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Pairwise Linkage Analysis of 11 Loci on Human Chromosome 4

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Summary

New RFLPs are described for INP10 and interleukin 2. The 55 pairwise genetic linkage relationships for these two loci and nine additional loci on the long arm of chromosome 4 (4q) are reported. Fifteen new linkages are established, and new data are added to the four previously reported linkages on 4q. Tight linkage of interleukin 2 (T-cell growth factor), epidermal growth factor, and alcohol dehydrogenase is described. Significant differences were observed between male and female recombination rates. The female rate was estimated to be 1.27 times the male rate. On the basis of these pairwise results, the order for the 11 loci is D4S35-GC-(ALB/AFP)-MT2P1-D4S1-INP10-ADH3-(EGF/IL2)-(FBB/FBA/FBG)-MNS. This preliminary order can serve as a starting point for more detailed multipoint analysis.

Introduction

Linkage maps in experimental organisms such as bacteriophage T4, *Escherichia coli, Caenorhabditis elegans, Drosophila melanogaster,* and *Mus musculus* have provided insights into gene organization, genetic recombination, population genetics, and developmental genetics in these species. The availability of RFLPs and large reference pedigrees has now made it possible to construct a comprehensive genetic map in humans. Such a map can identify multiple linked markers, which can be used as surrogates in the diagnosis of linked Mendelian disorders. Linkage maps also serve as reference points for studying chromosome-specific abnormalities that have been reported in certain cancers (Cavenee et al. 1983; Reeve et al. 1984) and multiple-malformation syndromes (de la Chapelle et al. 1981; Ledbetter et al. 1982). Genes and markers lying closest to regions of interest can then be quickly identified and studied. In addition, establishment of clusters of tightly linked loci may identify previously unknown gene families and provide insight into their function, regulation, and evolutionary origins. Furthermore, phenomena such as interference, genetic and physical distance relationships, and changes in the recombination rate due to sex or age can now be studied in humans. We have studied the pairwise linkage relationships of 11 markers on the long arm of chromosome 4 (4q). This region contains 4.3% of the DNA in the total human genome, making a genetic map of this region an important step in defining a complete linkage map in humans (Edwards 1956; Renwick 1969; Botstein et al. 1980).

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Material and Methods

Families

The families used in this study consisted of 40 3-generation pedigrees with large sibships (average size 7.6) and grandparents made available through the

Centre d'Etude du Polymorphisme Humain (CEPH) (White et al. 1985). (CEPH is a collaborative organization founded by Jean Dausset to help in the establishment of a complete human linkage map. Further information is available from CEPH at 3, rue d'Ulm, 75005, Paris.) The families have been extensively typed to rule out apparent nonpaternity.

Markers

Table 1 presents a description of the markers used in the linkage analysis. GC (including GC subtyping) and MNS typing were done using standard procedures, and the results were provided to us by CEPH. For three of the marker loci (D4S35, ALB, and FBB/FBG), multiple RFLPs detected within a single locus were studied as haplotypes to increase linkage information. This is justified by the absence of recombinants between single-locus RFLP sites and, in two instances, by the presence of significant linkage disequilibrium. Specifically, D4S35 has two separate *Msp*I RFLPs (Scambler et al. 1985), which are an unknown physical distance apart; but in 190 individuals typed, no recombinants have been observed. The ALB *Pst*I and *Eco*RV sites and the fibrinogen beta (FBB) and gamma (FBG) RFLPs are ~25 (Minghetti et al. 1986) and ~30 (Kant et al. 1985) kb apart, respectively. There is significant association between RFLPs within both loci (Murray et al. 1984, 1985*a*), and to date no recombinants have been observed within either the albumin or fibrinogen loci.

Two additional genes on 4q, alpha-fetoprotein (AFP) and alpha fibrinogen were considered in the analysis. AFP is known to lie 15 kb from albumin (Urano et al. 1984). The above-described ALB RFLPs and AFP RFLPs (Murray et al. 1985*c*) show high linkage disequilibrium (Murray et al. 1985*b*), and, to date, no recombinants have been observed between ALB and AFP. In the analysis, these two genes could therefore be considered as a single locus. Fibrinogen alpha (FBA) is within 10 kb of FBG (Kant et al. 1985), and RFLPs for these two genes show significant association (Humphries et al. 1984; Murray et al. 1985*a*). Again, no recombinants have been observed between FBA and FBG, allowing the three genes (FBB-FBA-FBG) to be considered as a single locus in the analysis. The high association of AFP

Table 1

Description of Markers

Symbol (Name)	Source of Polymorphism	Physical Position	Reference(s)
D4S35 (G9)	*Msp*I (2)	4pter-4q26	Scambler et al. 1985; Varshney et al. 1984
GC (Group-specific component)	Protein	4q12-4q21	McCombs et al. 1986
ALB (albumin)	*Pst*I, *Eco*RV	4q11-4q21	Harper and Dugaiczyk 1983; Murray et al. 1984
MT2P1 (metallothionein II)	*Eco*RI	4p11-4q21	Lieberman et al. 1985; Pakstis et al. 1986
D4S1 (3.6)	*Bgl*II	4pter-4q26	Gilliam et al. 1984
INP10 (γ IFN-induced cDNA)	*Bcl*I	4q21	Luster et al. 1985; Luster et al. 1987
ADH3 (alcohol dehydrogenase)	*Xba*I	4q21-4q25	Smith et al. 1985
IL2 (interleukin 2 [T-cell growth factor])	*Kpn*I	4q26-4q28	Seigel et al. 1984; Paetkau 1985
EGF (epidermal growth factor)	*Hinc*II	4q25-4q27	Morton et al. 1986; Murray et al. 1986
MNS (MNSs blood group)	Red cell	4q28-4q31	Kidd and Gusella 1985
FBB (fibrinogen-beta)	*Bcl*I	4q28-4q31	Humphries et al. 1984
FBG (fibrinogen-gamma)	*Kpn*I/*Sac*I	4q28-4q31	Marino et al. 1986; Murray et al. 1985*a*; Chung et al. 1983*a*, 1983*b*

NOTE.—Symbols are those of Human Gene Mapping IX. Physical position is the consensus position established by the listed references.

with ALB RFLPs and of FBA with FBG RFLPs obviated the use of AFP and FBA in the haplotypes described above and subsequently used in the linkage analysis.

RFLP Analysis

Restriction-enzyme digestions were performed according to instructions of the manufacturer, using 2X enzyme excess and allowing all digests except *Bcl*I to proceed overnight. *Bcl*I was incubated at 55 C for 3 h. Digests were run on 0.6%–1.2% agarose gels and blotted onto Zeta-Bind® (AMF Cuno) according to manufacturer's recommendations. Prehybridization, hybridization, washing, and autoradiography were modifications of published procedures (Murray et al. 1983).

New RFLPs for INP10 and IL2 were identified by screening 10 unrelated Caucasians with the enzymes *Ava*II, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hinc*II, *Hinf*I, *Kpn*I, *Msp*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Taq*I, *Xba*I, and *Xho*I.

Family studies to demonstrate Mendelian inheritance were performed using the CEPH families and allele frequencies and the Hardy-Weinberg proportions obtained from the CEPH software.

Linkage Analysis

Pairwise linkage analysis was performed using the lod-score method of Morton (1955) and the LINKAGE programs (version 3.4) MLINK and ILINK (Lathrop et al. 1984, 1985). Allele frequencies used in this analysis were obtained by gene counting from the parents. An 11-point univariate lod table ($\theta = \theta_{male} = \theta_{female} = .0, .001, .05, .10, .15, \ldots, .45$) was calculated and summed for each of the informative families for all of the 55 possible pairwise locus combinations by using MLINK. Maximum-likelihood estimates of the univariate and bivariate ($z[\theta_m, \theta_f]$) recombination values were obtained using ILINK. Significance of the observed results was evaluated using the standard pairwise-linkage-test criterion of $Z(\theta) \geq 3$ to accept linkage at this θ and the standard pairwise-linkage-test criterion $Z(\theta) \leq -2$ to reject linkage (Morton 1955). Sex-specific differences in recombination were evaluated by contrasting the log likelihoods under the univariate and bivariate analyses. Twice the difference between these two values is a large sample approximation of the χ^2 with 1 df. The relationship between θ_m and θ_f was investigated by assuming a constant female:male ratio and fitting the model $\theta_f = c\theta_m$ by using unweighted least squares with the pairs of male and female estimates.

An order for the loci was derived from the pairwise recombination estimates by using a seriation algorithm (Gelfand 1971; Buetow et al. 1987*a*). In brief, the seriation algorithm generates a locus order by sequentially considering each row of a matrix of all possible pairs of recombination values. Within each row, the ordering process begins by choosing the locus pair with the smallest recombination value and placing them next to each other. The locus in the row with the next smallest recombination value is then selected for placement. Its values with the two previous loci are examined, and it is placed next to the locus with which it is most closely linked. This process is repeated until all loci in the row have been placed. After orders have been derived for each row, a final order can be determined by using a continuity index (Gelfand 1971; Buetow et al. 1987*a*).

Results

New RFLPs for INP10 and IL2

An RFLP for INP10 was identified with *Bcl*I (see fig. 1*A*). An insertion/deletion RFLP for INP10 was also identified with *Eco*RI and *Pst*I, one that has been reported elsewhere (Luster et al. 1987). This new *Bcl*I RFLP is not in complete association with the *Eco*RI and *Pst*I RFLPs (J. C. Murray and K. H. Buetow, unpublished data) and thus provides additional family information. Allele frequencies in 100 unrelated Caucasians are .35 for the 5.5-kb allele and .65 for the 15.0-kb allele. Genotypes fit Hardy-Weinberg proportions (data not shown).

An RFLP for IL2 was identified by using *Kpn*I (see fig. 1*B*). Genotypic frequencies fit Hardy-Weinberg proportions (data not shown). Allele frequencies in 100 unrelated Caucasians were .96 for the 19-kb allele and .04 for the 16-kb allele. Southern blotting procedures were carried out as above except that restriction-enzyme digests were electrophoresed for 24 h on a 0.6% agarose gel. The probe used was a 3.4-kb genomic fragment of the IL2 locus cloned into pUC13 at *Eco*RI sites that included the 5′-untranslated region and exons 1 and 2 of IL2.

Pairwise Linkages

Table 2 shows the 15 new significant linkages. New data were added to two established linkages (ALB-GC [Keats et al. 1979; Keats 1981] and GC-

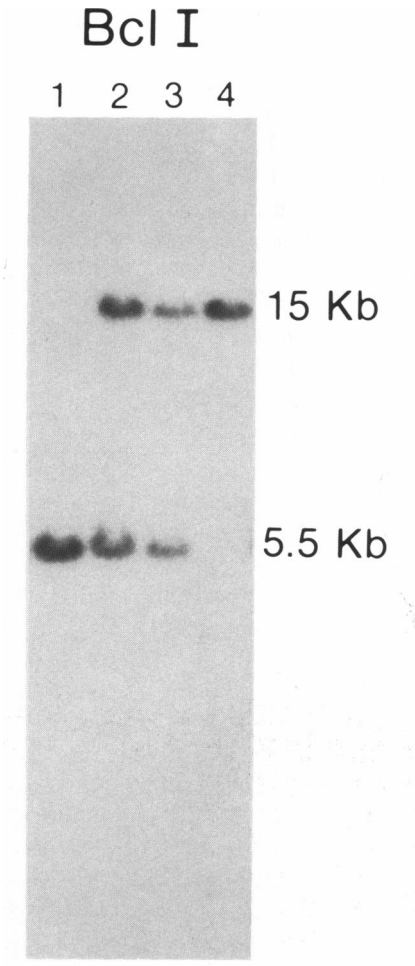

Table 2

New Significant Linkages on Chromosome 4

Pairwise Comparison	$\hat{\theta}$	Z_{max} ($\theta_m = \theta_f$)
D4S35-GC	0.12	10.17
D4S35-ALB	0.12	11.62
D4S35-MT2P1	0.17	4.17
D4S35-D4S1	0.15	4.64
GC-D4S1	0.09	12.21
GC-INP10	0.16	6.18
ALB-MT2P1	0.08	14.08
ALB-D4S1	0.08	14.94
ALB-INP10	0.08	13.09
ALB-ADH3	0.21	5.33
MT2P1-D4S1	0.05	13.04
MT2P1-INP10	0.12	8.44
D4S1-INP10	0.13	6.17
ADH3-EGF	0.04	12.32
ADH3-IL2	0.00	3.78

NOTE.—Linkages with $Z_{max} \geq 3.00$ for pairs of loci not previously reported to be linked are listed with θ at Z_{max} (full table of θ values available on request). $Z_{max} \geq 3.00$ was also observed for MT2P1-GC, GC-ALB, and FBB/FBG-MNS, which are pairs that have had previous linkage data reported.

MNS [Keats 1981]) and confirmed two other linkages (MT2P1-GC [Pakstis et al. 1986] and MNS-FBG [Olaisen et al. 1982]). (Complete tables of lod scores are available on request.)

Table 3 shows the results of the bivariate lod-score analysis. Of the 55 pairs examined, seven showed significant differences between θ_m and θ_f values. In all seven cases, the θ_f value was observed to be greater than the θ_m value. Regression analysis showed a significant relationship between θ_m and θ_f values. After excluding five values for which either the θ_m or θ_f value reached the boundary value of $\theta = .5$, the analysis showed that $c = 1.27$ with 82% of the variance explained by the regression. The fit due to regression was highly significant ($P < .00001$).

Seriation was performed on the univariate recombination matrix (available on request) and excluding IL2. This decision was made based on limited data

Figure 1 *A*, RFLPs for INP10 with *BclI*. Alleles of 15.0 and 5.5 kb are shown. Lane 1 is a father homozygous for the 5.5-kb allele, lane 4 is a mother homozygous for the 15.0-kb allele, and lanes 2 and 3 are their heterozygous offspring. The probe used for INP10 called IFN31.7 has been described elsewhere (Luster et al. 1985). *B*, RFLPs for IL2 with *KpnI*. Alleles of 19 and 16 kb are shown. No homozygotes for the rare 16-kb allele have been observed. Lane 1 is a homozygous mother, and lane 2 a heterozygous father. Lanes 3–5 show their children demonstrating Mendelian inheritance of the 16-kb allele. The probe used is described in the text.

Table 3

θ_m And θ_f Data

	D4S35	GC	ALB	MT2P1	D4S1	INP10	ADH3	EGF	IL2	FBB/FBG	MNS
D4S35		5.0*/24.6	8.9*/27.3	7.3/28.5	14.0/16.0	12.6/28.0	20.2/49.2	29.0/49.0	30.2/30.2	17.9*/50.0	50.0/50.0
GC	11.76		2.6/7.7	7.6/13.3	8.7/12.12	0.0*/25.2	29.9/39.4	34.5/43.7	19.4/49.2	46.8/46.8	36.2/44.4
ALB	12.48	31.63		2.5/13.1	8.3/8.1	6.6/8.5	17.1/26.2	24.2/50.0	21.7/0.0	39.3/49.8	43.3/49.8
MT2P1	5.03	12.82	14.86		0.0*/9.1	0.0*/19.1	18.6/38.9	25.9/49.8	0.0/0.0	35.7/49.3	50.0/50.0
D4S1	4.65	12.26	19.96	14.08		11.0/14.5	19.0/36.0	18.5*/43.5	15.7/49.4	44.8/48.6	40.0/50.0
INP10	1.93	8.00	13.12	9.74	6.20		0.3/20.5	0.3/24.6	14.2/14.2	49.7/33.9	42.7/36.3
ADH3	1.94	1.10	5.60	1.60	1.68	1.86		6.9/0.0	0.0/0.0	35.7/50.0	38.9/30.7
EGF	0.96	0.43	2.34	0.62	2.49	2.44	12.73		0.0/0.0	26.2/44.9	26.8/43.7
IL2	0.31	0.97	0.71	0.25	0.87	0.56	3.78	2.71		31.7/49.5	24.1/24.1
FBB/FBG	2.42	0.05	0.35	0.16	0.08	0.18	0.50	1.38	0.24		8.3/19.5
MNS	0.00	0.50	0.01	0.15	0.01	0.24	0.42	0.41	0.01	7.24	

NOTE.—For each reported comparison, in the upper right are θ_m/θ_f values and in the lower left are $z(\theta_m, \theta_f)$ values.
* Significant difference between θ_m and θ_f at $\alpha = .05$.

available for IL2. The order obtained from seriation of the male matrix was D4S35-GC-(ALB/AFP)-MT2P1-D4S1-INP10-ADH3 - (EGF/IL2) - (FBB/FBA/FBG)-MNS, with IL2 placed by using physical data (R. Shiang, unpublished results) and with no order implied for the loci presented in parenthesis.

Discussion

As part of a long-term study to examine the relationship of genetic and physical distance in humans, we are constructing a detailed genetic map of the long arm of chromosome 4. Three linkage groups are clearly established on 4q. The first is a cluster of six closely linked loci (D4S35, D4S1, MT2P1, GC, ALB/AFP, and INP10) that, on the basis of their physical assignments, provide a saturated genetic map in the vicinity of the centromere on 4q (table 1). The second, reporting close linkage of ADH3 at 4q21-q25 to EGF at 4q25-27 and to IL2 at 4q26-4q28, provides a linkage group in a region for which there has not previously been genetic map data. This grouping of three important genes provides not only a genetic reference point but also the opportunity to examine their function as a cluster in tumors or abnormalities of development. Finally, a third set of loci (MNS-FBB/FBA/FBG) is confirmed as a linkage group in the vicinity of 4q26-4q31. Selecting appropriate markers from each of these three groupings would provide an efficient search strategy for new 4q linkages to new polymorphic loci of genetic disorders.

The chromosome 4 map presented here will have the following practical applications:

1. Examination of table 3 shows that while only a relatively small percentage (12.7%) of the values show significant sex-specific differences in recombination, the general trend is for females to have higher θ values than do males (Weitkamp 1976). While no consistent region of very different rates is observed, individual pairwise comparisons show striking differences with sex. For example, the INP10-GC and INP10-MT2P1 comparisons show ~20% recombination in females while males show no recombination. Conversely, the ADH3-EGF comparison shows the opposite relationship, with no recombination observed in females and 6.9% observed in males. However, the good fit of the regression equation suggests that a constant difference adequately describes the overall set. All of the above examples fall within the 95% confidence limits of the predicted values.

The order derived from the seriation of the pairwise results, while preliminary, serves an an important first step in deriving a complete multipoint map of 4q. The map presented here has a high probability of being correct within a single inversion of adjacent loci (Buetow et al. 1987*b*). Additional confidence in the order is generated by its consistency with known physical localizations of loci included in the map. Availability of preliminary maps such as this greatly reduces the number of orders that must be considered in true multipoint analysis. Such multipoint analysis of these data is in progress and will be reported in a later paper.

2. Dentinogenesis imperfecta (DGI) (Ball et al. 1982), anterior segment mesenchymal dysgenesis (ASMD1) (Ferrell et al. 1982), and Rieger's syn-

drome (Ligutic et al. 1981) have all been proposed as lying on 4q. DGI is 13 cM from GC with a lod score of 6.9, and, since D4S1, MT2P1, ALB/AFP, and INP10 all lie within 10 cM of GC, they should prove useful in identifying the genetic position of DGI more precisely. ASMD1 has probable linkage with MNS ($Z = 2.36$) at a θ value of .09 (Ferrell et al. 1982). The physical map would suggest that MNS is flanked by EGF and possibly FBB/FBA/FBG, thus providing the opportunity to more specifically identify the portion of 4q containing the gene underlying ASMD1. Rieger's syndrome, which includes abnormalities of eye and tooth development, maps to the region that includes both epidermal and T-cell growth factors, so that linkage analysis with these loci would provide the chance to identify a possible role for growth-factor abnormalities in inherited patterns of malformation. Placing a disease locus within a saturated genetic map provides a step toward identification of specific mutations that lack an identifiable biochemical phenotype (Royer-Pokora et al. 1986).

3. The map will help to establish the relationship of recombination to physical distance. By combining the detailed genetic map with the large number of chromosome 4 rearrangements that are available, cytogenetic distances can be related to genetic distances. Use of cell-sorting techniques that identify the DNA content of human chromosomes (Harris et al. 1986) on a panel of chromosome 4's containing deletions or insertions could identify the DNA content of particular bands and help define explicit genetic-physical distance comparisons. Since the relationships of DNA content to recombination have been proposed to vary over short distances at hot spots (Chakravarti et al. 1984), their identification over longer cytogenetic distances should provide insights into the basic mechanisms of recombination in humans.

4. Chromosomal changes have been associated with the development of a variety of malignancies. Cancer phenotypes such as retinoblastoma (Cavenee et al. 1983) and Wilms tumor (Reeve et al. 1984) have shown loss of heterozygosity in tumor tissue when compared with normal tissue from the same individual. A detailed genetic map allows one to establish the boundaries of these changes and may be useful in localizing oncogenic or anti-oncogenic loci. Two candidate loci are included in the current genetic map. EGF and IL2, both shown to play an important role in cell growth, are found to be tightly linked to one another. A more detailed analysis of this clustering of growth factors is now feasible. In addition, a 4;11 translocation commonly observed in acute lymphocytic leukemia has its breakpoint at 4q21, and this map provides markers with genetic positions on either side of this point (Sacchi et al. 1986).

The map we report encompasses 75% of the mitotic 4q and 3.2% of the human genome. It demonstrates the feasibility of using large reference pedigrees such as those established by CEPH to establish detailed human genetic maps. Such maps may be easily extended by other investigators using the same reference panel.

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