A seven-base-pair deletion in an intron of the albumin gene of analbuminemic rats

(mRNA splicing/gene cloning/intron mutation)

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ABSTRACT Analbuminemic rats, which genetically lack serum albumin, have a mutation affecting albumin mRNA processing. Serum albumin genes were cloned from normal parental Sprague–Dawley rats. Structural analyses of the two albumin genes showed that the gene from analbuminemic rats had a seven-base-pair deletion in an intron. The deletion extended from base 5 to base 11 from the 5’ end of intron III of the albumin gene. This deletion converted the sequence, G-T-A-G-C-T, which is normally located at the 5’ end of intron HI, to G-T-A-G-C-G. RNA blot hybridization of analbuminemic and normal rat liver nuclear RNA using a DNA fragment containing the intron III as a probe showed that this intron sequence persisted in albumin mRNA precursors of analbuminemic rats.

Recent structural analyses have shown that many eukaryotic mRNAs are produced from split genes. The mechanisms of mRNA processing have been found to be complicated, involving capping, modification of nucleosides, poly(A) tail addition, and splicing. Information on the molecular mechanisms of splicing is increasing, and the involvement of U1, U2, and U6 small nuclear RNAs in splicing has been proposed (1–4) from sequence data. Yang et al. (5) confirmed experimentally the role of U1 ribonucleoprotein in splicing by using antisera against U1 ribonucleoprotein present in the sera of patients of systemic lupus erythematosus. The mechanisms of splicing of adenovirus mRNAs, ovalbumin mRNA, and globin mRNA have been studied extensively (6–9). However, the precise mechanism(s) of splicing is still unknown.

mRNA splicing is of course indispensable in producing mRNA in many eukaryotic mRNAs, and the importance of splicing in biological controls has been demonstrated in the production of IgM and IgD (10). Therefore, the mechanisms of mRNA splicing are currently important problems in molecular biology.

Experiments on directed mutation provide useful information on mRNA splicing. Although many important findings have been obtained by this artificial alteration of cloned genes, the biological activities of these altered genes usually have been assayed in artificial systems, except in studies on adenovirus. However, natural mutants that have a mutation affecting mRNA splicing should be ideal models for studies on the mechanisms of mRNA splicing. Human β+-thalassemia is one such mutant, suggested to have a blockade of mRNA processing and found to have a single-base-pair substitution (11, 12).

Analbuminemic rats, which lack serum albumin, genetically have been found also to have a mutation(s) affecting mRNA processing (13). Here we report a seven-base-pair deletion in an intron of the albumin gene of analbuminemic rats and evidence that albumin mRNA splicing is blocked in analbuminemic rats.

MATERIALS AND METHODS

Animals. Analbuminemic rats are a mutant of Sprague–Dawley rats maintained in Sasaki Institute, Tokyo, Japan. Control Sprague–Dawley rats of the same stock from which the first analbuminemic rat was isolated (14) were obtained from CLEA Japan.

Chemicals. (32P)dATP, (32P)dCTP, (32P)dGTP, and (32P)dTTP were purchased from the Radio Chemical Centre, Amersham. Restriction endonucleases EcoRI, HindIII, BamHI, Pst I, Sal I, HindI, Hae III, and Sau3AI were from Takara Shuzo, Kyoto, Japan. DNA polymerase I and a large fragment of DNA polymerase I were products of Boehringer Mannheim, Mannheim, Federal Republic of Germany. T4 DNA ligase was obtained from New England BioLabs. Nitrocellulose paper and amiono-benzoyloxymethylcellulose paper were products of Schleicher & Schuell. Other chemicals were reagent grade.

Rat Genomic Libraries. Genomic libraries from analbuminemic and Sprague–Dawley rats were made with phage λ Charon 4A as a vector. Approximately 15- to 20-kilobase-pair DNA fragments generated by partial digestion with EcoRI were selected by sucrose density gradient centrifugation and ligated to EcoRI-digested Charon 4A DNA under the conditions described by Maniatis et al. (15). Ligated DNA was packaged in vitro by the method of Hohn and Murray (16). Packaged phages were propagated once by using Escherichia coli, strain K802, as an indicator to make a stock phage library. Approximately 5 × 107 independent clones were made and screened by the plaque hybridization method (17) with 32P-labeled, cloned albumin cDNA, designated pAlb-1 (18), as a probe. Labeling of the cloned cDNA with 32P was carried out by nick translation (19) with DNA polymerase I.

Subcloning of DNA fragments of isolated phage DNA containing the albumin gene was carried out with pKH47 (20), a derivative of pBR322, as a vector through its EcoRI site or HindIII site. All procedures involving recombinant DNA were carried out in P3 biocontainment at the National Cancer Center Research Institute in compliance with the Guidelines for Research Involving Recombinant DNA Molecules issued in March 1979 by the Ministry of Education, Science and Culture of Japan.

Southern Blot of Cloned Albumin Gene. Phage DNAs containing the cloned albumin gene were cleaved with restriction enzymes, subjected to electrophoresis on 1% agarose gel, and transferred to nitrocellulose paper by the method of Southern (21). Nitrocellulose paper was processed and hybridized by the method of Wahl et al. (22) with the 32P-labeled, cloned albumin

Abbreviations: R, purine; Y, pyrimidine.
cDNA prAlb-1 as a probe. Bands were located by autoradiography with a Dupont Cronex Lightning Plus intensifier.

RNA Blot Hybridization. RNAs were subjected to electrophoresis on 1% agarose gel containing 7.5 mM methymercuric hydroxide by the method of Bailey and Davidson (23). RNAs were transferred to diazobenzyloxymethylcellulose paper by the method of Alwine et al. (24). The prehybridization and hybridization conditions were as described (13). The cloned albumin cDNA prAlb-1 and the approximately 300-base-pair EcoRI fragment containing the intron HI sequence were used as probes.

DNA Sequence Determination. The DNA base sequence was determined by the method of Maxam and Gilbert (25). DNA fragments of subcloned albumin gene were end-labeled either by 3' addition under the conditions described by Sanger et al. (26) or by 5' phosphorylation with polynucleotide kinase.

RESULTS

Molecular Cloning of Albumin Genes from Normal Sprague-Dawley Rats and Analbuminemic Rats. Rat albumin genes were cloned by the method of Maniatis et al. (15) by using partial digests of genomic DNAs with EcoRI. Approximately 1.2 × 10⁶ independent clones were screened in both cases, and 15 and 16 plaques were selected, respectively, from analbuminemic rat and normal Sprague-Dawley rat libraries. Digestion patterns with EcoRI of representative clones from the normal Sprague-Dawley rat (prAlb-1, pAlb-2, and pAlb-3) and from the analbuminemic rat (pAlb-1, pAlb-2, and pAlb-3) are shown in Fig. 1. Their hybridizations to the cloned cDNA prAlb-1 are also shown. Cross restriction maps were constructed for these six clones and are shown in Fig. 2. The data of restriction maps and hybridization to cloned cDNA showed that pAlb-1 and pAlb-2 contained the 5’-flanking sequence and the 5’ half of the coding sequence, pAlb-2 and pAlb-3 contained the middle portion of the coding sequence, and pAlb-3 and pAlb-3 contained the 3’ half and the 3’-flanking sequence (data not shown).

Altogether, cloned genes were extended about 30 kilobase pairs in both analbuminemic and normal Sprague-Dawley rats.

The molecular structure of the rat albumin gene has been reported by Sargent et al. (27, 28). The structure of the rat albumin gene that we cloned in the present work coincided well with that reported previously.

Because all of the fragments present in Southern blot hybridization of the albumin gene with total genomic DNAs and cloned albumin cDNA were found to be contained in the cloned albumin gene and to be contiguous in one albumin gene, there was no indication of the presence of an albumin pseudogene in either analbuminemic or normal Sprague-Dawley rats.

The above data also indicate that the albumin genes from analbuminemic and normal Sprague-Dawley rats were indistinguishable in gross structure. The digestion patterns of the two genes with BamHI, SalI, and PstI, respectively, were also indistinguishable (data not shown). Thus, there was no large deletion, insertion, or rearrangement in the albumin gene of analbuminemic rats.

Structural Organization of the Albumin Gene of Normal Sprague-Dawley and Analbuminemic Rats. Nucleotide sequence determination revealed that the albumin gene was split into at least 15 exons. Because the nucleotide sequence of the 5' end of the albumin mRNA has not yet been determined, we could not define the 5' end of the albumin gene. Therefore, the number of exons is tentative. The structure of the albumin gene has been reported by Sargent et al. (27, 28), our observations on the structure of albumin genes of normal Sprague-Dawley rat and analbuminemic rat were well consistent with the published data (28). As reported by Sargent et al. (28), we detected the T-A-T-A-T-A sequence and C-C-A-A-T sequence at approximately 30 and 85 base pairs upstream from the putative transcription initiation site, respectively, in both normal Sprague-Dawley and analbuminemic rat genes.

The gross structural organization is shown in Fig. 3. All of the exons of the albumin gene of normal Sprague-Dawley rats also were present in the gene of analbuminemic rats, and the structural organizations of the genes of normal Sprague-Dawley and analbuminemic rats were indistinguishable.

Nucleotide Sequences of Rat Albumin Genes. We determined the sequence of cloned rat albumin genes from normal Sprague-Dawley and analbuminemic rats up to about 10,000 base pairs. Because we found that analbuminemic rats have a mutation affecting mRNA processing (13), we determined in particular the nucleotide sequences around splicing junctions.

We found (Fig. 4) a seven-base-pair deletion in the 5' end of intron HI of the analbuminemic rat albumin gene. The nu-
cleotide sequence of the 5' end of the intron HI is 5'-G-T-A-G-G-T-T-T-C-C-G-C-G-C-A-G 3' in the normal rat gene, but is 5'-G-T-A-C-C-G-A-G 3' in the analbuminemic rat gene. Therefore, the seven-base DNA fragment 5'-G-T-T-T-C-C-G-C-G-C-A-G 3' is deleted in the gene of analbuminemic rats.

The nucleotide sequences around the splicing junctions of the analbuminemic rat gene were identical with those of the normal rat gene, except at the 5' end of intron HI. As has been pointed out (29, 30), the 3' end of each intron has a nucleotide sequence with a pyrimidine (Y)-rich stretch—A-G and the 5' end has G-T-R-R (R = purine). So far as has been determined, the nucleotide sequences of the albumin genes of analbuminemic and normal Sprague–Dawley rats in regions other than splicing junctions are identical (data not shown). As we determined only about 10,000 base pairs of the 15,000 base pairs, the possibility of additional mutations was not completely eliminated, but digestions with several kinds of restriction endonucleases indicated that there is no deletion, insertion, or rearrangement in the gene of analbuminemic rats large enough to be recognized by polyacylamide gel electrophoresis.

**Nuclear RNA Blot Hybridization.** From data on the nucleotide sequence of the albumin gene of analbuminemic rats, only one mutation was detected. This mutation was located very close to the splicing junction. Therefore, it seemed very probable that this seven-base-pair deletion blocks albumin mRNA processing, probably splicing. However, it was essential to determine whether the intron HI that contains the deletion is removed during maturation of albumin mRNA in analbuminemic rats. To answer this question, we carried out RNA blot hybridization, using the intron HI as a probe in comparison with that with cDNA.

When eDNA was used as a probe, cytoplasmic RNA of normal Sprague–Dawley rat liver gave a single strong band in the position of mature mRNA; this was not found with cytoplasmic RNA of analbuminemic rat liver (Fig. 5). No band was observed when cytoplasmic RNA of analbuminemic rats was used, but many bands were observed when nuclear RNA of analbuminemic rat liver was used, as with nuclear RNA of normal Sprague–Dawley rats. When the DNA fragment of the HI intron was used as a probe, the band of mature mRNA disappeared as expected. Most of the bands of nuclear RNA of normal Sprague–Dawley rats also disappeared, and only bands of high molecular weight were detected. In contrast, nuclear RNA of analbuminemic rats gave many bands from the large to the

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**FIG. 4.** List of the nucleotide sequences at the splicing junctions. Underlined is the DNA sequence deleted in the albumin gene of analbuminemic rat. A, Position of the seven-base-pair deletion.
small molecular weight region, and the relative intensities of the bands were much stronger than those of normal rats. This clearly indicates that the intron HI sequence persisted in “albumin mRNA precursors” in analbuminemic rats.

DISCUSSION

In the present work, we clearly demonstrated that there is a seven-base-pair deletion in the HI intron of the albumin gene of analbuminemic rats. This deletion is located at the 5′ end of the HI intron of the albumin gene. The nucleotide sequence of the 5′ end of introns is strongly conserved in many eukaryotic genes (30) and is considered to be important in mRNA splicing (31). The proposed model of mRNA splicing by Lerner et al. (1) and Rogers and Wall (2) are based on the fact that the nucleotide sequence of the 5′ end of introns can hybridize to U1 RNA (32). The alternative models proposed by Ohshima et al. (3) and Mount and Steitz (33) are also based on the same fact. Recently Yang et al. (5) demonstrated that U1 ribonucleoprotein indeed plays an important role(s) in mRNA splicing of adenovirus. From the above data, it is widely accepted that the nucleotide sequence of the 5′ end of introns is critical for mRNA splicing.

Our data on the nucleotide sequence of the albumin gene of analbuminemic rats clearly demonstrated that a seven-base-pair deletion in HI intron replaces the normal nucleotide sequence of the 5′ end; that is, 5′ G-T-A-G-T-T-C-3′ is replaced by 5′ G-T-A-G-G-A-C-G-T-3′. Therefore, it is reasonable to conclude that this mutation blocks albumin mRNA splicing in analbuminemic rat liver. This conclusion is supported by the fact that the intron HI sequence persisted in “albumin mRNA precursors” in the nuclei of the liver of analbuminemic rats. We also recently found restriction fragment length polymorphism in the 3′-flanking sequence of the albumin gene in a stock of Sprague–Dawley rats. Using this polymorphism, we found that analbuminemia is closely linked to the albumin structural gene (to be published elsewhere). All of our data strongly suggest that the seven-base-pair deletion that we found in the present work in the intron HI of the albumin gene of analbuminemic rats blocks albumin mRNA splicing. To determine the biological significance of the mutation that we found in the present work on the splicing of mRNA, it is important to examine if the mutation blocks mRNA splicing in an artificially constructed test system with simian virus 40 (34) or the thymidine kinase gene (35).

We determined the 13-nucleotide sequence of the 3′ end of the introns of the rat albumin gene and found that the nucleotide sequence of the 3′ end of all introns was 5′ Y-A-G-3′ as has been noticed in many eukaryotic genes (30). We also determined the nucleotide sequence of the 5′ end of the introns of rat albumin gene. All 5′ ends of introns had 5′ G-T-3′ as noticed (30). Previously there were reports that the consensus sequence of the 5′ end of introns is 5′ G-T-A-A-G-T-3′ (36), 5′ G-T-A-A-G-T-A-3′ (2), or 5′ G-T-R-A-G-3′ (31), but no 5′ G-T-A-A-G-T-A-3′ was found at the 5′ end of the rat albumin gene. Only one 5′ G-T-A-A-G-T-3′ and seven 5′ G-T-R-A-G-3′ sequences were observed in the rat albumin gene. A completely conserved sequence, deduced from our sequence data on the rat albumin gene was 5′ G-T-R-3′. However, if we postulate that the signal sequence for mRNA splicing at the 5′ end of introns is 5′ G-T-R 3′, it is very hard to understand why the mutation of analbuminemic rats blocks albumin mRNA splicing. Therefore, we tentatively propose that the Y-A-G-T-R consensus sequence is essential but not sufficient for mRNA splicing. The nucleotide sequence around the splicing junctions must be important in the regulation of mRNA splicing.

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