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Construction of the physical map for three loci in chromosome band 13q14: Comparison to the genetic map

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ABSTRACT **Pulsed-field gel electrophoresis (PFGE) and deletion mapping are being used to construct a physical map of the long arm of human chromosome 13. The present study reports a 2700-kilobase (kb) *Not* I long-range restriction map encompassing the 13q14-specific loci D13S10, D13S21, and D13S22, which are detected by the cloned DNA markers p7D2, pG24E2.4, and pG14E1.9, respectively. Analysis of a panel of seven cell lines that showed differential methylation at a *Not* I site between D13S10 and D13S21 proved physical linkage of the two loci to the same 875-kb *Not* I fragment. D13S22 mapped to a different *Not* I fragment, precluding the possibility that D13S22 is located between D13S10 and D13S21. PFGE analysis of *Not* I partial digests placed the 1850-kb *Not* I fragment containing D13S22 immediately adjacent to the 875-kb fragment containing the other two loci. The proximal rearrangement breakpoint in a cell line carrying a del13(q14.1q21.2) was detected by D13S21 but not by D13S10, demonstrating that D13S21 lies proximal to D13S10. Quantitative analysis of hybridization signals of the three DNA probes to DNA from the same cell line indicated that only D13S10 was deleted, establishing the order of these loci to be cen–D13S22–D13S21–D13S10–tel. Surprisingly, this order was estimated to be 35,000 times less likely than that favored by genetic linkage analysis.**

The use of restriction fragment length polymorphisms (RFLPs) in human pedigree analysis is a powerful tool in the search for human disease loci (1). By following the inheritance of RFLP genotypes in families in which a disease state is segregating, the genes responsible for a large number of genetic disorders have been localized to specific chromosomal subregions (2–6). In the absence of cytogenetic abnormalities that may help to identify the position of the affected gene (7–10), the limits of a disease locus can be defined by RFLP markers on either side (11–13). This is generally carried out by multilocus linkage analysis (14), which predicts the most likely order of loci and estimates the map distances separating them. The nonuniformity of recombination frequency along a chromosome and between sexes necessitates refinement of genetic maps by physical methods before designing a rational approach to cloning a disease gene. In this regard, pulsed-field gel electrophoresis (PFGE) (15) and long-range restriction mapping techniques (16) are now being used extensively to generate physical maps of various human chromosomes (17) or chromosome segments to which disease loci have been genetically mapped.

We are constructing a physical map of the long arm of human chromosome 13, which contains ≈100 megabase pairs of DNA. PFGE has been used previously to establish a long-range restriction map surrounding the *RB1* locus and to localize the breakpoints of three retinoblastoma-associated translocations within the *RB1* gene (18). This map is being extended in both directions by making use of cloned DNA probes derived from several chromosome 13-specific libraries (19–21) and a panel of cell lines carrying overlapping deletions of chromosome 13.

In addition to *RB1*, a number of other cloned DNA segments have been mapped to chromosome band 13q14 by deletion mapping or by *in situ* hybridization (22–24). Two of these loci, D13S21 and D13S22, have been shown to lie proximal to *RB1* following analysis, by *in situ* hybridization, of a retinoblastoma patient bearing a balanced chromosomal translocation (25) that disrupts the *RB1* gene (18, 26). In this report, we have used a combination of PFGE and deletion mapping to construct a physical map encompassing D13S21 and D13S22 and a third 13q14 locus, D13S10. This has allowed a comparison with the genetic linkage map of this region (27). We have found that the order established by physical methods, cen–D13S22–D13S21–D13S10–tel, differs from that predicted by genetic linkage analysis (cen–D13S21–D13S22–D13S10–tel) despite a very large likelihood odds ratio supporting the latter order (27).

MATERIALS AND METHODS

Cell Lines. Lymphoblastoid cell lines from a normal male (GM07048) and female (GM06991) and from two patients with sporadic bilateral retinoblastoma (GM01484, GM07312) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The karyotypes of the patients showed deletions in one chromosome 13 at q14.1–q22.1 and q14.1–q21.2, respectively. Rb131 is a fibroblast cell line derived from a patient with retinoblastoma and carries a balanced translocation described as t(13;18)(q14;q12) (47). Fibroblast cell lines 414/73K, which has a normal karyotype except for a der(7)(7q32;13q14), and 502/86K, carrying a balanced t(4;13)(q21q14), are derived from phenotypically normal individuals and were obtained from W. Cavenee (Ludwig Institute, Montreal). Lymphoblastoid cells were grown in RPMI 1640 medium, and fibroblast lines were grown in Dulbecco's modified Eagle's medium. Both media were supplemented with 17% fetal bovine serum.

DNA Probes. The single copy cloned DNA fragments p7D2 (19), pG24E2.4 and pG14E1.9 (21), and H2-26 (20) have been given locus designations D13S10, D13S21, D13S22, and D13S28, respectively, by the Human Gene Mapping Committee (28). Except for D13S28, which maps to 13q12–q13 (23), these loci have been mapped to 13q14 (19, 24). Human

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Abbreviations: RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis.

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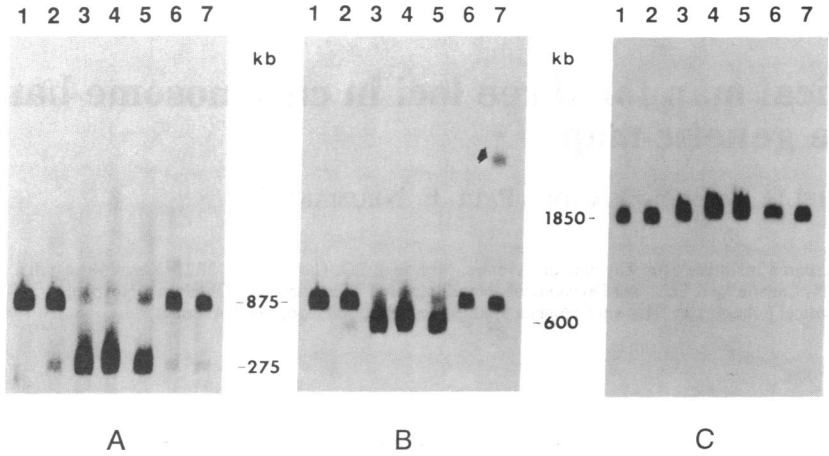

FIG. 1. Physical linkage of D13S10 (p7D2) and D13S21 (pG24E2.4) by PFGE analysis of cell lines showing differential methylation. *Not* I-digested DNA from different cell lines was electrophoresed under conditions to achieve resolution over a wide size range (220–5700 kb). A Southern blot of one gel was hybridized with D13S10 (*A*) and then D13S21 (*B*). A second filter from a similar gel (*C*) carrying the same digests was hybridized with D13S22. The cell lines used were GM07048 (lanes 1), GM06991 (lanes 2), Rb131 (lanes 3), 414/73k (lanes 4), 502/86k (lanes 5), GM01484 (lanes 6), and GM07312 (lanes 7). The deletion junction fragment detected by D13S21 and estimated to be ≈4000 kb is indicated with an arrow.

DNA inserts were isolated from plasmids in low-melting point agarose and radiolabeled by the random primer method (29).

Genomic DNA Preparation and Restriction Enzyme Digestion. High molecular weight human DNA was prepared in agarose plugs from cells suspended in phosphate-buffered saline (10^7 cells per ml) as described (18, 30). Restriction enzyme digests were carried out using the buffer conditions recommended by the supplier except that spermidine was added (to a final concentration of 5 mM) to buffers with >50 mM NaCl. Complete restriction digests were incubated for 4 hr with two additions of enzyme (20 units per plug). Partial digests were carried out by combining all reaction components (including different amounts of restriction endonuclease) on ice and maintaining the reaction mixtures overnight at 4°C before incubation at the appropriate temperature for 90 min. Agarose plugs were loaded directly into the wells for pulsed-field gels but were melted and mixed with loading buffer prior to standard continuous electrophoresis for deletion mapping.

PFGE and Southern Hybridization. The one-dimensional PFGE system used was developed on the basis of a theoretical model of the electrophoretic process (30, 31) with alternating fields applied by a computer-controlled power supply designed and built at the Xerox Research Centre Canada. All gels were 0.8% agarose and were run in a horizontal gel electrophoresis chamber (model H1; Bethesda Research Laboratories) in either 1× TBE (89 mM Tris borate/89 mM boric acid/2 mM EDTA, pH 8.3) (Figs. 1 and 2*A*) or 1.5× TBE (Fig. 2*B*).

For the gels in Fig. 1, the duration of the forward (+) polarity pulse was varied linearly (in 30 steps) from 75 to 3000 sec while the field strength in the forward direction was decreased from 2 V/cm to 1.3 V/cm. The ratio of the inverse (−) polarity pulse duration and field strength relative to those of the forward were kept constant at 1:2.5 and 1:2, respectively, over the 144-hr duration of electrophoresis. The gels in Fig. 2 were run in a similar manner except that the length of the forward polarity pulse was varied from 250 to 6000 sec, and the entire pulse sequence was repeated in a cyclic fashion over 180 hr. The electrophoresis conditions used for gels in Fig. 1 give good resolution of both the *Saccharomyces cerevisiae* chromosome size markers [220–2200 kilobases (kb)] and the three chromosomes of *Schizosaccharomyces pombe* (3500, 4600, and 5700 kb; ref. 32). The electrophoresis parameters used in Fig. 2 increase the separation in the 2000- to 5000-kb size range (C.T., unpublished data).

The procedure for Southern transfer to GeneScreen*Plus* (New England Nuclear) membrane have been described in detail (18). For sequential hybridization with different probes, filters were stripped in 0.1× TE/0.1% SDS at 75°C for 30 min (33). Autoradiography was performed (1-week exposures) to ensure complete removal of the probe.

RESULTS

PFGE Mapping of D13S10, D13S21, and D13S22. Our strategy for the physical mapping of the long arm of chromosome 13 by PFGE initially involved hybridization of regionally mapped DNA probes to Southern blots of normal

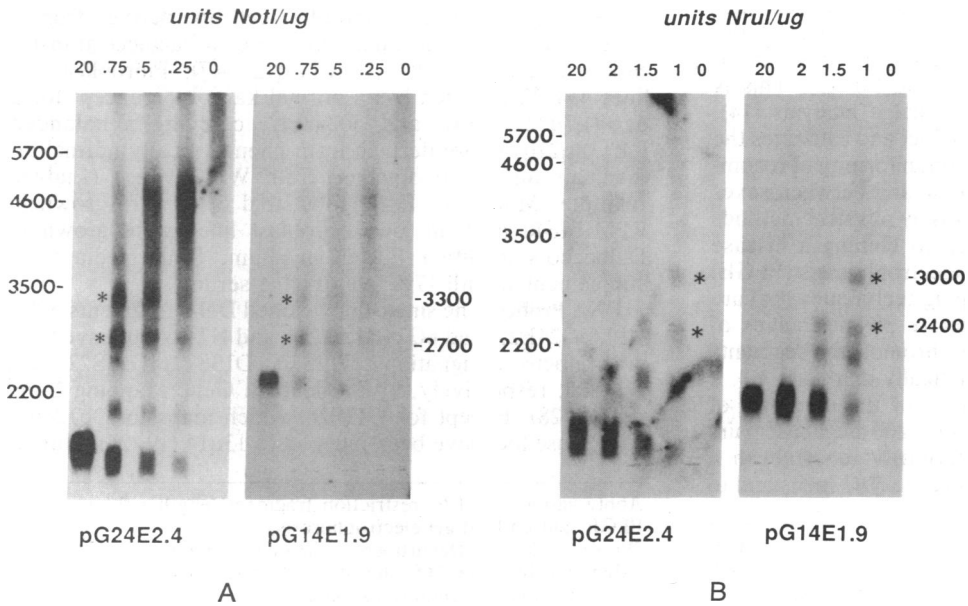

FIG. 2. Physical linkage of the D13S21, D13S10, and D13S22 loci by partial digest analysis. DNA from cell line GM07048 was digested to completion (20 units) or partially by incubating it with decreasing amounts (indicated above each lane) of *Not* I (*A*) or *Nru* I (*B*). After electrophoresis under conditions that increase the resolution between 2200 and 5700 kb, the gels were transferred to nylon membranes, which were then hybridized sequentially with D13S21 (pG24E2.4) and D13S22 (pG14E1.9). The positions of the largest chromosome of *S. cerevisiae* (2200 kb) and the three chromosomes of *Sc. pombe* (3500, 4600, and 5700 kb) used as molecular size markers are indicated. The estimated sizes of partial digestion fragments shared by the two loci (indicated with asterisks) are shown on the right of each panel. Sizes are in kb.

Table 1. Relative number of copies per genome (±SD) of the D13S10, D13S22, and D13S21 [compared with DNA from a normal individual (GM07048)] in cell lines (GM01484, GM07312) with deletions in chromosome 13

Locus (probe)	DNA source (cell line)			
	GM07048	GM06991	GM01484	GM07312
D13S10 (p7D2)	2.00	1.93 ± 0.26	0.90 ± 0.14	0.79 ± 0.17
D13S22 (pG14E1.9)	2.00	2.33 ± 0.53	1.00 ± 0.29	2.16 ± 0.38
D13S21 (pG24E2.4)	2.00	2.13 ± 0.50	0.98 ± 0.22	1.96 ± 0.52

Each lane in Fig. 3 and two other equivalent sections of the same Southern blot (i.e., three independent lanes for each DNA sample) were scanned densitometrically. The ratio of the integrated optical density of the bands detected by D13S10, D13S22, and D13S21 in the DNA of two deletion lines and a cell line from a second normal individual (GM06991) relative to the intensity of the band in the DNA of GM07048 was calculated, with all values normalized with respect to the signal intensity obtained with probe H2-26 (D13S28). The values given are the mean ± SD copy number determined by independent analysis of the three lanes for each DNA sample assuming two copies of each locus in GM07048. These data demonstrated that only D13S10 is deleted in GM07312.

male DNA (GM07048) digested with several infrequently cleaving restriction enzymes. After hybridization of D13S10, D13S21, and D13S22 to DNA digested with *Not* I, *Bss*HII, *Mlu* I, or *Nru* I, no restriction fragments in common were observed between these three 13q14-specific loci with the possible exception of an 875-kb *Not* I fragment shared by D13S10 and D13S21 (data not shown).

When *Not* I-digested DNA from a number of cell lines with chromosomal rearrangements within 13q14 was probed with D13S10 (Fig. 1*A*), each lane showed hybridization to an 875-kb fragment and, with the exception of GM07048, to a 275-kb fragment. After hybridization of the same filter with D13S21, a 600-kb fragment was detected in addition to the 875-kb fragment (Fig. 1*B*). A fragment of ≈4000 kb was also observed in *Not* I-cleaved GM07312 DNA hybridized with D13S21 (Fig. 1*B*, lane 7). This represents the junction fragment in the deletion-bearing cell line GM07312 (see below). Since the sum of the smaller fragments (275 plus 600 kb) detected by D13S10 and D13S21, respectively, equals the size of the fragment seen in common, and the relative signal intensity of the 875-kb fragment to that of the smaller fragments is consistent in each of the seven cell lines, these results support the conclusion that D13S10 and D13S21 are located within 900 kb of each other and flank a *Not* I site that is differentially methylated.

When the same digests were hybridized with D13S22, a fragment of 1850 kb was detected in each lane (Fig. 1*C*). These results preclude the possibility that D13S22 lies between D13S10 and D13S21, thereby disproving the gene order predicted by linkage analysis (27).

Physical Linkage of D13S21, D13S22, and D13S10 by the Analysis of Partial Digests. Since no restriction fragments generated by complete digestion by *Not* I, *Bss*HII, *Mlu* I, or *Nru* I were found in common between D13S22 and either D13S10 or D13S21, partial analysis was carried out in an attempt to physically link these loci. When the same blot was hybridized with D13S21 and then D13S22, the smallest *Not* I partial digest product in common was estimated at 2700 kb (Fig. 2*A*), which is approximately equal to the sum of the individual *Not* I fragments detected by these probes. These results suggest that the 875- and 1850-kb *Not* I fragments detected by D13S22 and D13S21, respectively, are immediately adjacent to one another in the genome. This close physical linkage is supported by the finding that both probes identified a *Nru* I partial digest product of ≈2400 kb (Fig. 2*B*).

Deletion Breakpoint Mapping and Dosage Hybridization Establish the Probe Order cen–D13S22–D13S21–D13S10–tel. The cell line GM07312 carries a heterozygous deletion of chromosome 13 reported as del13(q14.1q21.2). As mentioned above, D13S21 detected an additional fragment of ≈4000 kb in *Not* I-digested DNA from this cell line (Fig. 1*B*, lane 7), which represents the deletion junction fragment. Since D13S10 and D13S21 share the 875-kb *Not* I fragment disrupted by this deletion in GM07312, the breakpoint in 13q14.1 must be between D13S10 and D13S21 with D13S10 on the deleted side. To confirm these findings, quantitative hybridization analysis of *Hin*dIII or *Eco*RI digests of DNA from GM07312 was performed with D13S10, D13S21, and D13S22 (Table 1; Fig. 3). D13S10 detects only one copy per genome in the DNA of cell line GM07312 (Fig. 3*A*; Table 1) while both D13S21 and D13S22 hybridize with two copies per genome intensity (Fig. 3*B*; Table 1). These results confirm that D13S10 lies distal to D13S21 and support the conclusion that the *Not* I hybridization fragment detected by D13S21 (Fig. 1*B*, lane 7) corresponds to the deletion junction fragment of cell line GM07312. The fact that D13S22 is not deleted in GM07312 (Fig. 3*B*; Table 1) and the previous conclusion that D13S22 cannot be between the other two loci establishes the following order: cen–D13S22–D13S21–D13S10–tel. A summary map of this region, together with the genetic map predicted by genetic linkage (1), is presented in Fig. 4.

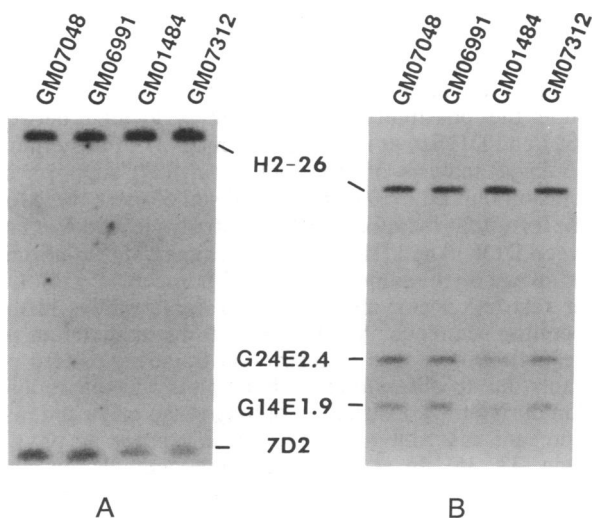

FIG. 3. Deletion mapping of D13S10, D13S21, and D13S22 with respect to GM07312. DNA probes p7D2 (D13S10), pG24E2.4 (D13S21), and pG14E1.9 (D13S22) were hybridized to Southern blots of *Hin*dIII-digested (*A*) or *Eco*RI-digested (*B*) DNA from the indicated cell lines. Cell lines GM07048 and GM06991 are from a normal male and female, respectively. GM07312 has a heterozygous del13(q14.1q21.2) and GM01484 has a heterozygous del13(q14.1q22.1). GM01484 serves as a positive control for deletion of these loci since all three are present in only one copy. As an internal control for the amount of DNA in each lane, probe H2-26, which is not deleted in either GM01484 or GM07312, was hybridized to the same filter. Representative sections of a blot containing three samples of each cell line DNA are shown.

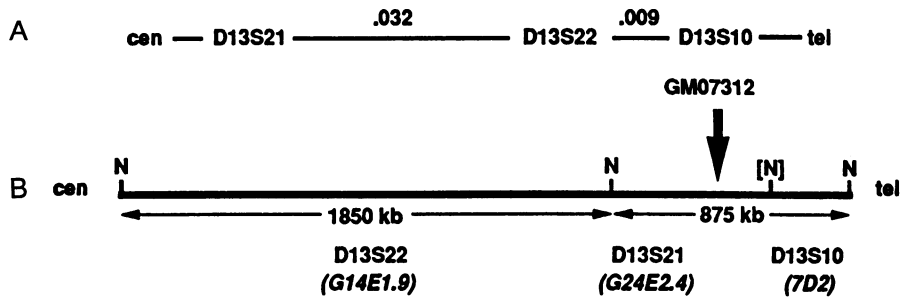

FIG. 4. Comparison of genetic and physical maps around D13S10, D13S21, and D13S22. (*A*) Segment of the genetic map determined by Bowcock *et al.* (27) showing the relative positions of the three loci and the sex-averaged recombination fractions between them. (*B*) Physical map as established by restriction digest and deletion breakpoint analysis. The proximal deletion breakpoint in cell line GM07312 is shown above the *Not* I restriction map and the three 13q14 loci (the associated probe designations are given in parentheses) are indicated below the map. Their exact locations between *Not* I sites have not been determined. The *Not* I site, which was cleaved to different extents in various cell lines, is shown in brackets. The orientation with respect to the centromere (cen) and telomere (tel) is indicated.

DISCUSSION

Using a combination of long-range restriction and deletion mapping, we have constructed a 2700-kb physical map around three 13q14-specific polymorphic loci. Two of the loci, D13S10 and D13S21, are present on the same 875-kb *Not* I fragment. In some cell lines, this fragment is cleaved with *Not* I into 275- and 600-kb fragments containing D13S10 and D13S21, respectively (Fig. 1). D13S22 resides on a different *Not* I fragment (1850 kb; Fig. 1), which was shown to be immediately adjacent to the 875-kb *Not* I fragment shared by D13S10 and D13S21 by analysis of partial digests (Fig. 2). The deletion breakpoint in cell line GM07312 (del13q14.1–q21.2) was shown to occur between D13S21 and D13S10 (Figs. 1 and 3; Table 1). Since previous studies have shown that D13S21 and D13S22 map to proximal 13q14 (30, 31) and since D13S22 is present in two copies in the GM07312 cell line (Fig. 3; Table 1), the order of the three loci is cen–D13S22–D13S21–D13S10–tel. This conclusion is largely dependent on analysis of a single cell line (GM07312) bearing a chromosomal rearrangement. The GM07312 deletion may be complex or associated with an inversion polymorphism, so that all alternative gene orders have not been absolutely excluded. On the other hand, the results of the PFGE analysis (Figs. 1 and 2) exclude the possibility that D13S22 is located between D13S21 and D13S10 as in the genetic map (27). Furthermore, the linkage analysis of Bowcock *et al.* (27) supports our conclusion that D13S10 is the most distal of these three loci.

The fortuitous location of a differentially cleaved *Not* I site between D13S10 and D13S21 was instrumental in confirming their linkage on the same 875-kb *Not* I fragment (Fig. 1). Like other restriction enzymes with CpG dinucleotides in their recognition sequence, *Not* I appears to be methylation sensitive (34). Differences in restriction fragment pattern presumably due to differential methylation is a frequent observation during the construction of long-range maps and, as in the present case, have been useful in confirming physical linkage (33, 35). In at least one study, methylation status appeared to be tissue specific, with blood DNA being more highly methylated than sperm DNA, which, in turn, was more methylated than DNA from lymphoblastoid cell lines (36). In the present report, although the sample size is small, the *Not* I site between D13S21 and D13S10 was cleaved to a greater extent in DNA from the three fibroblast cell lines compared to the lymphoblastoid lines.

It is generally assumed that 1% recombination in humans is, on average, equivalent to 1000 kb (37). The relationship between map distance and physical distance is not linear because crossover frequency and the degree of interference are not constant throughout the genome (37–39), and significant differences are seen in the rate of crossing over between sexes (37, 40). Genetic distance generally exceeds that determined by long-range restriction mapping (e.g., see refs. 17, 36, 41, and 42). Such was the case in the present study, where multipoint linkage analysis (27) predicted that D13S21 and D13S10 were separated by 4 centimorgans while the physical distance was shown to be <900 kb. In at least one case, the distance determined by long-range mapping was much greater than expected from genetic linkage analysis (41).

The difference in distances determined by genetic and physical methods is of only minor importance compared to differences in locus order. Bowcock *et al.* (27) favored an alternative gene order (cen–D13S21–D13S22–D13S10–tel) and reported the odds in favor of this order over the order indicated by our physical mapping to be 35,000:1, as determined by three-locus linkage analysis. However, the demonstration that D13S10 and D13S21 are located on the same *Not* I fragment and that D13S22 was not deleted in GM07312 contradict the gene order favored by Bowcock *et al.* (27).

The disparity in the gene order determined by the two approaches led to a reexamination of the linkage analysis that resulted in the construction of the multilocus map (21) and raises questions about confidence levels for gene orders in three-locus and four-locus linkage analysis. The confidence levels associated with log-likelihood differences and odds ratios have not been determined. A logarithm of odds (lod) difference score of 3.0 for the maximal likelihood estimate of recombination frequency between two loci means that the data are 1000 times more likely to be obtained if the recombination frequency is that estimate rather than 0.5. That does not mean that these loci are 1000 times more likely to be linked than not linked because the *a priori* probability of linkage is low. An odds ratio of 1000:1 in pairwise linkage analysis is equivalent to ≈20:1 *a posteriori* odds in favor of linkage, which corresponds to the 5% confidence level (37, 43–45). In other words, if the lod score associated with the maximal likelihood estimate of recombination frequency is >3.0, the probability of linkage is >95%. To the best of our knowledge, similar tests of significance of odds ratios in multilocus mapping are not available. We believe that the odds ratios in multilocus analysis of gene order will have to be much greater than 1000:1 to reach the 5% confidence level.

Another indication of the disparity between odds ratio and *a posteriori* odds is the contradictory results of pairwise, three-locus, and four-locus analyses reported by Bowcock *et al.* (27). The confidence limits of recombination frequencies from pairwise analysis are quite large so there is considerable uncertainty about gene order. Four-locus analysis suggested a different gene order than three-locus analysis. Consider two additional loci included in their linkage study, D13S1 and ESD. D13S1, detected by DNA probe p7F12, has been mapped to 13q12–q13 by *in situ* hybridization (46) and is not deleted in either GM07312 or GM01484 (data not shown). Therefore, physical mapping dictates an order of cen–

D13S1–D13S22–D13S21–tel, which is the order slightly favored by three-locus genetic analysis (odds ratio, 6:1). On the other hand, the four locus analysis, incorporating ESD, favors reversing the positions of D13S22 and D13S21, with an odds ratio of 1.7×10^{12}. The significance of an odds ratio of this magnitude is uncertain.

These results suggest that caution should be taken when interpreting multipoint linkage analysis carried out with closely linked markers. As the number of markers increases so does the number of possible orders. The lack of markers exhibiting a high degree of polymorphism and the paucity of recombination events between closely linked markers makes conclusive results difficult to obtain. While long range mapping may be useful in eliminating ambiguities in linkage maps (e.g., see ref. 41), the results presented here demonstrate that the two techniques can yield different orders as well.

The implications of disparities in the physical and genetic maps are 2-fold. First, the finding that D13S21 and D13S10 are <900 kb apart, rather than the much larger 4 megabase pairs suggested by genetic linkage, would drastically alter one's approach to cloning a disease gene between the two loci. More importantly, if the order determined by genetic linkage is incorrect, so-called "flanking markers" would not accurately define the boundaries within which the search for a disease locus should take place.

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