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Linkage Disequilibrium of Plasminogen Polymorphisms and Assignment of the Gene to Human Chromosome 6q26-6q27

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SUMMARY

Linkage disequilibrium was observed between newly identified DNA polymorphisms and a previously described protein polymorphism for plasminogen. This finding implies that the two types of polymorphisms describe variation at the same locus. The plasminogen gene was mapped to chromosomal bands 6q26-q27 using somatic-cell hybrids and in situ hybridization. Linkage disequilibrium between protein and DNA polymorphisms has utility in substituting for protein typing in instances where only DNA samples are available, such as from deceased individuals or extinct species. The technique may be useful when cross-hybridizing sequences make the interpretation of Southern blot patterns difficult and may obviate the need for extensive DNA sequencing. In some cases, disequilibrium may provide information useful for determining the appropriate direction for chromosome walks from a marker locus to a target locus.

INTRODUCTION

Linkage disequilibrium is the nonrandom association of an allele at one locus with an allele at another locus. A marked degree of linkage disequilibrium

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provides strong evidence that the two markers describe variation at physically close loci. Linkage data derived from family studies of protein-polymorphism segregation may be directly related to data from DNA-polymorphism segregation patterns if the protein and DNA polymorphisms are in linkage disequilibrium. This type of analysis will be useful in gene families such as the serine proteases, in which nonsyntenic pseudogenes or related functional genes may create complex hybridization patterns on Southern blots.

Plasminogen (PLG) is a glycoprotein in the family of serine proteases. Abnormalities of PLG may result in disordered hemostasis in affected individuals (Aoki et al. 1978). The gene for PLG had been provisionally mapped to chromosome 4 (Eiberg et al. 1981; Eiberg and Mohr. 1982) by family studies linking PLG protein polymorphisms (Raum et al. 1980) to a group-specific component (GC). Markers specific to particular chromosomal regions are useful in gene-mapping studies for unmapped genetic disorders and in studying chromosome rearrangements. To further define the PLG locus we used somatic-cell hybrids and in situ hybridization to establish the PLG chromosomal location and determined the degree of linkage disequilibrium between protein and DNA polymorphisms for this gene.

MATERIAL AND METHODS

Probes

PLG probes used in the present study were genomic DNA subclones derived from λ phage isolates previously reported (Malinowski et al. 1984). Probe JES-VI-56-5 was an 800-bp *Hin*dIII-*Bam*HI fragment in pUC8. It was derived from isolate λ3 and contained an exon encoding the amino-terminal half of kringle 4, PLG residues 346–399 (Malinowski et al. 1984). Probe III-4-1-A6 was a 2.4-kb *Eco*RI fragment in pBR322 derived from the 3′ end of isolate λ2 (Malinowski et al. 1984). The 3′-*Eco*RI site was from a linker. Probe III-4-1-A6 contains at least one exon, as determined by hybridization to cloned cDNA for PLG. Probe III-4-1-A6 is ~15 kb 3′ of probe JES-VI-56-5 (Malinowski et al. 1984).

Populations

Individuals studied were normal individuals from the greater Seattle area. Blacks were American blacks, except for one African black. Asians were eight native Japanese, eight native Chinese, and three native Koreans. Blood samples were obtained with informed consent.

RFLP Detection

Southern blot analysis and DNA preparation were performed as previously described (Murray et al. 1983) using nick-translated probes in plasmid vectors, except that blots were washed at 65 C for 1 h in 0.1 × SSC and 0.1% sodium dodecyl sulfate. DNAs from 10 unrelated Caucasoids were used to screen for restriction-fragment-length polymorphisms (RFLPs) using the restriction enzymes *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*NI, *Eco*RI, *Eco*RV, *Hae*III, *Hgi*AI, *Hin*dIII, *Hinf*I, *Msp*I, *Mst*II, *Nci*I, *Pst*I, *Pvu*II, *Rsa*I, *Sac*I, *Sau*IIIA, *Sph*I,

*Stu*I, and *Taq*I. Once RFLPs had been identified, family studies were done to confirm Mendelian segregation and conformance with Hardy-Weinberg equilibria was assessed.

Protein Polymorphisms

Detection of protein polymorphisms was performed as reported (Raum et al. 1980) using serum from normal individuals on whom DNA was also available.

Linkage Disequilibrium

Linkage disequilibrium is defined as the deviation of observed haplotype (gamete) frequencies from those expected from the products of their individual frequencies. For a pair of loci with two alleles, standardized linkage disequilibrium (expressed as r) can be calculated as follows:

		Site B: +	Site B: −	
Site A	+	$g_1 = p_1p_2 + \epsilon$	$g_2 = p_1q_2 - \epsilon$	p_1
	−	$g_3 = q_1p_2 - \epsilon$	$g_4 = q_1q_2 + \epsilon$	q_1
		p_2	q_2	

$$r = \frac{g_1p_1p_2}{p_1q_1p_2q_2} = \frac{\epsilon}{p_1q_1p_2q_2},$$

where p_j and q_j are the frequency of + and − alleles at site j, g_i is the observed frequency of the ith haplotype, and ϵ is the deviation in frequency due to association (Li 1976).

The measure calculated above is the correlation coefficient between uniting gametes at two loci and is largely independent of gene frequencies. Significance can be obtained by taking the tan-hyperbolic inverse of the values, which is the large-sample normal deviant. Given the range of our samples' sizes and the structure of our data, an alternative in evaluating significance is Fisher's exact probability test. In this test the probability of obtaining the observed distribution of haplotypes and all more extreme distributions is calculated. Two-locus haplotypes were derived either from individuals homozygous at one locus or from family studies.

Somatic-Cell Hybrids

A panel of human-mouse somatic-cell hybrids was used. Human chromosomes and marker enzymes for each chromosome as well as genomic DNA were analyzed on the same cell passage. Human-mouse somatic-cell hybrids were isolated and characterized as reported elsewhere (Shows et al. 1984). Human chromosomes were analyzed as previously described (Shows et al.

1978). Human chromosomal translocations have been well characterized in cell hybrids. Enzyme markers for each chromosome were studied as described elsewhere (Shows et al. 1982; Shows 1983).

In Situ Hybridization

In situ hybridization of both PLG probes was done as previously described (Deeb et al. 1986). Probes were used separately, and results were pooled.

RESULTS

Three common DNA polymorphisms were demonstrated. These were seen with *Msp*I, *Sac*I, and *Rsa*I digests and are shown in figure 1. Gene frequencies for several populations are shown in table 1. Mendelian inheritance was shown for all markers in several families, and Hardy-Weinberg equilibria were demonstrated (data not shown). Cross-hybridizing bands were seen with most probe-enzyme combinations used (fig. 1).

Linkage disequilibrium of the three common RFLPs and the protein polymorphism is shown in table 2. There was significant disequilibrium in Caucasoids for five of the six pairwise comparisons. No significance was seen for *Rsa*I-PLG A/B, but data were limited because of small sample size (29 assigned haplotypes). *Msp*I (+) and *Sac*I (−) were completely associated in the Caucasoids studied, although this was not observed in a smaller, black population studied, in which association was high but not complete (data not shown). Very high association values were present for PLG A/B with both *Sac*I and *Msp*I RFLPs. The lack of complete association of *Sac*I and *Msp*I alleles with PLG A/B implied that neither of these RFLP sites represented the nucleotide site of the DNA variation causing the protein polymorphism.

A sample of data from the experiments with somatic-cell hybrids is shown in figure 2. Table 3 shows the complete data set. A single *Sac*I RFLP allele was found in all 13 cell lines containing chromosome 6 and in zero of 19 cell lines not containing chromosome 6, for 0% discordancy. Discordance for other chromo-

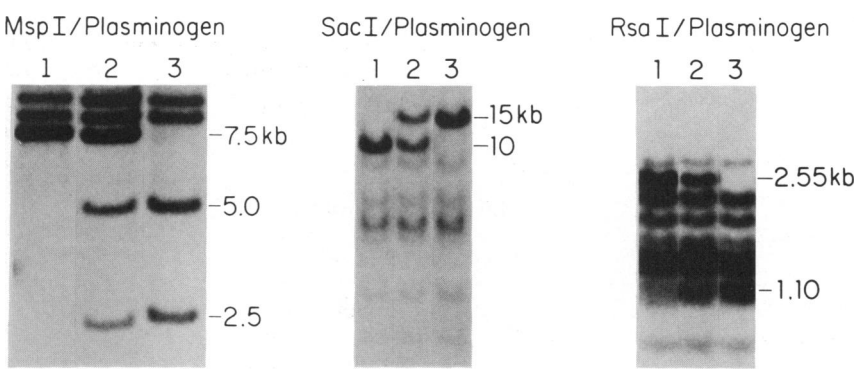

FIG. 1.—Southern blot analysis of DNA polymorphisms using plasminogen-specific probes. *Sac*I and *Rsa*I digests probed with JES-VI-56-5 from the 5′ end of locus. *Msp*I digest was probed with III-4-1-A6 from the 3′ end of locus. Lanes 1, 2, and 3 are father, child, and mother, respectively. Several nonpolymorphic and cross-hybridizing bands are seen in all lanes.

TABLE 1

PLG RFLP ALLELE FREQUENCIES

RACIAL GROUP	*Sac*I +/− (*N*)	*Msp*I +/− (*N*)	*Rsa*I +/− (*N*)
Caucasoid	.33/ .67 (231)	.67/.33 (205)	.24/.76 (177)
Black	.17/ .83 (35)	.90/.10 (42)	.09/.91 (32)
Asian	.00/1.00 (38)	1.00/.00 (38)	.54/.46 (28)

NOTE.—*N* = Number of unrelated chromosomes studied.

somes ranged from 19% to 56%, with a minimum of 28 informative cell lines studied per chromosome. There were 12 (38%) discordances in 32 cell lines studied for chromosome 4.

To sublocalize the PLG locus, in situ hybridization studies were performed using the two different PLG probes in separate experiments. The results for each probe were similar and were pooled. A total of 47% of 446 metaphase cells exhibited between one and four autoradiographic grains, the rest of the metaphase cells having no grains. Forty-nine (33%) of 149 informative hybridization sites localized PLG to the distal two bands of the long arm of chromosome 6 (fig. 3). The other sites of hybridization were scattered over the genome. Of the grains located on chromosome 6, 20 (41%) were at band q26 and 15 (31%) were at band q27. Thus, the PLG gene locus is on chromosome 6 at band q26 or q27.

DISCUSSION

Previous linkage results had tentatively assigned PLG to chromosome 4 (Eiberg et al. 1981; Eiberg and Mohr 1982), with a pooled lod score of 2.91 at $\hat{\theta} = .43$ in males. Not all studies supported this assignment (Bissbort et al. 1983), however. The combination of somatic-cell hybrids and in situ hybridization reported here reassign the PLG gene to the long arm of chromosome 6. This new assignment will aid in establishing linkage relations for genes and disease loci on 6q, such as alpha-L-fucosidase (FUCA2), which has been previously assigned to the same linkage group as PLG (Eiberg et al. 1984).

TABLE 2

PLG LINKAGE DISEQUILIBRIUM

	*Msp*I	*Sac*I	*Rsa*I	PLG A/B
*Msp*I		−1.00	+.204	+.844
*Sac*I	<.0001		−.233	−.869
*Rsa*I	.049	.023		+.221
PLG A/B	<.0001	< .0001	.175	

NOTE.—Values of *r* are presented above the diagonal; values of *P* (Fisher's exact probabilities) are below the diagonal. Sample sizes ranged from 29 to 85 chromosomes in which the pairwise haplotypes were determined from homozygotes or family studies. All chromosomes studied were from unrelated U.S. Caucasoids.

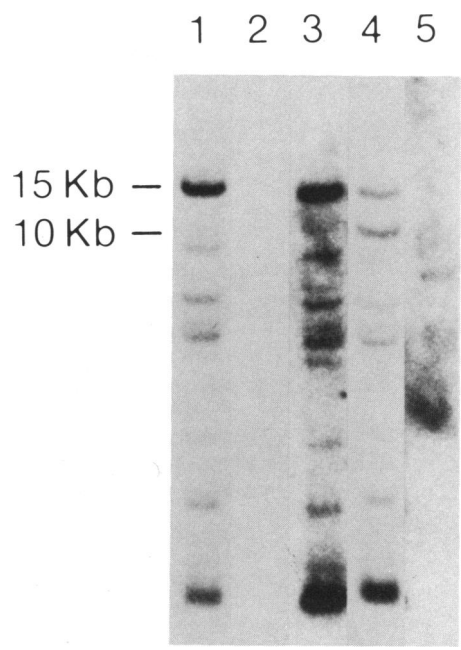

FIG. 2.—Southern blot hybridization of PLG gene probe (JES-VI-56-5) to somatic-cell hybrids and control DNA digested with *Sac*I. Lane 1, human genomic DNA from an individual homozygous for the 15-kb *Sac*I allele; lane 2, mouse genomic DNA; lane 3, cell line DUM-13 (see table 3), which contains chromosome 6 and is missing chromosome 4; lane 4, genomic human DNA from an individual heterozygous for 15-kb and 10-kb alleles; lane 5, cell line REW-8D (see table 3), which contains chromosome 4 and lacks chromosome 6.

The linkage disequilibrium observed between the previously reported PLG protein polymorphism and the newly reported RFLPs demonstrates the utility of nonrandom association studies. Owing to the evolutionary relationship of linkage disequilibrium to recombination (Lewontin 1974), strong linkage disequilibrium is typically only observed either between very tightly linked markers or for loci within the same gene cluster. Therefore, markers found in linkage disequilibrium may generally be assumed to be syntenic and in close physical proximity. Since the DNA and protein polymorphisms describe variation at the same locus, the protein variant for PLG can be assumed to arise from a locus on 6q as well. The DNA polymorphism data can therefore be directly compared to previously reported data derived only from the protein polymorphism.

Linkage disequilibrium has been commonly reported at human gene loci (Bech-Hansen et al. 1983; Chakravarti et al. 1984*a*, 1984*c*; Murray et al. 1984; Cox et al. 1985; Ohlsson et al. 1985) and is generally related to the distance between markers (Chakravarti et al. 1984*a*). Such disequilibrium has been use-

TABLE 3

DISTRIBUTION OF THE *SacI* RFLP FOR PLG WITH HUMAN CHROMOSOMES IN HUMAN-MOUSE HYBRIDS

		HUMAN CHROMOSOME																							
	PLASMINOGEN	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	TRANSLOCATIONS
Hybrid:																									
DUA-3BSAGA ...	−	−	+	−	−	−	−	+	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	...
DUA-5BSAGA ...	−	−	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	−	−	+	−	−	...
DUM-13	+	+	+	+	−	−	+	+	−	−	+	+	+	−	+	t	+	+	+	+	+	+	+	t	X/15 15/X
EXR-5CSAz	+	+	−	+	+	+	+	+	+	+	+	t	+	+	+	+	−	+	+	+	+	+	+	+	X/11 (& 1/30 X)
ICL-15	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	−	−	+	+	−	−	...
JSR-14	+	−	+	+	+	+	+	−	−	−	−	−	+	+	−	−	−	+	−	−	+	+	−	+	...
JWR-26C	+	t	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	1/2
REW-5	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	−	+	+	+	...
REW-8D	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	+	+	+	+	...
REW-11	−	−	−	−	+	−	−	+	−	−	−	+	+	+	−	−	+	−	−	−	+	+	+	+	...
REX-11BSAgB ...	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	...
REX-26	−	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	t	t	22/X
SIR-8	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	...
TSL-1	−	−	−	+	+	−	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	+	−	−	...
TSL-2	+	−	+	t	−	+	+	−	−	−	+	−	+	−	−	−	−	t	+	−	+	+	−	+	17/3 3/17

VTL-6	+	−	+	−	−	−	+	+	+	−	+	+	−	−	−	+	−	+	−	+	+	+	+	−	...
VTL-7	−	−	−	−	−	−	−	+	−	−	−	+	−	−	+	−	−	+	−	+	+	+	+	−	7q-
VTL-8	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+	+	+	−	...
VTL-17	−	−	−	−	−	−	−	+	−	−	+	+	−	+	+	−	−	+	−	−	+	+	−	−	...
WIL-1	−	−	−	−	−	−	−	−	+	−	−	−	+	−	+	−	−	+	+	−	−	+	−	+	...
WIL-2	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	+	−	+	−	−	−	+	−	+	...
WIL-5	−	−	−	−	+	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−	−	+	−	+	...
WIL-6	+	−	+	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	...
WIL-7	+	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	...
WIL-8X	−	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	...
WIL-8Y	+	−	−	−	−	−	+	+	−	−	+	+	−	−	+	+	+	+	+	+	+	+	+	+	...
WIL-14	−	+	−	+	−	+	−	+	+	−	+	−	+	−	+	+	−	+	−	−	−	−	−	+	...
WIL-15	+	−	+	+	+	−	+	+	−	−	+	+	+	+	+	−	−	+	+	−	+	+	−	+	...
XER-11	+	+	−	+	+	−	+	+	+	−	+	t	+	+	−	+	+	+	+	+	+	+	+	t	11/X X/11
XTR-2	−	−	−	t	−	+	−	−	+	−	+	−	+	+	−	−	−	−	+	−	+	+	−	t	3/X
XTR-3CSAZK ...	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	−	−	+	−	−	−	+	−	−	...
XTR-22	+	−	+	−	+	+	+	−	+	−	+	+	−	−	−	+	−	−	+	+	+	+	+	+	X/3 (& 1/34 X)
Plasminogen/ Chromosome:																									
	(+/+)	4	10	8	8	8	13	9	7	2	12	9	8	6	8	7	4	11	10	8	11	13	7	10	
	(+/−)	8	3	4	5	5	0	4	6	11	1	2	5	7	5	5	9	1	3	5	2	0	6	1	
	(−/+)	3	3	7	7	5	0	8	12	3	10	8	10	7	12	6	4	16	9	3	9	10	5	8	
	(−/−)	16	16	11	12	14	19	11	7	16	9	11	9	12	7	13	15	3	10	16	10	3	13	9	
% Discordancy		35	19	37	38	31	0	37	56	44	34	33	47	44	53	35	41	55	37	25	34	50	35	32	

NOTE.—Cell lines described in Results. t = A translocation. A + in the "Plasminogen" col. indicates that one of the two *Sac*I alleles was seen on a Southern blot of DNA from that cell line. A + or − in the "Human Chromosome" col. indicates the presence or absence, respectively, of that human chromosome in that cell line.

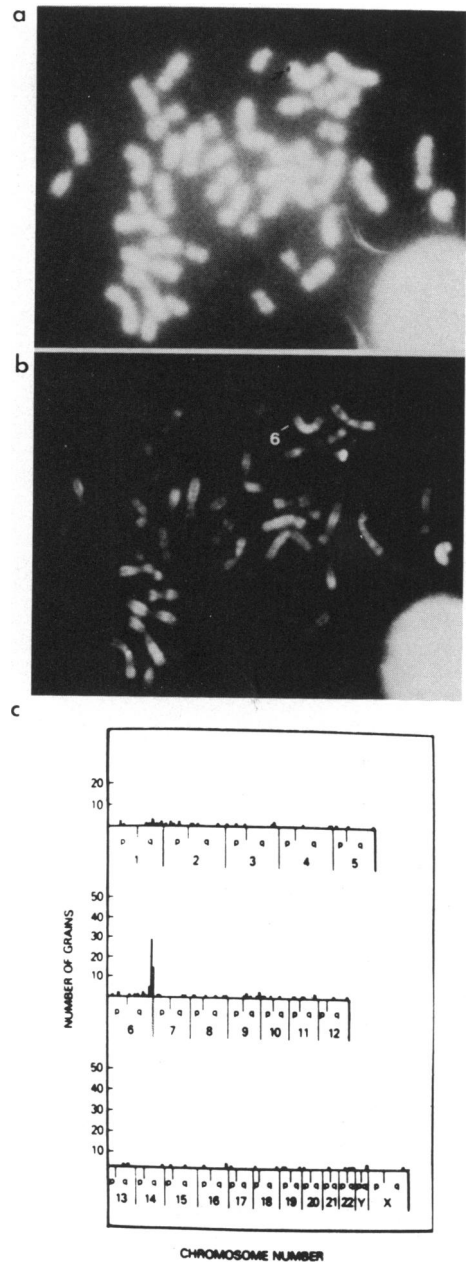

FIG. 3.—In situ hybridization of the PLG gene probes to metaphase chromosomes. *a*, Example of metaphase cell from a human male showing a single grain on chromosome 6 (arrow); *b*, same metaphase cell stained by Q-banding for chromosome identification; *c*, histogram showing the distribution of autoradiographic grains over human chromosomes after in situ hybridization with plasminogen probes labeled with ^{3}H nucleotides to a specific activity of $\sim 7 \times 10^{7}$ cpm/μg. Data from hybridization using two different probes (JES-VI-56-5 and III-4-1-A6) were pooled.

ful in assaying relative recombination rates (Chakravarti et al. 1984*a*) and is an important consideration in genetic counseling and in determining the information content for sets of markers (Chakravarti et al. 1984*b*). Disequilibrium is not always found between physically close markers (e.g., <10 kb apart), even in situations in which markers on either side of internal markers show disequilibrium (Barker et al. 1984). Thus, failure to find disequilibrium is not evidence against close linkage or physical proximity. The exact physical distance between our PLG RFLPs is presently unknown.

Establishing the equivalency of protein and DNA polymorphisms is useful in several contexts. First, such linkage disequilibrium can assist in the localization of changes at the DNA level that are responsible for variation observed at the protein level. Observation of complete association of a protein variant with an RFLP for a particular DNA probe obviates the need for large amounts of DNA sequence data as a criterion for showing that the DNA polymorphisms and the protein variant arise from the same locus. Since the genes for large proteins may encompass tens of kilobases, such sequencing studies can be a significant undertaking.

Second, this approach may allow indirect—but highly accurate—studies of presumed protein types in which samples containing the specific protein are no longer available. DNA from cell lines and tissues of deceased individuals—or even from extinct species—would still provide critical data (Higuchi et al. 1984).

Third, it will allow easy fusion of the existing large body of population data, derived from protein polymorphisms in racially or ethnically distinct groups, with DNA data now available on contemporary samples (Mourant et al. 1976). Stored specimens on individuals studied previously and now lost to ascertainment may contain usable DNA long after protein and red cell antigens have been exhausted or can no longer be typed.

As we observed for PLG, when DNA probes create complex hybridization patterns on Southern blots, associations between protein and DNA variants may aid in identifying RFLPs of interest. This finding is frequently observed when a particular gene has related pseudogenes or is a member of a gene family with members at other loci. It may be difficult to find hybridization conditions or a probe structure with which to obtain reproducible and easily interpretable patterns on a Southern blot. If, however, one can establish that particular RFLPs are in nonrandom association with the protein polymorphisms at that locus, such RFLPs (and not pseudogene RFLPs or RFLPs for other genes in the family) can be inferred to describe variation at the structural locus responsible for the observed phenotype. These RFLPs can then be used in linkage, somatic-cell, and other genetic studies of that locus. This task is made easier by the fact that the linkage disequilibrium does not need to be complete—only significant enough to establish both synteny and proximity. Since significant associations may extend 100 kb or more (Chakravarti et al. 1984*a*) and (Ohlsson et al. 1985) and since RFLPs may occur, on average, every 100–300 bp (Cooper et al. 1985), it should rarely be difficult to find a nonrandomly associated RFLP.

This use of linkage disequilibrium may be logically extended not only to

RFLPs that are surrogates for protein markers but also to localization of DNA changes resulting in disease phenotypes. For a given genetic disorder with a low spontaneous mutation rate and relatively few origins, association between a tightly linked marker and disease locus may be used in combination with chromosome "walking" or "hopping" (Collins and Weissman 1984) in determining the direction of future walks.

This procedure would be employed by first measuring the association between a linked marker with an existing probe and the disease phenotype. After walking or hopping to locations both 5′ and 3′ to the previous location, polymorphisms in these fragments would be tested for association with the disease locus. Since association is evolutionarily related to recombination (Lewontin 1974) and, theoretically, to physical distance (Chakravarti et al. 1984*a*), movement away from the disease locus should result in a decrease in association while movement closer to the disease locus should result in an increase in association—with some exceptions, as noted above. The fragment with the higher association would then be taken as a new reference point, and another hop or walk would be taken. Walks would continue until no increase in disequilibrium was to be found. Depending on the distance between adjacent jumps, smaller intervals could then be employed to find polymorphisms progressively closer (more associated) than previous sites. In humans, linkage disequilibrium has been demonstrated over distances as great as 100 kb. This is a distance that is amenable to current walking techniques. When the interval between associated polymorphisms is small enough, the entire region may be sequenced in normal and affected chromosomes.

Detailed analysis of DNA variation in a region localized by associated flanking markers could allow identification of the causal mutation by virtue of its complete association with a disease phenotype. This technique has been used to identify DNA variations potentially associated with nondeletion forms of hereditary persistence of fetal hemoglobin (Waber et al. 1985). Such disequilibrium determinations may be the only way to locate mutation sites causing phenotypes for which the underlying mechanisms are unknown.

In summary, using somatic-cell hybrids and in situ hybridization, we have established the position of the PLG gene at chromosomal bands 6q26-q27. Linkage disequilibrium for RFLPs and protein polymorphisms was found, establishing that the newly assigned locus on 6q is identical to that previously tentatively assigned to chromosome 4 on the basis of protein polymorphisms. Such disequilibrium determination may be useful in bridging the gap between cytogenetic and molecular maps in humans.

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