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Molecular genetics of human serum albumin: Restriction enzyme fragment length polymorphisms and analbuminemia

(human chromosome 4 markers/DNA variants/alloalbuminemia/prenatal diagnosis)

JEFFREY C. MURRAY*†, CHRISTINA M. DEMOPULOS*†, RICHARD M. LAWN‡, AND ARNO G. MOTULSKY*†§

Departments of *Medicine (Medical Genetics) and §Genetics and †Center for Inherited Diseases, University of Washington, Seattle, WA 98195; and ‡Genentech Inc., Department of Molecular Biology, 460 Pt. San Bruno Boulevard, South San Francisco, CA 94080

Contributed by Arno G. Motulsky, June 20, 1983

ABSTRACT By using cDNA probes for the human albumin gene, four restriction enzyme fragment length polymorphisms (RFLPs) were discovered that were transmitted by codominant autosomal inheritance. Among Caucasians, the gene frequencies were 0.04/0.96 for *Msp* I/5′, 0.43/0.57 for *Hae* III/3′, 0.44/0.56 for *Hae* III/5′, and 0.04/0.42/0.54 for *Pst* I/5′. These common variants provide a marker for chromosome 4 (q11–q13). A calculation of the extent of DNA variation at the albumin locus revealed that 1/95 nucleotide sites was affected by a RFLP, a figure similar to that found in the globin system. Restriction enzyme fragment study of the DNA of a human analbuminemic individual revealed no gross structural rearrangements of the albumin locus. The exact nature of the abnormality will require more study.

Genetic variation of human serum albumin can manifest as alloalbuminemia or as analbuminemia. Both conditions are clinically benign. In alloalbuminemia, electrophoretic variation in serum albumin is caused by mutations in the coding areas of the albumin locus resulting in amino acid substitutions. About 20 such variant albumins exist. Most are rare; a few appear to be more frequent because of genetic drift or founder effects (1, 2).

Human analbuminemia is transmitted by autosomal recessive inheritance. Maintenance of oncotic pressure in affected individuals is mediated by compensatory increases of other serum proteins. Our group (3) demonstrated the presence of greatly decreased quantities (17 mg/dl or less than 1/200 of normal) of immunologically normal serum albumin in an American Indian girl with analbuminemia. We postulated that the basic defect would be a "thalassemia-like" lesion that left the albumin locus intact but interfered somehow with gene expression.

The availability of cDNA probes for albumin gene coding regions allows analyses of the albumin locus at the molecular level (4). We have used cDNA probes and restriction enzyme mapping techniques on the DNA of this analbuminemic individual and her consanguineous parents. There were no differences detectable between their albumin locus and that of normal controls, thus supporting the hypothesis that the molecular defect in our analbuminemic patient was *not* caused by a gross gene deletion. Furthermore, in normal individuals we have detected four different restriction enzyme fragment length polymorphisms (RFLPs) in the albumin region.

MATERIALS AND METHODS

DNA for restriction enzyme analysis was prepared from whole blood by the method of Poncz *et al.* (5). Fifty-six unrelated Caucasian individuals, including 5 sets of parents whose children were available, were studied for the detection of polymorphisms. Samples were obtained from all individuals after obtaining signed informed consent.

Aliquots (10 μg) of DNA were digested with various restriction enzymes (Bethesda Research Laboratories and P-L Biochemicals), using buffers recommended by the supplier or described by Maniatis *et al.* (6). Digestions were carried out overnight and the products were electrophoresed on 0.8–1.2% agarose gels (Bethesda Research Laboratories) for 16 hr in Tris acetate/EDTA buffer (6) at 60 mA. After staining in ethidium bromide, DNA was transferred to nitrocellulose paper by using a modified Southern procedure (7). Hybridization probes were prepared by nick-translation (8) with [^{32}P]dCTP as a substrate. Probes were derived from plasmids containing cDNA sequences complementary to the albumin mRNA (4) and consisted of (*i*) a 900-base-pair (bp) probe to the 5′ end of the human serum albumin coding region and including about 30 bp of 5′ noncoding sequence (F47); (*ii*) a 1,000-bp probe to the 3′ end of the albumin coding region including about 300 bp of 3′ noncoding sequence (B44); and (*iii*) a 250-bp probe (B44-mid) to the middle of the albumin coding region generated from the 5′ end of B44 by *Pst* I digestion of the plasmid B44 (see Fig. 1). The only *Pst* I site found within the cDNAs used was that separating B44 from B44-mid. After overnight prehybridization in Stark's buffer and 1% glycine, between 2 and 4 × 10^6 cpm of labeled probe was hybridized in Starks buffer and 10% dextran sulfate (9) for 2–4 days at 42°C and then washed 3 times for 5 min each with 0.1% sodium dodecyl sulfate/0.3 M sodium chloride/0.03 M sodium citrate at room temperature and twice for 15 min each with 0.1% sodium dodecyl sulfate/0.015 M sodium chloride/0.0015 M sodium citrate at 50°C (10). Blots were dried and autoradiographed with Kodak X-Omat AR film and DuPont Cronex Lightning Plus intensifying screens.

The number of nucleotide bases screened with restriction enzymes refers to the total number of bases cut by a given restriction enzyme that generated one or more DNA fragments. This number was computed for each restriction enzyme by the equation

$$\mathrm{NBS} = (\mathrm{BR})(\mathrm{NBD} + 1),$$

in which NBS = no. of bases screened, BR = no. of bases recognized by the restriction enzyme, and NBD = no. of bands detected.

The total number of bands detected (NBD) was determined by counting the number of bands of different lengths found with all probes used for a given restriction enzyme. Problems estimating the frequency of DNA variants from restriction enzyme data include: (*i*) Fragments of similar size are not separable, thus giving a falsely low estimate for the number of bases screened. (*ii*) Two restriction sites that lie close together will generate a small fragment (<500 bp) that either runs off the gel

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Abbreviations: RFLP, restriction enzyme fragment length polymorphism; bp, base pair(s); kb, kilobase(s).

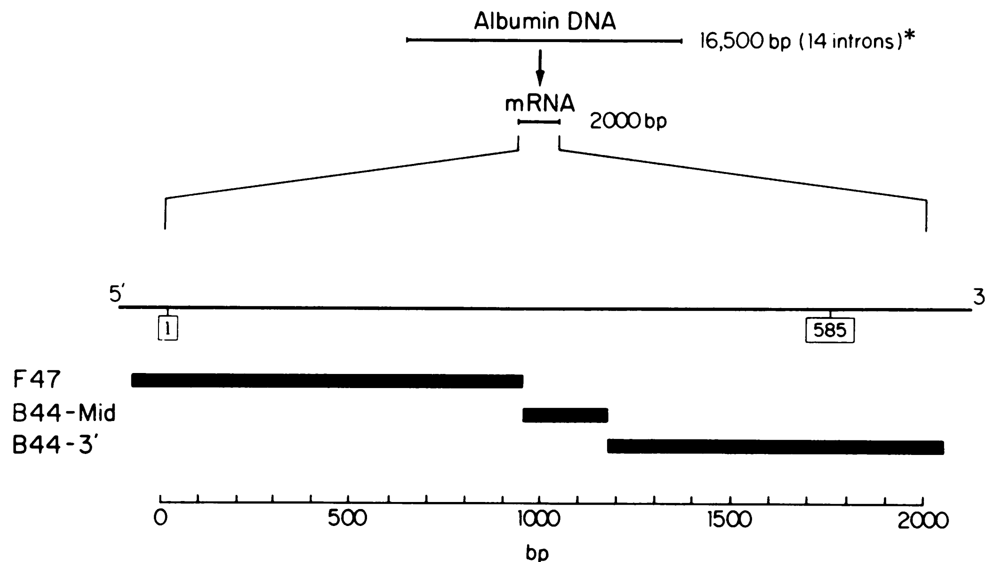

FIG. 1. Diagram of albumin locus in man. The 16,500 bp of albumin genomic DNA code for mRNA of about 2,000 nucleotides encoding the 585 amino acids in the mature albumin molecule. Three separate cDNA probes—F47, B44-mid, and B44—were used in mapping studies with positions shown here.
* Fourteen introns is based on the number found in rat albumin; the number has not yet been verified for humans.

or may not transfer effectively and thus will be missed. (*iii*) There may be some overlap of restriction enzyme sites with different enzymes so that the number of bases screened may be falsely increased. However, all of these effects are small and should result in an error of no more than 10%—an estimate based on the ability to distinguish between fragments that differ in length by as little as 5%. Furthermore, overlap between restriction enzyme sites is unlikely because only a small fraction of nucleotides of the albumin gene are recognized by the restriction enzymes used in this study (see *Results*).

RESULTS

Analbuminemia. The enzymes and probe combinations listed in Table 1 were used to screen DNA from the analbuminemic individual. In comparison with normal controls, no differences in fragment lengths were detected in either the affected individual or her heterozygous parents. It can therefore be inferred that the albumin gene was present in this analbuminemic individual and that no large (>100 bp) structural rearrangements (deletions, additions, or inversions) of the DNA existed. We cannot rule out smaller rearrangements or single base-pair changes.

RFLPs at the Human Serum Albumin Locus. Common variants at the albumin locus detectable by restriction enzymes were

Table 1. Restriction enzyme analysis of analbuminemia

Albumin probe	Enzymes tested
5′	*Ava* II, *Bam*HI, *Bgl* I, *Bst*EII, *Hae* III,* *Hin*cII, *Hin*dIII, *Hin*fI, *Msp* I,* *Pst* I,* *Pvu* II, *Sst* II, *Taq* I, *Xho* I
3′	*Bam*HI, *Bcl* I, *Eco*RI, *Hae* III,* *Hin*fI, *Msp* I, *Pst* I, *Taq* I

All enzyme/probe combinations were tested on normal individuals, an analbuminemic woman, and her parents. No differences in fragment sizes were noted in any case.
* Parents and the affected individual were homozygous for the fragment length polymorphism detected with this enzyme in the population.

searched for in normal individuals and in the analbuminemic family. Table 2 lists each enzyme used, the number of different bands detected, the sizes of the bands, and the number of bases screened with each enzyme. The equation given in *Materials and Methods*¶ indicates that the total number of bases (NBS) that were recognition sites for all restriction enzymes used was 380. At least nine unrelated individuals were used for each enzyme tested with a given probe, providing a 95% probability of detecting variants with a population incidence of 15% or greater (11). Because we detected four polymorphisms the frequency of RFLPs at the albumin locus with a frequency of 15% or more was estimated to be about 0.01 (4/380) or 1 polymorphic site in 95 nucleotides.

One polymorphism was detected with the enzyme *Msp* I by using either the probe for the 5′ side of the cDNA (F47) or that for the middle portion of the cDNA (B44-mid). Two allelic fragments could be identified: either a single 18-kb fragment or a double band consisting of a 13- and a 5-kb fragment (see Fig. 2). Study of a normal family showed Mendelian codominant inheritance of the variant DNA. Thus, among three offspring of a heterozygote (*18/13*) × homozygote (*18/18*) mating, two were *18/18* homozygotes and one was an *18/13* heterozygote. Four of 48 Caucasians (8.9%) and 2 of 11 North American Blacks were heterozygous for the *13* allele. The gene frequency for the *18* allele among 76 Caucasians was 0.96 and that for the *13* allele was 0.04. These gene frequencies followed the distribution expected with random matings of the different genotypes by the Hardy–Weinberg law.

A second polymorphism was detected with the enzyme *Hae* III and a probe to the 3′ portion of the albumin locus (B44). Two allelic forms were generated: either a 1.15-kb fragment or a 0.75-kb fragment (see Fig. 3). The *Hae* III forms were also inherited as Mendelian codominant alleles (data not shown). In 24 unrelated Caucasians examined to date, 6 individuals had a single 1.15-kb allele, 9 individuals had a 0.75-kb fragment only, and 9 individuals had both the 1.15-kb band and the 0.75-kb band. These distributions were those expected by random mating of the different genotypes and followed Hardy–Weinberg

¶ When polymorphic bands are detected, NBS is found by multiplying bases recognized with the number of restriction enzyme sites.

Table 2. Fragments detected in normal individuals with the albumin probes and number of bases screened for RFLPs

Enzyme (nucleotides recognized)	Albumin probe	Fragment sizes, kb (individuals tested)	Total band(s) detected	Nucleotides screened
Alu I (4)	5′	0.6 (9), 0.8 (10)	2	12
Ava II (4.5)	3′	2.8 (10), 5.0 (10)	3	18
	5′	9.6 (10)		
*Bam*HI (6)	3′	8.0 (12)	1	12
Bgl I (6)	3′	4.0 (10), 10 (10)	3	24
	5′	23 (10)		
*Bst*EII (6)	3′	15 (8)	1	12
	5′	15 (2)		
*Eco*RI (6)	3′	10.6 (14)	1	12
Hae III (4)	3′	4.1 (59), 1.8 (58), 1.5 (16), 1.15 (18),* 0.75 (20),* 0.4 (4)*	12*	48
	5′	4.1 (28), 3.8 (21),* 3.6 (23),* 2.2 (10), 1.4 (10), 0.25 (10)		
*Hin*cII (5)	5′	4.3 (11), 4.0 (10), 2.8 (11)	3	20
*Hin*dIII (6)	3′	1.8 (9), 4.2 (25), 6.2 (33)	3	24
	5′	6.2 (10)		
*Hin*fI (4)	3′	2.5 (9)	2	12
	5′	2.0 (10)		
Kpn I (6)	Mid	5.6 (17), 9 (17)	3	24
	5′	14 (9), 5.6 (8)		
Msp I (4)	3′	6.8 (10), 18 (10)	5*	20
	Mid	5.0 (5),* 6.8 (49), 13 (5),* 18 (56)*		
	5′	3.7 (7), 6.8 (8), 18 (8)		
Pst I (6)	3′	2.5 (22), 4.0 (23), 4.7 (23)	5*	42
	5′	4 (28), 24 (2),* 18 (12),* 14 (10)*		
Pvu II (6)	3′	16 (10)	2	18
	5′	7.2 (12), 16 (12)		
Sst II (6)	5′	50 (9)	1	12
Taq I (4)	3′	1.0 (18), 2.6 (18), 5.5 (20)	6	28
	5′	1.8 (10), 2.6 (10), 4.1 (12), 0.6 (8)		
Xba I (6)	3′	3.8 (9), 3.6 (9)	6	42
	5′	1.5 (9), 1.9 (9), 3.0 (9), 14 (9)		
Totals			59	380

Enzymes used are listed with number of nucleotides in their recognition site in parentheses. Fragment sizes in kilobases (kb) are shown for each enzyme/probe combination used, with the number of individuals observed with a particular fragment size in parentheses. Total bands detected reflect all bands of different sizes found with a given enzyme, including all polymorphic bands. Nucleotides screened calculation was described in the text.

* Includes bands that are polymorphic.

proportions. The frequency of the 1.15 allele was 0.43 and that of the 0.75 allele was 0.57.

The third polymorphism was also detected with the enzyme *Hae* III, but with a probe to the 5′ portion of the gene (F47). Alleles of either 3.6 or 3.8 kb were found in very tight linkage with those detected in the *Hae* III/3′ polymorphic system (see Fig. 3). Thus, the 3.8-kb *Hae* III/5′ allele was always associated with the 1.15-kb *Hae* III/3′ allele and the 3.6-kb *Hae* III/5′ allele was always found with the 0.75-kb *Hae* III/3′ allele. In 47 Caucasian individuals the frequencies of the *3.8* allele and *3.6* allele were 0.44 and 0.56, respectively.

The fourth polymorphism was detected by using *Pst* I and the 5′ probe (F47). Three alleles were detected in 46 Caucasians: a *24* allele with a gene frequency of 0.04, an *18* allele with a gene frequency of 0.54, and a *14* allele with a gene frequency of 0.42 (see Fig. 3). The lack of smaller bands associated with the shorter variants (18 and 14 kb) suggests that the polymorphic sites were located 5′ to the 5′ end of this probe.

The analbuminemic subject and both her parents were homozygous for identical alleles detectable with *Msp* I, *both Hae* III variant fragments, and the *Pst* I variant fragments [*Msp* I: *18/18* (father), *18/18* (mother), and *18/18* (daughter); *Hae* III/3′ probe: *1.15/1.15* (father), *1.15/1.15* (mother), and *1.15/1.15* (daughter); *Hae* III/5′ probe: *3.8/3.8* (father), *3.8/3.8* (mother), and *3.8/3.8* (daughter); *Pst* I: *14/14* (father), *14/14* (mother), *14/14* (daughter)]. These data therefore were uninformative regarding formal linkage of the gene locus for analbuminemia and of RFLPs at the albumin locus (see *Discussion*).

Minor Bands. A number of minor bands were observed. These bands were of about 1/10 the intensity of the major bands detected (see Fig. 2) and were observed consistently with certain enzyme/probe combinations. These bands were not included in computations of the number of bases screened (NBS) at the albumin locus. Faint bands detected included a 20-kb band with *Bgl* I/3′ probe; a 25-kb band with *Bgl* I/3′ probe; a 4.0-kb band with *Eco*RI/3′ probe; a 3.7-kb band with *Hin*dIII/3′ probe; a 6.6-kb band with *Hin*dIII/5′ probe; an 8-kb band with *Msp* I/mid probe; a 10-kb band with *Msp* I/mid probe; and an 11-kb band with *Taq* I/3′ probe. These minor bands may represent albumin pseudogenes (for which there is no evidence) or more likely cross-hybridization with the α-fetoprotein (*AFP*) locus. Studies of albumin and α-fetoprotein have shown significant

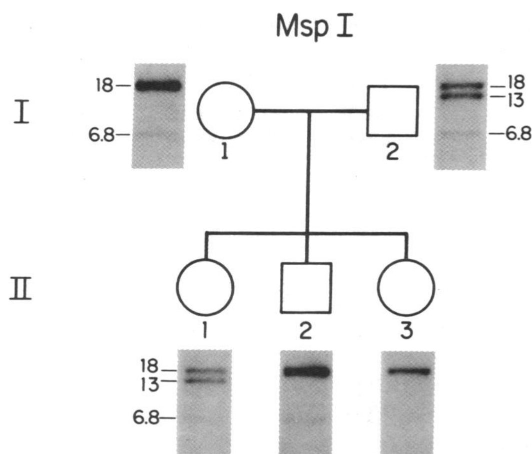

FIG. 2. Pedigree of family with *Msp* I polymorphism. The 18- and 13-kb fragments are codominant alleles. The 6.8-kb fragment shown is a nonpolymorphic band detected with *Msp* I. A 5-kb band found only in association with the 13-kb band is not shown here, and two faint bands are seen between the 13- and 6.8-kb fragments.

homology between these two loci (12) so that cross-hybridization would not be unexpected.

Isoelectric Focusing of Albumin. Isoelectric focusing of albumin from heterozygotes for the four polymorphic markers demonstrated no obvious differences from normal. These data were compatible with the inference that the RFLPs arose outside of coding regions for albumin. The possibility of RFLPs affecting degenerate codons for electrophoretically equivalent amino acids was considered less likely.

DISCUSSION

Aberrant protein production has been best studied in the thalassemias that result from underproduction of hemoglobin. In the β^+ thalassemias there is decreased but measurable production of β-globin chains. Mutations affecting intron/exon splice junctions or the regulatory 5′ flanking regions underlie most mutants of this type (13–16). In the β^0 thalassemias there is no production of β-globin chains. Nonsense mutations causing nonfunctional shortened chains and, rarely, a large deletion of the coding regions of DNA have been shown to cause this phenotype. These disorders are excellent models for studies of mutations affecting the production of specific proteins.

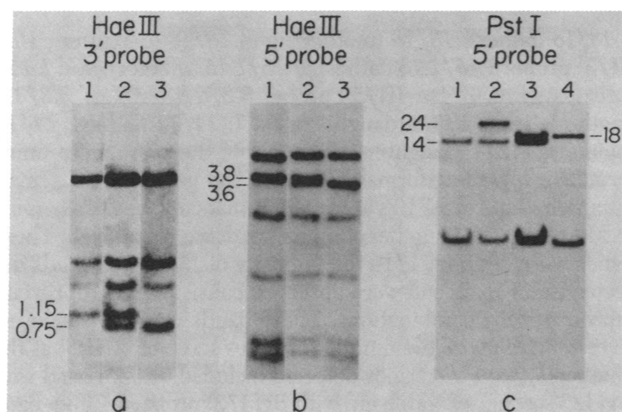

FIG. 3. (*a*) *Hae* III/3′ probe shows homozygotes for 1.15-kb (lane 1) and 0.75-kb (lane 3) bands and a 1.15-kb/0.75-kb heterozygote (lane 2). (*b*) *Hae* III/5′ probe shows homozygotes for 3.8-kb (lane 1) and 3.6-kb (lane 3) bands and a 3.8-kb/3.6-kb heterozygote (lane 2). (*c*) *Pst* I/5′ probe shows homozygotes for 14-kb (lane 1) and 18-kb (lane 4) bands and heterozygotes for 24-kb/14-kb (lane 2) and 18-kb/14-kb (lane 3) bands. Unlabeled bands are nonpolymorphic bands listed in Table 2.

Analbuminemia. Analbuminemia has been detected in rats. As in human analbuminemia, affected rats inherit the trait in an autosomal recessive fashion and appear to be physiologically unaffected. By examining two different RFLPs at the rat albumin gene, it was shown that the analbuminemic phenotype was tightly linked to the albumin gene locus (17). Furthermore, while large molecular weight RNA corresponding to albumin DNA was synthesized in the liver nucleus, no albumin mRNA was found in the cytoplasm (18), suggesting that the albumin gene was intact and transcribed but was not processed normally. Recently it was shown that analbuminemic rats have a 7-bp deletion in an intron that begins five bases from the site of an exon/intron splice junction (19). This deletion presumably resulted in aberrant albumin RNA splicing and decreased albumin production.

Because no serum albumin with normal mobility can be found in rare homozygotes for alloalbuminemia who exhibit a single abnormal albumin band, it has been suggested that the functional gene for serum albumin is a single locus and not duplicated (1). This is supported by recent evidence that multiple, independently isolated fragments of human DNA with homology to an albumin probe all have identical overlapping restriction enzyme maps suggesting a single-site origin (20). Thus, the trace amounts of normal albumin in our patient with analbuminemia probably came from a single albumin locus rather than from a minor locus located apart from the major locus. Furthermore, the human serum polymorphism for group-specific component (Gc) is linked to the human albumin locus (21), and our previous studies with this trait were compatible with linkage of the gene for human analbuminemia to the albumin locus. Thus, a defect in or near the albumin locus rather than an unlinked control defect was considered to be the most likely cause of analbuminemia. The molecular data shown here demonstrated the presence of a grossly intact albumin gene as was inferred earlier. Nevertheless, the presence of a small structural aberration (less than 100 bp) or a single base-pair change outside the relatively small proportion of sites that have been specifically analyzed by the restriction enzymes cannot be ruled out. It is likely that the mutation in analbuminemia will affect a region associated either with initiation of transcription/translation or with mRNA processing, as has been shown for the human thalassemias and for rat analbuminemia.

Twenty analbuminemic individuals have been described worldwide (22, 23). It is possible that a variety of different molecular abnormalities have caused their defects as has been shown for the thalassemias. Because our analbuminemic subject is the product of a consanguineous mating she presumably is a homozygote for an identical molecular defect inherited from a common ancestor. However, analbuminemics with unrelated parents may be compound heterozygotes for different defects and may show different patterns of polymorphisms at the albumin locus.

Albumin-Related DNA Variants. Not unexpectedly, the use of a gene-specific probe for the albumin locus disclosed the presence of RFLPs. Such variants in intergenic and intervening sequence DNA not involved in specification for protein structures have been frequently demonstrated. The present data, gathered by using a probe specific for the albumin gene, provide evidence for a set of previously unknown RFLPs. In view of the known chromosomal location of the albumin gene, these DNA variants can be assigned to a specific region (q11-q13) of the long arm of chromosome 4 (24, 25).

DNA probes such as those for the albumin gene allow the systematic study of the frequency of RFLPs. Data generated

by Jeffreys (26) and Antonarakis *et al.* (27) indicate that about 1 in 100 noncoding nucleotide sites may be polymorphic—a very high degree of variation outside the coding portions of the globin gene. Jeffreys screened 60 unrelated individuals and would have detected relatively rare polymorphic sites—i.e., those with frequencies as low as 1%. Our screening involved a minimum of 9 unrelated individuals and had a 95% chance of detecting variant sites present in a frequency of 15% or more (11). We have shown that 380 nucleotides at the albumin locus exhibited recognition sites for various restriction enzymes used and detected 1 variant in 95 nucleotides sites. This estimate is based on the assumption that the observed variants resulted from single base-pair substitutions and not from insertions or other structural rearrangements. Because the fragment length of the two shorter bands detected equaled the length of the longer band in one case (i.e., 18 kb = 13 kb + 5 kb for the *Msp* I variant) and because there was no evidence of deletions with the many nonpolymorphic bands detected, this assumption appeared reasonable. Furthermore, although the exact positions of the polymorphic sites detected within the albumin locus are not yet known, it is likely that these RFLPs arose in noncoding regions, particularly since less than 10% of the albumin locus contains coding sequences.

The number of bases examined for DNA variants is limited to those sites recognized by the specific restriction enzymes used. Since the human albumin locus has 16,500 bp [including introns, exons, and 5′ and 3′ flanking sequences (20)] we have sampled about 2.3% of the total albumin genome by restriction enzyme techniques. This figure is a maximal estimate because some of the restriction enzyme sites counted could lie several thousands of bases outside of the 16,500 bp of the albumin locus. Nevertheless, our observed frequency of RFLPs is very similar to that of Jeffreys and Antonarakis and is compatible with the suggestion that about 1/100 noncoding nucleotide sites in humans will show common DNA variants. The actual amount of variation between the nucleotide sequences of any two specific individuals may be considerably less than 1 in 100 nucleotides and will be dependent on the allele frequencies of the polymorphic sites being studied. These data provide quantitative evidence for the remarkable molecular heterogeneity of DNA in humans.

Use of Albumin-Related RFLPs. The detection of albumin-related RFLPs at a specific chromosomal site will be useful for a variety of studies. Their relatively high frequency makes the variant fragments good markers for future investigations of genetic disorders of the long arm of the fourth chromosome. For instance, genetic linkage of analbuminemia and dysalbuminemic hyperthyroxinemia (28) to albumin-related polymorphic loci could be used to prove that these conditions affect the albumin locus directly and are not caused by unlinked genetic defects distant from the albumin gene. Dentinogenesis imperfecta is a disease known to be linked to the albumin locus, and the genes for other conditions such as dihydropteridine reductase deficiency (atypical phenylketonuria) and dysfibrinogenemia have been tentatively mapped to chromosome 4. The current map for chromosome 4 suggests that the genes for these conditions may be linked to the albumin locus as well (24). Linkage studies using albumin-related RFLP to localize these genes with respect to the albumin locus are therefore indicated. A potential close linkage of an albumin-related polymorphic locus with a disease such as atypical phenylketonuria would allow prenatal diagnosis as well as carrier detection in certain families and therefore may be of potential practical importance. Furthermore, it has been calculated that the availability of 200–400 restriction fragment polymorphic sites randomly distributed over the human chromosome map would ultimately produce linkage markers for all monogenic genetic diseases (29, 30). The albumin-related polymorphisms described here provide a set of markers for these purposes.

Note Added in Proof. We have now screened 602 bases at the albumin locus. Two additional polymorphisms have been detected: one with *Hae* III and B44-mid probe giving bands at 4.05 and 4.1 kb and a second with *Eco*RV and a 3′ probe giving bands of 9.0 and 6.2 kb.

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