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The Friedreich Ataxia Critical Region Spans a 150-kb Interval on Chromosome 9q13

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Summary

By analysis of crossovers in key recombinant families and by homozygosity analysis of inbred families, the Friedreich ataxia (FRDA) locus was localized in a 300-kb interval between the X104 gene and the microsatellite marker FR8 (D9S888). By homology searches of the sequence databases, we identified X104 as the human tight junction protein ZO-2 gene. We generated a large-scale physical map of the FRDA region by pulsed-field gel electrophoresis analysis of genomic DNA and of three YAC clones derived from different libraries, and we constructed an uninterrupted cosmid contig spanning the FRDA locus. The cAMP-dependent protein kinase γ-catalytic subunit gene was identified within the critical FRDA interval, but it was excluded as candidate because of its biological properties and because of lack of mutations in FRDA patients. Six new polymorphic markers were isolated between FR2 (D9S886) and FR8 (D9S888), which were used for homozygosity analysis in a family in which parents of an affected child are distantly related. An ancient recombination involving the centromeric FRDA flanking markers had been previously demonstrated in this family. Homozygosity analysis indicated that the FRDA gene is localized in the telomeric 150 kb of the FR2-FR8 interval.

Introduction

Friedreich ataxia (FRDA) is an autosomal recessive degenerative disease involving the central and peripheral nervous systems and the heart, with a worldwide prevalence of 1–2/100,000 (Harding 1984). It is clinically characterized by relentlessly progressive trunk and limb ataxia usually with onset before age 25 years, loss of deep sensation and of tendon reflexes, dysarthria, kyphoscoliosis, pes cavus, hypertrophic cardiomyopathy, and increased risk of diabetes mellitus (Geoffroy et al. 1976; Harding 1981). The disease leads to substantial physical handicap at a young age and reduces life expectancy mainly as a consequence of heart disease (Leone et al. 1989). The first pathologic changes are thought to occur in the dorsal root ganglia, with loss of large sensory neurons, followed by thinning of the spinal cord because of atrophy of the posterior columns and spinocerebellar tracts. The primary defect of FRDA has not yet been identified, despite extensive biochemical studies, and all claims of a specific metabolic abnormality have not held up to subsequent investigations.

As for many genetic disorders of unknown cause, a positional cloning approach has been attempted for FRDA. The gene was mapped to chromosome 9q13, at zero recombination from the marker loci D9S15 (Chamberlain et al. 1988) and D9S5 (Fujita et al. 1989). These markers were physically linked to each other by pulsed-field gel electrophoresis (PFGE) analysis of genomic DNA (Pandolfo et al. 1990). The distance between D9S15 and D9S5 was estimated to be 260 kb by analysis of YAC clones (Fujita et al. 1992). Expansion of sampled families and availability of additional highly polymorphic markers then allowed identification of recombination events (Chamberlain et al. 1993) and performance of linkage disequilibrium studies, both in small inbred populations (Sirugo et al. 1992) and in large outbred samples (Sirugo et al. 1993), leading to the determination of the following gene order: 9cen-FRDA-D9S5-D9S15-9qter. However, flanking markers that define a candidate FRDA region have only recently been identified (Rodius et al. 1994). The "FR" series of microsatellites, described in Rodius et al. (1994), were of crucial importance for this accomplishment. By analysis of crossings-over in key recombinant families (Duclos

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et al. 1994; Rodius et al. 1994), by homozygosity analysis of inbred families (Monros et al. 1994) and by exclusion of the candidate gene X104 by mutation analysis (Duclos et al. 1994), the FRDA locus was localized between the microsatellite markers FR2 (D9S886) and FR8 (D9S888). The distance between FR2 and FR8 was estimated to be ~300 kb by physical mapping of a single YAC. In this report, we confirm the accuracy of the physical map of the FRDA region by PFGE analysis of genomic DNA and of additional YAC clones; and we describe the construction of a cosmid contig spanning the FRDA locus, the identification of X104/CSFA1 as the human ZO-2 gene, and the localization of the cAMP-dependent protein kinase γ-catalytic subunit gene within the candidate region. We have also identified new polymorphic markers in the FR2-FR8 interval. Homozygosity analysis with these markers indicates that the critical region for FRDA is limited to a 150-kb interval between FR2 and the new marker F8101.

Material and Methods

YAC Library Screening

The CEPH YAC library screening was described by Rodius et al. (1994). The St. Louis and the ICI YAC libraries were screened by PCR, using primers derived either from polymorphic markers linked to FRDA or from ends of previously isolated YACs. The ICI YAC library contains inserts of an average size of 300 kb cloned into the *Eco*RI site of the pYAC4 vector. We screened a copy of this library, which is maintained at the Department of Biotechnology of the San Raffaele hospital, Milan.

YAC Analysis

Analysis of the CEPH YACs was described in Rodius et al. (1994). DNA from the St. Louis and ICI YACs was prepared in agarose plugs using standard methods. The size of the cloned DNA was determined by PFGE, using a contour clamped homogeneous field (CHEF) apparatus. A physical map of each YAC was obtained by partial and complete digestion with a few rare-cutting restriction enzymes (*Not*I, *Eag*I, and *Mlu*I), followed by hybridization of blots of the PFGE-separated fragments with end-vector probes. The 2.7-kb *Pvu*II and 1.7-kb *Bam*HI fragments of pBR322 were used as probes for the left and right arm of the pYAC4 vector, respectively. YAC ends were isolated by *Alu*-vector PCR, were cloned, and were sequenced to generate sequence-tagged sites (STSs). Both ends of 37FA12 were shown to map on chromosome 9 by amplification of the corresponding STS from a chromosome 9–only human-hamster somatic cell hybrid.

YAC Subcloning into Cosmids

Subcloning of CEPH YACs 22E4 and 700F10 into the Spcos2 cosmid vector was described by Rodius et al. (1994). ICI YACs 37FA12 and 22AC2 were subcloned into the Scos1 cosmid vector. Agarose plugs containing YAC DNA were subjected to partial digestion with *Sau*3AI, and fragments were dephosphorylated using alkaline phosphatase and recovered by agarase digestion of the plugs. Ligation of the YAC fragments to the Scos1 arms, packaging and infection of *Escherichia coli* NM544 strain were done according to standard techniques and followed the packaging extract manufacturer's recommendations (GigaPack Gold II, Stratagene).

Cosmid Contig Construction

Cosmids containing rare restriction sites (*Eag*I, *Not*I, *Mlu*I, *Sac*II, and *Bss*HI) were identified by digestion with the specific enzyme and used as anchors to construct contigs. Assembly of the cosmid clones into contigs was done by hybridization of Southern blots of *Eco*RI, *Eag*I, *Not*I, or *Mlu*I digested cosmids with entire cosmid probes, using competition with sheared total human DNA and short exposure times to minimize nonspecific hybridization signals.

PFGE Analysis of Genomic DNA

High-molecular-weight genomic DNA sample was prepared in agarose plugs from the peripheral blood lymphocytes of a healthy subject, using standard methods. DNA samples were digested with *Not*I and with *Mlu*I, run on a CHEF gel, and blotted onto a nylon membrane (Hybond N+). A single blot was sequentially hybridized with the cosmids A3 and B1, which span the two *Not*I sites that were identified in the FRDA critical region by YAC analysis. The cosmid probes were preannealed with sheared total human DNA to prevent hybridization of repeated sequences.

Screening of Arrayed cDNA Libraries

Sixty thousand clones from human placental, heart, and liver unamplified cDNA libraries constructed in lambda ZAP II vectors by using *Not*I linkers were deposited into 96-well trays for storage and retrieval. A primer pair flanking the *Not*I cloning site of lambda ZAP II was used to amplify the insert of each clone by PCR, and these PCR products were spotted at high density onto nylon filters by using a robotic device. Clones containing repeat elements, as *Alu* or long interspersed sequence, were identified by hybridization of the filters with human Cot-1 DNA and shown to constitute 5%–10% of the libraries. We used pools of cosmids in the FRDA critical region as probes to hybridize the libraries. Knowledge of the grid positions of repetitive sequence clones allowed us to identify the signals that were likely to be due to hybridization of unique sequences. The corresponding clones were retrieved from the 96-well plates, mapped back to the region of interest by hybridization to Southern blots of YACs and cosmids, and sequenced.

Table 1

New Polymorphic Markers in the FRDA Region

Marker	Polymorphism	No. of Alleles	Heterozygosity	PCR Primers	Annealing Temperature (°C)	Size (bp)
FR6	$(TC)_{10}CTCT(TC)_3T(TC)_9$	5	.65	CCTTTCCTGGATGCATTGTGT TCATGAGGGTGGGACTCTCA	58	175
F71	$(CAA)_{13}$	3	.38	ATCGCGGCACTGCACTCC GGCATAATACACAACAGGCTAG	59	246
F5225	$(TCTA)_4(TCCA)_{12}$	3	.46	GACTGCAGCTTGAAGCCTAG ACTGCACTCCAGCCTGGG	58	162
F8101	A_{18}	2	.47	CGTGGAGACATGGCATAAGCA CTTATCATCCTGTTGGCTCTGTG	58	88
A3U1	SSCP	2	.42	GACTTCTGATGATACCCATCAAC CCTAGGTAAACAGTAGCATCC	55	152
CS2	*Aci*I or *Fnu*H4I RFLP	2	.42	CCCATCAAGCCTTTTCCATCACAG GCCTCCAAATAAGGTCACATTCTG	55	156

Sequence Analysis of Cosmid Fragments

Fragments from cosmids were subcloned using several restriction sites. Double-stranded and single-stranded plasmid DNA was sequenced using the dideoxynucleotide terminator method. Sequencing reactions were performed either with fluorescently labeled dideoxynucleotides or primers and analyzed on an ABI 373 automated sequencer, or with ^{35}S-labeled dATP and analyzed on traditional sequencing gels.

SSCP Analysis

Primers were designed to obtain fragments 200 bp to be analyzed by this method. Primers for the polymorphic sequences CS2 and A3U1 are listed in table 1. PCR reactions consisted of 30 cycles with the following parameters: 1 min at 94°C, 1 min at the appropriate annealing temperature for the specific primer pair (table 1), and 1 min at 72°C. PCR products (1–2 ml) were diluted 1:5–10 in deionized formamide; heated for 2 min at 80°C; quickly chilled on ice; and separated on 16 cm long, 1 mm thick, 6% polyacrylamide, 10% glycerol, nondenaturing gels in 1 × TBE. Electrophoretic runs were done at 350 V, for 3 h at 4°C. Fragments were detected by silver staining. In some experiments, PCR was performed using end-labeled primers to obtain hot amplification products, that were separated on nondenaturing, 6% polyacrylamide sequencing gels, and detected by autoradiography, as described in Duclos et al. (1994).

Identification and Analysis of the CS2 RFLP

Amplification products from two individuals homozygous for the two SSCP variants of the CS2 sequence were directly sequenced on both strands. A T→C transition was observed at position 37 of the 156-bp CS2 sequence in the less frequent SSCP variant, generating new *Aci*I and *Fnu*H4I restriction sites. This *Aci*I/*Fnu*H4I RFLP was tested in additional individuals by digestion of 10 ml of PCR products with 0.5 U of one of the enzymes for 30 min. Digested fragments were separated on 2% agarose and visualized by ethidium bromide staining.

Short Tandem Repeat Polymorphisms Analysis

Sequences containing polymorphic short tandem repeats were amplified using the primers listed in table 1. One oligonucleotide primer was end-labeled as described elsewhere (Pandolfo et al. 1990). PCR reactions consisted of 30 cycles with the following parameters: 1 min at 94°C, 1 min at the appropriate annealing temperature for the specific primer pair (table 1), and 1 min at 72°C. Amplification products (3 ml) were separated on 6% denaturing, polyacrylamide sequencing gels and detected by autoradiography.

Results

Construction of a YAC Contig Spanning the FRDA Locus

A YAC contig spanning the FRDA critical region was constructed by screening the CEPH YAC library as described in Rodius et al. (1994). In this contig, a single clone, 700F2, spans the FR2-FR8 interval. Construction of an additional, independent YAC contig, was initiated by screening the St. Louis library with an STS derived from the anonymous DNA segment MCT112 (D9S15). Successive rounds of YAC library screening were performed using STSs derived from previously isolated YAC ends in order to extend the contig in the direction

indicated by genetic analysis. The screening with D9S15 yielded four positives, including a clone (A207F7) that contained both D9S15 and D9S5. The St. Louis YAC library was then screened using oligonucleotide primers derived from the right end of A207F7, which we had mapped 100 kb centromeric to D9S5. Three unrearranged clones were obtained: the largest one (A97C10) had a size of 260 kb, extending the cloned region to 330 kb beyond D9S5. The ICI library was then screened with the centromeric left end of A97C10, obtaining YAC 37FA12. This 300-kb YAC extended the cloned region a further 250 kb. The current telomeric flanking marker for FRDA, FR2, was found within 37FA12. Primers derived from the left end of 37FA12 were used to obtain YAC 22AC2 from the ICI library (290 kb). YAC 22AC2 contained FR8, FR7, and FR5. The entire FRDA minimum genetic region was therefore bridged by the clones 37FA12 and 22AC2. Overall, we cloned more than a megabase of chromosome 9q into YACs, including the entire minimum genetically defined candidate region for FRDA (fig. 1).

Large-Scale Physical Map of the FRDA Region

A large scale physical map was derived from the YACs by PFGE, using *Not*I and *Mlu*I restriction sites as landmarks, and it is shown in figure 1. In the critical FRDA region, the map derived from 37FA12 and 22AC2 is in agreement with that obtained from the CEPH YAC 700F10 (Rodius et al. 1994). The concordance between

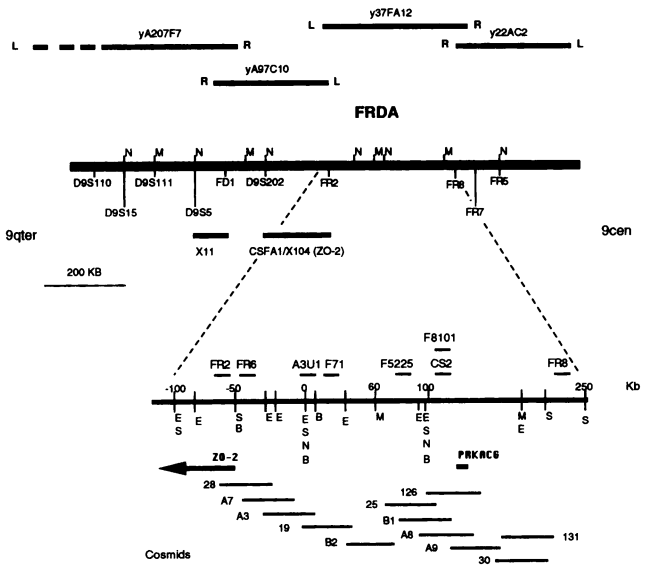

Figure 1 Large-scale map of the chromosome 9q13 region included in the YAC contig. The position of two cloned genes, X11 and CSFA1/X104 (ZO-2), is indicated. The critical FRDA region is enlarged to show the position of the new polymorphic markers and of the PRKACG gene. The minimum number of cosmids covering the region is shown below the map. M = *Mlu*I site; N = *Not*I site; E = *Eag*I site; S = *Sac*II site; and B = *Bss*HI site.

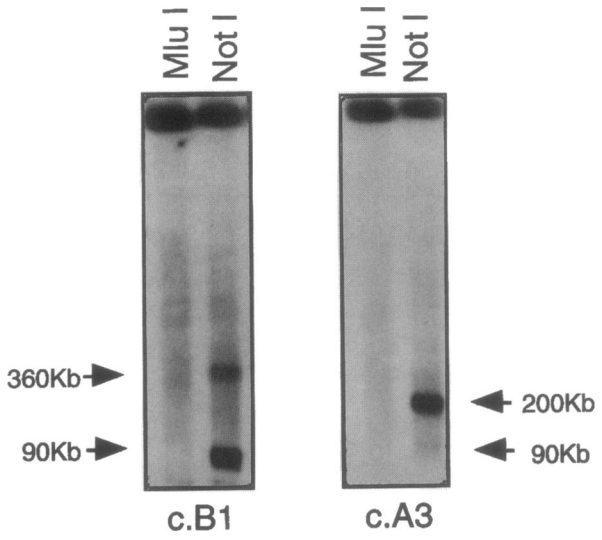

Figure 2 PFGE analysis of genomic DNA. See fig. 1 for the positioning of the cosmids used as probes within the FRDA critical region.

the physical maps of YACs derived from different libraries suggested that no major rearrangement had occurred in any of the clones. This observation was further confirmed by PFGE analysis of genomic DNA using YAC-derived probes (fig. 2). In particular, the same *Not*I fragments that had been identified by physical mapping of the YACs were detected in lymphocyte genomic DNA, indicating that no major deletion was present in our contig. The genomic PFGE analysis also revealed that all *Not*I sites in the critical FRDA region are unmethylated, at least in lymphocytes, while all *Mlu*I sites are methylated. Additional analysis of YACs and YAC-derived cosmids revealed that *Not*I sites were clustered in CpG island with additional rare CpG containing restriction sites (fig. 1).

Construction of a Cosmid Contig Spanning the Critical FRDA Region

The YAC clones 37FA12, and 22AC2 were subcloned into cosmids and a contig was assembled as described in Material and Methods. Cosmids from 700F10 (Rodius et al. 1994) were then added to the contig, obtaining complete, uninterrupted coverage of the critical FRDA region (fig. 1).

X104/CSFA1 Is the Human ZO-2 Gene

The critical FRDA region is flanked on the telomeric side by the polymorphic microsatellite FR2, which is localized within the previously identified X104 gene. X104 was isolated by exon amplification using 700F10-derived cosmids (Rodius et al. 1994). The same gene was also isolated by cDNA direct selection and called "CSFA1" (Pandolfo et al. 1994). A recent database search allowed identification of X104/CSFA1 as the hu-

man equivalent of the canine gene encoding the epithelial tight junction protein ZO-2 (Jesaitis and Goodenough 1994). X104/CSFA1 and ZO-2 show 91% sequence identity in the coding region and 81% identity in the 3′ UTR. A discordance in the position of the stop codon between the published sequences of X104 and ZO-2 was due to a few mistakes in the automatic sequencing of X104. The correct sequence has four additional nucleotides, three G's in positions 3336, 3354, and 3361; and an A in position 3367. These changes also make the corresponding segment of X104 perfectly identical to CSFA1. The predicted amino acid sequences of X104/CSFA1 and dog ZO-2 are 93% identical.

Identification of the PRKACG Gene in the Critical FRDA Region

Sixty thousand cDNA clones from human heart, liver, and placenta, spotted on high-density filters, were screened by hybridization with cosmids and YACs from the critical FRDA region. Because of the limited number of screened clones, only highly expressed transcripts could be identified by this approach. Three overlapping cDNAs were isolated corresponding to the α-catalytic subunit of the cAMP-dependent protein kinase (PRKACA). All three clones hybridized to the same 5.9-kb *Eco*RI fragment from y37FA12 and from the overlapping cosmids 126, 24, 141, A1, A8, A2, and A9. The chromosomal localization of the PRKACA gene, whose transcript is highly expressed in all tissues, is unknown, while the gene encoding the testis-specific γ-catalytic subunit of the same enzyme (PRKACG) had previously been mapped to chromosome 9q13, the same chromosomal band where FRDA is localized (Foss et al. 1992). Since the α and γ subunits have 90% sequence identity at the nucleotide level, we assumed that the gene localized within our contig was PRKACG. This assumption was confirmed by sequence analysis of cosmid 126, which additionally showed that the PRKACG gene has no introns. Lack of introns within the PRKACG gene facilitated the design of primers for amplification of portions of the gene from FRDA patients for mutation analysis. SSCP analysis of 15 patients did not reveal any variation. In order to determine whether PRKACA also maps to the region, we designed PRKACA-specific PCR primers. With these primers an amplification product of the expected size could be obtained from genomic DNA but not from any of the YACs and cosmids in the region.

New Polymorphisms in the FRDA Critical Region

We identified six new polymorphisms across the FRDA candidate region, all of which can be tested using PCR-based assays. The map position of the polymorphic fragments in indicated in figure 1. Information on these polymorphisms is summarized in table 1. Two polymorphisms, A3U1 and CS2, are two-allele systems, originally detected by SSCP. As the CS2 polymorphism changes *Aci*I and *Fnu*H4I restriction sites, a restriction enzyme assay was developed for this marker. The remaining polymorphisms, FR6, F71, F5225, and F8101, are of the STRP type.

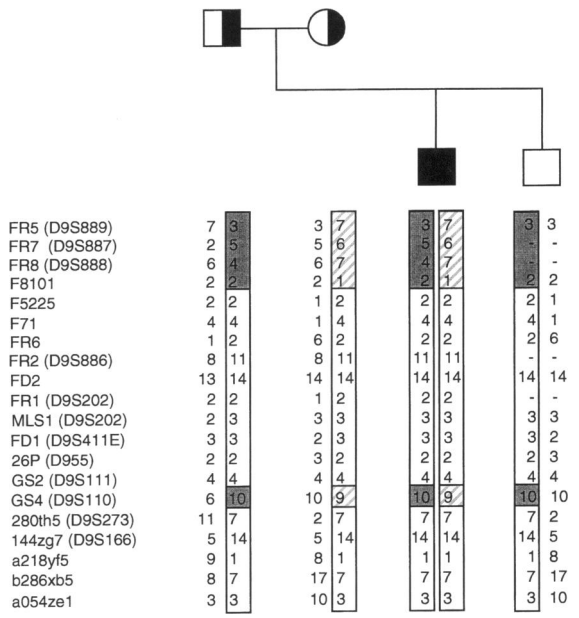

Figure 3 Pedigree diagram of the LF53 family. Haplotypes for FRDA-linked markers are indicated under each typed individual's symbol. Disease-associated haplotypes are boxed; regions of heterozygosity of paternal (*shaded*) and maternal (*hatched*) origin are indicated.

Homozygosity Analysis

Family LF53, described by Monros et al. (1994), was analyzed with the new markers, in an attempt to reduce the critical interval for the FRDA locus. The family pedigree and typing data are shown in figure 3. In family LF53, an affected subject with typical FRDA is the offspring of parents originating from the same small village in Spain. Homozygosity for five highly polymorphic markers telomeric to the FRDA critical region was previously observed in the affected subject of this family, a situation that may occur by chance with a probability of 3×10^{-3} and that was never found in 200 unaffected individuals from the same population (Monros et al. 1994). That strongly suggested that the affected individual in family LF53 is homozygous by descent at the FRDA locus, having inherited two copies of the same mutation from a common ancestor of his parents. However, the same individual was found to be heterozygous for the centromeric markers FR8 (D9S888), FR7 (D9S887), and FR5 (D9S889). This finding was interpreted as the result of an ancient recombination event that occurred just centromeric to the FRDA locus. Analysis with the new markers revealed heterozygosity for F8101 and homozygosity for all the other more telomeric markers. This result is fully consistent with

the hypothesis of a centromeric ancient recombination, suggesting that the FRDA mutation in this individual is localized telomeric to F8101. Accordingly, the critical interval for FRDA may be reduced to 150 kb between F8101 and FR2. In order to reinforce this conclusion by confirming homozygosity by descent in the affected individual, we typed family LF53 with additional telomeric markers that were not included by Monros et al. (1994). The affected individual was homozygous for all tested markers, with the exception of the $(CA)_n$ repeat D9S110 (GS4). Two lines of evidence, however, suggest that a mutation at this microsatellite is responsible for the observed heterozygosity. The first is the presence of homozygosity at highly polymorphic markers that are very close to D9S110 (fig. 3). These markers can be ordered according to the following map: 9cen–(FR7/FR8)–1 cM–(D9S273/D9S166/a218yf5/D9S110)–1 cM–b286xb5–1 cM–a054ze1–9qter. Homozygosity for all the markers distal to D9S110 has a probability of $\sim 1.5 \times 10^{-4}$ of occurring by chance. The second is the difference of only one CA repeat unit between the two D9S110 alleles in the affected individual, which is probably the most common result of mutation events at microsatellites, likely due to polymerase slippage. We directly observed a similar one repeat unit change for D9S110 in a family (CB-UN1) described in Chamberlain et al. (1993).

Discussion

Positional cloning of a disease gene requires the precise identification of a candidate region between flanking markers and the generation of a contig of genomic clones spanning the critical interval. Insufficient genetic information from crossings-over prevented the achievement of this goal for the FRDA locus for several years. Only recently, recombination information and linkage disequilibrium data became available, which allowed the positioning of the FRDA locus centromeric to a set of closely linked 9q13 markers. The successive development of the "FR" series of markers and the exclusion of X104/CSFA1 as candidate FRDA gene eventually allowed bracketing of the disease locus between flanking markers, within an estimated 300-kb interval (Duclos et al. 1994; Rodius et al. 1994). However, all FR markers were derived from a single YAC clone, y700F10, which was also the only source of physical map information for the FRDA region. In this report, we describe the isolation of additional YAC clones spanning the FRDA critical region, whose physical map has confirmed the map originally derived from y700F10. Furthermore, PFGE analysis of genomic DNA excluded major rearrangements, particularly large deletions, of the YACs. It is highly desirable to obtain a complete cosmid contig spanning the interval where an unknown disease gene is localized, in order to generate a finer physical map, to isolate additional markers and STSs, and to proceed toward gene isolation. We were able to reach this goal by subcloning the three YACs spanning the FRDA critical region into cosmids. Most of the region was covered by multiple cosmids derived from different YACs, which gives additional protection against small deletions or rearrangements that might have occurred within one of the YAC clones. One particular interval was apparently difficult to clone into cosmids, with corresponding candidate clones demonstrating signs of instability after growth in culture. This interval, which surrounds the *Mlu*I site at position 60 in the map (fig. 1), could eventually be spanned by the contig, but only coverage with a single cosmid could be obtained. Construction of this cosmid contig provides a critical reagent for attempts to isolate the disease gene.

We identified the X104/CSFA1 gene, isolated elsewhere (Duclos et al. 1994; Pandolfo et al. 1994), as the human homologue of the dog Z0-2 gene, which encodes an epithelial tight junction protein (Jesaitis and Goodenough 1994). ZO-2 is expressed in epithelial tissues and at low levels in the brain, and its transcript is undetectable in the heart, by northern hybridization. It is closely related to another tight junction protein, ZO-1, and it shows similarities to erythrocyte p55, to the *Drosophila lethal-disc-large* (*dlg*) gene, and to a synapse-associated protein from rat brain, PSD-95/SAP90 (Jesaitis and Goodenough 1994). The biological properties of ZO-2 make it an unlikely candidate for FRDA, and no mutation was found in 85% of the coding sequence of this gene in FRDA patients (Duclos et al. 1994).

By direct screening of arrayed cDNA libraries with YAC and cosmid clones, we identified an additional gene in the candidate FRDA region, PRKACG. This transcript has a very specific tissue distribution, being limited to the testis, and therefore it seems unlikely to be involved in the etiology of FRDA. The lack of introns within the PRKACG gene allowed a rapid screen for mutations in FRDA patients, and none were found. Screening of ~60,000 cDNA clones in arrayed libraries did not yield any additional transcript from the region, indicating that the level of expression of the FRDA gene is not very high, at least in the examined tissues, including heart—which is involved in the disease–liver, and placenta. As identification of additional transcripts from the region is in progress, we searched for polymorphisms associated with any isolated sequence that could be useful to further reduce the candidate interval. Six polymorphic systems were identified. These markers were used to determine the extension of loss of haplotype homozygosity in the affected offspring of the distantly related parents of family LF53 (Monros et al. 1994). This subject is highly likely to be homozygous by descent at the FRDA locus, as reported in Monros et al. (1994). However, he was shown to be homozygous for all mark-

ers telomeric to and including FR2 but heterozygous for FR8, FR7, and FR5, suggesting the occurrence of a recombination event between FRDA and FR8 in one of the generations separating his parents from their common ancestor who carried the disease-causing mutation. Analysis of the new markers demonstrated heterozygosity for the most centromeric one, F8101, and homozygosity for all the others, placing the recombination event telomeric to F8101, strongly suggesting that the FRDA locus lies within the 150 kb between F8101 and FR2. Narrowing the critical FRDA region to this relatively small interval, which is completely cloned into YACs and cosmids, is an important step toward the isolation of the disease gene.

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References

Chamberlain S, Farral M, Shaw J, Wilkes D, Carvajal J, Hillermann R, Doudney K, et al (1993) Genetic recombination events which position the Friedreich's ataxia locus proximal to the D9S15/D9S5 linkage group on chromosome 9q. Am J Hum Genet 52:99–109

Chamberlain S, Shaw J, Rowland A, Wallis J, South S, Nakamura Y, von Gabain A, et al (1988) Mapping of mutation causing Friedreich ataxia to human chromosome 9. Nature 334:248–249

Duclos F, Rodius F, Wrogemann K, Mandel J-L, Koenig M (1994) The Friedreich ataxia region: characterization of two novel genes and reduction of the critical region to 300 kb. Hum Mol Genet 3:909–914

Foss KB, Simard J, Bérubé D, Beebe SJ, Sandberg M, Grzeschik KH, Gagne R, et al (1992) Localization of the catalytic subunit Cg of the cAMP-dependent protein kinase gene (PRKACG) to human chromosome region 9q13. Cytogenet Cell Genet 60:22–25

Fujita R, Agid Y, Trouillas P, Seck A, Tommasi-Davenas C, Driesel AJ, Olek K, et al (1989) Confirmation of linkage of Friedreich's ataxia to chromosome 9 and identification of a new closely linked marker. Genomics 4:110–111

Fujita R, Sirugo G, Duclos F, Abderrahim H, Le Paslier D, Cohen D, Brownstein BH, et al (1992) A 500 kb YAC contig tightly linked to the Friedreich's ataxia locus contains five CpG clusters and a new highly informative polymorphic microsatellite. Hum Genet 89:531–534

Geoffroy G, Barbeau A, Breton G, Lemieux B, Aube M, Lepert C, Bouchard JP (1976) Clinical description and roentgenologic evaluation of patients with Friedreich's ataxia. Can J Neurol Sci 3:279–287

Harding AE (1981) Friedreich's ataxia: a clinical and genetic study of 90 families with an analysis of early diagnostic criteria and intrafamiliar clustering of clinical features. Brain 104:589–620

——— (1984) The hereditary ataxias and related disorders. Churchill Livingstone, Edinburgh

Jesaitis LA, Goodenough DA (1994) Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the drosophila discs-large tumor suppressor protein. J Cell Biol 124:949–961

Leone M, Rocca WA, Rosso MG, Mantel N, Schoenberg BS, Schiffer D (1988) Friedreich's disease: survival analysis in an Italian population. Neurology 38:1433–1438

Monros E, Smeyers P, Rodius F, Cañizares J, Moltò MD, Vilchez JJ, Pandolfo M, et al (1994) Mapping of Friedreich's ataxia locus by identification of recombination events in patients homozygous by descent. Eur J Hum Genet 2:291–299

Pandolfo M, Pizzuti A, Redolfi E, Munaro M, Di Donato S, Cavalcanti F, Filla A, et al (1994) Isolation of a new gene in the Friedreich's ataxia region on human chromosome 9 by cDNA direct selection. Biochem Med Metabol Biol 52:115–119

Pandolfo M, Sirugo G, Antonelli A, Weitnauer L, Ferretti L, Leone M, Dones I, et al (1990) Friedreich's ataxia in Italian families: genetic homogeneity and linkage disequilibrium with the marker loci D9S5 and D9S15. Am J Hum Genet 47:228–235

Rodius F, Duclos F, Wrogemann K, Le Paslier D, Ougen P, Billault A, Belal S, et al (1994) Recombinations in individuals homozygous by descent localize the Friedreich ataxia locus in a clone 450-kb interval. Am J Hum Genet 54:1050–1059

Sirugo G, Cocozza S, Redolfi E, Brice A, Cavalcanti F, De Michele G, Dones I, et al (1993) Linkage disequilibrium analysis of Friedreich's ataxia in 140 Caucasian families: positioning of the disease locus and evaluation of allelic heterogeneity. Eur J Hum Genet 1:133–143

Sirugo G, Keats B, Fujita R, Duclos F, Purohit K, Koenig M, Mandel JL (1992) Friedreich ataxia in Louisiana Acadians: demonstrations of a founder effect by analysis of microsatellite-generated extended haplotypes. Am J Hum Genet 50:559–566