Splicing mutation in human hereditary analbuminemia

(genetic disorder/molecular defect/RNA processing)

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ABSTRACT We have identified a structural defect in the serum albumin gene in human analbuminemia. Sequence determination of 1.1 kilobases (kb) of the 5' regulatory region and of 6 kb across exonic regions revealed a single AG-to-GG mutation within the 3' splice site of intron 6 in the defective gene of an analbuminemic individual. In an *in vitro* assay on the RNA transcript this mutation causes a defect in splicing of the intron 6 sequence and in subsequent ligation of the exon 6–exon 7 sequences. Using polymerase-amplified genomic DNA and allele-specific oligodeoxynucleotide probes, we have also shown that the sequence of this intron 6/exon 7 splice junction is normal in a different, unrelated analbuminemic individual. Analbuminemia in humans is therefore the result of one of multiple defects in our genome.

Alalbuminemia is a genetic disorder that is characterized by the virtual absence of albumin in the serum of affected individuals. Considering that in normal individuals albumin is >50% of total serum proteins, a deleterious mutation in the albumin gene would be expected to be eliminated by the selective forces. Here we report on one such mutation that rendered the human albumin gene defective and yet survived in a homozygous individual.

Alalbuminemia is thought to be transmitted as an autosomal recessive disorder because of the 22 reported analbuminemic individuals, 8 are known to be from consanguineous matings (1–5). In two of these individuals both parents of the analbuminemic were hypoalbuminemic, possibly indicating a heterozygous state (1, 5). A limited family study of one of these two analbuminemic patients (5), a young American Indian female, suggested that the analbuminemic trait was linked to the group-specific component (GC) locus. The albumin gene itself is in close linkage with that of GC (6, 7), suggesting that the molecular defect of analbuminemia may lie at or near the albumin gene. Restriction fragment length polymorphism analysis revealed no differences between this analbuminemic gene and that of other control individuals (8). In the present study we examined the albumin gene of the analbuminemic American Indian girl and identified a structural defect in the form of a splicing mutation as a cause of her analbuminemia.

MATERIALS AND METHODS

Genomic Libraries. Genomic DNA was isolated from fibroblasts obtained from an analbuminemic American Indian individual (National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository; repository number GM6248), as well as from leukocytes of a Swiss analbuminemic individual and of a control individual with normal serum albumin levels. DNA was *Sst* I digested and enriched on agarose gels for the size fraction of 15–20 kilobases (kb), containing the albumin gene. The enriched

DNA was used to construct libraries in the phage vector λ 2001 (9). Albumin-specific phage clones λ Norm and λ Analb were isolated from these libraries by using human albumin cDNA clones (10). The genomic phage clones possess a 16-kb *Sst* I fragment spanning the albumin gene from 1 kb upstream of the transcriptional start site to just within exon 13. Using the Maxam and Gilbert (11) procedure, the American analbuminemic albumin gene was selectively sequenced in regions presumed to be essential for correct gene expression including 1.1 kb of the 5' flanking region, exons 1–13, and across all intron/exon splice junctions present on λ Analb.

Dot-Blot Hybridization. DNA was blotted onto Gene-ScreenPlus nylon membranes (New England Nuclear) as described (12). The filters were prehybridized in $5\times$ standard saline phosphate/EDTA (SSPE) ($1\times$ SSPE is 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) (13), $2\times$ Denhardt's solution (14), 0.5% sodium dodecyl sulphate at 40°C for 3 hr. Allele-specific oligonucleotide (ASO) probes D-41 and D-42 were end-labeled using [γ - 32 P]ATP and polynucleotide kinase to a specific activity of 5×10^8 cpm/ μ g. The filters were hybridized with $\approx 10^6$ cpm of either D-41 or D-42 for 9 hr at 42°C, then washed three times in 3 M tetramethylammonium chloride/50 mM Tris-HCl, pH 8.0/2 mM EDTA/0.2% sodium dodecyl sulphate (15, 16). The first and last washes were at room temperature for 20 min, and the second wash was at 60°C for 10 min. The filters were autoradiographed for 10 hr at -70°C with an intensifying screen.

In Vitro Splicing of RNA. Run-off transcripts of plasmids were prepared *in vitro* using [α - 32 P]CTP (ICN, 3000 Ci/mmol; 1 Ci = 37 GBq) and T7 RNA polymerase. The transcripts were gel purified and used for *in vitro* splicing by the HeLa extract as described (17). Splicing reactions were done in a 30- μ l volume containing 1 mM ATP, 20 mM creatine phosphate, 3 mM MgCl₂, 2.7% polyvinyl alcohol (vol/vol), 60% HeLa cell nuclear extract (18), 60 units of placental RNase inhibitor (RNA guard, Pharmacia), and labeled transcripts (2×10^7 cpm). Splicing was done at 30°C for 3 hr with 5- μ l aliquots being removed at different time points. The reaction was stopped by incubating the aliquots at 30°C for 10 min with 3 volumes of 100 mM Tris-HCl (pH 7.5)/20 mM EDTA/2% sodium dodecyl sulphate/proteinase K at 0.4 mg/ml calf thymus RNA at 0.1 mg/ml. All samples were phenol extracted and ethanol precipitated prior to electrophoresis on 4% acrylamide:bisacrylamide (30:1) sequencing gels.

RESULTS

Nucleotide Sequence of the Defective Albumin Gene. More than 7 kb of DNA was sequenced on λ Analb (Fig. 1), and only one difference was found between the albumin genes of

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Abbreviation: ASO, allele-specific oligonucleotide.

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FIG. 1. Map of human albumin gene as previously determined (19). Exons are indicated by closed boxes, and introns are indicated by open boxes. Phage and plasmid clones are shown schematically by lines beneath map. Phage clones λAnalb and λNorm were isolated from albumin-enriched *Sst* I libraries, prepared in λ2001 (9), with genomic DNA obtained from either an analbuminemic or a control individual. λAnalb was subcloned into the plasmid vector pT7/T3-19 (Bethesda Research Laboratories) to produce plasmid clones pAnalb 9.1 and pAnalb 9.4. pAnalb 9.1 possesses the 9.1-kb *Sst* I–*Kpn* I fragment, whereas pAnalb 9.4 possesses the 9.4-kb *Eco*RI–*Sst* I fragment of λAnalb. Partial sequence of analbuminemic albumin gene was determined from these subclones, and sequenced regions are indicated by broken line beneath map. Asterisk (above map), position of splice-junction mutation within analbuminemic gene.

the analbuminemic and of a control individual (19). No differences were found in the 5' regulatory region nor in the coding sequences of exons 1–13, including the intron/exon junctions. The only difference was detected at the 3' splice junction of intron 6. The penultimate nucleotide of intron 6 had undergone an adenine to guanine transition, thereby destroying the invariant AG dinucleotide (Fig. 2) known to be essential for correct splicing (20, 21).

To demonstrate that the splice-junction mutation detected

FIG. 2. Nucleotide sequence of 3' splice junction of intron 6 of normal and analbuminemic genes. Sequence was determined by the procedure of Maxam and Gilbert (11) using the G, G + A, T + C, and T specific reactions designated by G, A, T, and C. For the analbuminemic gene, the sequence of this region was determined from both strands. The characteristic sequence of a pyrimidine-rich tract followed by the invariant AG dinucleotide is shown for the normal gene, and position of intron/exon boundary is indicated by horizontal line. In the analbuminemic gene, adenine is replaced by guanine, and uncertainty in the location of intron/exon boundary is indicated by broken line.

in the cloned analbuminemic albumin gene was present in genomic DNA, the DNA was amplified by the polymerase chain reaction (12) and analyzed by hybridization with ASO probes. Genomic DNA was analyzed from both the American Indian girl and a control individual, as well as from a second analbuminemic individual of Swiss ancestry (22, 23). The albumin gene from the Swiss individual was previously shown by Southern analysis to be grossly intact (24). Oligodeoxyribonucleotides D-58 and D-59 were used to selectively amplify a 110-bp region encompassing the 3' splice junction of intron 6 (Fig. 3 Upper). The amplified DNA was examined by dot-blot hybridization with the ASO probes D-41 (normal) and D-42 (albuminemic) (Fig. 3 Lower). The normal probe (D-41) hybridized to both the normal and the Swiss analbuminemic genomic DNAs, whereas the analbuminemic probe (D-42) hybridized exclusively to the analbuminemic American Indian genomic DNA. This finding demonstrates that the splice-junction mutation was not an artifact of cloning and also that the analbuminemic American Indian is lacking a functional 3' splice junction in intron 6 in both alleles of the gene.

The allele-specific hybridization results demonstrate the presence of only the aberrant 3' splice junction in intron 6 in the American Indian analbuminemic. This finding is compatible with this individual being homozygous for the splice-junction mutation. The ASO results also demonstrate that the intron 6/exon 7 splice junction of the Swiss analbuminemic is normal, and therefore the cause of analbuminemia in this individual must be different from that of the American Indian girl. Because the albumin gene from the Swiss individual has been demonstrated by Southern analysis to be grossly intact (24), the cause of analbuminemia in this case is most probably also the result of a small lesion within the albumin gene. This finding suggests that analbuminemias result from one of several different mutations within the albumin gene.

Processing of the RNA Transcript. To determine whether the mutant splice junction of the gene affects processing of the RNA transcripts into mature mRNA, an *in vitro* splicing assay was done. RNA transcripts spanning the region from exon 6 through exon 7, of both the normal and the American Indian analbuminemic genes, were obtained *in vitro* from the plasmid constructs pNorm 0.7 and pAnalb 0.7 (Fig. 4 Upper). The transcripts were analyzed for their ability to be spliced by a HeLa cell splicing extract. After a short time lag, a 126-nt fragment was produced in both the normal and analbuminemic splice reactions (Fig. 4 Lower). This fragment is of the expected size of exon 6 and is probably the

FIG. 3. Polymerase chain reaction (PCR) amplification and ASO hybridization of genomic DNA. Genomic DNA from the American Indian and Swiss analbuminemic individuals (ANALB.) as well as a control individual was amplified by the PCR method as described (12). (Upper) Synthetic oligodeoxyribonucleotide primers D-58 and D-59 were used to selectively amplify a 110-nt region encompassing the intron 6/exon 7 splice junction. Two and one-half micrograms of each genomic DNA was amplified in 25 PCR cycles. (Lower) One twentieth of each of the amplified DNAs (corresponding to 125 ng of the original unamplified DNA) was blotted in duplicate onto GeneScreenPlus nylon membranes as described (12). Additionally, 40 ng of λ Analb or λ Norm were blotted onto the membranes; then the filters were hybridized with the D-41 or D-42 allele-specific probes as described.

result of cleavage at the 5' splice junction. Three additional bands are generated in the normal but not in the analbuminemic splice reaction. One of these migrates with a velocity expected for a 214-nucleotide (nt) species, which is the size of the correctly ligated product of exon 6 and exon 7. This band is unique to the normal RNA splicing reaction, and it migrates somewhat faster than a common band visible in this region for both the normal and the analbuminemic experiment. The two other bands in the normal RNA have an apparent size greater than the primary RNA transcript, and one may correspond to the lariat of intron 6, the other to the lariat of the uncleaved intron 6-exon 7 intermediate. This observation would then be in agreement with previous findings that the lariat structures seen among the products of mRNA splicing (25, 26) and branched DNA structures (27) exhibit anomalous electrophoretic mobilities in gels.

The splicing results demonstrate that the aberrant 3' splice junction prevents correct splicing *in vitro*. It is therefore unlikely that this mutant junction functions correctly *in vivo*. This is supported by studies of splice mutations of the β -globin gene (28, 29). In one study, it was demonstrated that any alteration of the 3' AG abolished splicing *in vitro* and elicited splicing at cryptic 3' junctions *in vivo* (28). In nature, there are at least two examples of this same AG-to-GG mutation at the 3' splice junctions that result in disease in man. The presence of this mutation within the β -globin gene of one individual was demonstrated to be the cause of β -thalassemia (30). In another case, the same defect within the gene for apolipoprotein E was shown to be the cause of an apolipoprotein E deficiency (31).

It is interesting to note that although analbuminemia is characterized by a "virtual" absence of albumin, traces of albumin have been detected by immunological methods in the serum of analbuminemics at 10^{-4} levels of normal amounts (5, 32). Two possibilities might account for the trace level of albumin in these individuals. It may represent an artifact of the serological determination, either a cross-reacting antigen or a background level of detection. Alternatively, it could result from leakiness of the mutated splice junction, such that some RNA molecules are correctly spliced despite the mutation, and the 10^{-4} -fold level results

from translation of these few normal mRNAs into *bona fide* albumin. Splicing is not a mistake-proof process, and indeed alternative splicing events are known to occur at low frequency at cryptic sites (33). So perhaps it is not unreasonable to speculate that some mutant splice junctions could give rise to trace amounts of correctly spliced mRNA.

DISCUSSION

Albunemia is paradoxical from both a clinical and a genetic standpoint. Albumin has long been accepted as an essential protein with many functions attributed to it. This belief is supported by the apparent low incidence of analbuminemia in the human population. In contrast, the rate of evolution of this protein is moderately high (34), as would be expected of a protein with no or limited vital importance; this is supported by the apparent lack of serious clinical manifestations of this condition. Two possibilities exist to explain this paradox: (i) Albumin is not essential as previously believed, and the apparent low incidence of analbuminemia is due to the lack of diagnosis. Although it can be argued that albumin is not essential because analbuminemia is almost asymptomatic, that a higher incidence would go undetected is unlikely with the current widespread use of automated serum protein determinations and serum electrophoresis. (ii) Albumin is essential, and the apparent lack of symptoms is a consequence of the time at which analbuminemia is diagnosed. Although analbuminemia is innocuous in adults, it may have profound effects during prenatal development. At this precarious time, analbuminemia may be life-threatening as a consequence of the resulting edema or bilirubinemia. Once past this critical stage of development the few surviving analbuminemics may lead a life uncomplicated by this condition. In fact, prenatal and neonatal mortality due to spontaneous abortion, nonthalassemic, non-immune fetal hydrops (35), and sudden infant death syndrome remains largely unexplained. It is interesting to note that a sibling of the analbuminemic American Indian girl died of an unknown cause 5 days after birth (5). Unfortunately no blood analysis was done to determine whether she also was analbuminemic.

FIG. 4. *In vitro* splicing of RNA transcripts. (Upper) Plasmids pAnalb 0.7 and pNorm 0.7 were prepared from clones pAnalb 1.7 and pNorm 1.7, respectively. Plasmids pAnalb 1.7 and pNorm 1.7 possess a 1.75-kb *Taq* I-*Bgl* II fragment spanning the region from exon 6 through exon 7; this fragment was inserted into *Acc* I/*Bam*HI-cleaved pT7/T3-19 (Bethesda Research Laboratories). Plasmids pAnalb 0.7 and pNorm 0.7 were prepared from plasmids pAnalb 1.7 and pNorm 1.7 by the removal of a 1-kb *Acc* I-*Mst* II fragment from within intron 6. The resulting incompatible staggered termini were filled in with dNTPs using Klenow fragment, and the plasmids were recircularized by blunt-end ligation with T4 DNA ligase. The site from which the *Acc* I-*Mst* II fragment was removed is indicated by -1034 bp. (Lower) *In vitro* splicing results. Run-off transcripts of *Eco*RI-digested pAnalb 0.7 and pNorm 0.7 were prepared *in vitro* with [α - 32 P]CTP (ICN, 3000 Ci/mM) and T7 RNA polymerase. Size standards are similarly prepared run-off transcripts of the same template that was cut with different restriction endonucleases to give 121-, 239-, and 453-nt markers. Before addition of HeLa extract, full-length Norm and Analb transcripts migrate as single bands at the indicated position of 769 nt, being as compact as the size marker bands. After addition of HeLa extract, various RNA splicing products appear at different electrophoretic positions on the gel, as indicated by arrows between the two gels.

Characterization of the splice-junction mutation as a cause of analbuminemia in humans opens up the possibility for future population studies. The ASO probes described here could be used to assess the genotypic frequency of this genetic defect in humans. A knowledge of the actual genotypic frequency might help resolve the apparent paradox of this disorder and, in a broader sense, provide insights into the rate of occurrence of new mutations and the persistence of genetic diseases.

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