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Cloning of the breakpoints of a deletion associated with choroideremia*

F. P. M. Cremers[1], F. Brunsmann[2], W. Berger[1], E. P. M. van Kerkhoff[1], T. J. R. van de Pol[1], B. Wieringa[1], I. H. Pawlowitzki[2], and H.-H. Ropers[1]

[1] Department of Human Genetics, University Hospital, University of Nijmegen, P.O.Box 9101, NL-6500 HB Nijmegen, The Netherlands
[2] Institut für Humangenetik der Universität, W-4400 Münster, Federal Republic of Germany

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Summary. In order to characterize a previously described submicroscopic deletion encompassing (part of) the choroideremia (tapetochoroidal dystrophy: TCD) gene, we have cloned a 10.5-kb *Eco*RI fragment from the patient's DNA; this fragment carries the junction between both deletion endpoints ("junction fragment"). The distal portion of this fragment defines a new marker within, or just distal to, the TCD gene. This marker has been employed to confirm the diagnosis in several affected family members, and to rule out carriership in a female at risk with conspicuous clinical signs.

Introduction

Tapetochoroidal dystrophy (TCD) is an X-linked disorder of the retinal pigment epithelium (RPE), choroid, and retina; it causes progressive nightblindness and visual field constriction in affected males (Goedbloed 1942; Waardenburg 1942; McCulloch and McCulloch 1948). Female carriers generally show characteristic pigment mottling in the midperiphery or posterior pole; this closely resembles fundus anomalies observed in affected young males (Pameijer et al. 1960; Krill 1967). In most cases, the fundus signs in TCD carriers are stationary and cause no visual field defects.

The gene locus for TCD has been assigned to Xq21 by analysis of deletions in male TCD patients with complex phenotypes (Hodgson et al. 1987; Nussbaum et al. 1987; Schwartz et al. 1988; Cremers et al. 1988, 1989a). Molecular characterization of the Xq21 region in patients with classical TCD revealed microdeletions that spanned the DXS165 locus, but none of the other loci tested (Cremers et al. 1987). Cloning of several new markers in the vicinity of the DXS165 locus by use of chromosomal walking and jumping techniques enabled us to define the size of a DNA segment that is spanned by four microdeletions, and to characterize the X-chromosomal breakpoint of a de novo X;13 translocation that was identified in a female with TCD (Cremers et al. 1989b).

Here we report on the cloning and characterization of a DNA fragment that carries the junction between the endpoints of a previously described microdeletion that encompasses (part of) the TCD gene (Cremers et al. 1987, 1989b). The junction fragment was employed to perform reliable carrier detection in the patient's family.

Materials and methods

TCD patients

The TCD family shown in Fig. 3 forms part of a five generation family previously described in detail by Diekstall and Demeler (1988). All members except individuals 7.2 and 7.12 were examined by funduscopy and functional ophthalmologic methods. TCD was diagnosed in three males (7.6, 7.7, 7.11). Two obligate carrier females (7.1, 7.4) showed the expected pigmentation alterations in the midperiphery of the fundus. In a possible female carrier (7.8), fundus characteristics were inconclusive (Diekstall and Demeler 1986, 1988).

Cloning of the 7.6 deletion-junction fragment

DNA from patient 7.6 (100 µg) was digested with *Eco*RI; fragments were ethanol precipitated, and resolved electrophoretically on a 0.7% low-gelling-temperature (LGT) agarose gel. Flanking lanes containing size markers were subsequently cut off and stained with ethidium. The unstained central part of the gel was cut into 2 mm-wide strips perpendicular to the running direction. A small piece of each strip, corresponding to one lane of DNA, was melted for 5 min at 95°C, and brought to 0.3–0.5 *M* NaOH. The samples were spotted onto a Hybond-N membrane by means of a slot-blot apparatus that had been prewarmed to 37°C. DNA (4 µg) from a male, female, and a cell line containing four X-chromosomes (LCL127) were used as controls. The membrane was neutralized by washing in 0.5 *M* TRIS-HCl pH 7.5, 1.5 *M* NaCl, 1 m*M* EDTA for 10 min, air-dried, and exposed to UV-light (305 nm) for 3 min.

The membrane was hybridized to probe p1bD5-II, a single copy sequence located just centromeric to p1bD5, and washed as recommended by the manufacturer. A positive hybridization signal at 10.5 kb was observed. Next, the LGT-agarose strip containing DNA from the 10.5 kb *Eco*RI fragment was rinsed three times

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Offprint requests to: F. P. M. Cremers

in 10 m*M* TRIS-HCl pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, melted at 65°C for 10 min, and incubated overnight with 50 U agarase/ml (Calbiochem) at 37°C. DNA (2 μg) was recovered by sequential phenol and chloroform extraction, and by ethanol precipitation in the presence of 0.3 *M* NaAc, pH 6. A portion (120 ng) of the 10.5 kb *Eco*RI fraction was ligated to 500 ng of *Eco*RI and *Sac*I doubly cleaved lambda DASH DNA (Stratagene, La Jolla, Calif). The ligation mixture (310 ng) was packaged in vitro using Promega packaging extract. After plating on *Escherichia coli* strain P2392, 95000 clones were obtained. Identification of p1bD5-II positive plaques and subcloning of DNA fragments into plasmid vectors pGEM3 (Promega) or pBluescript (Stratagene) were performed according to published procedures (Maniatis et al. 1982). The nucleotide sequences of DNA fragments p1bD5-I and pJ7.6 were determined in one strand employing the supercoil sequencing method, essentially as given by Chen and Seeburg (1985), and Hattori and Sakaki (1986).

Southern blot analysis

Chromosomal DNA was isolated as described by Aldridge et al. (1984), with minor modifications. DNA (10 μg) was digested with the appropriate restriction enzyme, fragments were resolved by agarose gel electrophoresis and blotted onto Gene Screen Plus (NEN) membranes as described (Cremers et al. 1989a). All probes employed have been described elsewhere (Cremers et al. 1989a, b). Insert DNAs were electrophoretically separated from the plasmid vector and isolated in LGT-agarose (Biorad). Radioactive probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein 1983, 1984). Prior to hybridization, probes containing repetitive sequences (p1bD5, p1bD5-I, p1bD5-II, pJ60, pJ7.6) were preassociated with sonicated human DNA as described by Litt and White (1985). Details of (pre)hybridization and washing of filters were as published (Church and Gilbert 1984).

Field inversion gel electrophoresis (FIGE) analysis

Methods for the preparation of high molecular weight LCL127 (48,XXXX) DNA in agarose blocks, restriction enzyme digestion, FIGE, and blotting, have been described in detail elsewhere (Cremers et al. 1989a).

Results

Cloning of the 7.6 deletion junction fragment

In a previous study, we isolated a cosmid spanning the DXS165 locus (c237; Cremers et al. 1989b). Single- or low-copy fragments were subcloned, gel-purified, and used as probes. The proximal endpoint of the deletion in patient 7.6 was mapped between p1bD5 and a more centromeric subclone of c237, p1bD5-IV (Cremers et al. 1989b). With probe p1bD5-I or -II, Southern analysis of *Eco*RI digested DNA from patient 7.6 and a male control revealed a normal band of 7.8 kb in the control male, and an altered (junction) fragment of 10.5 kb in patient 7.6 (Fig. 1b). Probe p1bD5-I detects a 0.9-kb *Hin*dIII band in DNA of patient 7.6, whereas in healthy controls, a 0.5-kb band is seen (results not shown). This indicates that the proximal endpoint of this deletion must be located within the p1bD5-I sequence (Fig. 1a).

The 10.5-kb *Eco*RI fragment was gel-purified and cloned into lambda DASH phage vector DNA. Probes p1bD5-I and -II detected 4 clones out of 95000 recombinants, one of which was analyzed in detail. The restriction pattern of this clone (λ J7.6; Fig. 1a) agreed with that expected for the rearranged chromosome segment. The authenticity of the junction clone was determined by Southern analysis of genomic DNAs from the deletion patients 3.5, 7.6, 25.6, and a control male, and by detailed analysis of λ J7.6 plasmid-borne subclones. The 6.0-kb *Hin*dIII-*Eco*RI fragment originating from the telomeric part of λ J7.6 (Fig. 1a) predominantly contains

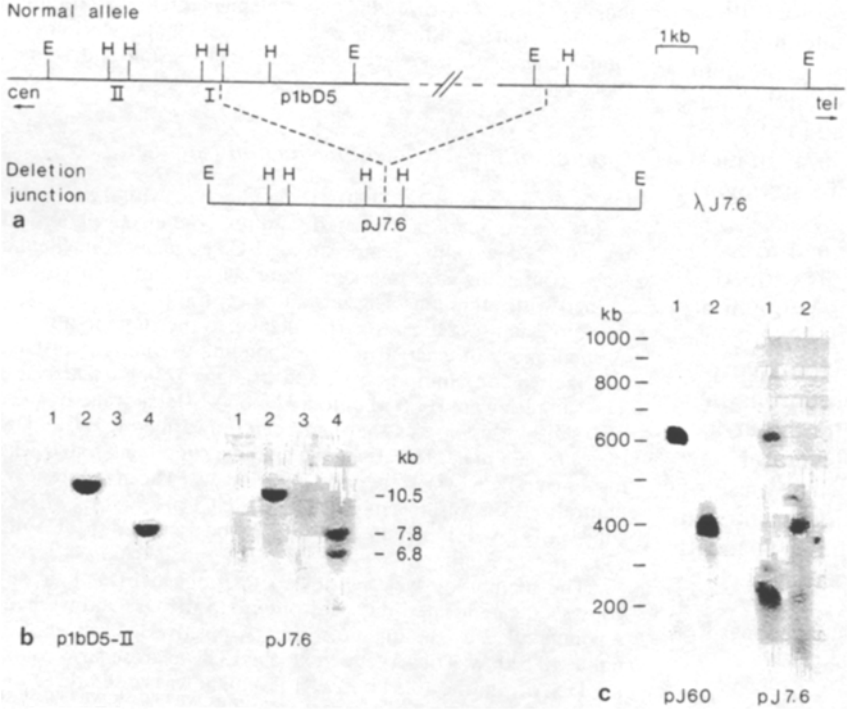

Fig. 1a–c. Cloning and characterization of the deletion junction fragment in TCD patient 7.6. **a** Restriction maps of the DNA segments that span the 7.6 deletion endpoints, and of the deletion junction clone λ J7.6. Only *Eco*RI *(E)* and *Hin*dIII *(H)* sites are indicated. **b** Southern blot analysis of *Eco*RI-digested DNA of TCD patients 3.5 *(lanes 1)*, 7.6 *(lanes 2)*, 25.6 *(lanes 3)*, and a male control *(lanes 4)*, employing a probe located just centromeric to deletion 7.6 (p1bD5-II), and the deletion junction clone pJ7.6. The weak hybridization signal detected with pJ7.6 in patient 3.5 *(lane 1)*, is the result of a smaller amount of DNA in this lane compared with the others. **c** FIGE analysis with probes pJ60 and pJ7.6. LCL127 DNA was digested with *SfiI (lanes 1)* or *SalI (lanes 2)*. The scale shown on the *left* is based on the migration of the *Saccharomyces cerevisiae* chromosomes that were employed as size markers

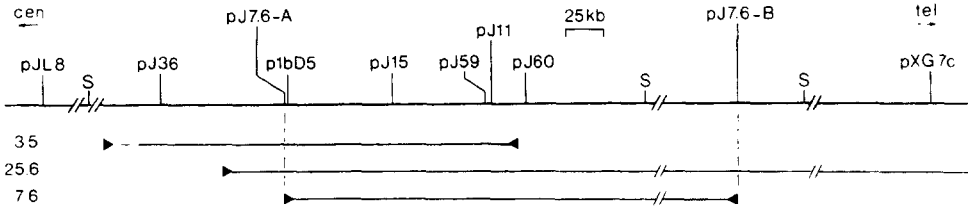

Fig. 2. Deletion map of the TCD locus at Xq21.2. Deleted regions are depicted between *arrowheads*. The centromeric endpoint of the deletion in patient 3.5 is not accurately mapped as indicated by a *dashed line*. Three *Sfi*I *(S)* sites are indicated to underscore the finding that pJ7.6-B is located on a 230-kb *Sfi*I fragment that does not span any of the other DNA probes shown here

moderate or highly repetitive sequences (not shown). The actual deletion-breakpoint containing subclone pJ7.6 (0.9-kb *Hin*dIII) proved to be a low-copy probe, and subsequently was used in Southern analysis. In addition to the bands detected by p1bD5-II, pJ7.6 hybridizes to a 6.8-kb *Eco*RI fragment in patient 3.5 and control male (Fig. 1b). This *Eco*RI band is detected by the part of clone pJ7.6 (pJ7.6-B) that is located distal to the breakpoint junction, telomeric to the 3.5 deletion, and completely contained within the 25.6 deletion (Fig. 2). Nucleotide sequencing of clones pJ7.6 and p1bD5-I revealed that the junction fragment contains 466 bp of the region flanking the centromeric deletion endpoint, and approximately 430 bp flanking the telomeric deletion endpoint.

FIGE analysis

To determine the physical location of pJ7.6-B, we hybridized pJ7.6 to a FIGE blot containing *Sfi*I- and *Sal*I-digested control DNA. As expected, because approximately half of the clone (pJ7.6-A) originates from the DXS165 locus, pJ7.6 detects the *Sfi*I and *Sal*I bands of 625 kb and 400 kb, reported earlier (Cremers et al. 1989a). In addition, pJ7.6 detects a 230-kb *Sfi*I band, and two faint *Sal*I bands of 230 kb and 125 kb (Fig. 1c). From these data, combined with the data from deletion analysis, we conclude that pJ7.6-B is located at an unknown distance telomeric to pJ60, but centromeric to pXG7c (Fig. 2).

Carrier detection in family 7

Hybridization of probe p1bD5 to a blot containing *Eco*RI-digested DNA from members of family 7 (see Fig. 3), identifies a deletion in the affected males 7.6, 7.7, and 7.11, as expected. Dosage analysis carried out in order to determine TCD carriership in individuals at risk was hampered by different factors, such as insufficient availability of DNAs, blotting conditions, and nonspecific degradation of DNA. With the pJ7.6 probe, unambiguous proof could be obtained for the carrier status of females 7.1 and 7.4, as they display the junction 10.5-kb *Eco*RI band next to the normal 6.8-kb and 7.8-kb *Eco*RI fragments (Fig. 3). Two individuals at risk for carrying the TCD deletion, namely female 7.8 and male 7.5, showed only the normal 6.8-kb and 7.8-kb *Eco*RI bands.

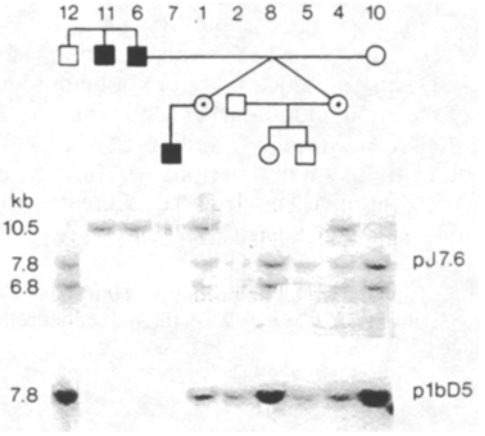

Fig. 3. Southern blot analysis of *Eco*RI-digested DNA of individuals from family 7 with probes pJ7.6 and p1bD5

Discussion

As a prerequisite for cloning of the TCD gene, we isolated several clones from the relevant DNA region by chromosomal jumping (Cremers et al. 1989b). In addition, we have obtained markers from a library highly enriched for sequences from the TCD locus by preparative FIGE (Pol et al. 1990). As a third approach, described here, we have cloned the junction fragment (pJ7.6) of a submicroscopic deletion associated with TCD. FIGE analysis of control DNA indicated that the junction clone detects two different *Sfi*I fragments. Therefore, the corresponding deletion is not confined to the 625-kb *Sfi*I fragment, which was previously shown to carry part of the TCD gene, and we have to conclude that deletion 7.6 extends further distally, encompassing at least one, and possibly several, other *Sfi*I fragment(s).

Cloning of the proximal deletion breakpoint and the corresponding junction fragment enabled us to provide a reliable diagnostic test for relatives of patient 7.6. The presence of a deletion-specific junction fragment confirmed the diagnosis of TCD in three affected males and the obligate carrier state of two females. The absence of the junction fragment in the DNA of a boy (7.5) and a girl (7.8), both at risk of inheriting the deletion from their carrier mother, ruled out TCD and carriership of TCD in these individuals, respectively. It is noteworthy that at the age of 8 years, the fundi of individual 7.8 showed some small midperipheral pigmentations that were taken as being indicative of TCD carriership. Therefore, our finding illustrates that, whenever possible, carrier diagnosis in TCD should be corroborated by DNA analysis. Recent data from our laboratory indicate that

similar deletions encompassing part of the TCD gene can be detected in at least 10% of the patients (unpublished results). In most other families, unambiguous diagnostic results can be obtained with closely linked probes (Pol et al. 1990).

As depicted in Fig. 2, pJ7.6-B is located between the most telomeric jump clone (pJ60) and the anonymous probe pXG7c (DXS95), and is therefore useful as a new molecular entry site for the chromosomal segment that carries the TCD locus. Indeed, employing pJ7.6 as a probe, we have recently detected two microdeletions associated with TCD, one of which had not yet been identified with any of the anonymous markers and jump clones available from the Xq21.2 region (Cremers et al., 1990). Characterization of these small deletions will further assist in defining the genomic size of this locus and eventually help to clone the TCD gene itself.

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