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Amino acid substitutions in genetic variants of human serum albumin and in sequences inferred from molecular cloning

(alloalbuminemia/bisalbuminemia/albumin polymorphism/cDNA sequence/anomalous migration in NaDodSO$_4$/PAGE)

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ABSTRACT **The structural changes in four genetic variants of human serum albumin were analyzed by tandem high-pressure liquid chromatography (HPLC) of the tryptic peptides, HPLC mapping and isoelectric focusing of the CNBr fragments, and amino acid sequence analysis of the purified peptides. Lysine-372 of normal (common) albumin A was changed to glutamic acid both in albumin Naskapi, a widespread polymorphic variant of North American Indians, and in albumin Mersin found in Eti Turks. The two variants also exhibited anomalous migration in NaDodSO$_4$/PAGE, which is attributed to a conformational change. The identity of albumins Naskapi and Mersin may have originated through descent from a common mid-Asiatic founder of the two migrating ethnic groups, or it may represent identical but independent mutations of the albumin gene. In albumin Adana, from Eti Turks, the substitution site was not identified but was localized to the region from positions 447 through 548. The substitution of aspartic acid-550 by glycine was found in albumin Mexico-2 from four individuals of the Pima tribe. Although only single-point substitutions have been found in these and in certain other genetic variants of human albumin, five differences exist in the amino acid sequences inferred from cDNA sequences by workers in three other laboratories. However, our results on albumin A and on 14 different genetic variants accord with the amino acid sequence of albumin deduced from the genomic sequence. The apparent amino acid substitutions inferred from comparison of individual cDNA sequences probably reflect artifacts in cloning or in cDNA sequence analysis rather than polymorphism of the coding sections of the albumin gene.**

Although the human albumin gene is highly polymorphic (1–6), the protein product of the gene is rarely so (7). Serum albumin polymorphism (alloalbuminemia) that is detectable by electrophoresis is very rare in most human populations but does occur with a prevalence of 1–25% in certain ethnic groups (7–9). In Caucasians and in Japanese a double albumin peak (bisalbuminemia) is seen in only ≈1 in 3000 sera examined in clinical electrophoresis (10). In contrast, in the San Carlos Apache Indians the variant albumin Naskapi has an allele frequency of 1.6%, and albumin Mexico-2 has an allele frequency of 3.7% (8). More than 30 apparently different genetic variants of human serum albumin have been typed by electrophoretic analysis under various conditions (9), and up to 80 have been named and described (7); yet, the structural change has been identified in only a few. The structural types of variants include proalbumins of several kinds that retain a substituted propeptide (11, 12), several examples of single-point substitutions (13–17), and one with a COOH-terminal deletion (18).

In a systematic study of some 20 different albumin variants, we are attempting to identify the structural change by use of (*i*) tandem HPLC (17), (*ii*) HPLC mapping and isoelectric focusing of CNBr peptides, and (*iii*) amino acid sequence analysis of the variant peptides. Here we report analytical studies of albumins Naskapi, Mersin, Adana, and Mexico-2, for which several genetic and epidemiological studies have been published (7, 8, 14, 19).

To determine the structural change in a mutant protein, it is essential to know the complete amino acid sequence of the normal (wild type) protein, especially if a single-point substitution is expected. The human albumin gene is present as a single copy (20). However, when we began this work, no unambiguous amino acid sequence for human serum albumin existed. Two early structures obtained by protein sequencing had up to 10 discrepancies (21, 22). The amino acid sequences later inferred from two well-documented cDNA sequences had only two discrepancies (2, 3), but a preliminary report (4) listed three additional amino acid substitutions. Also, various groups studying cDNA sequence analysis (4), genomic sequence analysis (1), or restriction fragment length polymorphism (5, 6) have concluded that the human albumin gene is highly polymorphic.

If albumin gene polymorphism in the coding regions is indeed frequent, it would be difficult to establish the structural change in genetic variants. However, our results for individual specimens of albumin A and for some 14 genetic variants agree with the protein sequence deduced from the genomic sequence at all five amino acid positions where disagreement exists, with results based on one of the five cDNA sequences. We conclude that the albumin sequence deduced from the genomic sequence should be used as the reference standard and that effective polymorphism of the coding sections of the albumin gene is infrequent except in certain linked ethnic groups. The prevalence of albumin Naskapi in geographically dispersed North American Indians and its identity with albumin Mersin of Eti Turks is of anthropological interest and may have significance for an understanding of population migration.

MATERIALS AND METHODS

Sera from individuals with an albumin genetic variant were screened electrophoretically (7, 8). None of the eight specimens studied was from individuals known to be closely related. The phenotypes studied were Mexico-2 (Me-2) both homozygous (Me-2/Me-2) (sera C276683 and C276685) and heterozygous (A/Me-2) (sera C276681 and C276682) from the Pima population in Arizona (7, 23), heterozygous Naskapi (A/Na) (serum C276684) from the Pima population, homozygous Naskapi (Na/Na) (serum C12765) from the Montagnais population in Canada (24), and heterozygous Mersin (A/Mersin) (serum C180460) and Adana (A/Adana) (serum C180458) found in Eti Turks (19). As a reference standard we used a commercial human albumin supposed to

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be A/A (lot 102578, Calbiochem-Behring, La Jolla, CA). The purification of the albumin from serum samples was described by Takahashi *et al.* (17).

$NaDodSO_4$/PAGE of the carboxymethylated albumins and of the CNBr digests was done by a method modified from that of Laemmli (25) with a gradient gel from 12% to 30%. To localize the amino acid substitution in each albumin variant to a specific CNBr fragment, polyacrylamide gel isoelectric focusing of the CNBr digest of the carboxymethylated albumin was done as described by Galliano *et al.* (16) except that a pH interval of pH 3–10 was used.

Two procedures were used to isolate the peptide having an amino acid substitution. (*i*) A tryptic digest of the carboxymethylated albumin variant was subjected to preparative peptide mapping analysis by automated tandem HPLC (17), or (*ii*) the peptides of the CNBr digest of the carboxymethylated variant were separated by reversed-phase HPLC on a Vydac C_{18} column (0.46 × 25 cm; The Nest Group, Southboro, MA). The CNBr peptides were eluted from a Vydac column at a flow rate of 1.0 ml/min with a linear gradient from 30% to 50% of *n*-propanol/acetonitrile (1:2, vol/vol) containing 0.1% trifluoroacetic acid during 60 min. The CNBr fragments are designated CB1–CB7 in their order in the amino acid sequence. The peptides were identified by their amino acid composition or by their sequence, and the amino acid substitution was identified by amino acid sequence analysis of the aberrant peptide. Amino acid analysis was performed with a Beckman model 121M amino acid analyzer and sequence determination was done with a Beckman model 890C sequencer (17).

RESULTS AND DISCUSSION

Albumin Adana. Albumin Adana is a variant identified in a single Eti Turk patient of the Adana district (19). The Adana variant is a slow migrating albumin, similar to albumin B as if it had a change of an acidic to a basic amino acid; however, the CNBr fragment pattern of Adana and B differ in our isoelectric focusing system and also in the PAGE system of Franklin *et al.* (14, 19). We have localized the substitution site of albumin Adana to CB6 (residues 447–548) (Fig. 1) and have sequenced most of the tryptic peptides of this region, but we have not yet identified the site because of the small amount of serum available.

Albumin Mexico-2. Albumin Mexico-2 is relatively common in Indians of the southwestern United States and Mexico. The four individual specimens that we studied were from Pima Indians of the Gila River Community near Phoenix, AZ, in which this variant reaches polymorphic frequency (i.e., an allele frequency of 1% or greater). Franklin *et al.* (14) report that albumin Mexico-2 has a single amino acid substitution at position 550, where aspartic acid is replaced by glycine. By use of tandem automated HPLC we confirmed this substitution by isolation and sequence analysis of tryptic peptide T75 (residues 546–557) from a homozygous sample (Me-2/Me-2) and also from a heterozygous sample (A/Me-2) (17). In the present work, we extended the study to albumin Mexico-2 isolated from the serum of two more individuals with the phenotypes Me-2/Me-2 and A/Me-2, and we confirmed the substitution of aspartic acid-550 by glycine by sequence analysis of peptide T75. This substitution results from a single base mutation of the codon GAT to GGT. We found no evidence for any other substitutions in further study done by isoelectric focusing (Fig. 1) or HPLC of the CNBr fragments of phenotypes Me-2/Me-2 and A/Me-2. This single substitution results in an increase of one positive charge on albumin Mexico-2. This accords with the slow mobility of this variant, which is intermediate between that of albumin A and albumin B. The latter has an increase of two positive charges because of the exchange of lysine for glutamic acid-570, as reported by Winter *et al.* (13) and confirmed by T. Isobe in our laboratory. No pathological condition has been clearly associated with albumin Mexico-2 (26).

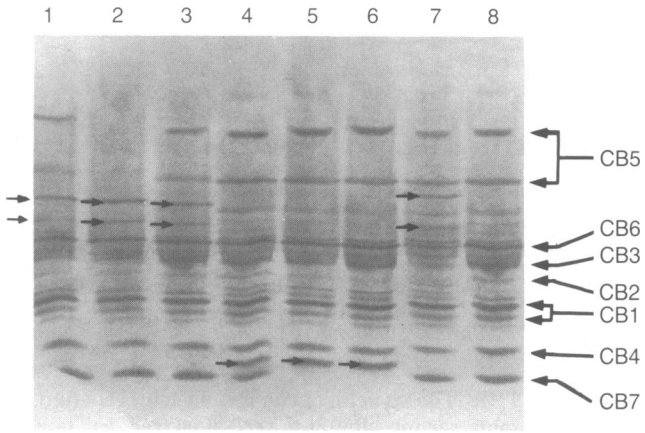

FIG. 1. Isoelectric focusing of CNBr fragments of normal human albumin A and of genetic variants: A/Na (lane 1), Na/Na (lane 2), A/Mersin (lane 3), A/Me-2 (lane 4), Me-2/Me-2 (lanes 5 and 6, different individuals), A/Adana (lane 7), commercial human albumin A (lane 8). Arrows indicate bands for CNBr fragments with an amino acid substitution resulting in a change in charge. CNBr fragments (e.g., CB5) may have two charge forms because of the homoserine–homoserine lactone equilibrium.

Albumins Naskapi and Mersin. Albumin Naskapi has a polymorphic frequency in North American Indians who are linguistically related but widely separated geographically, including the Northern Athabascans of Alaska and Canada and the Southern Athabascans, such as the Navajos and Apaches (7, 8). It is also found in high frequency in the linguistically different Algonkian speaking people of eastern United States and Canada. Albumin Mersin has been detected in polymorphic frequency in Eti Turks of southeastern Turkey (19). On the basis of two types of electrophoretic screening, Franklin *et al.* (19) concluded that albumins Naskapi and Mersin are identical or very similar and that the site of substitution is located between residues 330 and 446. By sequence analysis of tryptic peptides purified from heterozygous albumin Mersin and two specimens of albumin Naskapi (A/Na and Na/Na), we have established that both variants have an identical substitution, which is glutamic acid for lysine-372. This exchange results from a single base change in the codon AAA to GAA, and it accords with the fast mobility of albumins Naskapi and Mersin, which corresponds to an increase of two negative charges. Furthermore, the two variants exhibit identical mobility in cellulose acetate electrophoresis at pH 8.6 and in $NaDodSO_4$/PAGE in the systems we use (see Fig. 3). In addition, they have identical tryptic peptide maps in our automated HPLC system (17), and their CNBr digests exhibit identical HPLC peptide profiles and indistinguishable patterns in $NaDodSO_4$/PAGE (Fig. 2) and in isoelectric focusing (Fig. 1). These results support the recommendation of Franklin *et al.* (19) that the precedent name Naskapi be used for both variants.

Anomalous Migration of Naskapi and Mersin Variants in $NaDodSO_4$/PAGE. Unlike other genetic variants of serum albumin such as albumin B and Mexico-2 that differ by a single amino acid substitution, Naskapi and Mersin exhibit anomalous migration in NaDodSO4/PAGE (Fig. 3). They migrate more slowly, as if they had a higher molecular weight than albumin A. In all experiments, whether with serum or purified protein, heterozygote specimens of A/Na and A/Mersin clearly separated into two bands (Fig. 3). One band corresponded to albumin A (M_r, 66,000); the second band characteristic of the variant migrated more slowly with an apparent M_r ≈68,000. The homozygote albumin Na/Na migrated as a single band with apparent M_r of ≈68,000. By

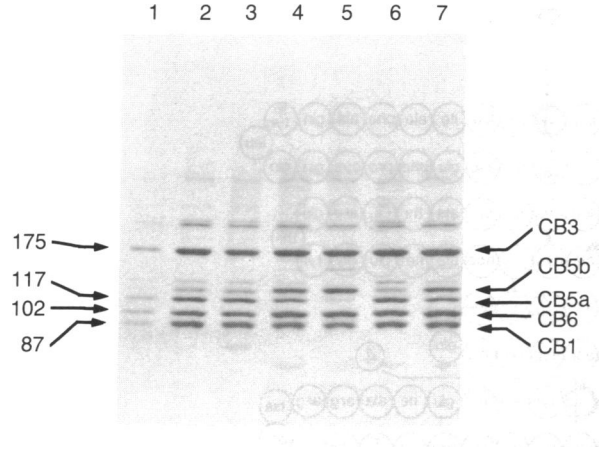

FIG. 2. NaDodSO$_4$/PAGE of CNBr fragments of normal human albumin and of genetic variants: A (lane 1), A/Me-2 (lane 2), Me-2/Me-2 (lane 3), A/Na (lane 4), Na/Na (lane 5), A/Adana (lane 6), A/Mersin (lane 7). The left ordinate gives the number of amino acid residues in each fragment. Note that fragment CB5 (residues 330–446) appears as a major band CB5a in normal albumin A. Two bands (CB5a and CB5b) are present in the heterozygote albumins A/Na and A/Mersin, but only CB5b is present in the homozygote Na/Na. In all samples, a secondary light band appears near the position of CB5b.

use of NaDodSO$_4$/PAGE, the factor causing the mobility change was localized in the CNBr fragment in which the substitution occurs; CB5b (residues 330–446) was missing in the homozygote Na/Na and was replaced by a band of apparently higher molecular weight (Fig. 2). The change in CB5 was also observed for the heterozygote specimens.

Despite various tests we could adduce no evidence for a true increase in molecular weight of the genetic variants because of covalent insertion or addition of a peptide or nonpeptide moiety or because of strong noncovalent binding. Therefore, we attribute the anomalous migration of the Naskapi and Mersin variants to a conformational change resulting from a single amino acid substitution. No other structural change of the variant albumins was identified by: (*i*) amino-terminal sequence analysis of the intact variant for 35–40 residues, (*ii*) HPLC profile analysis of the tryptic or CNBr peptides, (*iii*) extensive sequence analysis accounting for ≈85% of the Naskapi albumin. Various tests for the presence of nonpeptide substances that might be tightly bound or covalently linked to the variant were also negative. For example, no carbohydrate was identified by amino acid analysis under conditions that separate glucosamine and galactosamine; nor was carbohydrate detected by staining the electrophoretograms. Defatting with charcoal did not change the mobility of the variants in NaDodSO$_4$/PAGE. The mobility difference in NaDodSO$_4$/PAGE could not have been caused by a disulfide interchange, because the albumins and the CNBr fragments were completely reduced and alkylated.

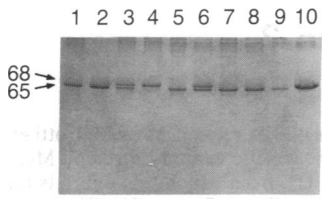

FIG. 3. NaDodSO$_4$/PAGE of reduced and carboxymethylated normal albumin and of genetic variants: Me-2/Me-2 (lane 1), A/Me-2 (lane 2), A/Na (lane 3), Na/Na (lane 4), A/Adana (lane 5), A/Mersin (lane 6), Me-2/Me-2 (lane 7), A/Me-2 (lane 8), commercial human albumin A (lane 9), commercial bovine albumin (lane 10). A double band (M_r, 65,000 and 68,000) is present in heterozygote specimens A/Na, and A/Mersin, but the homozygote Na/Na exhibits a single band of M_r 68,000. Normal human and bovine albumin have the same apparent M_r and have 80% homology in amino acid sequence (22). Numbers on left represent $M_r \times 10^{-3}$.

Although mobility in NaDodSO$_4$/PAGE has been routinely used for estimation of the molecular weight of proteins since the method was introduced by Weber and Osborn (27), anomalous results are often obtained for glycoproteins. Furthermore, several authors, including Weber, have reported that a single amino acid substitution may unexpectedly alter the mobility and result in an erroneous estimate of the molecular weight (28, 29). Similar examples of anomalous migration in mutants differing at a single site are emerging from studies of site-specific mutagenesis. The most probable explanation of the phenomenon for albumins Naskapi and Mersin is that the substitution of lysine-372 by glutamic acid causes a local difference in the binding of NaDodSO$_4$ that is reflected in the regional conformation. In the three-dimensional model of Brown and Shockley (30), the position homologous to the lysine-372 to glutamic acid exchange is close to the binding site for palmitate. The change from a positive to a negative charge at this site may result in a difference in local conformation that may affect the ability to bind the similar ligand dodecyl sulfate. The fact that the anomalous migration is exhibited only by the polymorphic variants Naskapi and Mersin suggests that the lysine-372 to glutamic acid exchange may convey some adaptive advantage. We have also observed anomalous migration by the polymorphic variant Yanomama-2, but not for a series of other rare single-point mutants. Furthermore, in our NaDodSO$_4$/PAGE system, three different proalbumins (Gainesville, Lille, and Takefu) have the same mobility as albumin A, although they have an additional hexapeptide at the amino terminus.

Warfarin Binding Affinity of Albumins Mexico-2 and Naskapi. Serum albumin has important binding and transport functions for many ligands and drugs (30, 31), and some albumin variants exhibit modified binding affinity for certain ligands (10, 24). Albumins Mexico-2 and Naskapi exhibit a small but significant decrease in binding affinity for the drug warfarin (26). Warfarin is bound by a CNBr fragment (CB3) (residues 124–298) and the drug interacts with the sole tryptophan at position 214, but the site includes a larger binding area (32). However, the substitutions in albumins Naskapi and Mexico-2 are in other regions of the protein (lysine-372 and aspartic acid-550, respectively). Although the substitutions differ in the two variants, each is located close to one of the two high-affinity binding sites for fatty acid. According to Peters (31), occupancy of these two sites by fatty acid causes a concerted conformational change that stabilizes albumin against heat and proteolysis and exerts favorable allosteric effects on bilirubin binding. Thus, it is conceivable that these polymorphic alleles result in an altered albumin conformation that may confer some advantage on closely knit populations with ancient cultural and dietary traditions.

Discrepancies in Amino Acid Sequences Derived from Structural Studies of Albumin and from Molecular Cloning. Although electrophoretically detectable mutations in serum albumin are very rare in most populations, an apparent amino acid substitution has been inferred for each of the five individual albumin clones for which the cDNA sequence has been reported (2–4), and the nucleotide variation has been estimated as >1 in 100 (4). Five of the nucleotide differences in the cDNA sequences would lead to an amino acid substitution (Table 1; Fig. 4). None of these apparent mutations occurs in some 14 individual specimens of albumin genetic variants we have analyzed thus far, or in the normal albumin A from the heterozygous individuals. Nor are these substitutions detectable in commercial pooled albumin from four different sources. In each of the five positions, the amino acid identified by our structural study of the normal albumin specimens and of the genetic variants is identical with that

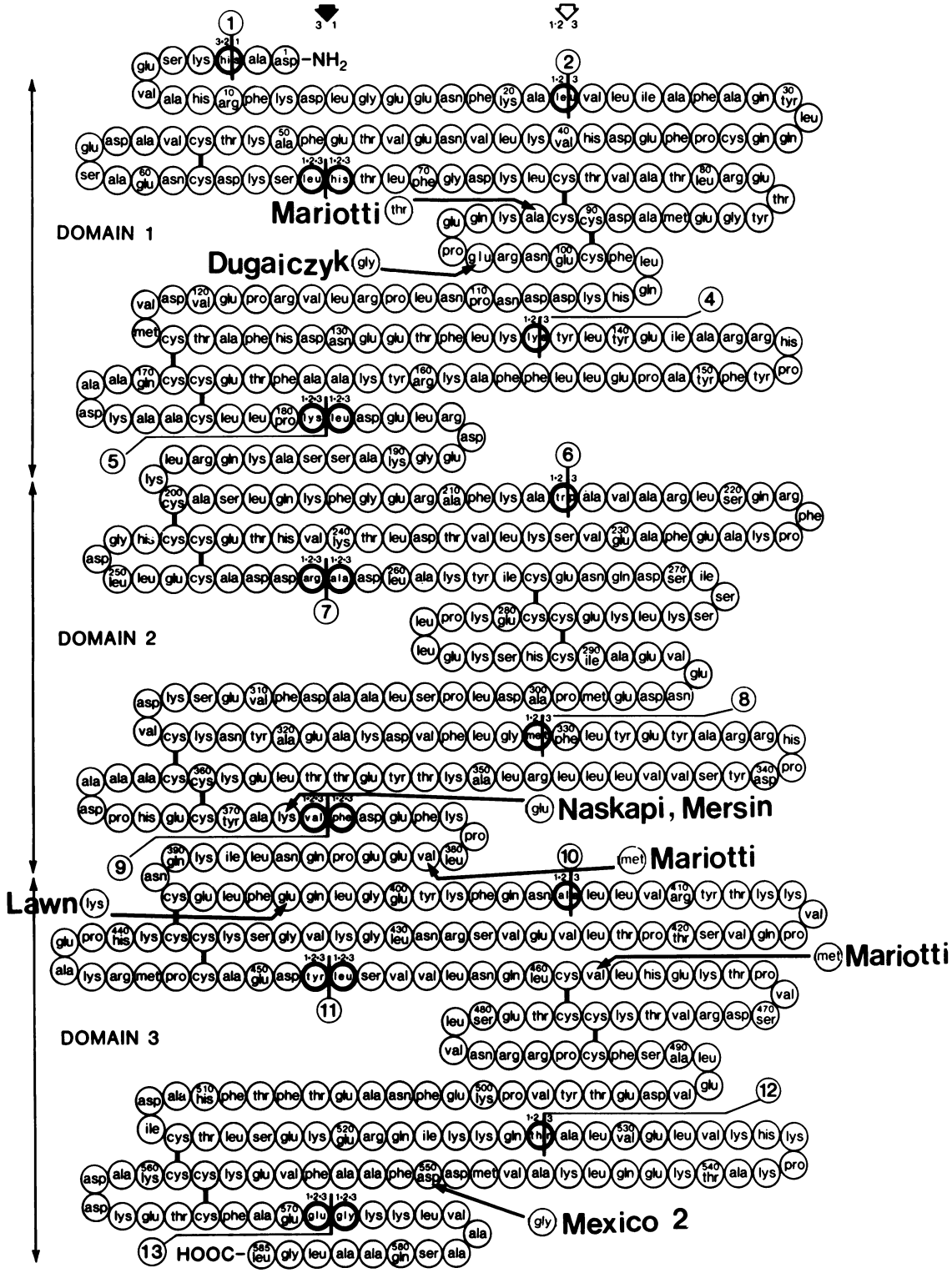

FIG. 4. Amino acid sequence of human serum albumin showing substitutions inferred from cDNA sequences by Mariotti *et al.* (4), Dugaiczyk *et al.* (2), and Lawn *et al.* (3), and also those determined by amino acid sequence analysis of the genetic variants Naskapi, Mersin, and Mexico-2. The protein sequence shown is based on the genomic-sequence analysis of Minghetti *et al.* (1), from which this figure is taken and modified. The layout of the polypeptide chain, including the domain structure and disulfide bonds, is according to Brown (22). The locations of the introns (designated 1–13) and of the codon positions at the introns are from Minghetti *et al.* (1).

inferred from the genomic sequence of the albumin gene reported by Minghetti *et al.* (1).

If the frequency of polymorphism of serum albumin is high, and if there are many alleles, the polymorphism would probably be undetectable in commercial albumin, which is always processed from a plasma pool of many donors. However, despite the low frequency of genetic variants in European and Japanese populations, mutations leading to a charge difference are easily detected because of daily routine clinical electrophoretic analysis of numerous individual serum samples. Tárnoky (10) estimates that the number of possible alleles from single-point mutations in the albumin gene is ≈2500, of which ≈1/3rd should separate on electrophoresis. Yet, in Caucasians and Japanese, the incidence of

Table 1. Amino acid substitutions in genetic variants of human serum albumin and in sequences inferred from molecular cloning

Source of sequence	Position in albumin amino acid sequence						
	92	97	372	381	396	462	550
Genomic (1)	Ala	Glu	Lys	Val	Glu	Val	Asp
cDNA (2)	Ala	*Gly*	Lys	Val	Glu	Val	Asp
cDNA (3)	Ala	Glu	Lys	Val	*Lys*	Val	Asp
cDNA (4)	*Thr*	Glu	Lys	*Met*	Glu	*Met*	Asp
Albumin variants							
Naskapi	Ala	Glu	*Glu*	Val	Glu	Val	Asp
Mersin	Ala	Glu	*Glu*	Val	Glu	Val	Asp
Mexico-2	Ala	Glu	Lys	Val	Glu	Val	*Gly*
Other variants	Ala	Glu	Lys	Val	Glu	Val	Asp
Normal albumin							
A	Ala	Glu	Lys	Val	Glu	Val	Asp
A (21)	Ala	Glu	Lys	Val	Glu	Val	Asp
A (22)	Ala	Glu	Lys	Val	Glu	Val	Asp

All data not referenced are from this work and refer to individual specimens of the albumin variants named. In addition, data are given for 12 other genetic variants and for the normal albumin from the heterozygous specimens. Substitutions are italicized.

bisalbuminemia is only in the range of 1:1000 to 1:10,000 (7–10), which is several orders of magnitude less than the apparent incidence of mutations in the cDNA clones.

None of the five substitutions inferred from cDNA sequence analysis accords with discrepancies in codon usage (silent mutations) noted in these studies (1–4). Furthermore, when we used the computer program ENZYME to identify all possible sequence-specific sites for the restriction enzymes used in the study of albumin genetic polymorphism (5, 6), we found only two such sites in the albumin exons; neither was related to the apparent amino acid substitutions. Therefore, we conclude that the apparent amino acid substitutions inferred from comparison of individual cDNA sequences probably arise from artifacts in cloning or in cDNA sequence analysis.

Significance for Biochemical Genetics and Gene Migration. A principal function of albumin is to bind and transport many drugs and metabolites; hence, any structural mutation causing a conformational change may affect its natural role. Yet, although albumin Naskapi is polymorphic in widely separated ethnic groups, no selective advantage has been adduced. Nonetheless, this albumin variant unlike most others is a useful genetic marker to trace migration pathways. We have not identified the albumin Naskapi substitution in 14 other genetic variants that we have studied, including five different Japanese variants and one from Taiwan. Albumin Naskapi also differs electrophoretically from four other Chinese variants (33). Albumin Naskapi occurs in high frequency in eastern Algonkian-speaking Indians of eastern Quebec (Naskapi, Montagnais), and in the Athabaskan-speaking Indians of Alaska, western Canada, and the southwestern United States (Apache and Navajo). It occurs in lower frequency in the Sioux of the northern Midwest. It has not been found in other North, South, and Central American Indians or in Europeans and Africans. Outside of North America, it has been found only in the Eti Turks and in a single Punjabi (India) (34). The Naskapi albumin specimens we analyzed originated in Labrador and Arizona and the identical albumin Mersin is found in Eti Turks. Although identical mutations may arise independently in unrelated populations, the structural identity of albumins Naskapi and Mersin supports the suggestion (19) that they may have arisen some 20,000 years ago from the same mutation in a population ancestral to both American Indians and Eti Turks.

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