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Single base mutation in the proα2(I) collagen gene that causes efficient splicing of RNA from exon 27 to exon 29 and synthesis of a shortened but in-frame proα2(I) chain

(RNA splicing mutation/type I procollagen/osteogenesis imperfecta)

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ABSTRACT **Previous observations demonstrated that a lethal variant of osteogenesis imperfecta had two altered alleles for proα2(I) chains of type I procollagen. One mutation produced a nonfunctioning allele in that there was synthesis of mRNA but no detectable synthesis of proα2(I) chains from the allele. The mutation in the other allele caused synthesis of shortened proα2(I) chains that lacked most or all of the 18 amino acids encoded by exon 28. Subclones of the proα2(I) gene were prepared from the proband's DNA and the DNA sequence was determined for a 582-base-pair (bp) region that extended from the last 30 bp of intervening sequence 26 to the first 26 bp of intervening sequence 29. Data from six independent subclones demonstrated that all had the same sequence as a previously isolated normal clone for the proα2(I) gene except that four subclones had a single base mutation at the 3′ end of intervening sequence 27. The mutation was a substitution of guanine for adenine that changed the universal consensus sequence for the 3′ splicing site of RNA from -AG- to -GG-. S1 nuclease experiments demonstrated that about half the proα2(I) mRNA in the proband's fibroblasts was abnormally spliced and that the major species of abnormal proα2(I) mRNA was completely spliced from the last codon of exon 27 to the first codon of exon 29. The mutation is apparently unique among RNA splicing mutations of mammalian systems in producing a shortened polypeptide chain that is in-frame in terms of coding sequences, that is used in the subunit assembly of a protein, and that contributes to a lethal phenotype.**

Defects in either the gene for proα1(I) chains or the gene for proα2(I) chains of type I procollagen are found in several heritable disorders of connective tissue in humans. The heritable disorders have heterogeneous phenotypes but are primarily classified as either osteogenesis imperfecta, a disease characterized by brittle bones, or Ehlers–Danlos syndrome, a related condition characterized by loose joints (see refs. 1 and 2). Several different kinds of mutations in the genes for type I procollagen have been found in probands with osteogenesis imperfecta or Ehlers–Danlos syndrome. Three of the mutations were partial gene deletions (3–6). Seven variants of osteogenesis imperfecta had single base mutations in coding sequences (refs. 7–12; C. D. Constantinou, K. B. Nielsen, and D.J.P., unpublished data). A series of additional mutations in probands with osteogenesis imperfecta or Ehlers–Danlos syndrome were observed to cause synthesis of shorter proα1 or proα2 chains of type I procollagen without any evidence as to whether the mutations were partial gene deletions or RNA splicing mutations (13–17).

We previously demonstrated that a lethal variant of osteogenesis imperfecta had two altered alleles for proα2(I) chains (18, 19). One mutation produced a nonfunctioning allele in that there was synthesis of mRNA but no detectable synthesis of proα2(I) chains from the allele. The mutation in the other allele caused synthesis of shortened proα2(I) chains that lacked most or all of the 18 amino acids encoded by exon 28. We demonstrate here that the mutation in the allele producing the shortened proα2(I) chains was a single base substitution that caused efficient splicing of RNA from the last codon of exon 27 to the first codon of exon 29.

MATERIALS AND METHODS

Isolation and Sequencing of Genomic Subclones. Skin fibroblasts were cultured (18) and the DNA from six 175-cm^2 flasks was extracted (20). The DNA was digested with *Hin*dIII and fragments of ≈6 kilobases (kb) were isolated by agarose gel electrophoresis and electroelution. The fragments were cloned into the bacteriophage vector Charon 21A and packaged with a commercial packaging extract (Promega Biotec, Madison, WI). About 5×10^6 individual clones were generated. The clones were screened with a *Hin*dIII/*Eco*RI fragment of 3.6 kb from NJ-3, a genomic clone for proα2(I) chains (21). A series of subclones of the 3.6-kb *Hin*dIII/*Eco*RI fragment from NJ-3 in the bacteriophage M13mp18 or M13mp19 were sequenced with the dideoxynucleotide method (22). The normal sequence was used to design four synthetic oligonucleotides that were then used to sequence the 3.6-kb *Hin*dIII/*Eco*RI fragment from the proband's proα2(I) genes.

S1 Nuclease Protection Experiments. Total RNA from six 175-cm^2 flasks of skin fibroblasts (18) was extracted with guanidinium isothiocyanate and poly(A)$^+$ RNA was isolated (20). Two single-stranded cDNA probes were prepared. One was an 846-base-pair (bp) fragment obtained by cleaving a full-length cDNA clone for human proα2(I) chains (23) with *Pvu* II. The *Pvu* II/*Pvu* II fragment extended from base pair 1583 to base pair 2428 of the cDNA clone (numbered from the cap site). The fragment was subcloned into the *Sma* I site of M13mp19. To synthesize uniformly labeled antisense DNA, the universal primer for M13 (0.5 pmol) was annealed to 1.1 μg of the template DNA in 13 μl of 15.4 mM $MgCl_2$/15.4 mM Tris·HCl, pH 8.0, by heating to 90°C for 10 min and cooling slowly to room temperature. Eleven microliters of the annealed template and primer were transferred to a tube containing 100 μCi of lyophilized [^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq). Two hundred picomoles each of unlabeled dATP, dGTP, and dTTP, and 170 pmol of dCTP were added to the reaction mixture. The volume was adjusted to 18 μl with water, and 2 μl of Klenow fragment of DNA polymerase I (1 unit/μl; United States Biochemical, Cleveland, OH) was added. The mixture was incubated at room temperature for

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Abbreviation: nt, nucleotide(s).

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10 min, 500 pmol of each nucleotide was added, and the incubation at room temperature was repeated. The sample was heated to 65°C for 5 min and digested with *Hin*dIII. The labeled probe was electrophoretically separated from the template strand in a 5% polyacrylamide gel containing 8 M urea and electroeluted (24).

The second single-stranded DNA probe was a 172-bp *Ava* I/*Nco* I fragment extending from base pair 1693 to base pair 1864 of the proα2(I) cDNA (23). The fragment was labeled at the 5′ end by cleaving the cloned cDNA Hf-15 (23) with *Nco* I, treating with alkaline phosphatase, labeling with polynucleotide kinase and [γ-^{32}P]ATP, cleaving with *Ava* I, separating the strands on a polyacrylamide gel, and electroeluting the antisense DNA strand (20, 24).

For the probe protection experiments, ≈50,000 cpm of the 846-nucleotide (nt) probe (≈0.5 fmol) or 10,000 cpm of the 172-nt probe (≈3 fmol) was hybridized to 2 μg of poly(A)$^+$ RNA in 80% formamide/0.25 mM EDTA/300 mM NaCl/25 mM Hepes buffer, pH 7.0, at 56°C for 3 hr. The sample was then digested with 2 or 4 units of S1 nuclease (Seikagaku, Saint Petersburg, FL) for 30 min at 40°C in 300 μl of 50 mM NaCl/1 mM $ZnSO_4$/30 mM NaOAc buffer, pH 5.3. The products were precipitated with ethanol and separated on a 5% DNA sequencing gel (22). The size of the fragments was estimated by comparison with 5′-end-labeled fragments from a *Hae* III digest of ϕX174 DNA and DNA fragments sequenced by the dideoxy chain-termination method (22).

RESULTS

Structure of the Normal Proα2(I) Gene Around Exon 28. Subclones were prepared that contained a 3.6-kb *Hin*dIII/*Eco*RI fragment from the normal genomic clone NJ-3 (21), and DNA sequencing was carried out on a 582-bp region extending from the last 30 bp of intervening sequence 26 to the first 26 bp of intervening sequence 29. The coding sequences of exons 27, 28, and 29 were identical to the sequences for the same region previously obtained from cloned cDNAs (23). The structure of the intervening sequences of the region was not previously defined. The three 5′ ends of the intervening sequences were 87–100% similar to the consensus sequence -A($^{C}_{G}$)GT($^{A}_{G}$)AGT- (25, 26). The three 3′ ends of the intervening sequences were 66–93% similar to the consensus sequence of -(Y)$_{12}$N($^{C}_{T}$)AGG- (where Y is pyrimidine) (25, 26). Probable sites for lariat loop formation were present at base 126 of intervening sequence 27 and at base 171 of intervening sequence 28 (bases numbered 210 and 459 in Fig. 1). One possible cryptic 3′ splice site was in exon 28 and it was 81% similar to the consensus site of -(Y)$_{12}$N($^{C}_{T}$)AGG- (25, 26). One possible cryptic 5′ splice site was in intervening sequence 28. It contained the hexanucleotide sequence -GTAAAT- that is identical with the sequence for a 5′ splice site in several genes (25), including the 5′ splice site for intervening sequence 12 of the proα2(I) gene of human type I procollagen (40). The hexanucleotide sequence is preceded by -CT-, a dinucleotide sequence that was reported (25) to precede the 5′ splice site in only 9 of 139 intervening sequences. However, -CT- precedes the 5′ splice site in 15 of 43 intervening sequences in the human proα2(I) gene (23, 27). In the chicken proα2(I) gene (28), -CT- precedes the 5′ splice site in 14 of 46 intervening sequences.

Structure of the Proband's Proα2(I) Genes Around Exon 28. Four oligonucleotides were used as primers for sequencing six independent clones from the proband's genomic DNA. Two of the subclones generated a 582-bp sequence that was identical to the sequence obtained from the normal proα2(I) gene (Fig. 1). Four comparable clones from the proband's DNA had a sequence that was identical to the normal sequence for the same 582 bp except for a single base change at the 3′ end of intervening sequence 27. The single base change was a conversion of adenine to guanine that changed the last two nucleotides at the 3′ end from the universal consensus sequence of -AG- to -GG- (Fig. 2).

Structure of the Major Species of Abnormally Spliced mRNA. The single base change of the universal consensus sequence at the 3′ end of intervening sequence 27 can theoretically generate four species of abnormally spliced mRNAs from the normal and cryptic splice sites in the region containing exon 28 (Fig. 3): A, complete exon skipping so that the last codon of exon 27 is spliced to the first codon of exon 29; B, normal splicing out of intervening sequence 28 but no splicing of intervening sequence 27 so that the sequences of intervening sequence 27 are retained in the mRNA; C, use of the cryptic 3′ splicing site in exon 28 and the normal 5′ splicing site of intervening sequence 28 so that 8 nt of coding sequence from the 3′ end of exon 28 are retained in the mRNA; D, use of the cryptic 3′ splice site in exon 28 and the cryptic 5′ splice site in intervening sequence 28 so that the mRNA contains 8 nt of coding sequence from the 3′ end of exon 28 and 51 nt of noncoding sequence from the 5′ end of intervening sequence 28. To establish the structure of the major species of abnormally spliced mRNA in the proband's fibroblasts, two probe-protection experiments were carried out with single-stranded cDNA probes and S1 nuclease.

In the first experiment, a single-stranded probe containing 846 nt of coding sequence was used. The probe extended from the 15th codon of exon 25 to the 36th and last codon of

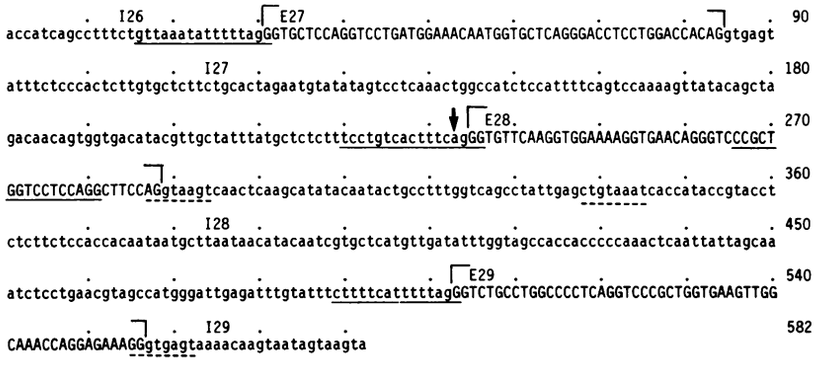

FIG. 1. Nucleotide sequence of 582 bp of the normal proα2(I) gene extending from the last 30 bp of intervening sequence 26 to the first 26 bp of intervening sequence 29. All three exons are 54 bp long, intervening sequence 27 is 150 bp, and intervening sequence 28 is 214 bp long. Arrow, position of the single base substitution in mutant allele; underlined sequences, consensus sequence for 3′ splicing sites; underlined with broken line, consensus sequences for 5′ splicing sites; capital letters, exon sequences; E27–E29, exons 27–29; I26–I29, intervening sequences 26–29.

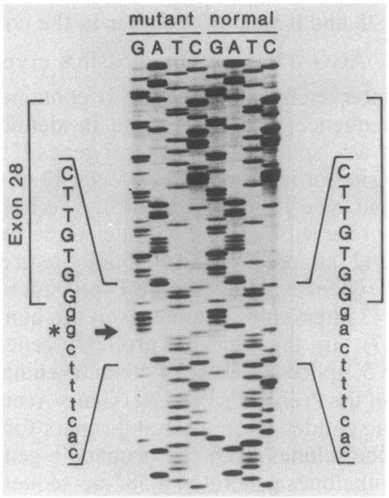

FIG. 2. Sequencing gel of nucleotide sequences from normal and mutant alleles for the 3' end of intervening sequence 27 and the 5' end of exon 28. Arrow, position of single base substitution; asterisk, substituted base; capital letters, exon sequence.

exon 37. As indicated in Fig. 4, 846 nt of the coding sequence in the probe were fully protected by mRNA from control fibroblasts. With mRNA from the proband's fibroblasts, part of the probe was protected and part was cleaved. The larger cleaved fragment was 631 nt. The smaller cleaved fragment was 161 nt (not visible in Fig. 4 but seen in a longer exposure of the same gel). Densitometry of films from several exposures of the gel indicated that the ratio of the protected 846-nt fragment to the cleaved 631-nt fragment was 1.32. Corrected for the different lengths of the two uniformly labeled fragments, the ratio was 0.98. Therefore, the results indicated that about half the proα2(I) mRNA in the proband's fibroblasts was abnormally spliced to generate the 631-nt fragment. Because the larger fragment observed with the 846-nt probe was 631 nt (Fig. 4), the results were consistent with possible RNA splice A in Fig. 3. Because the fragment was not 682 nt, the results were inconsistent with possible RNA splice B (Fig. 3 and Table 1).

In the second experiment, a single-stranded probe was used that contained 172 nt of coding sequence. The probe extended from the 1st nucleotide of exon 27 to the 10th nucleotide of exon 30. The probe was labeled at the 5' end of the antisense strand. The 172 nt of the probe was fully protected by mRNA from control fibroblasts (Fig. 5). With mRNA from the proband's fibroblasts, part of the probe was fully protected and part was cleaved to a major fragment of 68 nt and minor fragments of 65 and 66 nt. Again, the results were consistent with possible RNA splice A (Fig. 3 and Table 1). Since the major and minor cleaved fragments were <72 nt (8 nt of exon 28, 54 nt of exon 29, and 10 nt of exon 30), the results ruled out the possibility that a major fraction of the abnormally spliced RNA was generated from the cryptic 3' splice site in exon 28 with normal splicing of intervening sequence 28 (possible RNA splice C in Fig. 3 and Table 1). Since the major and minor cleaved fragments generated from the 172-nt probe (Fig. 5) were >64 nt (54 nt of exon 29 plus 10 nt of exon 30), the results were not consistent with the conclusion that a major fraction of the abnormally spliced RNA arose from use of the two cryptic sites (possible RNA splice D in Fig. 3 and Table 1). Also, since they altered the reading frame, possible RNA splices C and D (Fig. 3) were inconsistent with previous observations indicating that the proband's fibroblasts synthesized proα2(I) chains at a near normal rate and that all the proα2(I) chains contained an in-frame deletion of ≈18 amino acids (18). In addition, possible RNA splice D was inconsistent with previous data from R-loop analysis indicating that about half of the proα2(I) mRNA (31 of 49 molecules) had a single and enlarged R loop in place of the two R loops of intervening sequences 27 and 28 seen with proα2(I) mRNA from normal fibroblasts (19).

DISCUSSION

The exons encoding the α chain domains of fibrillar procollagens begin with a complete codon (27, 28). Therefore, a partial gene deletion or an RNA splicing mutation that efficiently removes all the coding sequences of one exon will give rise to an mRNA in which the codon reading frame is intact. The complete codon at the 5' end of each exon is always for glycine. Therefore, each exon encodes a discrete number of the repeating -Gly-Xaa-Yaa- sequences of amino acids found in the α chains of collagen. The triple-helical structure of the collagen molecule depends on repeating

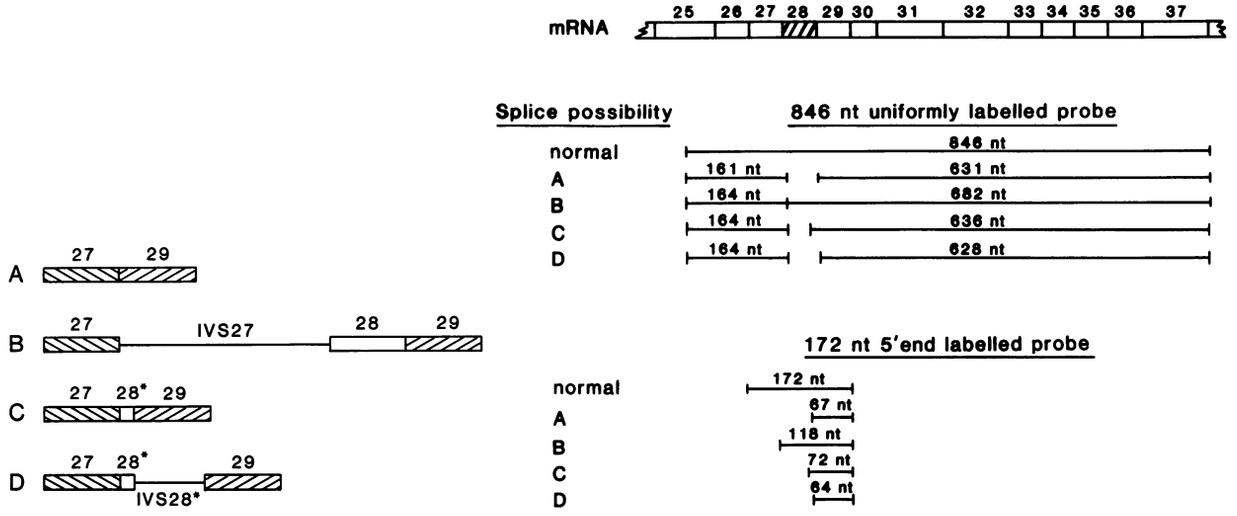

FIG. 3. Possible abnormal splicing of RNA in the proband and predicted results of S1 nuclease probe protection experiments. (*Left*) Possible abnormal splices: A, splicing from the last codon of exon 27 to the first codon of exon 29; B, splicing of intervening sequence 28 only; C, use of the cryptic 3' splice site in exon 28; D, use of the cryptic 3' splice site in exon 28 and the cryptic 5' splice site in intervening sequence 28. (*Right*) Probes used in probe protection experiments. The protected fragments of the two probes and their relationship to the exons in the mRNA are shown. As noted in Table 1, more than one possibility is predictable for possible splice A. Numbers, exons 25–37; IVS27, intervening sequence 27; 28*, last 8 nt of coding sequence from exon 28; IVS28*, first 51 nt of intervening sequence 28.

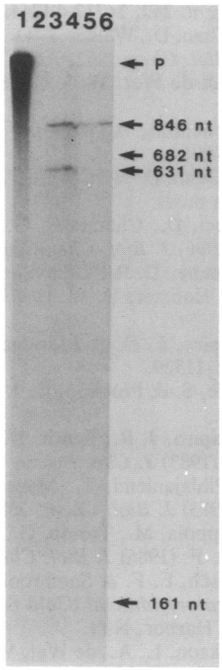

FIG. 4. Probe protection experiment with the 846-nt probe and S1 nuclease. Poly(A)$^+$ RNA was hybridized with the uniformly labeled and single-stranded cDNA probe, and the hybrids were digested with S1 nuclease as described in the text. Lanes: 1, probe alone; 2, unprotected probe digested with 2 units of S1 nuclease; 3, poly(A)$^+$ RNA from proband's fibroblasts digested with 4 units of S1 nuclease; 4, poly(A)$^+$ RNA from proband's fibroblasts digested with 2 units of S1 nuclease; 5, poly(A)$^+$ RNA from control fibroblasts digested with 4 units of S1 nuclease; 6, poly(A)$^+$ RNA from control fibroblasts digested with 2 units of S1 nuclease. RNA from control fibroblasts protected the 846 nt of coding sequence in the single-stranded DNA probe. RNA from the proband's fibroblasts gave three fragments: fully protected 846 nt of coding sequence, 631 nt of a cleaved probe, and an additional fragment of 161 nt (not seen in this exposure of film). Longer exposure of films did not reveal a fragment of 682 nt, the size expected if a major species of mRNA contained intervening sequence 27 and exon 28 in addition to exons 29–37 (B in Fig. 3).

-Gly-Xaa-Yaa- sequences, since every third amino acid is in the center of the triple helix and the structure probably cannot accommodate an amino acid residue larger than glycine (30). However, all the hydrogen bonds stabilizing the triple helix are interchain bonds that link the peptide bonds

Table 1. Possible modes of RNA splicing from the mutated proα2(I) allele

Possible RNA splice	Probe protection experiments			
	Predicted		Observed	
	846 nt	172 nt	846 nt	172 nt
A*	628 or 631	64 or 67	631	65–68
B	682	118		
C†	636	72		
D‡	628	64		

*The last 3 nt of exon 27 are identical to the last 3 nt of exon 28 (23). Therefore, possible RNA splice A predicts a fragment of either 628 or 631 nt from the probe containing 846 nt of coding sequence: 628 nt from exons 29 to 37 with or without 3 nt from exon 27. It predicts either a 64-nt or 67-nt fragment from the 172-nt probe: 54 nt from exon 29 and 10 nt from exon 30 with or without 3 nt from exon 27.

†As discussed in text, possible RNA splices C and D are inconsistent with previous biosynthetic data (18) since they alter the reading frame.

‡As discussed in text, possible RNA splice D is inconsistent with R-loop data (19).

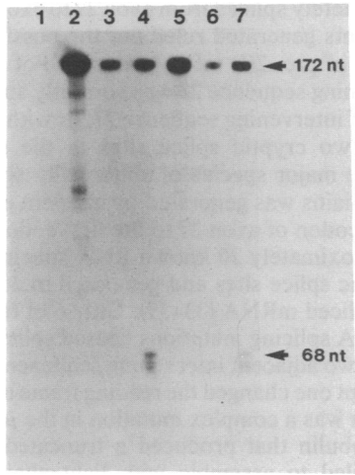

FIG. 5. Probe protection experiment with the 172-nt probe and S1 nuclease. Lanes: 1, unprotected probe; 2 and 5, poly(A)$^+$ RNA from control fibroblasts; 3 and 6, poly(A)$^+$ RNA from an unrelated line of osteogenesis imperfecta fibroblasts (RMS-42); 4 and 7, poly(A)$^+$ RNA from the osteogenesis imperfecta variant studied here. Lanes 1–4 were digested with 2 units of S1 nuclease and lanes 5–7 were digested with 4 units of S1 nuclease. With poly(A)$^+$ RNA from the proband's fibroblasts (lanes 4 and 7), a major band of 68 nt and two minor bands of 66 and 65 nt were seen. The minor bands are a result of S1 nuclease "nibbling" from the ends of heteroduplexes (29). The band of the 68-nt fragment is not as intense as expected if half the mRNA is abnormally spliced, apparently because of differential loss during ethanol precipitation.

at the center of the structure. Therefore, slippage or misregistration of the tripeptide units in one chain relative to the other two can readily occur (31, 32). As a consequence, a mutation that efficiently removes all the codons of one or more exons can generate a shortened proα chain that is incorporated into the triple helix of procollagen (1–4, 13–18).

Previous observations (18, 19) on the osteogenesis imperfecta variant studied here demonstrated that all the proα2(I) chains synthesized by the proband's fibroblasts were shortened because of an in-frame deletion of ≈18 amino acids. The rate of synthesis of the shortened proα2(I) chains was high, since the ratio of newly synthesized proα1(I)/proα2(I) chains was 3:1, or only slightly different from the normal ratio of 2:1. R-loop analysis and probe protection experiments with a double-stranded DNA probe showed that about half the mRNA for proα2(I) chains in the proband's fibroblasts lacked most or all of the codons of exon 28 (19). Therefore, the results indicated that the proband had a mutation in one proα2(I) allele that produced an mRNA that either was not translated or was very inefficiently translated. The other allele had a mutation that was efficiently expressed as shortened proα2(I) chains lacking most or all of the amino acids encoded by exon 28.

Sequencing of genomic DNAs spanning the region around exon 28 demonstrated that one proα2(I) allele from the proband had a single base mutation that converted the universal consensus dinucleotide at the 3′ end of intervening sequence 27 from -AG- to -GG-. The consensus sequence -AG- is found at the 3′ end of all intervening sequences analyzed to date (25, 26). Only three previous mutations were found to alter the consensus -AG- dinucleotide at the 3′ end of an intervening sequence, one in the β-globin gene, one in the dihydrofolate reductase gene, and one in the apolipoprotein E gene (see refs. 33–36). All three mutations prevented use of the site for RNA splicing.

The probe protection experiments with mRNA from the proband's fibroblasts indicated that the major species of abnormally spliced proα2(I) mRNA consisted of an mRNA

that was completely spliced from exon 27 to exon 29. The size of the fragments generated ruled out the possibilities that a major species of proα2(I) mRNA consisted of transcripts in which intervening sequence 28 was correctly spliced without any splicing of intervening sequence 27, or with use of one or both of the two cryptic splice sites in the same region. Therefore, the major species of abnormally spliced mRNA for proα2(I) chains was generated by efficient exon skipping from the last codon of exon 27 to the first codon of exon 29.

Of the approximately 20 known RNA splicing mutations, 14 used cryptic splice sites and generated multiple forms of incorrectly spliced mRNA (33–39). Only 6 of the previously described RNA splicing mutations caused splicing across an exon and the two adjacent intervening sequences as was seen here. All except one changed the reading frame of the mRNA. The exception was a complex mutation in the μ heavy chain of immunoglobulin that produced a truncated polypeptide chain that failed to assemble with light chains (37). The mutation described here, therefore, is one of a limited class of mutations that produce complete and efficient splicing across an exon and two intervening sequences. Also, it is apparently unique among RNA splicing mutations in producing a protein that was both in-frame in terms of coding sequences and in terms of structural properties of the protein. The polypeptide chains produced were used for assembly of a triple-helical procollagen molecule, and the altered primary structure of the proα2(I) chain probably contributed to the lethal phenotype (2, 18, 19).

Because of the large number of similar exons found in procollagen genes (27, 28), RNA splicing mutations are difficult to detect. They may, however, be relatively frequent in individuals with heritable disorders of connective tissue as well as some seemingly normal individuals.

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