A Peptidase Gene in Chromosome 8q Is Disrupted by A Balanced Translocation in a Duane Syndrome Patient

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PURPOSE. To identify the gene disrupted by a de novo reciprocal balanced translocation t(6;8)(q26;q13) in a patient with Duane retraction syndrome (DURS). The break point in chromosome arm 8q is positioned within the DURS1 critical region.

METHODS. Fluorescence in situ hybridization (FISH) analysis using cosmid and BAC clones covering the DURS1 locus was performed to define the break point position and its relationship with expressed sequence tags (ESTs) in the region. Once the interrupted gene was identified, the full-length cDNA was sequenced and the genomic organization defined. Eighteen patients with sporadic DURS without cytogenetic abnormalities involving the DURS1 region were screened for point mutations in the candidate DURS1 gene.

RESULTS. A carboxypeptidase gene (CPAH) was directly interrupted between the first and second exons in a patient with DURS who carried a de novo reciprocal balanced translocation t(6;8)(q26;q13) involving the DURS1 region on chromosome arm 8q13. The gene was transcribed in at least two alternative mRNA forms, with different start and stop codons.

CONCLUSIONS. The CPAH gene was interrupted in a patient with DURS carrying a translocation break point in the DURS1 region on chromosome 8q13. CPAH is therefore a likely candidate for this abnormality, even if the possibility that other genes are involved, either by direct effects on transcription units present in the first CPAH intron or by position effects, cannot be ruled out. Functional studies of the influence of this gene on the morphogenesis of eye muscles and their innervation may clarify this question.


Duane retraction syndrome (DURS; Mendelian Inheritance in Man [MIM] 126800) is a congenital eye-movement disorder characterized by failure in the development of cranial nerve VI, resulting in restriction or absence of abduction, restricted adduction, and narrowing of the palpebral fissure, with retraction of the globe on attempted adduction.1 Approximately 0.1% of the general population shows this anomaly (5% of all cases of strabismus).2 DURS is mostly a sporadic disorder. However, genetic causes have been recognized. A locus on chromosome arm 2q31 has been linked to dominant DURS in a few families (DURS2)3–5 and a contiguous gene syndrome adding DURS to the branchio-oto-renal (BOR) syndrome has been associated with deletions of a locus on 8q13.6–8 Analysis of a patient with isolated DURS and an 8q13 microdeletion, positioned the DURS locus (DURS1) centromeric to the BOR gene within a 3-centimorgan (cM) region between markers D8S533 and D8S1767.9

We report the molecular analysis of a patient with DURS with a reciprocal balanced translocation t(6;8)(q26;q13). The break point in 8q in this patient involved the DURS1 critical region and directly disrupted a gene identified as a carboxypeptidase family member.

METHODS

Patients

The study protocol adhered to the tenets of the Declaration of Helsinki. A 31-year-old man carrying a de novo reciprocal translocation t(6;8)(q26;q13) exhibited strabismus, amblyopia, and narrowing of palpebral fissures.5 Eighteen patients with sporadic DURS without any chromosome aberration were also enrolled in the study.

FISH Analysis

Metaphase chromosome spreads and nuclei were obtained from phytohemagglutinin-stimulated peripheral blood cells and from a lymphoblastoid cell line. Fluorescence in situ hybridization (FISH) experiments were performed as previously described.10 YAC clones from contig WC08.8 (Genethon, provided in the public domain by the French Association against Myopathies, Evry, France, and is available at http://www.genethon.fr; and the Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA, available at www.wi.mit.edu), BAC clones from an RPCI-11 library (Research Genetics, Huntsville, AL), and cosmid clones obtained from YAC subcloning were used. Probes were biotinylated (biotin-14-dATP; Life Technologies, Gaithersburg, MD) and/or digoxigenated (digoxigenin-11-dUTP, Roche, Mannheim, Germany) using a nick-translation kit (Bionick kit; Life Technologies). FISH color images were collected with a charge-coupled device (CCD) camera (Photometrics; Roper, Ojai, CA) operated by an image analyzer (Metasystem; Carl Zeiss, Jena, Germany). Each FISH result was confirmed by at least two series of experiments on different healthy control individuals.

YAC Subcloning

Yeast DNA embedded in agarose plugs was partially digested with Sall3A restriction enzyme and ligated to BamHI digested vector (SuperCoE; Stratagene, La Jolla, CA). Ligations were packaged using a lysate (Gigapack Gold; Stratagene). After infection of Escherichia coli XL1 blue cells, 5000 cosmids were plated on Luria-Bertani (LB) agar plus ampicillin, in 35-mm Petri dishes. Clones were hybridized twice to

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Supported by Telethon-Italy Grant E689 and by the Italian Ministry of Health.

Submitted for publication March 11, 2002; revised July 8, 2002; accepted July 16, 2002.

Commercial relationships policy: N.

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6 The 8q13 break point was mapped by subcloning one of the YAC clones spanning the cosmid clone contig between the two markers, by using PCR screening, until the genomic region between the markers was saturated.

**Isolation of Cosmid Clones Containing Single Gene Exons**

The YAC 925D9 cosm library was PCR screened with oligonucleotide pairs flanking single exons of the identified gene.

**DNA Sequencing**

All the nucleotide sequences were automatically performed on a sequencer (model 373) with a fluorescent dye terminator kit (both from Applied Biosystems Inc., Foster City, CA).

**Northern Blot Analysis**

Northern blot analyses were purchased (BD Biosciences-Clontech, Palo Alto, CA) and hybridized with the CPAH 3′ untranslated region (UTR) cDNA after radioactive labeling by the random primer method, according to the manufacturer’s recommendations. The blots contained approximately 2 μg of polyA+ RNA per lane. The G3PDH cDNA probe was used as a hybridization control. Filters were exposed to x-ray films for up to 30 days.

**Mutation Screening**

Genomic DNA was amplified by PCR with oligonucleotide pairs designed on CPAH exon flanking sequences. PCR products were directly sequenced.

**RESULTS**

Metaphase chromosomes of a patient with DURS and a de novo reciprocal balanced translocation t(6;8)(q26;q13) involving the DURS1 locus, were analyzed by FISH, using YAC clones hybridizing to 8q13. These preliminary experiments restricted the DURS1 gene position to a 40-kb critical region between markers WI-4901 and SHGC37325.6 We constructed a seven-cosmid clone contig between the two markers, by subcloning one of the YAC clones spanning the DURS1 locus (YAC 925D9, CEPH MegaYAC library).6 The 8q13 break point was mapped by FISH analysis within a single cosmid clone (cosmid 53.3). In silico search of the cosmid region for expressed sequences tags (ESTs) derived from the same cDNA clone. The 1494-bp cDNA was sequenced (GenBank accession number AY044833; GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and characterized as the transcript of a carboxypeptidase gene (CPAH according to the nomenclature of the National Center for Biotechnology Information [NCBI], Bethesda, MD). Comparing genomic sequences to the full-length retina cDNA showed that the CPAH gene is composed of eight exons, is approximately 200 kb long and is entirely contained in the DURS1 critical region. The first ATG of the longest open reading frame (ORF) of the retina cDNA is positioned in the fourth exon. The resultant 193-amino-acid peptide shows homologies to the mature form of many carboxypeptidases—in particular, zinc carboxypeptidases.

To confirm that the carboxypeptidase gene was interrupted by the reciprocal translocation t(6;8)(q26;q13), clones from the RPCI-11 human genomic BAC library (Research Genetics) were selected, by using cDNA sequence information, and used for a first set of FISH experiments. BAC 185G5 (containing exons 3–8 of the CPAH gene, as demonstrated by PCR) and BAC 7F18 (containing exon 8 only) showed signals on both the normal chromosome 8 and the der(8) chromosome, whereas BAC 319N16 (containing exons 1 and 2) showed an additional signal on the der(6) chromosome. To refine the break position within the gene, FISH analysis was performed with cosmid subclones from YAC 925D9.6 The Co1 cosmid subclone containing only the first CPAH exon showed two signals, one on the normal chromosome 8 and the other on the der(6) chromosome (Fig. 1A). The same result was obtained with a 5-kb...
genomic fragment containing the first exon. On the contrary, a cosmid clone containing the second exon (Co3) still hybridized the normal chromosome 8, whereas another signal was seen on the der(8) chromosome instead of the der(6) chromosome. Therefore, the break in chromosome arm 8 separated the first exon from the rest of the gene. The first exon was translocated onto the der(6) chromosome.

Northern blot experiments were performed using the CPAH 3'UTR as a probe to avoid cross-hybridization to highly homologous carboxypeptidase mRNAs. A hybridization control probe was used on the same blots. No signal for the CPAH probe was detected after long exposure of the blots (up to 1 month), suggesting that the expression levels of the CPAH gene in the analyzed tissues (adult brain, liver, lung, kidney, placenta, skeletal muscle, heart, and pancreas; fetal brain, liver, lung, and kidney) were either absent or very low. Control probe hybridization was detectable after 2 days. Afterward, RT-PCR experiments were performed using a primer pair designed on the gene 3'UTR and cDNA from different human tissues (brain, skeletal muscle, heart, placenta, kidney, and liver). Positive PCR products were obtained only from brain samples. Actually, besides the retina cDNA that we sequenced, only two CPAH ESTs from a brain cDNA library have been deposited so far in GenBank, in addition to a full-length hematopoietic progenitor cell cDNA sequence, representing an alternatively spliced product of the same gene (GenBank accession number AF221584).

Structure comparison of the retina and the hematopoietic CPAH cDNAs showed major differences. The hematopoietic CPAH cDNA is 1537 bp long and contains an additional exon sequence (exon 2b) between the second and the third exons of the retina transcript.

Insertion of the exon 2b sequence shifts the first ATG of the longest ORF from the fourth to the first exon. Exon 2b skipping in the retina mRNA results in a 148-amino-acid shorter peptide at the NH2 terminus, without the activation peptide. The retina mRNA-derived peptide is therefore translated as a mature carboxypeptidase instead of a preprocarboxypeptidase (Fig. 2B). Moreover, the hematopoietic cell mRNA uses exon 7 as the last exon, instead of exon 8. Exon 7 is longer, however, with an alternative stop codon and 3'UTR. The COOH tail of the resultant protein is different and shorter than the retina product (Fig. 2B).

Eighteen patients with sporadic DURS were analyzed for microdeletions and point mutations of the CPAH gene. Several polymorphic variants, also detected in a series of 50 control samples, were found, but no pathogenic abnormality was noted.

**DISCUSSION**

The DURS1 locus was mapped at 8q13 by molecular characterization of two patients carrying an 8q microdeletion, and a number of anomalies including DURS. More recently, we narrowed the DURS1 locus analyzing a de novo balanced translocation t(6;8) in a patient with DURS. Fine molecular analyses showed that a carboxypeptidase gene CPAH was disrupted by

**Figure 2.** (A) Alignment of the zinc carboxypeptidase domain (PROSITE number P00132; provided in the public domain by Swiss Institute of Bioinformatics, Geneva, Switzerland and available at http://www.ncbi.nlm.nih.gov/GENE/prosite) of CPAH with other carboxypeptidase sequences. (B) Alignment of the CPAH peptide sequences inferred from the retina (bold) and from the hematopoietic progenitor cell cDNAs. Asterisks mark the identical regions. The retina form has no NH2 terminus with the activation domain (underscored). The COOH portions of the proteins are also different. ^, zinc carboxypeptidase domain.

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the chromosome rearrangement. 

Expression studies by Northern blot and RT-PCR suggest an absent or very low level of the CAH gene transcription in most tissues, confirmed by the poor representation in the EST database. RT-PCR experiments show that the gene is expressed in brain. The brain expression of CAH has been recently confirmed by Wei et al., who characterized the gene protein product as a member of the A/B metallocarboxypeptidase subfamily. The gene is also expressed in the retina, as demonstrated by two sequences in the EST database. cDNA from orthologue genes are absent in GenBank databases.

Data on the effect of the CAH gene on eye muscle development and innervation are not available. Many regulatory carboxypeptidases are present in the central nervous system and have important functions in protein and peptide processing. Unlike the digestive forms, many regulatory carboxypeptidases have no pro region, a phenomenon we have observed in the retina CAH putative peptide when compared with the blood cell form. No CAH gene mutation was found in a series of 18 patients with DURS. Because these patients occur sporadically in families, it is difficult to figure out the percentage of true cases of genetic DURS in the sample. The DURS locus assignment at 8q13 is based only on two sporadic cases with deletion of the same region. No DURS-affected family showing linkage to DURS1 has been described. The negative mutation screening demonstrates that CAH gene mutations are not a common cause of DURS.

We cannot rule out the possibility that the break in the CAH gene acts on a close gene by positional effect, as demonstrated in other genetic disorders. We have also analyzed the CAH 120-kb first intron sequence for the presence of possible inner genes. Three ESTs have similarities with intron sequences. All of them are single entries in the database without a multiple exon organization, and two of them are flanked by genomic polyA stretches, suggesting a cloning artifact from heterogeneous nuclear RNA retrotranscription during the cDNA synthesis step. Moreover, three two-exon putative genes have been deduced by the GenomeScan program (created by Christopher Burge and provided in the public domain by the Massachusetts Institute of Technology, Cambridge, MA; available at http://genes.mit.edu/genomescan.html). Two of them incorporate either the first or the second CAH exons and are transcribed from the same strand of CAH. However, no real transcript of that type has been cloned so far. The same is valid for the third putative intronic gene transcribed by the opposite CAH DNA strand. RT-PCR experiments with primers designed on the intron putative transcripts from the minus strand did not show amplification signals.

Theoretically, disruption of a gene by the break in chromosome arm 6q could be the cause of the syndrome in patients with DURS, because another case of rearrangement involving both the DURS1 region and 6q has been described. However, the 6q break points in the two patients occurred on different chromosomal bands (6q25 and 6q26). We tested both patients with a probe for band 6q25 (LiStarFISH; Affinity Research Products, Ltd., Marnhead, UK), confirming the cytogenetic results. The 6q probe signals were relocated distal to the 8q insertion region in the previously reported case, whereas they appeared at the normal position—proximal to the 8q region translocated onto 6q—in the present case. Moreover, although several pieces of evidence associate DURS with abnormalities in chromosome 8 (e.g., its occurrence in trisomy 8 mosaics), no evidence of isolated abnormality on chromosome 6 in patients with DURS has been described so far. Analysis of the CAH gene function during development would help clarify its pathogenic role in the morphogenesis and innervation of the eye musculature.

Acknowledgments

The authors thank the YAC Screening Center, Milan, Italy, for providing YAC clones.

References