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A nucleotide insertion and frameshift cause analbuminemia in an Italian family

(hypoalbuminemia/serum albumin/frameshift mutation/heteroduplex/single-strand conformation polymorphism)

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ABSTRACT **In analbuminemia, a very rare inherited syndrome, subjects produce little or no albumin (1/100th to 1/1000th normal), presumably because of a mutation in the albumin gene; yet, they have only moderate edema and few related symptoms owing to a compensatory increase in other plasma proteins. Because of the virtual absence of albumin the defect must be identified at the DNA level. In this study the mutation causing analbuminemia in an Italian family was investigated by analysis of DNA from a mother and her daughter. The mother was homozygous for the trait and had a serum albumin value of <0.01 g/dl (about 1/500th normal); the daughter was heterozygous for the trait and had a nearly normal albumin value. Molecular cloning and sequence analysis of DNA from both mother and daughter showed that the mutation is caused by a nucleotide insertion in exon 8; this produces a frameshift leading to a premature stop, seven codons downstream. The methods of heteroduplex hybridization and single-strand conformation polymorphism were used to compare the DNA of the mother and daughter to the DNA of two unrelated analbuminemic individuals (one Italian and one American). This showed that all three analbuminemic individuals had different mutations; these also differed from the mutation in the only human case previously studied at the DNA level, which was a splicing defect affecting the ligation of the exon 6–exon 7 sequences. Thus, analbuminemia may result from a variety of mutations and is genetically heterogeneous.**

Serum albumin is the most abundant secreted protein in the body; it comprises about 50% of the total protein in serum where it has a normal concentration of 3.5–4.5 g/dl (1). Human serum albumin (HSA) consists of a single polypeptide chain of 585 amino acid residues cross-linked by 17 disulfide bonds into a series of loops (1). A principal function of albumin is to bind and transport many metals, metabolites, and ligands including pharmaceutical drugs. A second function is to maintain the oncotic pressure and volume of the blood; hence, albumin or plasma are commonly administered intravenously to treat shock and loss of blood. Analbuminemia (or hypoalbuminemia) is a very rare syndrome in which subjects produce little or no albumin (about 1/100th to 1/1000th normal); yet, they exhibit only moderate edema or other symptoms, apparently because of a compensatory increase in other plasma proteins such as IgG and lipoproteins (2–10). Because millions of electrophoretic analyses of blood plasma or serum are done annually, analbuminemia is readily detected; yet, only about 30 cases have been reported. Its occurrence is probably $<1 \times 10^6$, for no cases have ever been identified at the Mayo Clinic, where about 40,000 electrophoretic analyses of serum are done annually (Robert Kyle, personal communication). Nor has it been reported in Sweden, where about one million electrophoretic analyses have been made (Carl-Bertil Laurell, personal communication). The paradox is that the albumin gene is subject to many neutral mutations (11–16); yet, analbuminemia, though tolerable, is extremely rare. It has been postulated (17) that the presence of albumin may be critical in fetal life and only a few analbuminemic individuals survive past the neonatal state; this could explain why analbuminemia is so rare in adults.

By use of heteroduplex hybridization and single-strand conformation polymorphism (SSCP) analysis, molecular cloning, and DNA sequence analysis, we have proved inheritance and have identified the mutation in the albumin gene in an Italian family that has been followed clinically and biochemically for several decades (4–6). Analbuminemia in a brother (R.U.) and a sister (R.R.) was reported by Fabiani and Pauluzzi (4) in an electrophoretic study of the family in 1971. At that time albumin was reported as absent, although a trace amount was detected by radial immunodiffusion. The parents were first-degree cousins. A subsequent genealogical and biochemical study in 1983 demonstrated associated hyperlipidemia, but only about 0.01 g of albumin per dl in the serum of the two subjects (5). A later study focused on the hyperlipidemia but reported no detectable albumin (6). In our investigation, blood used as the source of DNA was obtained with informed consent from the sister (R.R.) (designated here as Roma) and also from her daughter (Daughter of Roma) (see Fig. 1). Because of the greater amount available, most of the work was done on the DNA of the daughter, whose serum albumin level approached normal.

Albumin is encoded on chromosome 4 by a single autosomal gene for which the complete genomic sequence has been published (16). The gene spans 16,961 nucleotides and is split into 15 exons by 14 intervening introns. The mutation in the analbuminemic gene in the Italian family was localized to exon 8 by heteroduplex hybridization and SSCP analysis. Molecular cloning and double-stranded DNA (dsDNA) sequencing revealed the insertion of a single adenine in the normal AAAA sequence of nucleotides 9156–9159 of genomic DNA (16). This produced a frameshift followed by a stop codon seven codons downstream. The mutated gene would have produced a truncated albumin of only 273 residues, but no evidence for this product is available. This mutation differs from the exon splicing defect found in the only other case of human analbuminemia studied at the gene level (17) and from the 7-bp deletion observed in the albumin gene of Nagase analbuminemic rats (18). Further studies with DNA from several other human subjects indicated that each had a different, as yet unidentified, mutation.

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Abbreviations: HSA, human serum albumin; SSCP, single-strand conformation polymorphism; dsDNA, double-stranded DNA.

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MATERIALS AND METHODS

High Molecular DNA Preparation. Five to 10 ml of whole blood was diluted with 20 mM Tris·HCl, pH 7.5/5 mM EDTA to a total volume of 25 ml and centrifuged at 3000 × *g* for 7 min. The leukocytes were washed two or three times as described above, resuspended in a solution of 10 mM Tris, pH 8.0/150 mM NaCl/10 mM EDTA, and incubated overnight at 37°C after addition of SDS and proteinase K to final concentrations of 0.5% and 250 μg/ml, respectively (19). After adding 1/10th volume of 6 M sodium perchlorate, the mixture was incubated for 2 hr at 37°C. One phenol/chloroform/isoamyl alcohol (25:24:1) and two chloroform/isoamyl alcohol (24:1) extractions were then performed. Finally, the DNA was precipitated by the addition of 1/10th volume of 3 M sodium acetate (pH 6.0) and 3 volumes of 100% EtOH followed by spooling of the DNA onto a glass rod.

PCR Amplification. Twenty-eight primers, encompassing the 14 coding exons of HSA and their intron–exon junctions, were synthesized by the DNA synthesis facility of the Indiana University Institute for Molecular and Cellular Biology using an Applied Biosystems DNA synthesizer (model 394) for use in PCR amplification. These primer sets were used to PCR amplify regions of the HSA gene ranging from 288 to 464 bp in length (Table 1). PCRs were performed in a 100-μl reaction volume similar to the manufacturer's directions for "hotstart" PCR using AmpliWax PCR Gems (Perkin–Elmer/Cetus) [10 mM Tris·HCl, pH 8.3/50 mM KCl/1.0–2.0 mM $MgCl_2$/0.1 μM of each primer/200 μM of each deoxynucleotide/1 μg of genomic DNA/2.5 units of AmpliTaq DNA polymerase (Perkin–Elmer/Cetus)] (20). With a Perkin–Elmer/Cetus DNA thermal cycler, the reactions were amplified for 40 cycles using the following protocol: 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with a 5-sec increase in extension time for each successive polymerization step.

Heteroduplex Analysis. After PCR amplification, 0.5 M EDTA (pH 8.0) was added to the samples producing a final concentration of 5 mM (21, 22). For analysis of homozygous DNA samples, 15 μl of both wild-type and variant PCR products were combined and then denatured at 95°C for 3 min before slow cooling to room temperature for 2–3 hr. The annealed DNA (10 μl) and a tracking dye (2 μl) (AT Biochem, Malvern, PA) were mixed and loaded onto a 1.0-mm-thick mutation detection enhancement gel (AT Biochem) followed by electrophoresis in 0.6× Tris/borate buffer (1× = 89 mM Tris/89 mM boric acid/2 mM EDTA) at 800 V for 14–15 hr. The gel was stained in ethidium bromide (1 μg/ml) and photographed on a UV light box.

SSCP Analysis. PCR amplification was performed as described above except that the concentration of each cold nucleotide was reduced to 70 μM and the reactions were supplemented with 10 μCi of dATP[^{35}S] (>1000 Ci/mmol; 1 Ci = 37 GBq; Amersham) (23, 24). A 1- to 2-μl aliquot of the PCR reaction was then mixed with 9 μl of a tracking dye containing 95% formamide, 20 mM EDTA, and 0.05% of both bromophenol blue and xylene cyanol. The sample was heated to 95°C for 2 min, quenched on ice, and loaded immediately onto a 0.4-mm-thick mutation detection enhancement gel (AT Biochem). The gel was electrophoresed at 8 W for 8 hr at 4°C in 1× Tris/borate buffer (see above). Finally, the dried gel was exposed to x-ray film (Kodak XAR) for 48 hr.

Subcloning and DNA Sequencing. The genomic DNA was PCR-amplified using exon 8-specific primers (A15B and A16B, Table 1) with the PCR reaction conditions listed above. The 399-bp PCR product was digested with *Asp* 700 (Boehringer Mannheim), and the resulting 254-bp fragment was ligated into pBluescript (Stratagene) cut with *Sma* I. Transformation, screening, and dsDNA sequencing were performed as described (14).

RESULTS AND DISCUSSION

Family Study. Analbuminemia was reported in a brother (R.U.) and a sister (R.R.), whose parents were first-degree cousins (4–6) (Fig. 1). The patients were not given albumin intravenously, and over several decades the circulating albumin remained at a trace level (<0.01 g/dl of serum, or about 1/500th normal). Their parents and one sibling had normal serum albumin values of 4.6–4.9 g/dl; one sibling died perinatally of unknown cause. The analbuminemia was associated with hyperlipidemia and hypercholesterolemia. Other symptoms were mild, and edema was slight—probably because of a compensatory increase in other plasma proteins (total protein = 6 g/dl). The analbuminemic sister (R.R., here designated Roma) was in good health and had a daughter (Daughter of Roma), whose albumin level was nearly normal. Blood samples for DNA analysis were donated by the mother and daughter. Blood was also obtained and DNA was prepared from two other unrelated subjects with analbuminemia who have been followed clinically for some years: Codogno (Italy) (7) and G.M. (United States) (2, 3).

Of the 30 or so known cases of human analbuminemia, the only one explained at the molecular level is a mutation in an American Indian girl involving a splicing defect at the intron 6/exon 7 junction (17). The current work involved the molecular DNA analysis of analbuminemia in an Italian

Table 1. PCR primer sets used to amplify the exons of human serum albumin

Exon no.	Upstream primer			Downstream primer			Size of PCR product, bp
	Name	Sequence (5′ → 3′)	Coordinates	Name	Sequence (5′ → 3′)	Coordinates	
1	A01A	TGACAAGGTCTTGTGGAGAAA	(−195)–(−175)	A02A	GATAACCTTGTAAGACTTCAC	269–249	464
2	A03A	TGTAGGAATCAGAGCCCAATA	695–715	A04A	GAATCTGAACCCTGATGACAAG	982–961	288
3	A05A	GTTCTCTAGCGTAGCAACCTGT	2268–2289	A06A	CAATGGCTTATCAGTCTATGAG	2623–2602	356
4	A07A	CCTGACCAAGCTTAACCAGTATA	4203–4225	A08A	TAGAGTGTTGGCCTATGGAGTTA	4583–4561	381
5	A09A	TTCTGGGGAGAATGTCGATTAC	4983–5004	A10A	GTATCAGACACTCAGGTTCCCT	5291–5270	309
6	A11A	CTGAGCTTATGGAGGGGTGTTTC	5953–5975	A12B	GACAGCGTCCTCTAAATTATTCAAC	6348–6324	396
7	A13A	GCCCTAAGGATAAGTGATTACC	7539–7560	A14A	CAACCCACTGTCAGCTATCACCA	7932–7910	394
8	A15B	GGTCTGAGGAGAAAGTGTAGCA	9067–9088	A16B	GATGAAGAAACATTCTGGGCAG	9465–9444	399
9	A17A	ACCCCAAGTCCTTAGCTACTAAG	10598–10620	A18A	GATTCCAGAATTGAAACCATCTC	11013–10991	416
10	A19B	CATGTGGCTTTGAGTAGGAAGAAG	11852–11875	A20C	TAGACTACATTCAAATATAGACCTCTCC	12257–12230	406
11	A21A	ACATCTTAGTTGATTCCGGCCAA	13146–13168	A22A	ACATGCACACACACACATTACT	13488–13467	343
12	A23A	CACCTCTTTTGAATTTCTGCTC	13759–13780	A24A	GGCAACACTCCAATACTTTCCTC	14144–14122	386
13	A25C	CATGCAGATGAGAATATTGAGAC	15037–15059	A26B	CCTAAGCCCTAGCCTAACCAAAC	15417–15395	381
14	A27A	CAACTATGTCCGTGAGCTTCCA	15792–15813	A28A	GTGGTCGGTGCTGGTCTATATG	16130–16109	339

The optimal magnesium concentration for exons 2, 3, 7, 10, and 12 was 1.5 mM; the optimal concentration for exon 6 was 2.0 mM; all other exons had an optimum of 1.0 mM $MgCl_2$. Nucleotide coordinates are based on the genomic DNA sequence (16).

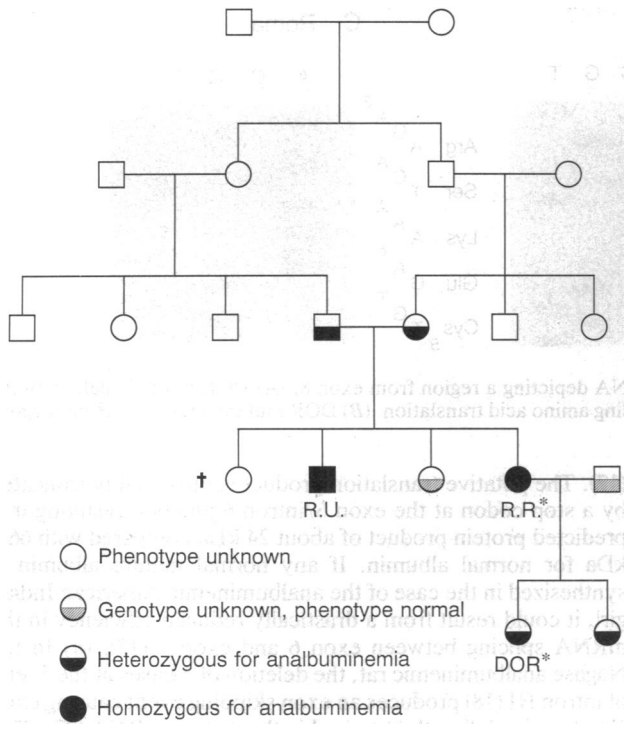

FIG. 1. Analbuminemia in an Italian family. The family tree is based on figure 1 of Baldo *et al.* (5) and has been extended to members of the fifth generation. Asterisks (*) mark the subjects whose DNA was sequenced in this study—i.e., the mother R.R. (Roma) and her daughter (DOR). The cross (†) identifies a sibling of R.R. who died of unknown cause as a neonate.

family. In our study, using primers D41 and D42 (17) as allele-specific oligonucleotide probes under stringent hybridization conditions, we examined DNA from human placenta (wild-type), Roma, Daughter of Roma, Codogno (an unrelated Italian analbuminemia), and pAnalb 9.4 (a plasmid containing the splice junction from an analbuminemic American Indian girl) (17). The dot blots clearly indicated that neither the Roma nor Codogno mutations were identical to the previously characterized analbuminemia in an American Indian girl (data not shown). The overall scheme for identification of the molecular defect in the Roma allele included (*i*) fine structure mapping of the mutation to a specific exon by heteroduplex analysis and SSCP analysis, (*ii*) PCR amplification of the mutant exon, (*iii*) subcloning of the PCR product by restriction digestion and ligation into an appropriately cut plasmid vector, and (*iv*) dsDNA sequencing to determine the precise nature of the mutation.

Heteroduplex Analysis. Heteroduplex analysis (21, 22) is based on the principle that when dsDNA fragments from normal and mutant alleles are denatured and reannealed, they can pair in several ways. They can form either perfectly complementary DNA duplexes called homoduplexes, or two similar (but not identical) DNA strands can anneal to form mixed duplexes referred to as heteroduplexes. The conformational differences of these dsDNA molecules can be resolved in a specific nondenaturing gel electrophoresis system. Heteroduplex analysis was performed for all of the 14 albumin-coding exons from Roma and Daughter of Roma, including 195 nucleotides upstream from the transcriptional start site. The heteroduplex results showed that the only detectable change in the albumin gene from these individuals was located in exon 8 (Fig. 2). The gel for exon 7 (Fig. 2) is representative of the results seen for the other 12 exons indicating that no other changes were found.

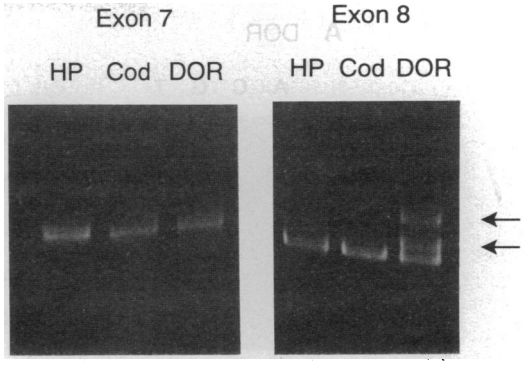

FIG. 2. Ethidium bromide-stained mutation detection enhancement (AT Biochem) gel of the heteroduplex analysis of exons 7 and 8 with DNA from human placenta (HP) (wild-type), Codogno (Cod, an unsolved Italian analbuminemia mutation), and Daughter of Roma (DOR) (see text for explanation). Arrows indicate the heteroduplex bands.

SSCP Analysis. A complementary analytical procedure, SSCP (23, 24), can detect fine structural differences between folded, single-stranded DNA molecules that differ by only a single-base substitution. The SSCP technique is dependent on the ability of nondenaturing acrylamide gels to discriminate between subtle alterations in the secondary structure of single-stranded DNA molecules. SSCP analysis of all 14 exons from the Daughter of Roma clearly indicated that the only detectable change occurred in exon 8 (Fig. 3). The results for exon 2 (Fig. 3) are representative of the data for the other 12 normal exons. Because heteroduplex and SSCP analysis examine slightly different physical parameters of the DNA, the complementary use of both these techniques can detect subtle single-base substitutions in almost any mutation (25).

DNA Sequence Analysis. DNA sequence analysis of clones from Roma DNA for exon 8 indicated that the change involved only a single "A" insertion (Fig. 4). In the normal serum albumin gene, nucleotides 9156–9159 (16) are listed as four A residues, whereas in the case of the Roma allele there

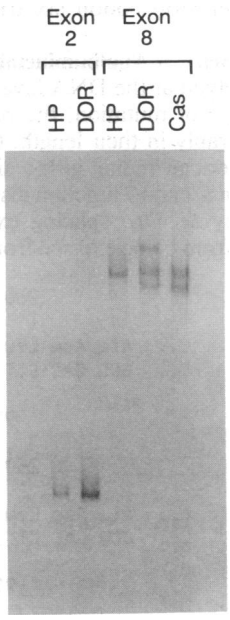

FIG. 3. Autoradiogram of a mutation detection enhancement gel of the SSCP analysis for exons 2 and 8 with DNA from human placenta (HP) (wild-type), Daughter of Roma (DOR), and Caserta (Cas) (an unpublished single-point substitution in exon 8 for comparison).

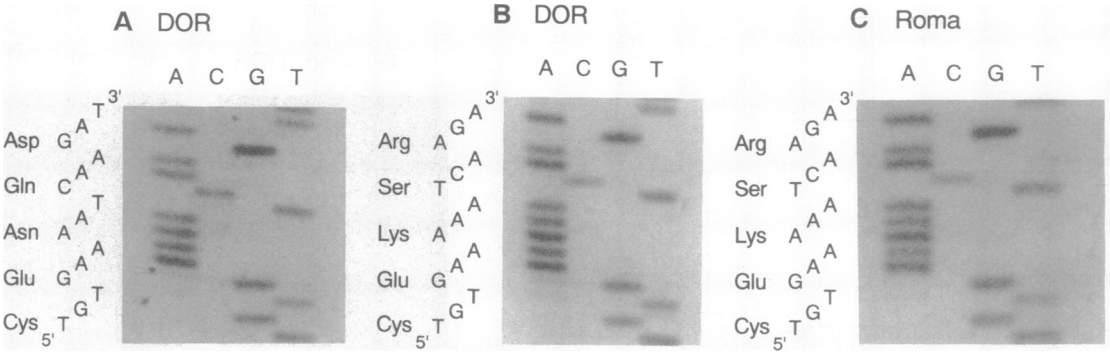

FIG. 4. Sequence ladders from Roma and Daughter of Roma (DOR) DNA depicting a region from exon 8. (*A*) DOR normal allele with the nucleotides grouped in appropriate codon triplets along with the corresponding amino acid translation. (*B*) DOR mutant allele. (*C*) Homozygous Roma mutant allele.

is a string of five A residues encompassing nucleotides 9156–9160. The additional base leads to a frameshift mutation causing a premature stop codon 7 amino acids downstream (Fig. 5). The result was substantiated by DNA sequencing of three variant and two normal clones from the Daughter of Roma. This individual was clearly heterozygous, possessing both the wild-type serum albumin allele and the mutant Roma allele (Fig. 4). The predicted translation product from the Roma allele would consist of only 273 amino acid residues instead of the normal 585 amino acid residues found in mature serum albumin. It is not known whether this putative truncated protein is synthesized, and, if so, if it is (*i*) broken down intracellularly, (*ii*) secreted poorly, or (*iii*) rapidly degraded upon release into the bloodstream. Further analysis would require a liver biopsy or very large amounts of serum, neither of which could be obtained.

Is Any Albumin or Albumin Fragment Made in Analbuminemia? Whether any mature albumin or albumin fragments are made by subjects with analbuminemia is difficult to answer because no such protein has been isolated and characterized structurally in any case. The type of genomic mutation found for Roma seems inconsistent with the synthesis of any mature normal albumin because of the presence of a stop codon followed by another stop codon six triplets further downstream (not shown).

Genetic Heterogeneity of Analbuminemia. Of the three analbuminemia cases solved at the DNA level, each defect results from a different type of mutation, the predicted products of which vary considerably in their length. (*i*) In the case of the analbuminemic American Indian girl, a single nucleotide substitution at the intron 6/exon 7 junction disrupts normal splicing (as demonstrated by *in vitro* splicing experiments), thereby preventing intron 6 from being excised from the mature mRNA (17). The putative translation product is presumably truncated by a stop codon at the exon 6/intron 6 junction resulting in a predicted protein product of about 24 kDa compared with 66.5 kDa for normal albumin. If any normal mature albumin is synthesized in the case of the analbuminemic American Indian girl, it could result from a drastically reduced efficiency in the mRNA splicing between exon 6 and exon 7 (17). (*ii*) In the Nagase analbuminemic rat, the deletion of 7 bases at the 5′ end of intron HI (18) produces an exon skipping event causing exon G to be joined directly to exon I in the mature mRNA (27). This mutation results in a frameshift causing a premature termination seven codons into exon I, leading to a protein of about 38 kDa. (Exons G, H, and I in the rat are equivalent to exons 8, 9, and 10 in humans.) A detailed analysis of the protein synthesis and transport within the hepatocytes of the Nagase rats indicated that several different sizes of albumin-like proteins accumulate within certain organelles but fail to be secreted in significant amounts (28). (*iii*) In the analbuminemic Roma individual described here, DNA sequence analysis has demonstrated that a single-base insertion leads to a frameshift mutation causing premature termination 7 amino acid residues downstream with a predicted protein product of about 31 kDa.

There is also evidence of heterogeneity in other cases of analbuminemia for which the mutation in the DNA has not yet been identified. A Swiss-type of analbuminemia was shown to be different from that found in the American Indian analbuminemia by allele-specific oligonucleotide probing (17). Furthermore, by use of heteroduplex and SSCP analysis, we have evidence that the gene defect causing analbuminemia in Roma differs from that in Codogno (Italian) (7) and G.M. (United States) (2, 3).

Inheritance. The findings for the Daughter of Roma clearly demonstrate the inheritance of the Roma allele. The albumin

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                        260                                 270
                         ^                                   ^
Normal      ...  Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu ...
Albumin     ...  GCG GAC CTT GCC AAG TAT ATC TGT GAA AAT CAA GAT TCG ATC TCC AGT AAA CTG AAG GAA ...
                          *                   *                   *
                       9140                9160                9180

                        260                                 270
Putative                 ^                                   ^
Roma        ...  Ala Asp Leu Ala Lys Tyr Ile Cys Glu Lys Ser Arg Phe Asp Leu Gln  -
Albumin     ...  GCG GAC CTT GCC AAG TAT ATC TGT GAA AAA TCA AGA TTC GAT CTC CAG TAA ACT GAA GGA ...
                          *                   *                   *
                       9140                9160                9180
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FIG. 5. DNA sequence and amino acid translation of exon 8 from HSA for a wild-type allele (16) and the mutant Roma allele. The underlined nucleotides indicate the region where a single A residue has been inserted in the Roma allele. The amino acids in boldface type represent the putative sequence for the Roma albumin. Note that the nucleotide at position 9163 is an A residue for both Roma and DOR, which agrees with the genomic sequence of HSA (16) but differs from the cDNA sequence given in Lawn *et al.* (26), who reported a G residue at that position. The numbering above the line refers to the amino acid residue in mature albumin, whereas the numbering below the line refers to the nucleotide number from the genomic sequence (16).

values reported for the parents and the surviving sister of Roma indicate that one normal allele appears to be sufficient to produce a normal or nearly normal level of serum albumin (4–6). Hence, the detection of heterozygous individuals may be difficult at the protein level using cellulose acetate electrophoresis or similar methods, especially since the condition is asymptomatic. Previous genealogical studies indicated that consanguinity was a frequent feature of analbuminemia (2–10). Heretofore, it had not been shown that first-degree relatives carried the mutant allele. Our study proves that analbuminemia is recessive and heritable.

Analbuminemia or Hypoalbuminemia? A cardinal question in any hereditary trait is whether homozygosity results in a true null mutation, i.e., a condition in which no protein product—truncated or not—is expressed. As yet, there is no unambiguous answer for human analbuminemia. As a result, some authors prefer the term hypoalbuminemia or give it as an alternative. Bennhold *et al.* (29), who reported the first case, named the condition analbuminemia because they could detect no albumin by the methods used. This distinguishes the trait from idiopathic hypoalbuminemia, for which several cases have been described (2, 30). For example, in the case of a 4-year-old child the circulating albumin level was 1–2 g/dl and increased over time (2). In another case the albumin concentration ranged from 0.85 to 1.1 g/dl (30) but in later life it approached normal (Elliot Vesell, personal communication).

Analbuminemia as described here has the following characteristic features that distinguish it from idiopathic hypoalbuminemia: (*i*) analbuminemia results from a heritable mutation in the albumin gene; (*ii*) it appears to remain constant throughout life; (*iii*) no mature albumin is demonstrable although traces of albumin-immunoreactive protein may be detectable; (*iv*) it results from a defect in albumin synthesis rather than increased catabolism of albumin; (*v*) edema is moderate or absent. Albumin metabolism in heritable analbuminemia differs from that in idiopathic hypoalbuminemia. In a number of cases of analbuminemia, clinically and biochemically similar to that described here, the metabolic half-life of infused ^{131}I-labeled albumin is prolonged (2, 3, 31), whereas in the case of idiopathic hypoalbuminemia in the 4-year-old child, the labeled albumin was rapidly degraded and had a half-life one-sixth of normal (2). Increased catabolism of albumin is also a characteristic of familial hypercatabolic hypoproteinemia, which exhibits a marked reduction of both albumin and IgG (32).

In conclusion, analbuminemia is an autosomally inherited trait that is clearly recessive. As described above, the cause is apparently heterogeneous in nature, resulting from a variety of genetic defects. In humans there is little maternofetal transfer of albumin (33). There may be a critical need for albumin in fetal and neonatal life; this may be compensated for in the growing child by increased plasma protein synthesis. Analbuminemia in young children and in adults is essentially asymptomatic, but the types of mutations causing it are similar to those found in diseases such as muscular dystrophy or hemophilia B, for which many different mutations have been identified.

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