larger alleles by PFGE after *Eco*RI digestion of high molecular weight genomic DNA derived from lymphoblastoid cell lines of affected and unaffected members of family FSH21 (fig 4). PFGE separation allows the identification of at least three types of larger alleles, in the range of 140 kb, 85 kb, and 45 kb, but some bands appear to be doublets and one cannot assign these alleles with sufficient precision to either one of the chromosomes. Digestion with *Bln*I (fig 3, panel B) drastically changes the p13E-11 hybridisation pattern and indicates unequivocally the 4q35 origin of the multiple alleles, since the 10q26 fragments are cleaved. Fig 4 shows the pedigree of family FSH21[8] and the segregation of large and small p13E-11 alleles in association with 4q35 and 10q26 markers. It appears that the 4q35 rearrangement giving origin to the 23 kb fragment associated with the disease has occurred on the grandmaternal chromosome characterised by the 4q haplotype 85-11.5-8.2 (thin lined box) and is the result of a deletion of about 62 kb, corresponding to nearly 20 *Kpn*I repeat units. The rearranged chromosome 4q35 has been transmitted to the affected daughter and her sibs, while the unaffected sister has inherited the chromosome characterised by the haplotype 140-8.8-7.2. At the same time the affected daughter has inherited the 10qter chromosome characterised by the haplotype 13-2-3 (thick lined box) carrying the small 13 kb fragment, while the unaffected sister has inherited from the mother the 10qter chromosome characterised by the haplotypes 85-1-3 carrying the large allele. In conclusion, *Bln*I digestion of genomic DNA converts the p13E-11 pattern from two loci (four alleles) to one locus (two alleles) and clearly identifies the

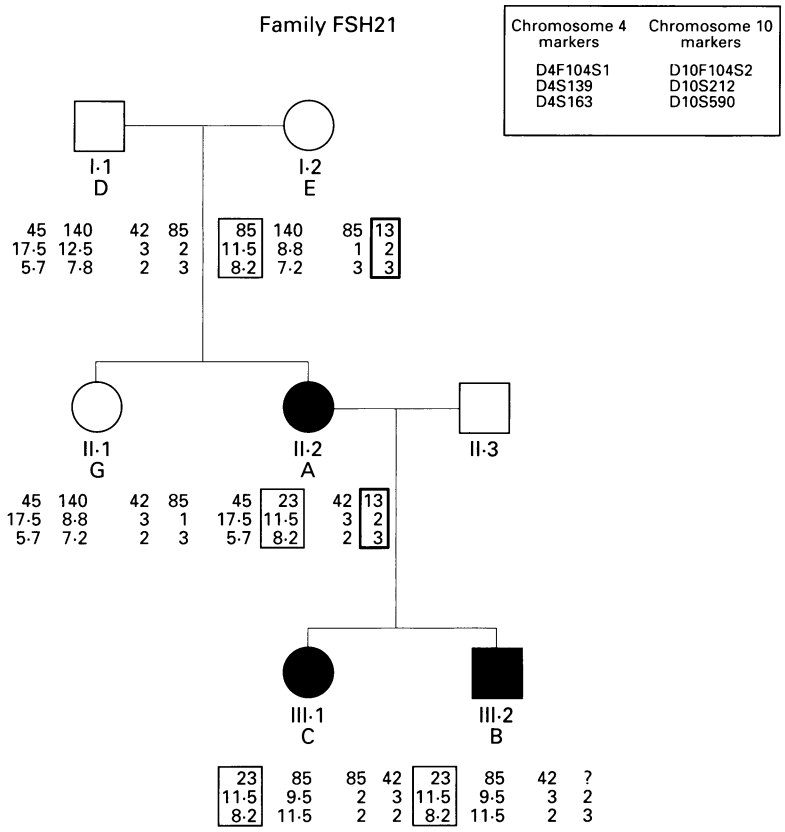

Figure 4 Segregation of 4q35 and 10q26 markers in family FSH21. The two loci identified by p13E-11 are named D4F104S1 (chromosome 4) and D10F104S2 (chromosome 10). The thin lined boxes indicate the 4q35 haplotype associated with the disease, the thick lined box indicates the 10q26 haplotype segregating with the 13 kb fragment. The sizes of p13E-11 alleles were determined by pulse field gel electrophoresis as shown in fig 3. The 23 kb p13E-11 fragment originates from a 62 kb deletion occurring on the 85 kb grandmaternal allele.

62 kb deletion occurring on one of the two 4q35 alleles of subject A as the chromosomal cause of disease development.

EFFECT OF *Eco*RI-*Bln*I DIGESTION ON THE p13E-11 HYBRIDISATION PATTERN IN BOTH SPORADIC AND FAMILIAL CASES OF FSHD

Twenty three affected subjects from 19 familial and four sporadic FSHD pedigrees were chosen on the basis of the presence of a small 4q35 fragment ranging in size between 13 kb and 25 kb. Genomic DNA was digested with either *Eco*RI alone or *Eco*RI-*Bln*I and electrophoresed for 48 hours in adjacent lanes to compare the size change with the best accuracy. In most cases the results of p13E-11 hybridisation show a better definition of bands after double digestion, with disappearance of 10q26 interfering alleles (fig 5). The blurred pattern at the top of the gel resulting from superimposition of larger multiple alleles disappears, while the small rearranged band running ahead in the gel is better defined than that observed after *Eco*RI digestion. As shown in fig 5A rearranged 4q35 alleles of 17, 23, and 26 kb become shorter, producing fragments of 14, 20, and 23 kb respectively. The reduction in size is compatible with the 3 kb predicted by restriction analysis of phage clones, but this change cannot be determined with accuracy in the upper portion of the gel. In panel B, double *Eco*RI-*Bln*I digestion of genomic DNA from an affected subject results in the cleavage of the 27 kb 10qter allele, while the 4q35 large allele is preserved and the small 17 kb allele is changed into a 14 kb allele. In panel C, the p13E-11 pattern of a normal female displays a mixture of large and small alleles that cannot be interpreted after *Eco*RI digestion. *Eco*RI-*Bln*I digestion results in the disappearance of the two 10q26 alleles, 27 kb and 9·5 kb, leaving the large 4q35 alleles at the top of the gel. The experiment shows that 10qter alleles can be as short as the Y specific 9·5 kb fragment. In panel D, a fragment of 23 kb is carried by the

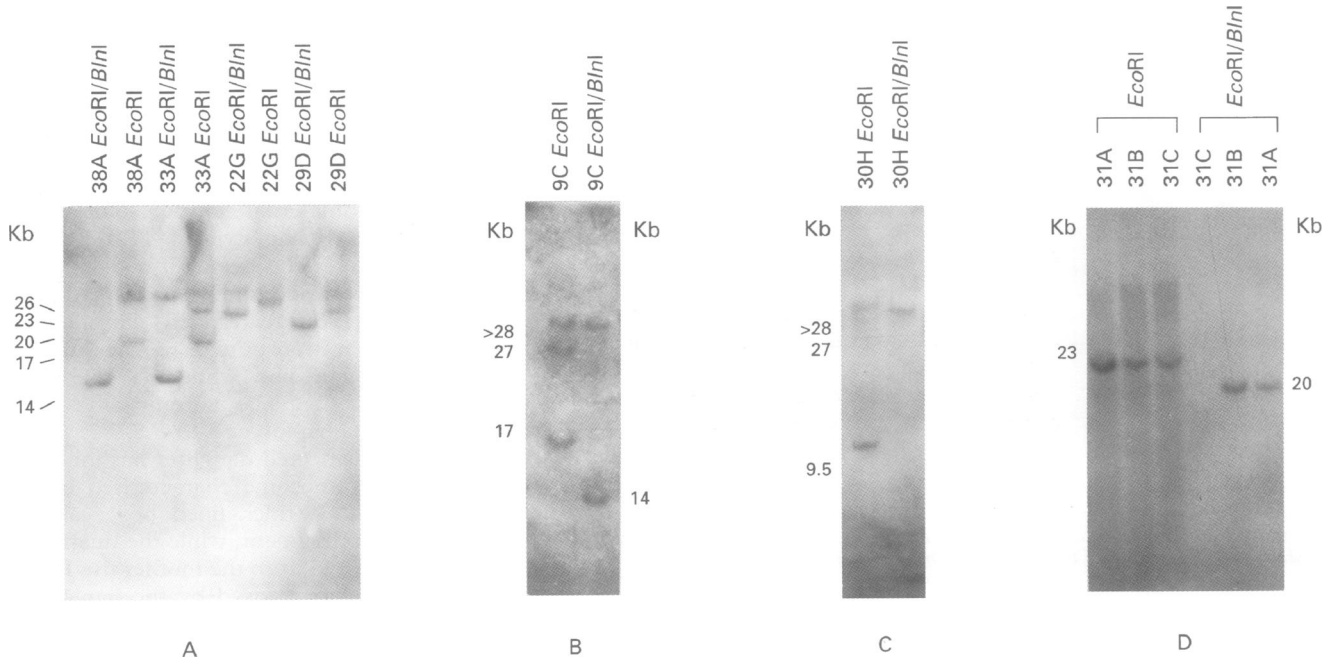

Figure 5 Effect of double digestion with EcoRI and BlnI on the sizes of p13E-11 alleles as determined by conventional agarose gel electrophoresis. Each panel refers to multiple alleles observed in subjects from different FSH families (see Results section for details).

unaffected mother (C) of the proband (A) and shows the same electrophoretic mobility of the rearranged 4q35 fragment transmitted from the affected father (B). The affected subject (A) appears to be homozygous for the fragment (see the double intensity of the signal) and this would prevent any prediction based on p13E-11 hybridisation pattern in the case of fetal DNA analysis. *Eco*RI-*Bln*I digestion cleaves the 23 kb allele derived from 10qter and only the 23 kb 4q35 fragments related to disease are preserved, allowing an unequivocal interpretation in the case of prenatal diagnosis. In conclusion, the conversion of p13E-11 hybridisation pattern to a single pair of 4q35 alleles implies a marked improvement in the interpretation of the results, advantageous for diagnostic and genetic counselling accuracy.

Discussion

The use of p13E-11 for the diagnosis of FSHD found its specific application in the case of sporadic FSHD patients. The appearance of a small p13E-11 fragment in the proband, in the absence of any rearrangement in the unaffected parents, provided convincing evidence for a strict correlation between 4q35 rearrangements and the development of disease. In the familial cases, the results are not always reliable owing to the segregation within the same family of small fragments of different chromosomal origin (10qter mainly) in at least 10% of the world population. After cloning, restriction mapping, and partial sequencing of *Kpn*I repeat units derived from the 10qter region, a major diagnostic breakthrough was achieved by differential *Bln*I restriction of genomic DNA derived from affected subjects: the p13E-11 hybridisation pattern is changed from two loci (four alleles) to one locus (two alleles) with the removal of 10qter alleles interfering with the 4q35 disease related fragments. After double digestion with *Eco*RI and *Bln*I one can assume that the alleles migrating in the gel are exclusively 4q35 specific and the small allele undergoing a constant reduction in size (3 kb) is the rearranged fragment correlated with the disease.

Differential *Bln*I restriction can also be applied to investigate the molecular mechanism of the 4q35 rearrangements. Since we did not find, in any of the subjects examined, a size reduction larger than 3 kb, we can exclude that the DNA rearrangements are the result of cryptic translocations of 10qter sequences on the tip of chromosome 4. If this were the case, the size reduction would be increased proportionately to the number of 10qter *Kpn*I units present in the hybrid rearrangement. It appears that the most likely mechanism is a 4q35 intralocus recombination between homologous *Kpn*I repeat units. As far as genetic counselling is concerned, the presence of a small 4q35 *Bln*I fragment will definitely prove that a deletion has occurred in a critical portion of the FSHD locus and the subjects carrying this fragment have a 95% risk of manifesting the disease in the first two decades of their life, taking into account the non-penetrance of the gene in 5% of people. Linkage analysis with other 4q35 probes such as D4S163 and D4S139 will still be required to exclude the rare cases of FSHD unlinked to 4q35,[2 10] to ascertain paternity, and to study the segregation of 4q35 haplotypes in the rare families where no small rearranged fragments are present.

It is worth noting that *Eco*RI-*Bln*I digestion will be particularly useful in genetic studies of small sized FSHD families where it is not possible to establish a significant linkage between small 4q35 fragments and the disease.

An extended application of *Bln*I restriction analysis in a larger number of FSHD families and normal subjects will confirm the association between short 4q35 fragments and the disease and the essential role of telomeric positional effect in the pathogenesis of the disease.

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