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Assignment of Human β-, γ-, and δ-globin genes to the short arm of chromosome 11 by chromosome sorting and DNA restriction enzyme analysis

(gene mapping/restriction endonuclease/fluorescence-activated cell sorter/hemoglobin)

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ABSTRACT **Normal human metaphase chromosomes isolated from fibroblasts were resolved into 14 peaks based on total Hoechst 33258 fluorescence and sorted with the fluorescence-activated cell sorter. The chromosomal DNA was extracted and characterized by *Eco*RI analysis. As expected, analysis of the peak containing chromosomes 16 and 18 detected the α-globin genes and of the peak containing chromosomes 9, 10, 11, and 12 detected the β-, γ-, and δ-globin genes. Translocations were then used to localize further the β-, γ-, and δ-globin genes. The first translocation t(11;22)(q25;q11), which moved nearly all of chromosome 11 to a different peak, confirmed that the β-, γ-, and δ-globin genes are on this chromosome. The second, t(4;11)(q25;q13), which moved the distal portion of the long arm of chromosome 11 to a new peak, showed that the genes are not in this segment. The third, t(X;11)(q11;p13), moved the distal region of the short arm of chromosome 11 to a peak which now contained the β-, γ-, and δ-globin genes. Therefore, the β-, γ-, and δ-globin genes reside on the distal portion of the chromosome 11 short arm including bands p13, p14, and p15. This sorting method may be used generally to assign other genes to chromosomal segments of the entire chromosome complement.**

Adult human hemoglobin molecules are principally composed of hemoglobin A ($\alpha_2\beta_2$) with a minor hemoglobin A_2 ($\alpha_2\delta_2$) component; between 3 and 9 months' gestation, the hemoglobin is principally hemoglobin F ($\alpha_2\gamma_2$) (1). Numerous pedigree analyses first indicated that the α- and β-globin genes are not linked (2, 3), and more recent somatic cell hybridization experiments have described the independent chromosomal segregation of the α- and β-globin genes (4). In contrast, the β-, γ-, and δ-globin genes were first considered to be linked after the discovery of the gene fusion products designated the Lepore (5) and Kenya (6, 7) hemoglobins. Recently, the close linkage of these genes has been defined by restriction endonuclease mapping (8–10) and the isolation of cloned globin genes (11). Different laboratories have tried to localize the globin genes by *in situ* hybridization (12–18) but, because of the specific activity of the probe, the results are open to controversy (19, 20). At the same time, somatic cell hybridization studies indicated that the human α-globin gene is on chromosome 16 and the human β- and γ-globin genes are on chromosome 11 (21–23). Gene mapping using partially purified chicken chromosomes prepared by zonal centrifugation has been reported (24, 25).

Herein we report the results of gene mapping by sorting normal (26) and translocated human chromosomes and analyzing the extracted DNA by restriction endonuclease digestion (27). Data obtained by this procedure indicate that the β-, γ-, and δ-globin genes are located on the distal portion of the short arm of chromosome 11. This method may be applied generally to assign other genes to chromosome segments.

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MATERIALS AND METHODS

Chromosome Preparation and Sorting. The Human Genetic Mutant Cell Repository (Camden, NJ) provided human fibroblast cultures with the following chromosome translocations: GM 980:45, XX,−22,t(11;22)(11pter→11q25::22q11→22qter); GM 380:46, XX,t(4;11)(4pter→4q25::11q13→11qter;11pter→11q13::4q25→4qter); and GM 2859:46, X,t(X;11)(Xpter→Xq11:11p13→11pter;11qter→11p13::Xq11→Xqter). Control chromosome suspensions from Lawrence Livermore Laboratory (LLL) cell strain 761 were prepared as reported (26). Less vigorously growing translocation-bearing fibroblasts were cultured in RPMI 1640 medium containing 2.0 g of $NaHCO_3$ per liter, autoclaved 50 mM Hepes·NaOH (pH 7.33), and 30% fetal bovine serum to stimulate suboptimally dividing cells. Fibroblasts with translocations were inoculated at 6×10^6 cells per 660-cm^2 Wheaton glass roller bottle and cultured for 48 hr. The medium was then replaced by fresh medium containing Colcemid (60 ng/ml) for an additional 14 hr. The rounded mitotic cells were harvested by swirling the medium inside the roller bottle 10 times. After filtration through a 37-μm mesh (Small Parts Inc., Miami, FL), aliquots (8×10^6 mitotic cells) were centrifuged at $200 \times g$ for 6 min, and the cell pellet was resuspended in 75 mM KCl with Colcemid and incubated for 30 min at 37°C. After recentrifugation, the mitotic cells were resuspended in 0.5 ml of isolation buffer (26) and placed at 37°C for 10 min and 0°C for 3 hr. The mitotic chromosomes were released by using a VirTis "45" microhomogenizer. The resulting chromosome suspension was stored at 4°C until sorting; then, it was resuspended with an 18-gauge needle and syringe, filtered through a 37-μm mesh, and stained with Hoechst 33258 (2–4 μg/ml).

Chromosomes were sorted by using a modified Becton-Dickinson FACS II cell sorter equipped with a Spectra-Physics 171-05 argon ion laser delivering 1.0 W of power in the ultraviolet region (λ = 351–364 nm) (28). The sorter directs stained chromosomes through the ultraviolet laser light and records the intensity of the emitted fluorescent light. Single droplets containing particles of the designated fluorescence intensity were electronically deflected to the right or left of the unsorted chromosomes (29). Chromosomes for DNA isolation were sorted into Beckman polyallomer centrifuge tubes immersed in ethanol/dry ice at −78°C and then stored at −80°C. To identify the region of the translocated chromosomes in the fluorescence distribution, 20,000 chromosomes were sorted from each region

of interest, and 50-μl aliquots of chromosomes sorted for DNA analysis were removed to monitor sort purity. Chromosome suspensions were prepared for cytological analysis by using Lief buckets (30), and the chromosomes were identified by banding patterns (26). For analysis of the chromosomal DNA, 7.5×10^5 chromosomes of each type were sorted in 2–4 hr.

DNA Isolation and Identification. One thawed tube from each sorted fraction was centrifuged at 55,000 × *g* for 3 hr, 90% of the supernatant was removed, and the contents of a second tube were transferred to the first tube and centrifuged as before. This procedure was repeated until all fractions had been pooled and centrifuged and the entire supernatant had been removed. The chromosomal protein was digested in a solution containing 275 μg of proteinase K (E. Merck Biochemicals, Darmstadt, Germany), 20 μg of depurinated salmon testis DNA (Sigma), 100 mM NaCl, 50 mM Tris·HCl (pH 7.5), 1 mM Na_2 EDTA (pH 7.4), and 0.5% sodium dodecyl sulfate in a final volume of 1.0 ml. This mixture was rotated at 200 rpm in a 52°C water-bath for 16 hr. After phenol extraction and reextraction of the phenol phase, pooled supernatants were added to 120 μl of 2.0 M NaOAc and 2 vol of ethanol. After 2 hr at −80°C and 3 days at −20°C the precipitated DNA was collected by centrifugation. The pellet was transferred to an Eppendorf tube, washed with 12 changes of 70% ethanol, and lyophilized. Approximately 50% of the sorted chromosomal DNA was recovered.

The DNA was digested with 30 units of *Eco*RI (New England Biolabs) (27), washed with 70% ethanol, and lyophilized. The entire sample from one sorted fraction was dissolved and applied to a single well of agarose gel (27). After electrophoresis, the DNA was transferred to nitrocellulose filters and the filters were hybridized with ^{32}P-labeled cDNA (specific activity, 3–4 × 10^8 cpm/μg) prepared by reverse transcription of human globin mRNA. The filters were then washed, dried, and autoradiographed for 3 days as described (27).

RESULTS

Normal chromosomes were first sorted to determine whether the α-, β-, γ-, and δ-globin genes could be detected in sorts containing chromosomes to which the genes had been assigned. When analyzing a suspension of normal human metaphase chromosomes isolated from cell strain LLL 761, the fluorescence-activated cell sorter generated a fluorescence intensity distribution containing 14 distinct peaks (Fig. 1). The chromosomes contained in each peak were sorted and identified by quinacrine banding analysis (26). *Eco*RI restriction analysis of the chromosomes sorted in the three designated areas revealed that the α-globin genes are in the chromosome fraction with chromosomes 16 and 18 (fraction 761-1) and the β-, γ-, and δ-genes are with chromosomes 9, 10, 11, and 12 (fraction 761-2), as expected from somatic cell hybridization studies (21–23). To determine the purity of these sorts, fraction 761-2 was sorted at the same time as a control fraction (fraction 761-3) which did not contain intact globin gene-bearing chromosomes. This fraction contained only a trace amount of globin genes resulting from broken interphase nuclei and randomly fragmented and aggregated metaphase chromosomes.

By using a translocation that involves one of two homologous chromosomes, which contributes to a derivative chromosome of sufficiently different size, a portion of one homolog may be sorted from its normal counterpart. The extracted chromosomal DNA is then tested to determine whether half the genes have been moved to a fraction of chromosomes in which they did not previously appear. Having localized the β-, γ-, and δ-globin genes to a peak that normally contains chromosomes 9, 10, 11, and 12, we confirmed that these genes are on chromosome 11 by sorting a translocation involving nearly all of chromosome

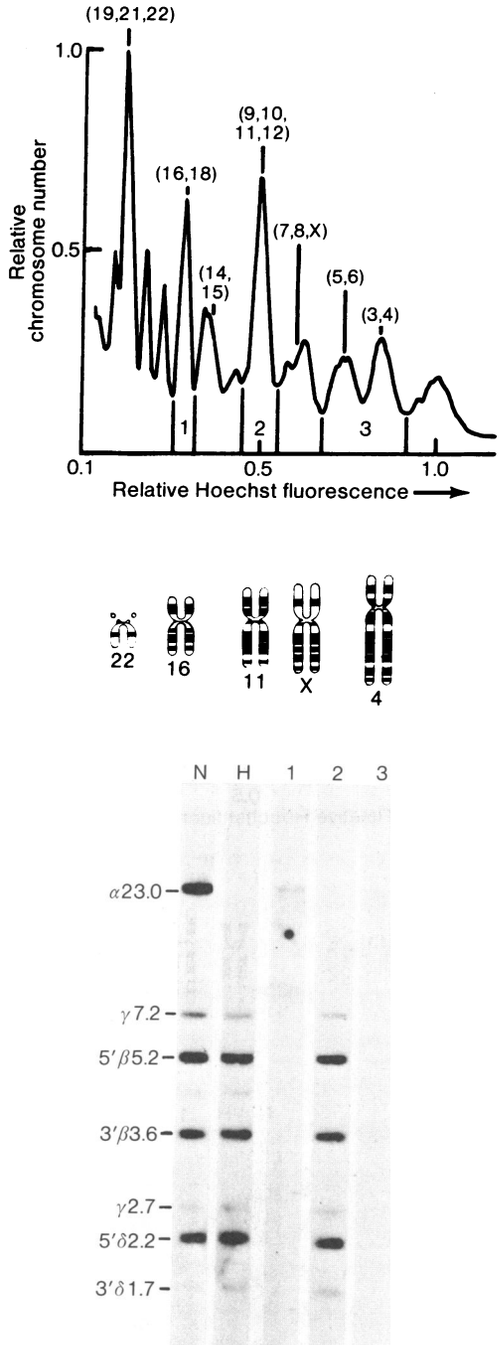

FIG. 1. (*Top*) Fluorescence distribution, generated by the fluorescence-activated sorter, of normal human chromosomes from LLL strain 761. Chromosomes within the peaks of interest are labeled above the distribution; the sorted fractions 1, 2, and 3, are indicated beneath the peaks. (*Center*) Diagrams of selected chromosomes directly below the peaks in which they are present illustrate that the relative fluorescence intensity generally increases with relative chromosomal size. Chromosomes 11 and 16 contain the hemoglobin genes; chromosomes 22, X, and 4 are involved in translocations of chromosome 11 used in this study. [Reproduced from the Paris Conference (31), by permission.] (*Bottom*) *Eco*RI analysis of total normal DNA (lane N), total hydrops DNA lacking α-globin genes (lane H), and the three sorted fractions of DNA indicated in *Top*. The identification of the gene bands and the length of the DNA restriction fragments in kilobase pairs are indicated at the left. Fractions 761-2 and 761-3 were sorted simultaneously. Fraction 761-1 contains the α-globin genes, fraction 761-2 contains the β-, γ-, and δ-globin genes, and fraction 761-3 contains a small amount of contaminating gene material.

11 (Fig. 2). This translocated chromosome now appeared in a peak without normal chromosomes 9, 10, and 12. Both fraction 980-2 containing the translocated chromosome 11 and fraction 980-1 containing the normal chromosome 11 sorted from GM 980:t(11;22)(q25;q11) were found to contain the β-, γ-, and δ-globin genes. Cytological analysis of the sorted peaks revealed that the normal chromosome 11 was sorted in fraction 980-1 but not in fraction 980-2, whereas the translocated chromosome was sorted in fraction 980-2. The intense β-, γ-, and δ-globin gene bands in fraction 980-2 compared to fraction 980-1 indicates that the genes were sorted with the translocated chromosome. If the bands were due to background hybridization in the absence of intact chromosome 11, their intensity would be much lighter as is observed in fraction 761-3 relative to 761-2. Therefore, the hemoglobin genes that were detected in the right sorted fraction 980-2 are located on translocated chromosome 11.

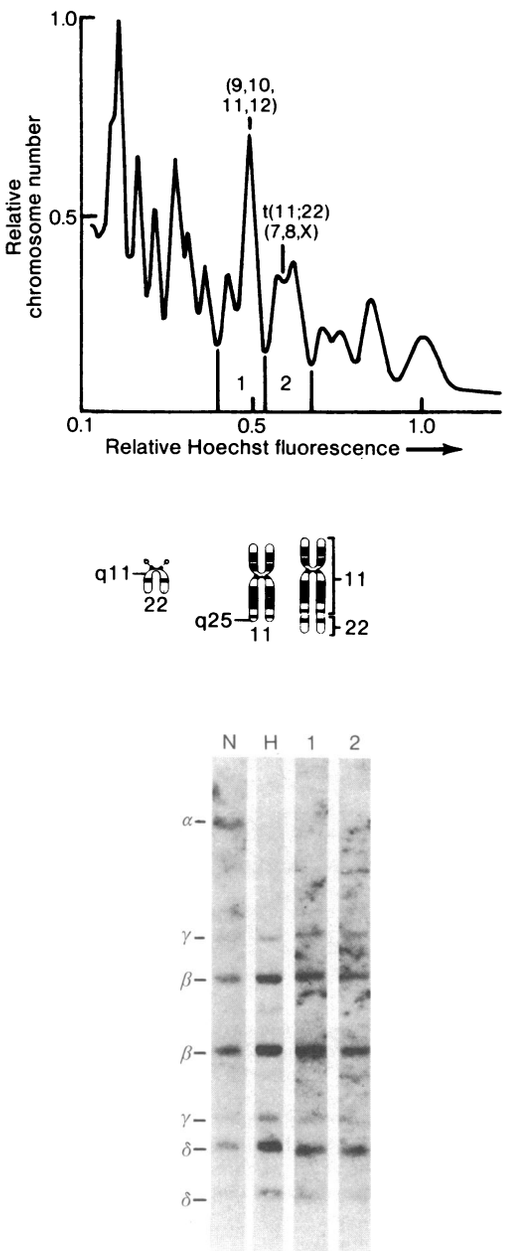

FIG. 2. Analysis of cell strain GM 980 containing the translocation t(11;22)(q25;q11). The chromosome content of the designated peaks is indicated, peaks of interest are labeled, and the sorted fractions are labeled 1 and 2. The chromosomes involved are diagramed and the translocation breakpoints are indicated on chromosomes 22 and 11. Below is the *Eco*RI analysis of normal DNA (lane N), hydrops DNA (lane H), and the two sorted fractions. The β-, γ-, and δ-globin genes are in both sorted fractions.

Other translocations were analyzed to determine which portion of chromosome 11 bears the β-, γ-, and δ-globin genes. The next translocation from cell line GM 380 moved a large portion of the long arm of chromosome 11 to a region of larger chromosomes (Fig. 3 *left*). Analysis of the sorted chromosomes from GM 380:t(4;11)(q25;q13) indicated that the β-, γ-, and δ-globin genes are not present in fraction 380-2 and thus do not reside on the distal portion of the long arm (q) of chromosome 11 between region 1 band 3 and the end.

The final translocation from repository cell line GM 2859 moved the distal portion of the short arm of chromosome 11, including bands 11p13, 11p14, and 11p15, to the centromere and short arm of the X chromosome (Margaret M. Aronson, Human Genetic Mutant Cell Repository, personal communication). This smaller chromosome translocation (X;11)-(Xpter→Xq11::11p13→11pter) was sorted with chromosomes 14 and 15 (Fig. 3 *right*), and the other derivative chromosome translocation (X;11)(Xqter→Xq11::11p13→11qter) was sorted with chromosomes 5 and 6. DNA analysis of GM 2859:t(X;11)(q11;p13) after simultaneous sorting of the translocated chromosome 11 short arm (fraction 2859-1) and the normal chromosome 11 (fraction 2859-2) indicated that each fraction had an equivalent number of globin genes. As a further control, we found that the normal chromosomes sorted from LLL 761 contained the β-, γ-, and δ-globin genes in fraction 761-2′ with chromosomes 9, 10, 11, and 12 but not in fraction 761-1′ with chromosomes 14 and 15. In a further sort of GM 2859, the fraction containing the large chromosome translocation (X;11)(Xqter→Xq11::11p13→11qter) contained only background levels of globin genes and the simultaneously sorted small translocation (X;11)(Xpter→Xq11::11p13→11pter) contained the β-, γ-, and δ-globin genes (data not shown). Therefore, the GM 2859 data indicated that the β-, γ-, and δ-globin genes are located on the distal portion of the short arm of chromosome 11.

DISCUSSION

We have assigned the β-, γ-, and δ-globin genes to the distal portion of the short arm of chromosome 11 by fluorescence-activated cell sorting and *Eco*RI restriction enzyme analysis. First, one identifies the group of sorted chromosomes in which the gene is located. Then, translocations of large chromosome segments are used to define the chromosome of interest within the larger group. Finally, additional translocations are studied to determine within which smaller chromosome segment the gene locus may be detected. This method may be used to determine the location of any gene or DNA sequence that may be identified by restriction enzyme analysis and hybridization to a gene-specific probe. After hybridization and autoradiography of the radiolabeled nitrocellulose filters, the filter may be reused for hybridization to other gene probes without further chromosome sorting. These probes may be prepared from the RNA of any differentiated tissue or from a cloned library of any organism's DNA. This chromosome-sorting procedure may be preferred to cell–cell hybridization because the genes located on chromosomes without selectable enzyme markers may be separated directly. Constructing unstable hybrid cells with a host genome that may interfere with gene characterization is unnecessary.

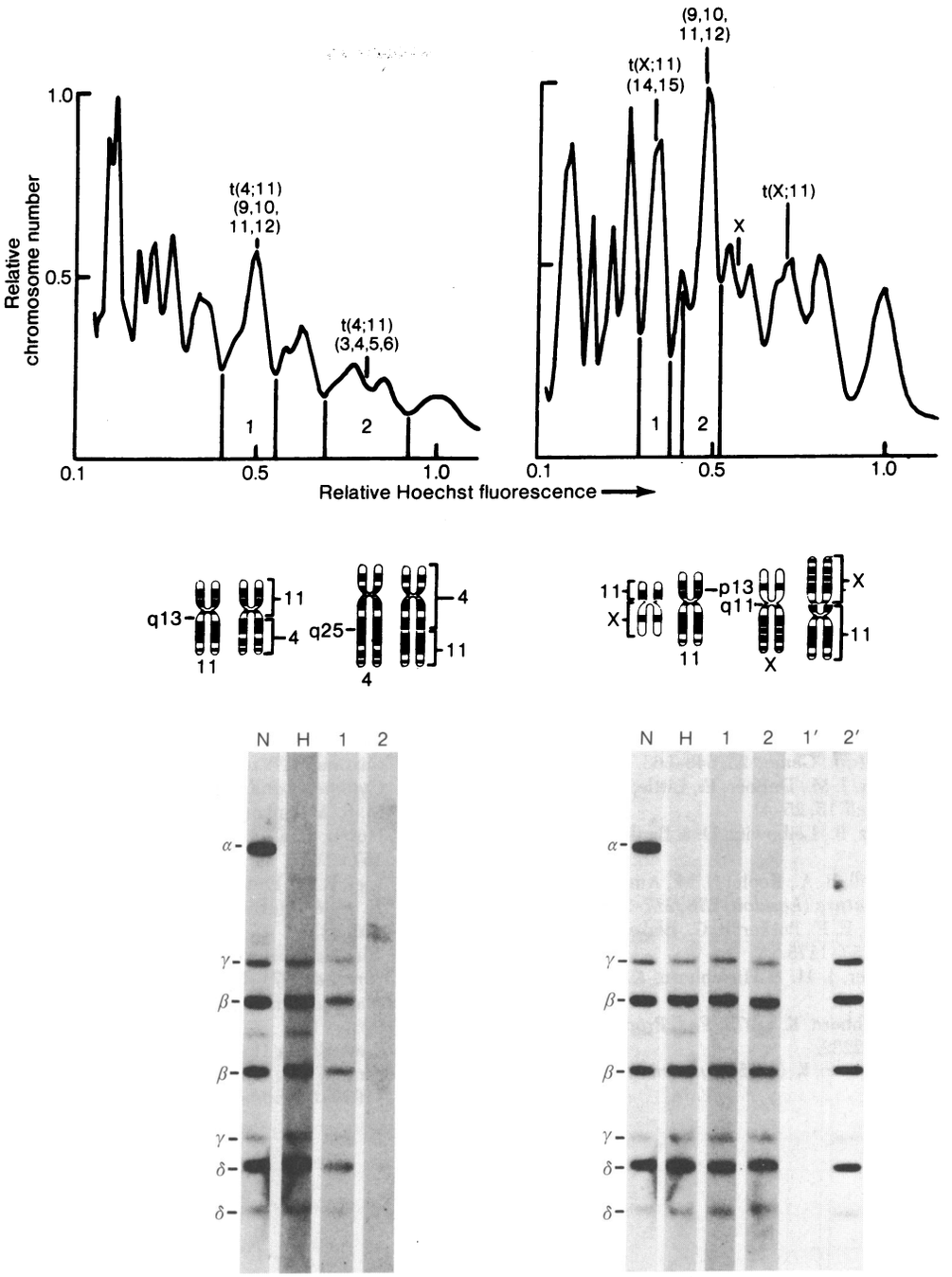

FIG. 3. (*Left*) Analysis of GM 380 containing the translocation t(4;11)(q25;q13). *Eco*RI analysis reveals that the β-globin genes remain in fraction 380-1 containing the short arms of both chromosome 11 homologs. The translocation breakpoints for translocation GM 380 are indicated on chromosomes 11 and 4. (*Right*) Analysis of GM 2859 containing the translocation t(X;11)(q11;p13). Fraction 2859-1 indicates that the genes moved to a new fraction, with chromosomes 14 and 15, containing the distal portion of the short arm of chromosome 11 (11pter → Xpter). Normal chromosomes 14 and 15 (fraction 761-1′) sorted from strain LLL 761 do not contain the β-, γ-, and δ-globin genes whereas normal chromosomes 9, 10, 11, and 12 in fraction 761-2′ sorted from strain LLL 761 do contain these genes. The breakpoints for translocation GM 2859 are indicated on chromosomes 11 and X.

Spontaneous chromosome rearrangements—including translocations, deletions, and inter- or intrachromosomal insertions—often change the size of the resulting chromosomes. The size of the subchromosomal fragment to which the gene may be assigned is dependent upon the availability of rearrangements with derivative chromosomes sufficiently different in fluorescence intensity to be sorted from the normal homolog. The Human Genetic Mutant Cell Repository, with its increasing library of cell cultures from patients, will remain an important source of chromosome abnormalities. In addition, new translocations and other rearrangements may be generated by irradiating (32) transformed cell cultures.

The greater the sorting resolution of chromosomes of similar fluorescence intensity, the greater the number of existing chromosome rearrangements that may be used to sort chromosome fragments from the normal homolog. At the same time, increased separation of similar chromosomes would allow any gene to be assigned to a single whole chromosome more directly. It has already been demonstrated that, with improvements in cytochemistry and instrumentation, the human karyotype can

be resolved into additional fluorescence categories (33). These considerations suggest that the full potential of this approach is yet to be realized.

Note Added in Proof. Recent karyotype analysis by Margaret Aronson at the Human Genetic Mutant Cell Repository now places the translocation in band 11p11. Therefore, the β-, γ-, and δ-globin genes may be assigned to bands 11p12, 11p13, 11p14, or 11p15.

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