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Xp21 Contiguous Gene Syndromes: Deletion Quantitation with Bivariate Flow Karyotyping Allows Mapping of Patient Breakpoints

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Summary

Bivariate flow karyotyping was used to estimate the deletion sizes for a series of patients with Xp21 contiguous gene syndromes. The deletion estimates were used to develop an approximate scale for the genomic map in Xp21. The bivariate flow karyotype results were compared with clinical and molecular genetic information on the extent of the patients' deletions, and these various types of data were consistent. The resulting map spans >15 Mb, from the telomeric interval between DXS41 (99-6) and DXS68 (L1-4) to a position centromeric to the ornithine transcarbamylase locus. The deletion sizing was considered to be accurate to ± 1 Mb. The map provides information on the relative localization of genes and markers within this region. For example, the map suggests that the adrenal hypoplasia congenita and glycerol kinase genes are physically close to each other, are within 1–2 Mb of the telomeric end of the Duchenne muscular dystrophy (DMD) gene, and are nearer to the DMD locus than to the more distal marker DXS28 (C7). Information of this type is useful in developing genomic strategies for positional cloning in Xp21. These investigations demonstrate that the DNA from patients with Xp21 contiguous gene syndromes can be valuable reagents, not only for ordering loci and markers but also for providing an approximate scale to the map of the Xp21 region surrounding DMD.

Introduction

Patients have been described with a variety of contiguous gene syndromes involving different portions of Xp21, and DNA from these patients has been important in mapping genes and markers within this chromosomal region. A patient, BB, with Duchenne muscular dystrophy (DMD), McLeod phenotype (XK), and chronic granulomatous disease (CGD) evidenced a visible chromosomal deletion permitting colocalization of the genes for these disorders to Xp21 (Francke et al. 1985). Subsequently, the BB DNA was used to clone the genes for DMD and CGD, the latter referred to as "CYBB" (Kunkel et al. 1985; Royer-Pokora et al. 1986). The brothers, CM and SM, originally reported with glycerol kinase deficiency (GKD) (McCabe et al. 1977; Guggenheim et al. 1980), manifest a contiguous gene syndrome, known as "complex GKD," characterized by deletions involving the adrenal hypoplasia congenita (AHC), glycerol kinase (GK), and DMD loci (Bartley et al. 1982, 1986; Hammond et al. 1985; Wieringa et al. 1985; Dunger et al. 1986; Saito et al. 1986; Francke et al. 1987; McCabe 1989; McCabe et al. 1989). Additional patients with AHC and GKD or with GKD and DMD permitted the ordering of these loci (Davies et al. 1988; McCabe 1989).

DNA specimens from patients with complex GKD, as well as their unique deletion breakpoints, have facilitated the mapping of probes, yeast artificial chromosome (YAC) human inserts, and sequence-tagged sites in this region (Francke et al. 1987; Davies et al. 1988; McCabe et al. 1989; Towbin et al. 1989, 1990; Love et al. 1990; Worley et al. 1992*b*). Although the order of loci and probes has been determined, estimates of

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distances within this portion of Xp21 have remained limited. The absence of complete pulsed-field gel electrophoresis (PFGE) or YAC contig coverage in this region has prevented the development of information regarding the distances between probes and loci.

In order to determine whether it would be possible to develop a scale for the genomic map in Xp21, we investigated a series of patients with contiguous gene syndromes involving this region. The sizes of the patients' deletions were estimated using bivariate flow karyotyping (Trask et al. 1989*a*, 1989*b*; Carter et al. 1990; Trask et al. 1990), and the flow estimates of deletion sizes were correlated with clinical and molecular genetic data on the deletions. The resulting map extends over a distance of >15 Mb, telomerically from the interval between DXS41 (99-6) and DXS68 (L1-4) to a position centromeric to the ornithine transcarbamylase (OTC) locus, with an accuracy of ±1 Mb.

Patients and Methods

Patients

Chromosomes for bivariate flow karyotyping were obtained from cell lines of the following patients who were selected because of phenotypic and cytogenetic evidence indicating that they had Xp21 deletions of varying sizes. The patients are ordered here as they appear in figures 1 and 2, from largest to smallest estimated deletion size.

Patient KC. — This is a female reported to be heterozygous for an Xp21 deletion and for CGD and OTC

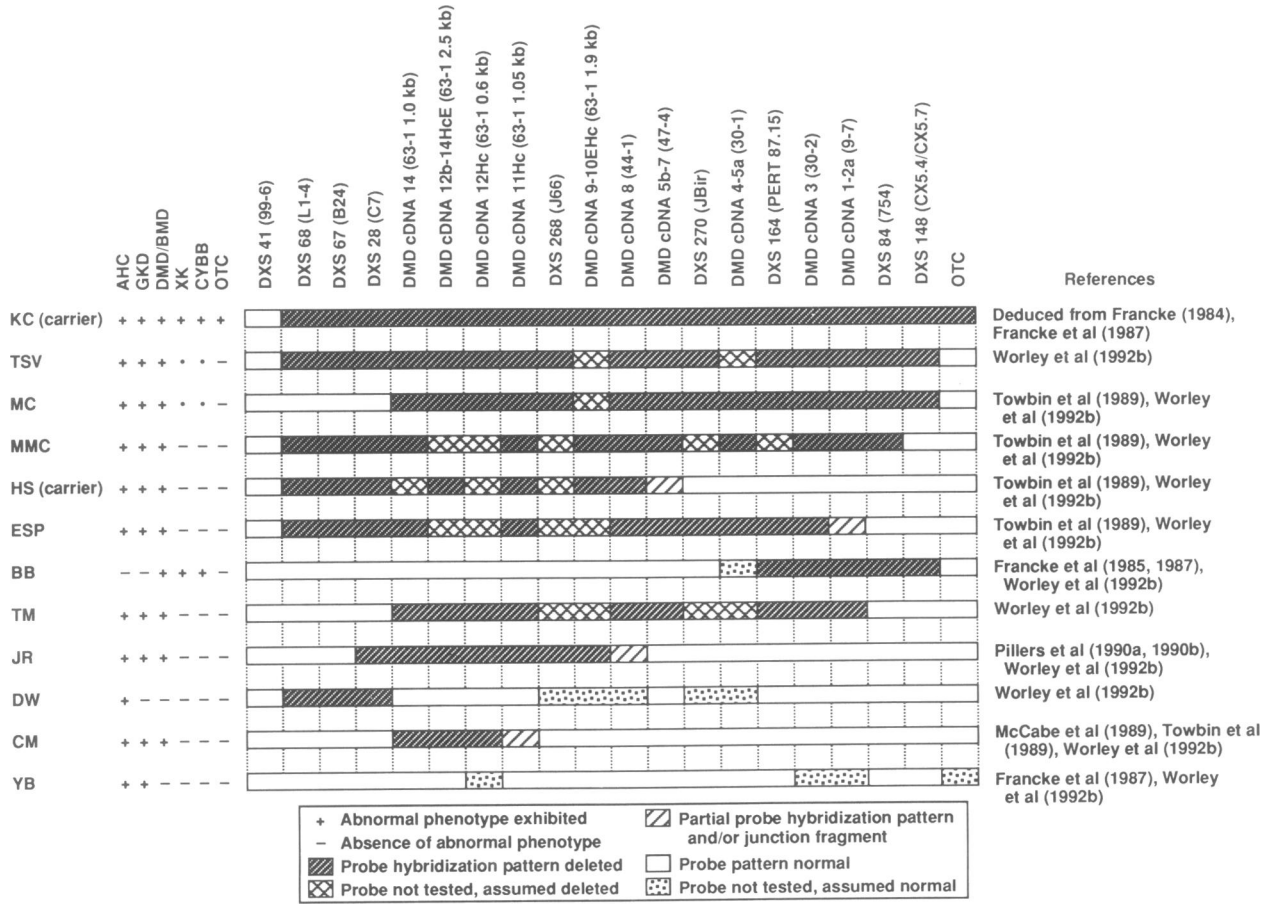

Figure 1 Phenotypes and molecular genetic analysis of individuals with Xp21 deletions

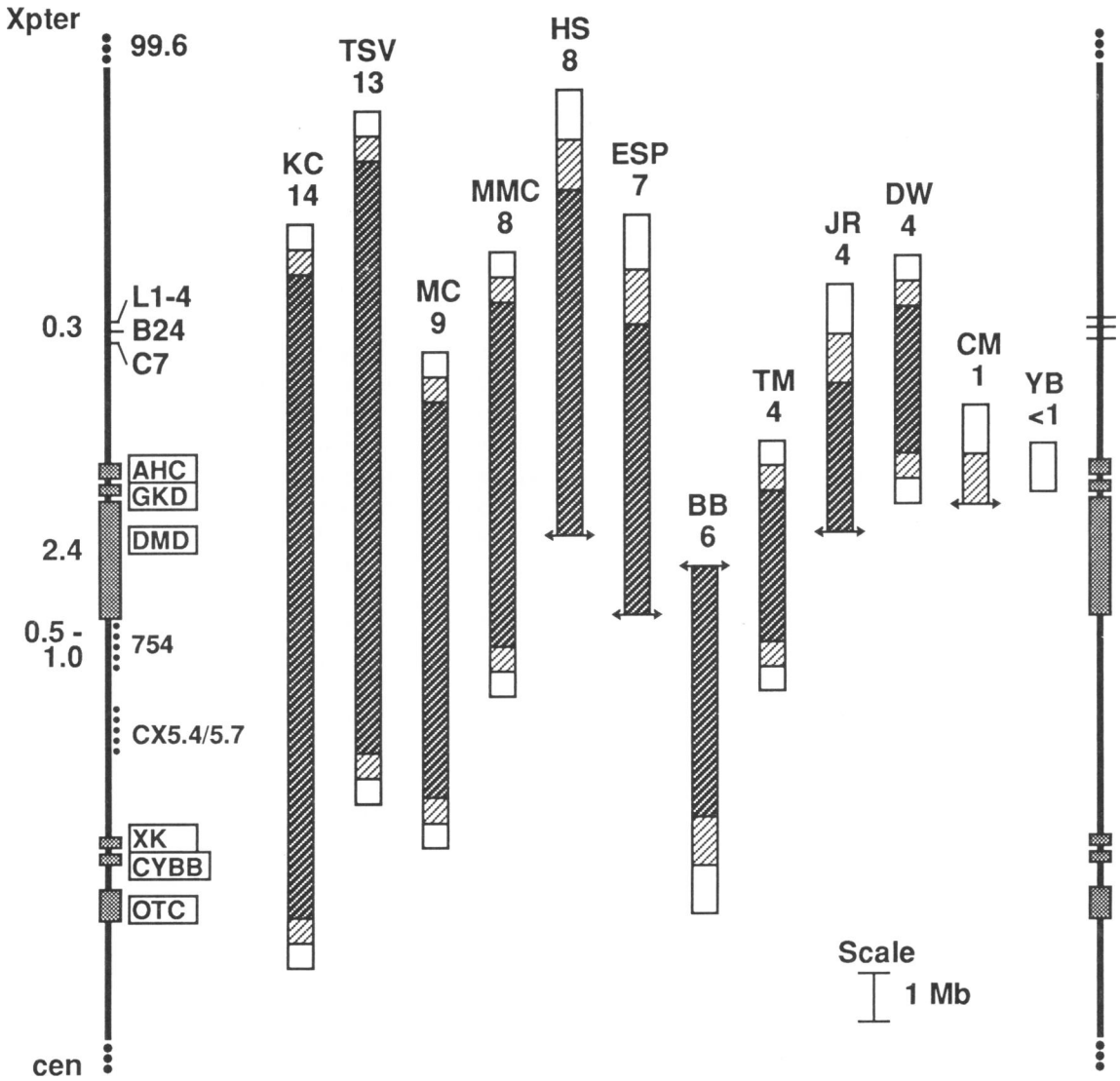

Figure 2 Summary of bivariate flow karyotyping estimates of patients' deletion sizes in Xp21 extending over 15 Mb. The deletions are correlated with phenotypes and/or genotypes for loci (boxed designations on left axis) and with hybridization results with genomic probes (unboxed designations on left axis) (see fig. 1). The numbers to the left of this axis indicate physical size estimates: 0.3 Mb for the size of the L1-4-B24-C7 probe group, 2.4 Mb for the DMD genomic region, and ~0.5–1.0 Mb for the distance from DMD to 754. The bars represent the patient deletions, with the sizes (in megabases) shown below the patient designations and above the bars. A double-headed arrow indicates a breakpoint which is localized ("anchored") within the DMD locus. The variance is estimated at ± 1 Mb. If an individual has one breakpoint anchored within the DMD locus, this variance is distributed to the opposite breakpoint (in patients HS, ESP, BB, JR, and CM). For the other patients this variance is distributed equally at both breakpoints. The mean estimate is shown by the line between the open bar and light diagonal hatching. The lower limit of resolution is 1 Mb.

(Francke 1984), with a chromosomal rearrangement that undoubtedly involved the AHC, GK, DMD, and XK loci as well (Francke et al. 1987).

Patient TSV.—This male, with a visible Xp21 interstitial deletion, has AHC, GKD, DMD, failure to thrive, developmental delay, microcephaly with cortical atrophy, and cryptorchidism (Worley et al. 1992*b*).

Patient MC.—This male was diagnosed with AHC,

GKD, and DMD (Towbin et al. 1989). He has developmental delay and seizures attributed to a respiratory arrest at 2 wk of age.

Patient MMC.—This male was the product of intellectually impaired parents and has AHC, GKD, DMD, failure to thrive, developmental delay, coarse facies, wide forehead, hypogonadotropic hypogonadism, bilateral cryptorchidism, coronal hypospadias, and a large phallus (Towbin et al. 1989).

Patient HS.—This is the carrier mother of two males (JS and GS) previously reported with AHC, GKD, DMD, mental retardation, episodic vomiting, bilateral cryptorchidism, and midface hypoplasia (Towbin et al. 1989).

Patient ESP.—This male has AHC, GKD, DMD, developmental delay, and plagiocephaly (Towbin et al. 1989).

Patient BB.—This male had a visible Xp21 chromosomal deletion and a clinical phenotype that included DMD, XK, and CGD (Francke et al. 1985). Additional findings included retinitis pigmentosa (Francke et al. 1985), but the patient did not have AHC or GKD (Francke et al. 1987).

Patient TM.—This male has AHC, GKD, DMD, developmental delay, failure to thrive, and a history of poor feeding, vomiting, and diarrhea (Worley et al. 1992*b*).

Patient JR.—This male, with a visible Xp21 interstitial deletion, was diagnosed with AHC, GKD, and DMD (Pillers et al. 1990*a*, 1990*b*). He also exhibited the ophthalmologic features, including characteristic electroretinogram (ERG), of Åland Island eye disease (AIED) (Weleber et al. 1989). However, AIED in the original Scandinavian population defined with this disease maps by linkage analysis to a different location, the pericentromeric region of X (Alitalo et al. 1991; Schwartz and Rosenberg 1991). Therefore, JR is considered to have a nonallelic, phenotypically similar disorder, designated "Oregon eye disease" (OED) by HGM 11 (Davies et al. 1991). Preliminary evidence suggests an association between OED and deficient expression of retinal dystrophin (Pillers et al., in press).

Patient DW.—This male has AHC and developmental delay without GKD or DMD (Worley et al. 1992*b*). Additional findings include left hydronephrosis, esotropia, and increased deep-tendon reflexes. Chromosome analysis was normal.

Patient CM.—This is the older of the two brothers originally reported with the complex GKD syndrome, with involvement of the AHC, GK, and DMD loci (McCabe et al. 1977, 1989; Guggenheim et al. 1980; Francke et al. 1987). His muscle disease is a mild form of Becker muscular dystrophy (McCabe et al. 1989). Additional findings include psychomotor retardation, growth failure, esotropia, spasticity, and osteoporosis with pathological fractures (Guggenheim et al. 1980).

Patient YB.—This male had three previously born brothers who died early in life (Francke et al. 1987). He presented with hypoglycemia and apnea in the neonatal period and was found to have AHC and GKD. There was no clinical evidence of DMD, and CPK was normal. Psychomotor development was reported to be normal. Interpretations of the karyotype have differed, with published reports indicating an interstitial deletion of Xp21.2 (Marlhens et al. 1987), while others have been unable to detect an Xp21 deletion cytogenetically (D. H. Ledbetter and S. Ledbetter, personal communication).

Flow Karyotype Analysis

Chromosomes were isolated from exponentially growing lymphoblastoid cell line cultures after a 12-h exposure to 0.2 μg colcemid/ml (van den Engh et al. 1988). Cells were pelleted and resuspended in a hypotonic buffer containing $MgSO_4$, to stabilize the chromosomes. Triton-X was added (final concentration 0.25% [v/v]), and the suspension was vortexed to lyse the cells and release chromosomes into suspension. The suspension of chromosomes, cellular debris, and intact interphase nuclei was then stained with Hoechst 33258 (HO; 3.8 μM), which binds preferentially to A-T base pairs, and chromomycin A3 (CA; 17 μM), which binds preferentially to C-G base pairs. The fluorescence intensity of these two dyes was quantified for each of ≃30,000 chromosomes from each cell line in a dual-beam flow cytometer (Trask et al. 1989*b*). The resulting HO versus CA bivariate flow karyotypes were displayed as contour plots at 256 × 256-channel resolution (e.g., see fig. 3). Contour lines indicated the frequency of events in each channel and were chosen for the figures at ~10% of the number of events in the highest peak of the distribution.

The most likely HO and CA coordinates, or peak position, for each of the 46 homologues were determined using an iterative least-squares fitting procedure as described elsewhere (Bevington 1969; Taylor 1982; van den Engh et al. 1990). This procedure fitted each homologue with a bivariate Gauss function by minimizing χ^2. The final value for χ^2 did not significantly

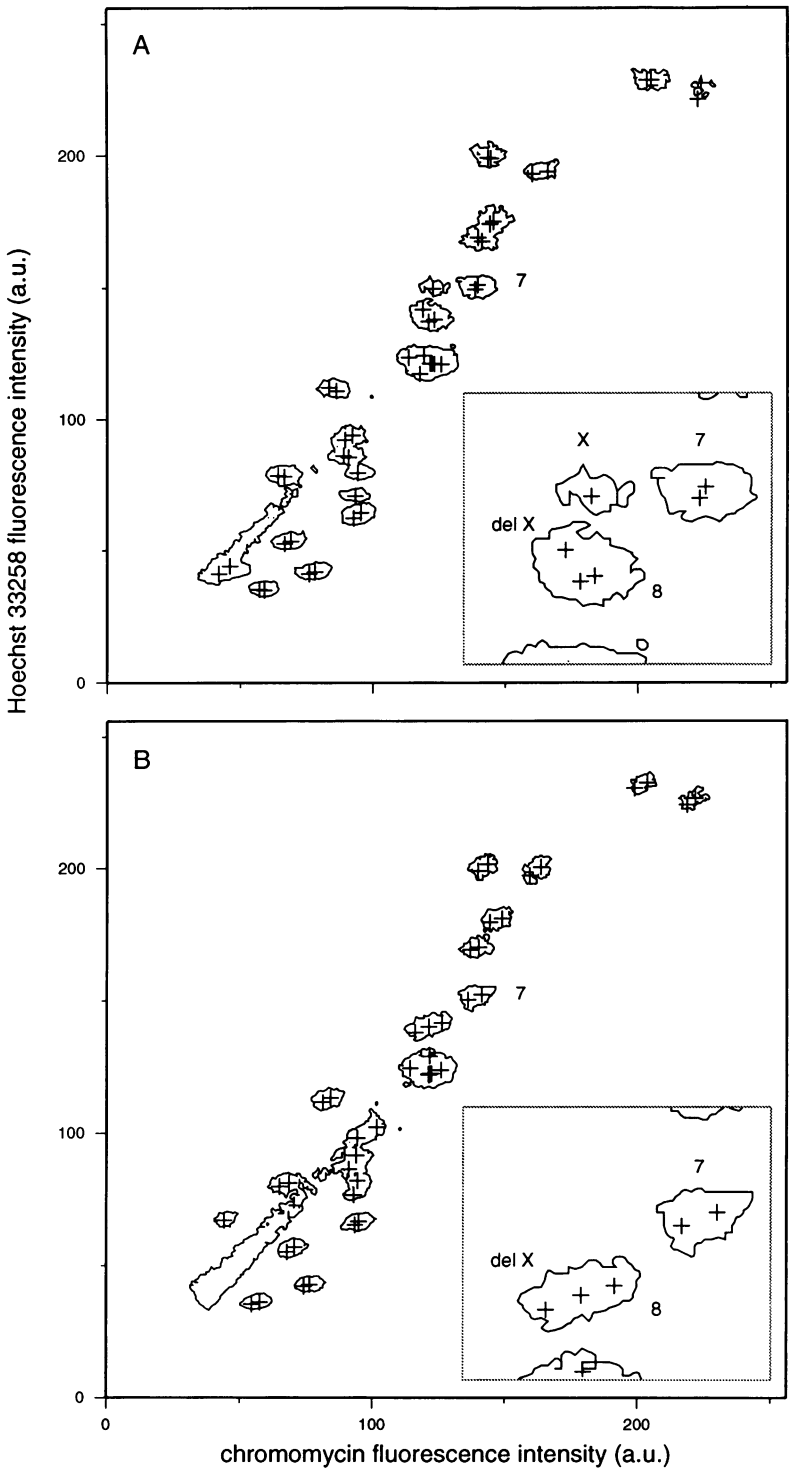

Figure 3 Bivariate flow karyotypes of (*A*) a heterozygous female (patient HS) and (*B*) a hemizygous male (patient TSV). The contour plot shows the peaks produced by measuring the Hoechst and chromomycin fluorescence intensities of chromosomes isolated from mitotic cells from these individuals. Crosses mark the most likely peak positions of each of the 46 homologues represented in the karyotype. The region of the karyotype containing the peaks produced by chromosomes 7, 8, X, and del X is shown in expanded form in the inset. In both karyotypes, the peak for the del X chromosome is displaced toward the origin, away from the position of the normal X. This displacement represents the decreased HO and CA fluorescence consequent with the reduced chromosomal DNA content of the deleted X homologue.

deviate from the number of data points in any fit. In karyotypes of female carriers, one Gauss distribution was fit to the peak representing the normal X homologue. The del X of all female carriers analyzed and all male patients except TM, CM, and YB formed a peak that merged to some degree with the peak for normal chromosomes 8. In these cases, three Gauss distributions (two for the two chromosome 8 homologues and one for the deleted X) were fit to the merged peak. In TM, CM, and YB, one Gauss distribution was fit to the peak representing the del X chromosome, which was well resolved from other peaks in the karyotype. The positions of both homologues of all chromosome types are shown as crosses in figure 3. To convert peak position to chromosomal DNA content, each karyotype was normalized to a reference karyotype by using the slope and Y-intercept of the relationship between the fitted peak positions of 12 different chromosome types in the two karyotypes determined by using least-squares linear regression (Trask et al. 1990). The 12 chromosome types used for normalization (i.e., 2–8, 13, and 17–20) were chosen because they showed little heteromorphic variation among normal individuals (Trask et al. 1989*a*) and because they represented an equal number of peaks on either side of the chromosome X peak. After normalization, the HO and CA coordinates of chromosome X were converted to DNA content (Trask et al. 1990). In brief, the relative distance between the origin and the projection of the normalized HO and CA coordinates on a line running through the origin and chromosome 4 was calculated as a relative measure of the DNA content of the chromosome. These values were converted to absolute DNA content, in megabases, by using the linear relationship between them and slide-based quantitation of chromosome DNA content and by assuming a haploid genome size of 3×10^9 bp (Trask et al. 1990).

The DNA contents of X chromosomes in the subjects studied were compared with the distribution of DNA content measured for the normal X chromosome of females carrying large deletions in the other X homologue or for normal males. This distribution was determined, as described above, on 18 individuals and has a mean of 159.7 Mbp and an SD of 0.8 Mbp. The accuracy of the fitting procedure, the normalization process, and the position determination of normal X chromosomes yielded ± 1 Mbp as a conservative estimate of the accuracy with which deletions can be estimated by flow karyotype analysis.

Results

The patients' phenotypes and the results of probe hybridization analyses are summarized in figure 1, with references to previous reports documenting the molecular genetic localization of the breakpoints in these individuals. Flow karyotype analysis was performed using lymphoblastoid cell lines from these patients. Representative bivariate flow karyotypes from two of these individuals, a heterozygous female (HS) and a hemizygous male (TSV), are displayed as contour plots (fig. 3). These plots show the difference in the position of the normal and deleted X chromosome in a carrier female (fig. 3*A*), whereas a single deleted X is seen in the affected male (fig. 3*B*). The DNA contents of the X chromosomes were calculated from their HO and CA fluorescence intensities. The sizes of the deletions ranged from 14 Mb in KC to below the level of sensitivity, or <1 Mb, in YB (fig. 2). Among these patients, CM had the smallest discernible deletion, 1 ± 1 Mb.

Discussion

Estimates of deletion sizes determined from karyotype analysis of 12 individuals—males affected with, or female carriers for, Xp21 contiguous gene syndromes—were correlated with phenotypes and probe hybridization analysis to develop an approximate scale for the genomic map in this region. The results of these investigations are summarized in figure 2. These correlations generate a map of the portion of Xp21 encompassed by the chromosome deletions. The map spans at least 15 Mb and permits estimates to be made of distances between genes and markers, with an accuracy of ± 1 Mb.

All 12 patients, with their unique breakpoints, were valuable in developing this map. The five individuals (HS, ESP, BB, JR, and CM) with breakpoints within the DMD locus should be noted particularly. The detailed knowledge of the DMD region, together with the localization of the patients' breakpoints by DNA analysis, served to "anchor" their deletions to a specific position in this locus. As a consequence of anchoring one of the breakpoints of these patients, the entire measurement variance was distributed to the breakpoint outside the DMD locus. Examination of the map shows that the mean estimate for each individual patient deletion is consistent with the clinical observations and probe analyses.

The map provides useful information regarding the relative localization of genes and markers. For example, this map suggests that AHC and GK are physically close to each other and are clearly closer to the DMD locus than to the distal probe group consisting of DXS68 (L1-4), DXS67 (B24), and DXS28 (C7). The TM, CM, and YB bivariate flow karyotype data suggest that the AHC and GK loci may be within 1–2 Mb of the telomeric end of the DMD gene. These conclusions are consistent with the observation that it is far more common for patients with the complex GKD contiguous gene syndromes to have involvement of the AHC, GK, and DMD loci together, rather than the contiguous pairs without involvement of the third locus, i.e., either AHC and GK or GK and DMD (McCabe 1989). The map also has been useful in developing a genomic strategy for cloning the GK and AHC loci, indicating that it would be far more reasonable to walk into this region with YACs from the telomeric end of DMD than from DXS28 (C7) (Worley et al. 1992*a*). As new probes and markers are developed in this region, their map positions with respect to the patient breakpoints will allow estimation of their distances from existing markers.

In contrast with the closeness of AHC and GK to the telomeric end of DMD, it appears that XK and CYBB are relatively more distant from the centromeric end of DMD and closer to OTC. We would estimate that the XK and CYBB loci are ~4–5 Mb centromeric from DMD and ~1–2 Mb telomeric from OTC. These results are consistent with the observation that patients with contiguous gene syndromes involving DMD, XK, and CGD appear to be seen less often than those with DMD and complex GKD, although it must be recognized that there may be factors other than size or distance alone that influence the frequency of interstitial deletions on the X chromosome.

The results summarized in the deletion map are consistent with information obtained by PFGE for Xp21. Previous PFGE work indicated that probe 754 (DXS84) was ≤0.5–1.0 Mb from the centromeric end of DMD (van Ommen et al. 1986; Burmeister et al. 1988); the deletion map, which includes MMC (deleted for 754) and TM (not deleted for 754), is consistent with this estimate. The PFGE data indicate that CX5.4/5.7 (DXS148) is ≥2.3 Mb centromeric from DMD and ≥1.5 Mb centromeric from 754; these distances are compatible with those obtained with bivariate flow karyotyping, particularly when TSV (deleted for CX5.4/5.7) and MMC (not deleted for CX5.4/5.7) are considered. Our results indicate that XK and CYBB are very close, in agreement with previous estimates (Bertelson et al. 1988; Ho et al. 1992). When we consider the region telomeric from DMD, the conclusion that L1.4 (DXS68) is ≥2.3 Mb from J66 is upheld by the deletion map (Burmeister et al. 1988). The assertion that C7 is also ≥4 Mb from the 3′ end of dystrophin (Love et al. 1990) is compatible with the estimated distance between these markers, as shown particularly by the flow karyotype of patient JR. This distal probe group, ordered ter→cen, L1-4 (DXS68), B24 (DXS67), and C7 (DXS28) by fluorescence in situ hybridization (Trask et al. 1992) and YAC data (Walker et al. 1991), has been reported to have a minimal size of ~0.3 by PFGE (Burmeister et al. 1988). The bivariate flow karyotyping results from JR (deleted for C7 but not for B24 or L1-4) and MMC, ESP, and DW (deleted for C7, B24, and L1-4) are compatible with these observations. Our estimate of the BB deletion at 6 ± 1 Mb also is consistent with previous evaluations of ≥3.3 Mb (van Ommen et al. 1986; Wilcox et al. 1986; Trask et al. 1990). We can conclude that the evaluation of distances within Xp21 by using bivariate flow karyotyping of patients' chromosomes with interstitial deletions yields results that are congruous with physical distances, which, to date, have been obtained primarily by PFGE. As YAC contigs become available across this region, we will be able to assess how well our approximations approach the actual linear DNA measurements.

DNA specimens from patients with Xp21 contiguous gene syndromes have proved to be valuable reagents for ordering probes and markers. The investigations reported here demonstrate that these patient reagents permit establishment of an approximate scale for mapping loci and markers within a region of 15 Mb surrounding DMD in Xp21.

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