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Chromosomal jumping from the *DXS165* locus allows molecular characterization of four microdeletions and a *de novo* chromosome X/13 translocation associated with choroideremia

(retinal dystrophy/X chromosome/gene mapping)

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ABSTRACT **Choroideremia (tapeto-choroidal dystrophy, TCD), an X chromosome-linked disorder of retina and choroid, causes progressive nightblindness and central blindness in affected males by the third to fourth decade of life. Recently, we have been able to map the *TCD* gene to a small region of overlap between five different, male-viable Xq21 deletions that were found in patients with TCD and other clinical features. Two families were identified in which classical, nonsyndromic TCD is associated with small interstitial deletions that are only detectable with probe p1bD5 (*DXS165*). To characterize these and two other deletions that were identified more recently, we have used the chromosome walking and jumping techniques to generate a set of five chromosomal-jumping clones flanking the *DXS165* locus at various distances. With these clones, we could localize four of the eight deletion endpoints and the breakpoint on the X chromosome of a female with a *de novo* X/13 translocation and choroideremia. These studies assign the *TCD* gene, or part of it, to a DNA segment of only 15–20 kilobases.**

Hereditary dystrophies of the retina and the choroid constitute a clinically heterogeneous group of Mendelian disorders that play an important role in the etiology of blindness in man. Individual diseases of this group have also been shown to be genetically heterogeneous. For retinitis pigmentosa (RP), autosomal dominant and recessive forms are known, and at least two distinct *RP* genes have been mapped to different sites on the short arm of the X chromosome (1–3). The gene defect underlying choroideremia, another member of this group, has been assigned to Xq13–q21 because of its tight linkage with various polymorphic probes from this region (4–6). Choroideremia is characterized by progressive degeneration of both retina and choroid leading to blindness by the third or fourth decade of life (7–9). Therefore, tapeto-choroidal dystrophy (TCD) is a more appropriate designation for this disorder. Female carriers, although normally asymptomatic, show characteristic patchy changes in the retinal pigmented epithelium that reflect random X chromosome inactivation.

To elucidate the molecular and, eventually, the biochemical defect underlying this disorder, we have set out to isolate the *TCD* gene by means of reverse-genetics strategies—i.e., by making use of its known chromosomal location. In general, very precise physical mapping, more accurate than can be achieved with linked DNA markers alone, is a prerequisite for the successful application of this strategy. Physical fine mapping of probes depends on the availability of chromosomal aberrations such as translocations, duplications, and deletions. Indeed, translocations and microdeletions were instrumental for the cloning of all four human genes that have been isolated in this way (10–13).

TCD and other clinical features have been described in various patients with cytogenetically visible or submicroscopic deletions spanning the Xq21 band (14–16). To determine the location of the *TCD* locus more precisely, we have characterized several of these deletions with anonymous cloned DNA sequences from proximal Xq (17). This has enabled us to divide the Xq21 band into seven different intervals and to assign the *TCD* locus to interval 3 spanning the probes pJL8 (*DXS233*), p1bD5, (*DXS165*), and pXG7c (*DXS95*) (18). Molecular characterization of patients with classical, nonsyndromic TCD revealed several additional deletions, part of which was confined to the *DXS165* locus (ref. 19, and unpublished data). This suggested to us that the distance between this locus and the *TCD* gene might be small enough to be bridged by genomic walking and jumping experiments.

Chromosome jumping is based on the circularization of long genomic DNA fragments and subsequent cloning of the junction fragments of these circles (20–27). In this way, it is possible to circumvent insert-size constraints of conventional cloning vectors, which limit the distance that can be covered by individual chromosome walking steps. Iterative screening of a previously described chromosomal-jumping library (21) has enabled us to isolate various new DNA sequences in the vicinity of the *DXS165* locus and to determine the size of a DNA segment that is spanned by four different deletions detected in TCD patients. In addition, we used these probes to map the X chromosomal breakpoint of a *de novo* X/13 translocation that has been described in a female with typical signs of choroideremia (28).

MATERIALS AND METHODS

TCD Patients. For the male patients 3.5, 7.6, 25.6, and LGL1134, the diagnosis of classical TCD had been established by detailed ophthalmologic examination (see, for instance, refs. 29 and 30). A female patient showed mild choroideremia and infertility secondary to premature ovarian failure (28). By using trypsin-Giemsa staining she was karyotyped as 46,X,t(X;13)(q21.2;p12). Replication studies with

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Abbreviations: TCD, choroideremia (tapeto-choroidal dystrophy); RP, retinitis pigmentosa.

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BrdUrd showed that the normal X was inactivated. Somatic cell hybrids formed between skin fibroblasts from this patient and mouse A9 fibroblasts yielded a mouse–human hybrid (CIII-1), which contained the derivative der(13) chromosome but not the reciprocal der(X) chromosome.

Southern Blot Analysis. Chromosomal DNA was isolated as described elsewhere (31) with minor modifications. DNA (10 μg) was digested with the appropriate restriction enzyme, and fragments were resolved by agarose gel electrophoresis and blotted onto GeneScreen*Plus* (NEN) membranes as described (18). Radioactive probes were prepared by random oligonucleotide priming (32, 33). Insert DNAs were electrophoretically separated from the plasmid vector and isolated in low-gelling-temperature agarose. Prior to hybridization, probes containing weak repetitive sequences (low-copy probes; p1bD5, p1bD5-I, pJ15-II, and pJ60) were preassociated with sonicated total human DNA as described (17, 34). Details of (pre)hybridization and washing of filters have been published (35).

Libraries. The construction of the 100-kilobase (kb) human chromosomal-jumping library has been described elsewhere (21). Approximately 2.5×10^6 chromosomal-jumping clones ("jump clones") of the amplified library (which, before amplification, comprised about 1.5×10^6 independent clones) were plated on the bacterial host MC1061 ($supF^-$) at a density of 100,000 plaques per 130-mm plate. Plating was performed in 0.7% top-agarose/Luria broth supplemented with 30% (vol/vol) glycerol for long-term storage as described by Klinman and Cohen (36). Plaques were lifted on Hybond-N membranes (Amersham) and subsequently screened as recommended by the manufacturer. The filters were hybridized three times with a combination of four probes. Master plates were stored at −70°C. Phage containing jump inserts were recovered from the master plates by drilling with a cut-off plastic tip at −20°C (36), and DNAs were purified by standard methods (37).

A phage λ EMBL 3 genomic library constructed from DNA of a human chronic myelogenous leukemia patient was provided by G. Grosveld (Erasmus University, Rotterdam); 5×10^5 plaque-forming units were plated on *Escherichia coli* LE392 and screened as described above for the chromosomal-jumping library. A c2RB cosmid library was constructed from a cell line containing four X chromosomes (GM1202; 49, XXXXY). Independent clones (5×10^5) were plated and screened as described elsewhere (38).

Subcloning and Mapping of Inserts. Phage DNA was digested with *Eco*RI, purified by phenol and chloroform extraction, and directly cloned into *Eco*RI–cleaved, phosphatase-treated pGEM vectors or ligated into *Eco*RI-cut, dephosphorylated phage λZAPII vector arms (Stratagene). The manufacturer's excision protocol was used to convert λZAPII constructs into plasmid (Bluescript) clones. Restriction maps of the inserts were generated by digestion with *Eco*RI, *Ava* I (which cuts in the middle of the *supF* gene), and *Eco*RI/*Ava* I, followed by Southern blotting and hybridization with *supF* and the starting clone.

RESULTS

Identification of a Third Deletion. In a previous report we showed that probe p1bD5 (*DXS165*) detected two deletions (patients 3.5 and 7.6) among 8 patients with classical TCD (19). Screening of an additional 22 TCD patients resulted in the identification of another deletion (patient 25.6) that not only spans p1bD5 (see Fig. 3*c*), but also pXG7c (*DXS95*), pXG8b, p722 (*DXS110*), pDP34 (*DXYS1*), and p47b (*DXYS5*) (data not shown). Therefore, the deletion in patient 25.6 encompasses part of interval 3, the entire interval 4, and part of interval 5 of the Xq21 band (18, 39).

Chromosomal Walking and Jumping. Employing p1bD5 as a probe on a human genomic cosmid library, clone c237 was isolated, which contained 36 kb of insert DNA (Fig. 1). Single- or low-copy fragments (p1bD5-I to -IV) were subcloned, gel-purified, labeled, and hybridized to nylon filters containing *Eco*RI-digested DNA of patients 3.5, 7.6, and 25.6 and a male control. In contrast to p1bD5, p1bD5-IV detects homologous sequences in the DNA of patient 7.6 (Fig. 3*b*, lane 2). Therefore, one end-point of the deletion in patient 7.6 must be located between p1bD5-IV and p1bD5. We have used this breakpoint as a specific marker for the presence or absence of a deletion in females at risk for carrying the TCD defect (39). For chromosomal jumping from the *DXS165* locus, we used p1bD5 and p1bD5-IV as starting points.

Screening the 100-kb human chromosomal-jumping library with p1bD5-IV resulted in the isolation of one jump clone, λJ36 (Fig. 2). The insert on this phage (7.5-kb *Eco*RI frag-

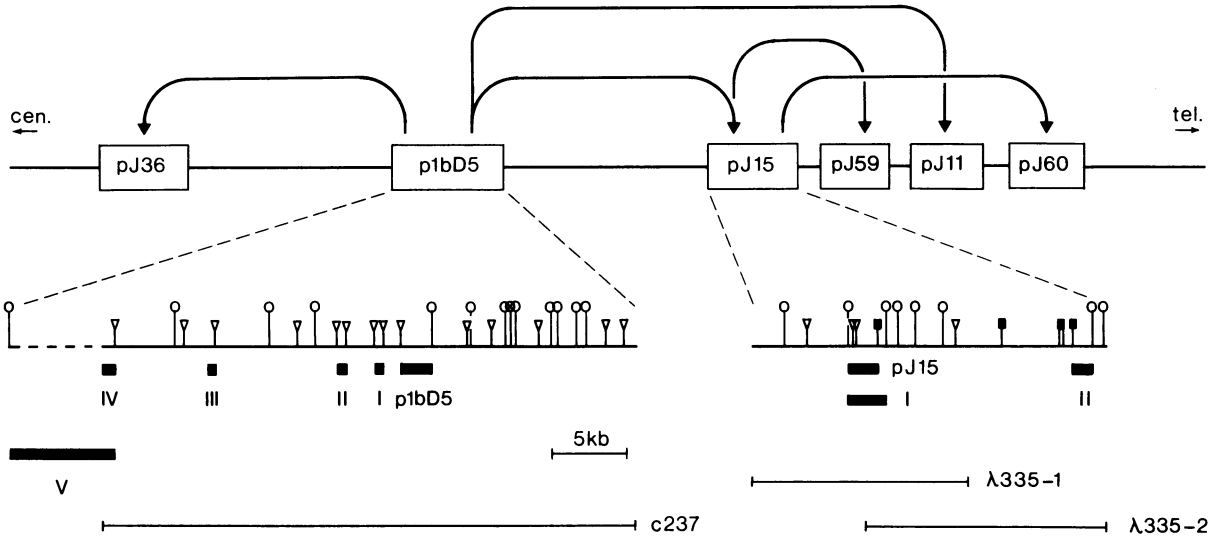

FIG. 1. Chromosomal walking and jumping centromeric and telomeric of the *DXS165* (p1bD5) locus. (*Upper*) The relative positions of the jump clones are based on the position of restriction-sites within the jump clones (Fig. 2), on Southern analysis of a deletion panel (Fig. 3), and on Southern analysis of normal DNA. (*Lower*) Restriction maps of the genomic clones c237 and λ335-1 and -2. The *Eco*RI (○) and *Hin*dIII (▽) sites are indicated for both c237 and the λ335 clones. *Bgl* II sites (■) are only given for the λ335 clones. p1bD5-V represents a sequence present in λJ36, which partly overlaps p1bD5-IV.

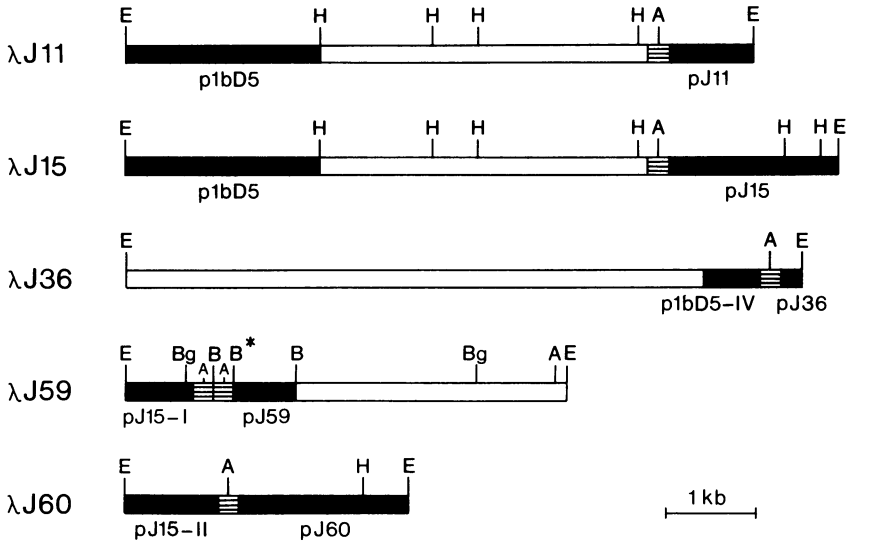

FIG. 2. Restriction maps of the phage λ inserts isolated from the chromosomal-jumping library. The *Eco*RI, *Hin*dIII, and *Ava* I sites are indicated in all inserts. For λJ59, we also indicated the *Bam*HI and *Bgl* II sites. The black boxes represent single- or low-copy sequences of the starting clones [at the left side of the *sup*F gene(s)] and the new jump clones (at the right side), whereas hatched boxes represent the *sup*F genes with their internal *Ava* I sites. The asterisk indicated in the restriction map of λJ59 indicates a *Bam*HI site that might be created in the cloning procedure. The *sup*F gene was originally flanked by *Bam*HI sites, and the genomic fragments, by *Sau*3AI sites (21).

ment; Fig. 2) consists almost entirely of a sequence (7.15-kb *Eco*RI/*Ava* I fragment) that spans part of p1bD5-IV and extends to an *Eco*RI site situated outside of c237 (denoted p1bD5-V, see Fig. 1). The remaining part of this insert, designated pJ36 (0.35-kb *Eco*RI/*Ava* I fragment), was used as a probe to screen a Southern blot carrying DNA from the three deletion patients. As shown in Fig. 3*a*, pJ36 is located outside the 7.6 and 25.6 deletions but still inside the 3.5 deletion. This indicates that with this jump we have crossed an endpoint of deletion 25.6. Since the 25.6 deletion encompasses several probes that are located distal to p1bD5, this breakpoint must be proximal to p1bD5. At the same time, this proves that the *TCD* gene must be located telomeric to the 7.6 deletion breakpoint.

With probe p1bD5, two different jump clones, λJ11 and λJ15, could be isolated (Fig. 2). The end fragments of both clones, pJ11 (1.05-kb *Eco*RI/*Ava* I fragment) and pJ15 (2.0-kb *Eco*RI/*Ava* I fragment), are devoid of repetitive sequences and are located within all three deletions (for pJ15, see Fig. 3*d*). To generate starting probes for further jumps in the telomeric direction, both single-copy probes were used to isolate corresponding sequences from a λ phage human genomic library. With probe pJ15, two clones with overlapping inserts were detected (λ335-1 and -2), spanning a total of 23 kb (Fig. 1). The orientation of these clones was deduced from the location of two *Hin*dIII sites situated within 500 base pairs (bp) of the *Eco*RI site of pJ15 (Figs. 1 and 2). In the λ335 inserts, two useful sequences were identified, a unique copy probe spanning pJ15, pJ15-I (2.5-kb *Eco*RI fragment), and pJ15-II—a low-copy probe (1.3-kb *Bgl* II/*Eco*RI fragment) located just centromeric from an *Eco*RI site (Fig. 1).

Screening the chromosomal-jumping library with pJ15-I yielded a λ phage (λJ59) that contained a fragment of 3.9-kb next to the pJ15-I sequences. From this fragment, a single-copy sequence (pJ59; 0.7-kb *Bam*HI fragment) was derived and subsequently used to screen the three deletions. Absence

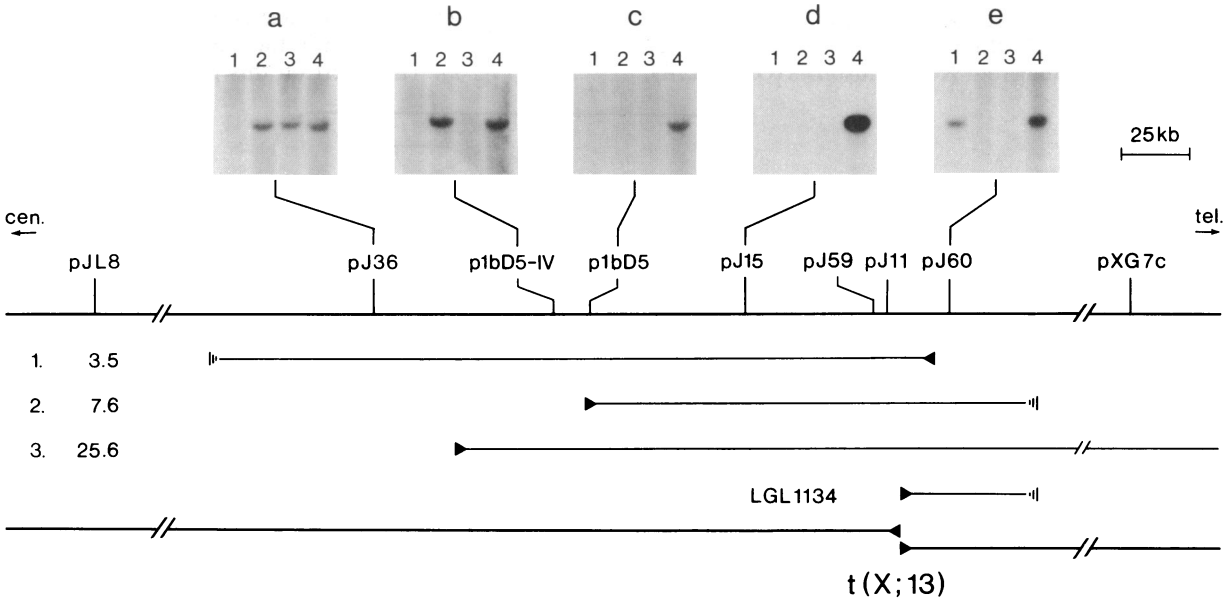

FIG. 3. Molecular analysis of deletions in patients with classical choroideremia with probes isolated by chromosome walking (p1bD5-IV) and jumping (pJ36, pJ15, and pJ60). (*Upper*) (*a–e*) *Eco*RI-digested DNA of patients 3.5 (lanes 1), 7.6 (lanes 2), and 25.6 (lanes 3) and a male control (lanes 4). The presence of comparable amounts of DNA in each lane was shown by using a probe located outside this region (pL2.98; Xq13; ref. 17). (*Lower*) Deleted regions of patients 3.5, 7.6, 25.6, and LGL1134 are depicted between arrowheads. The position of the centromeric deletion endpoint in patient 3.5 and the telomeric deletion endpoints in patients 7.6 and LGL1134 are unknown. The telomeric deletion endpoint in patient 25.6 is located in interval 5 of Xq21 (18, 39). The location of the X chromosomal breakpoint of a X/13 translocation in a female with choroideremia is shown at the bottom.

of hybridization signals revealed that pJ59, like pJ11, pJ15, and p1bD5, maps to the DNA segment that is deleted in all three patients. The sequences in λJ59 that are homologous to pJ15-I (0.7-kb *Eco*RI/*Ava* I fragment) contained a *Bgl* II site but no *Hin*dIII sites (Fig. 2), indicating a jump in the telomeric direction as depicted in Figs. 1 and 3.

Screening of the chromosomal-jumping library with pJ15-II yielded a λ phage (λJ60; Fig. 2) that contained a low-copy sequence, pJ60 (1.7-kb *Eco*RI/*Ava* I fragment). This sequence is located inside the 7.6 and 25.6 deletions but distal to the 3.5 deletion (Fig. 3*e*). Thus, this jump has provided us with the distal demarcation of the segment that all three deletions have in common.

The relative positions of pJ11 and pJ59 with respect to pJ60 were deduced from conventional Southern analysis. pJ59 and pJ11 hybridize to the same 16-kb *Hin*dIII fragment, whereas pJ11 and pJ60 are both located on a *Bam*HI fragment of approximately 36 kb (unpublished data). These results suggest the order Xcen-pJ59-pJ11-pJ60-pter. Field inversion gel electrophoresis analysis indicated that all jump clones, as well as *DXS165*, are located on a 400-kb *Sal* I and a 625-kb *Sfi* I fragment (unpublished data).

With these jump clones as probes, a fourth patient with TCD and a microdeletion was found. This deletion (LGL1134) spans pJ60, but none of the other jump clones nor the *DXS95* (pXG7c) locus (for pJ11, pJ15, and pJ60, see Fig. 4*a*). The *Hin*dIII fragment detected by pJ11 in DNA of patient LGL1134 is slightly smaller than that of a male control, which indicates that the centromeric deletion endpoint is located within this 16-kb *Hin*dIII fragment. Preliminary restriction mapping data indicate that the deletions in patients 3.5 and LGL1134 overlap a DNA segment of approximately 15–20 kb.

Mapping of the Translocation Breakpoint. DNA of the cell hybrid CIII-1 and a male control was analyzed with all jump clones from this region, and the anonymous probes p1bD5 (*DXS165*), pXG7c (*DXS95*), and p22.33 (*DXS11*), which maps further telomeric on Xq (40). As shown in Fig. 4*b*, probes p1bD5, pJ15, and pJ11 fail to hybridize to DNA from the CIII-1 hybrid, whereas sequences pJ60, pXG7c (not shown), and p22.33 are present on the der(13) chromosome of this cell line. Therefore, the X chromosomal breakpoint of this translocation can be positioned between pJ11 and pJ60, within or just proximal to the DNA segment that is deleted in the four patients described above (Fig. 3).

DISCUSSION

So far, there is only a limited number of reports dealing with the successful application of chromosome-jumping techniques (21, 23–25, 27). In this study, we made use of a 100-kb human chromosomal-jumping library that had been used previously to study the chromosomal region carrying the cystic fibrosis locus (21) and the Duchenne gene (24). One of the advantages of this library is that any sequence, unless located on a *Eco*RI fragment containing no *Sau*3AI restriction sites, can be used as a starting clone. Several different probes from one locus can be tested, and directional jumping can be achieved by using probes that are optimally positioned with respect to *Eco*RI sites (20, 26). Furthermore, the broad size range of the jumps (40–120 kb) results in "scanning" of neighboring genomic regions. Because of the relatively small average insert size (about 5 kb), approximately 2×10^6 independent phage must be plated to achieve a 95% coverage of the genome. To avoid repeated phage plating and lifting, we applied a long-term storage procedure for plated phage (38) in combination with plaque-lifting on nylon filters that can be rehybridized. Phage plated in this way remained viable for at least 1 year.

We used the *DXS165* locus as a starting point for jumps in the centromeric and the telomeric direction. The resulting jump clones were positioned with the help of four, partially overlapping, deletions present in the genome of classical TCD patients. The orientation of the probe cluster depicted in Figs. 1 and 3 was established by jump clone pJ36, which was located outside the 7.6 and 25.6 deletions, but inside the 3.5 deletion. As the 25.6 deletion does not only span *DXS165* (p1bD5) but also five loci that map telomeric to this marker, the order of loci in interval 3 (18) is firmly established as Xcen-*DXS233-DXS165-DXS95*-qter.

In most experiments, the direction of jumping could be predetermined by choosing starting clones that were located next to *Eco*RI sites (Fig. 1). The position of the jump clones was confirmed by Southern blot analysis of a deletion panel (Fig. 3). Jump clone pJ60 is located inside the 7.6 and 25.6 deletions but outside the 3.5 deletion. This means that we have jumped from one proximal deletion endpoint (patient 7.6) across another one that forms the distal demarcation of the deletion in patient 3.5. As one jump (pJ11) was almost sufficient to bridge this distance, we estimate that the region of overlap between these three deletions does not exceed 150 kb. The broad size range of the jumps performed with this

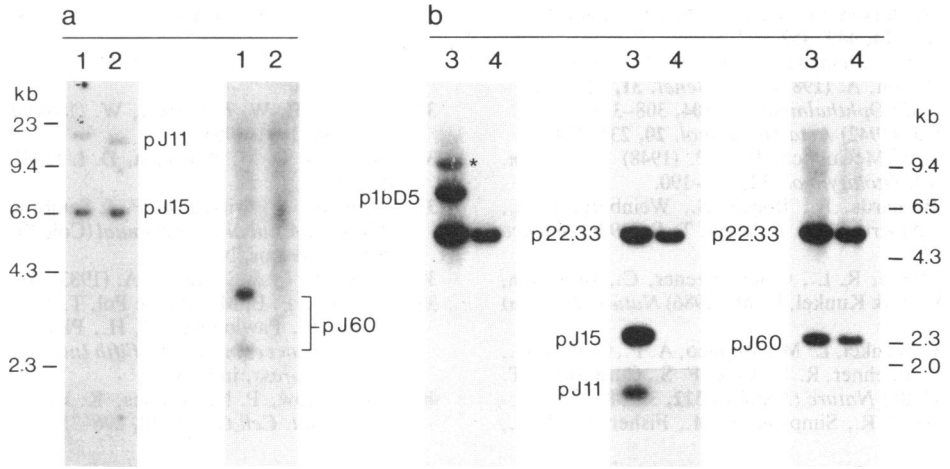

FIG. 4. (*a*) Southern blot analysis of *Hin*dIII-digested DNA of a control male (lanes 1) and patient LGL1134 (lanes 2). (*Left*) Southern blot hybridized with pJ11 and pJ15. (*Right*) The blot was stripped and rehybridized with pJ60, which detects *Hin*dIII fragments of 2.8 and 3.9 kb (see Fig. 1). (*b*) Southern blot analysis of *Eco*RI-digested DNA of a control male (lanes 3) and cell line CIII-1 (lanes 4). The six lanes were run in parallel and blotted as described. (*Center*) The lower part was exposed for a longer time to enhance the signal of probe pJ11 compared to pJ15. (*Left*) The asterisk indicates a second *Eco*RI fragment detected by p1bD5, which most likely is due to incomplete digestion of the control DNA.

library is illustrated by the fact that two successive jumps, pJ15 and pJ59, have resulted in bridging a distance comparable to that of one jump (pJ11).

Screening of additional TCD patients revealed another deletion (LGL1134), which is only detectable with the pJ60 probe. Preliminary data from Southern blot analyses indicate that the deletions in patients 3.5 and LGL1134 share a DNA segment of 15–20 kb that maps between pJ11 and pJ60. Furthermore, we were able to localize a translocation breakpoint, which presumably disrupts the *TCD* gene, between these two markers.

The size of the *TCD* microdeletions can only be estimated indirectly. The deletion in patient 25.6 contains six anonymous DNA loci and is estimated to span >5000 kb. The jump clones depicted in Figs. 1 and 3 are all located on a 625-kb *Sfi* I fragment and a 400-kb *Sal* I fragment, previously shown to contain the *DXS165* locus (18). For patients 3.5, 7.6, and LGL1134, deletion sizes cannot be determined with certainty, as the position of their centromeric (patient 3.5) or telomeric endpoints (patients 7.6 and LGL1134) relative to *Sal* I and *Sfi* I sites cannot be established.

In view of the apparent causal relationship between the microdeletions and the TCD phenotype of patients 3.5, 7.6, 25.6, and LGL1134, our studies have provided convincing evidence for (part of) the *TCD* gene to be located on a 15- to 20-kb segment between pJ11 and pJ60. This is corroborated by the fact that a translocation breakpoint associated with the TCD phenotype in a female is also located within or very close to this DNA segment.

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