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Recurrence of Lethal Osteogenesis Imperfecta Due to Parental Mosaicism for a Dominant Mutation in a Human Type I Collagen Gene (COL1A1)

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Summary

We have determined that two infants with perinatal lethal osteogenesis imperfecta in one family had the same new dominant point mutation. Although not detected in his dermal fibroblast DNA, the mutation was detected in somatic DNA from the father's hair root bulbs and lymphocytes. The mutation was also detected in the father's sperm, demonstrating that mosaicism in the father's germ line explains recurrence. The presence of both germ-line and somatic mosaicism indicates that the mutation occurred prior to segregation of the germ-line and somatic cell progenitors. About one in eight sperm carry the mutation, which implies that at least four progenitor cells populate the germ line in human males. The observation that the mosaic individual is clinically normal suggests that genetic diseases can have both qualitative and quantitative components.

Introduction

Germ-line mosaicism in humans can be suspected when normal parents produce multiple offspring affected with conditions that result either from dominant mutations in autosomal genes or from new mutations in X-linked genes. In these cases a fraction of the gametes of one parent carry a mutation that produces a clinical phenotype in offspring inheriting the mutant allele. The existence of a substantial population of gametes that carry a mutation or the presence of both germ-line and somatic mosaicism indicates that the mutation occurred early in development.

Germ-line mosaicism is thought to account for recurrence of several human disorders that result from autosomal dominant or X-linked mutations. The molecular basis of the disease is known for a few of these disorders (Duchenne muscular dystrophy [Bakker et al. 1987; Darras and Franke 1987; Monaco et al. 1987], perinatal lethal osteogenesis imperfecta [Byers et al. 1988*b*; Byers 1989], and a hemoglobinopathy [Bradley et al. 1980]), but in the majority clinical evidence alone supports mosaicism as the underlying cause of recurrence (David 1972; Ropers and Szliwowski 1979; Allanson 1986; Dodinval and Le Marec 1987; Hall et al. 1987). In Duchenne muscular dystrophy, an X-linked disorder, there is clear molecular evidence of germ-line mosaicism in female parents or male grandparents of affected individuals. However, female gametes are unavailable, and by the time grandparental germ-line mosaicism is recognized, the individual of interest may have died, making direct analysis of germ cells difficult.

The lethal form of osteogenesis imperfecta (OI), OI type II (Sillence et al. 1984), which usually results from new dominant mutations in the COL1A1 or COL1A2 genes, which encode the chains of type I collagen (Barsh et al. 1985; Chu et al. 1985; Cohn et al. 1986; Bateman et al. 1987; Vogel et al. 1987; Byers et al. 1988*a*; Willing et al. 1988), unexpectedly occurs among sibs of probands (Byers et al. 1988*b*; P. H. Byers, unpublished data). Here we show that mosaicism for a point mutation in the COL1A1 gene in paternal sperm explains recurrence of the phenotype in one family. About

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one in eight sperm carry the mutation, indicating that about one-quarter of the cells that contributed to the germ line in the father carried the mutation, thereby establishing a minimum number of four germ-cell progenitors in human males. Somatic mosaicism for the lethal mutation in the clinically normal father suggests that the phenotypic expression of a genetic disease can result from a combination of the nature of a mutation, its abundance, and its expression in target tissues.

Material and Methods

Clinical Summary

The proband, II-1 (our reference number 86-043), was identified by ultrasound at 17 wk gestation on the basis of markedly shortened extremities, sharply angulated femoral and humeral bones, and a small thorax with short ribs. The pregnancy was terminated and radiographic and clinical features compatible with a diagnosis of OI type II were noted. Infant II-3 (reference number 86-160) was the product of an uncomplicated term pregnancy with delivery at 36 wk gestation. Birth weight was 1,920 g, and the infant had dark blue scleras, craniotabes, multiple fractures, short and broad long bones, and a small, bell-shaped thorax. The infant died of respiratory failure in the first day of life. Radiographic and clinical features were compatible with a diagnosis of OI type II.

Labeling and Electrophoretic Analysis of Collagens

Dermal fibroblasts derived from single-punch biopsies were labeled and procollagens were prepared and analyzed as described elsewhere (Bonadio and Byers 1985). Procollagens labeled with [^{3}H]proline were harvested from the culture medium and cleaved with pepsin to produce collagen molecules (Bonadio and Byers 1985). For the cyanogen bromide (CNBr) peptide maps, the chains of type I collagen were separated by SDS-PAGE in 5% polyacrylamide gels in the first dimension, they were cleaved in the gel with CNBr, and the resulting peptides separated in a 12.5% gel in the second dimension (Bonadio and Byers 1985). Isoelectric focusing of CNBr-cleaved, [^{3}H]proline-labeled secreted proteins was carried out as described elsewhere (Benya 1981; Bonadio et al. 1985).

Gene Isolation and DNA Sequence Analysis

Sixty micrograms of genomic DNA was digested with *Eco*RI and size-fractionated by centrifugation through a 10-ml linear 5%–20% NaCl gradient in a Beckman SW41 rotor at 35,000 rpm for 5.5 h. The gradient was collected in 400-μl aliquots, 10% of each fraction was precipitated with ethanol, and DNA fragments were separated by electrophoresis in a 0.7% agarose gel. The DNA in the gel was transferred to nitrocellulose (Southern 1975), and fractions containing the fragment of interest were identified by hybridization with a 2.4-kb *Bam*HI-*Xho*I COL1A1 genomic probe (Barsh et al. 1984) labeled by random primer extension (Feinberg and Vogelstein 1984). The remainder of each hybridizing fraction was pooled and precipitated and 1 μg was ligated with 400 μg of *Eco*RI-cleaved pLAFR1 treated with calf-intestine alkaline phosphatase (Friedman et al. 1982). Ligated concatamers were packaged in vitro (Enquist and Sternberg 1979) using Gigapack Gold™ extracts (Stratagene), and recombinants were introduced into *E. coli* strain TB1. Clones carrying the COL1A1 gene were identified by colony hybridization (Grunstein and Hogness 1975) using the COL1A1 genomic probe described above. DNA sequence determination was by the chain-termination method (Sanger et al. 1977) using Sequenase® (United States Biochemical). The sequences of the oligonucleotide primers are 6-1, CATGCTGAGGGTACTGGCAT; 6-2, GCAGCAGACAAGGCTGTGGT; 6-3, AGGGAACTGGAGCCCAGCTA; 6-4, GGGGGACACAGCAGGGTACT; 6-5.2, CCTATCCCACAGCACAGCAT; 6-6, GTGAGCCTGGGCTTGGGGCT; 6-7, GCAGAGGGGATGAGGGGCTA; 6-C, CGAACCACATTGGCATCATC.

Gene Amplification

Somatic DNA samples were purified from fibroblast cultures with the exception of the sample from II-2, which was purified from lymphocytes. Sperm DNA was prepared from two individual ejaculates collected 15 mo apart. Reactions contained 500 ng genomic DNA in 100 μl of 50 mM KCl, 10mM Tris pH 8.3, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 400 μM of each deoxynucleoside triphosphate, 20 pmol of each oligonucleotide primer (Operon Technologies), and 5 units *Taq* polymerase (Perkin Elmer-Cetus). We performed 25–40 cycles of 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C. To increase the yield of the amplified fragment, products of the reaction were separated by electrophoresis through a 2% low-melting-point agarose gel (BRL) and the 225-bp fragment was resected and purified (Benson 1984). Ten nanograms of the purified amplified fragment was then reamplified under the conditions described above for 15 cycles, and the product repurified. Prior to restriction, samples were denatured and allowed to reanneal to ensure that heteroduplexes were present in proportion to the relative amounts of

the two alleles (see legend to fig. 5). Approximately 100 ng of purified amplified DNA was digested with *BglI* in a 25-μl reaction with 20 units of enzyme, using buffer provided by the manufacturer (BRL). Reaction products were separated by electrophoresis through a 6% acrylamide gel in TBE, stained with ethidium bromide and photographed. Negatives of the gel photographs were analyzed by quantitative densitometry. The sequences of the oligonucleotides are 6-3.2, CAGGTACAGGGAACTGGAGCCCAGC; 6-2R, TGACCACAGCCTTGTCTGCTGCTTC.

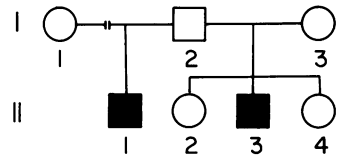


Figure 1 Pedigree of a family with recurrence of the OI type II phenotype. Solid symbols indicate affected infants, both of which died. Roman numerals identify each generation, while individuals within a generation are designated by arabic numerals. These designations are used to identify individuals in subsequent figures.

Results

Recurrence of Lethal Osteogenesis Imperfecta

To understand the molecular basis of recurrence of lethal OI we studied a family (fig. 1) in which two half-siblings with perinatal lethal OI were born to a common father and unrelated mothers. The family structure suggested that the father was mosaic in his germ line for the mutation that produced the OI phenotype. We analyzed type I procollagen molecules produced by dermal fibroblasts cultured from both affected offspring, their father, and the mother of one infant. Cells derived from both affected infants synthesized normal type I procollagen molecules and molecules that contained proα chains that migrated slower than normal chains (fig. 2*A*). Cells from controls and unaffected family members produced only normal type I procollagen molecules. The abnormal molecules synthesized by cells from both infants were secreted less efficiently than normal molecules. Chains synthesized in the presence of an inhibitor of posttranslational prolyl and lysyl hydroxylation (fig. 2*B*) had normal electrophoretic mobilities, which indicated that overmodification accounted for the slow mobility of the abnormal molecules. The α chains of type I collagen, produced by pepsin digestion of procollagen molecules, were also overmodified (fig. 2*C*), which demonstrated that overmodification affected the triple-helical domain.

A Point Mutation Results in Overmodification

All peptides produced by CNBr cleavage of the slowly migrating α1(I) chains migrated slowly, which showed that the entire triple-helical domain was overmodified (fig. 3). This finding suggested that a mutation that either delayed propagation of the triple helix from its start at the carboxyl-terminal end of the molecule or created an unstable triple helix resulted in prolonged exposure of the chains in abnormal molecules to the modifying enzymes. Separation of CNBr peptides by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension demonstrated that there had been an acidic charge change in α1(I)CB6 (residues 823–1014 of the triple helix) of some α1(I) chains synthesized by cells from both affected infants (fig. 3). The charge change in α1(I)CB6 was not present in collagens synthesized by cells from the father and the available mother.

We isolated both COL1A1 alleles from one of the affected infants (II-1) and determined the nucleotide sequences of the exons that encode α1(I)CB6 (fig. 4). The alleles were distinguished by the fortuitous finding of two DNA sequence polymorphisms (in introns) discovered during the sequence analysis (see fig. 4). The mutation that produced the charge shift was a single nucleotide change (G to A) which resulted in substitution of aspartic acid for the glycine at position 883 of the triple helix. The structure of this mutation is analogous to those seen for several sporadic cases of lethal OI (Cohn et al. 1986; Bateman et al. 1987; Vogel et al. 1987; Byers 1989).

Germ-Line and Somatic Mosaicism

Because the mutation disrupted a *BglI* restriction site we could recognize the abnormal allele in the other affected infant and in the unaffected members of the family. We used synthetic oligonucleotide primers to amplify (Saiki et al. 1988) a 225-bp fragment of genomic DNA that contained the exon with the mutation (fig. 5*A*). Both affected infants carried the normal and mutant alleles. The mutation was absent from fibroblast DNA from the father, the one available mother, and an unaffected sister of II-3. The mutation was present in sperm DNA from the father, confirming that germline mosaicism for the mutation explains recurrence of the phenotype in this family.

Following amplification of the target sequence in DNA from both affected infants, cleavage by *BglI* produced the 72-bp and 63-bp fragments in a 3:1 ratio.

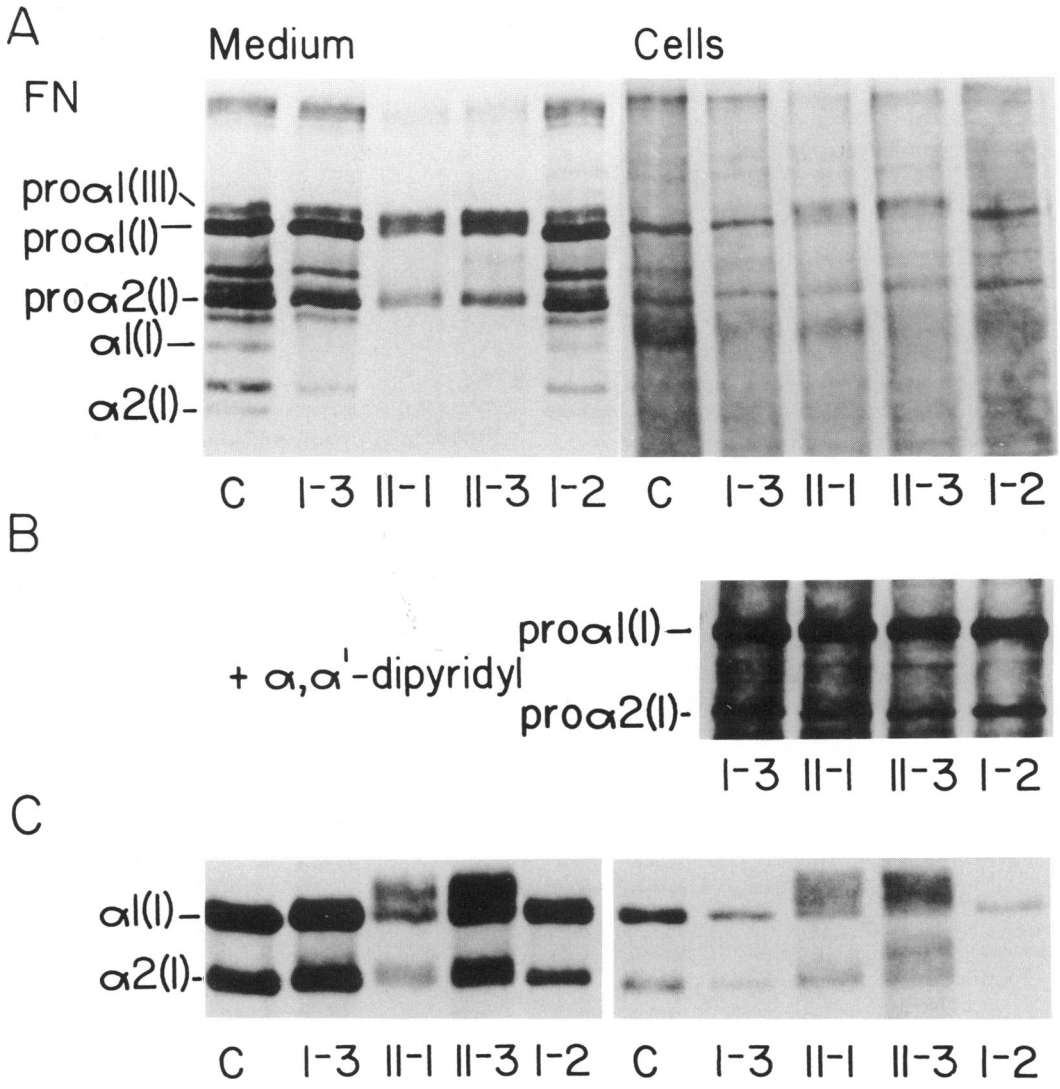


Figure 2 Electrophoretic analysis of ^{3}[H]proline-labeled procollagens and collagens. *A*, Proα chains from the medium and cell layers of a control (C), the mother (I-3) of II-3, the two affected infants (II-1 and II-3) and the father (I-2). Chains were separated under reducing conditions. Lanes are labeled as in fig. 1. *B*, Proα chains synthesized in the presence of α,α′-dipyridyl. Chains were separated under reducing conditions. Lanes are as in *A*. *C*, α Chains of type I collagen produced by pepsin digestion of procollagen samples. Chains were separated under nonreducing conditions. Lanes are as in *A*.

The unexpected ratio of abnormal to normal alleles in DNA from the infants was due to formation of interallelic heteroduplexes between strands of the amplified fragments during the last rounds of amplification (see legend to fig. 5). In amplified and cleaved DNA from the father's sperm, the ratio of the fragments was 1:3. If the relative efficiencies of amplification of the two alleles between fibroblast DNA and sperm DNA were the same, and if the ratio observed in the amplified DNA from the infants reflected a genomic ratio of 1:1, then approximately one in eight of the father's sperm carried the mutation.

We analyzed a second sperm sample and two additional somatic tissues from the father for the presence and abundance of the mutant allele (fig. 5*B*). The second sperm sample had a slightly higher level of the mutant allele than the original sample (one mutant allele per six normal alleles vs. one mutant per seven normal alleles in the first sample). DNAs from white blood cells and from a pool of 10 hair root bulbs each contained

the mutation in about 20% of the alleles (40% of the diploid cells). The mutant allele was detected in two of four DNA samples from individual hair root bulbs (data not shown). Thus, although the father has no clinical signs of OI, he carries the mutation in at least some of his somatic tissues.

Discussion

A Point Mutation Is Sufficient to Produce Lethal OI

We have demonstrated that germ-line mosaicism explains recurrence of lethal OI in this family. Furthermore, these data provide strong evidence to support the hypothesis (Byers and Bonadio 1985; Cohn et al. 1986) that point mutations in one allele that result in substitution for one triple-helical glycine residue in the type I collagen molecule are sufficient to produce the OI type II phenotype. It has been difficult to exclude the possibility that a background mutation in the population must contribute to the phenotype in sporadic cases, but the appearance of the same phenotype in these two infants argues strongly that only the point mutation is necessary. The construction of a transgenic mouse with an OI type II phenotype by introduction of a COL1A1 allele carrying a mutation that resulted in a cysteine-for-glycine substitution at triple-helical position 859 (Stacey et al. 1988) further supports the idea that such mutations are sufficient to produce the phenotype. In conjunction with population studies (Thompson et al. 1987; Byers et al. 1988*b*), our data suggest that recessive forms of OI type II (deWet et al. 1983) are rare and that germ-line mosaicism provides a preferred explanation for recurrence in families.

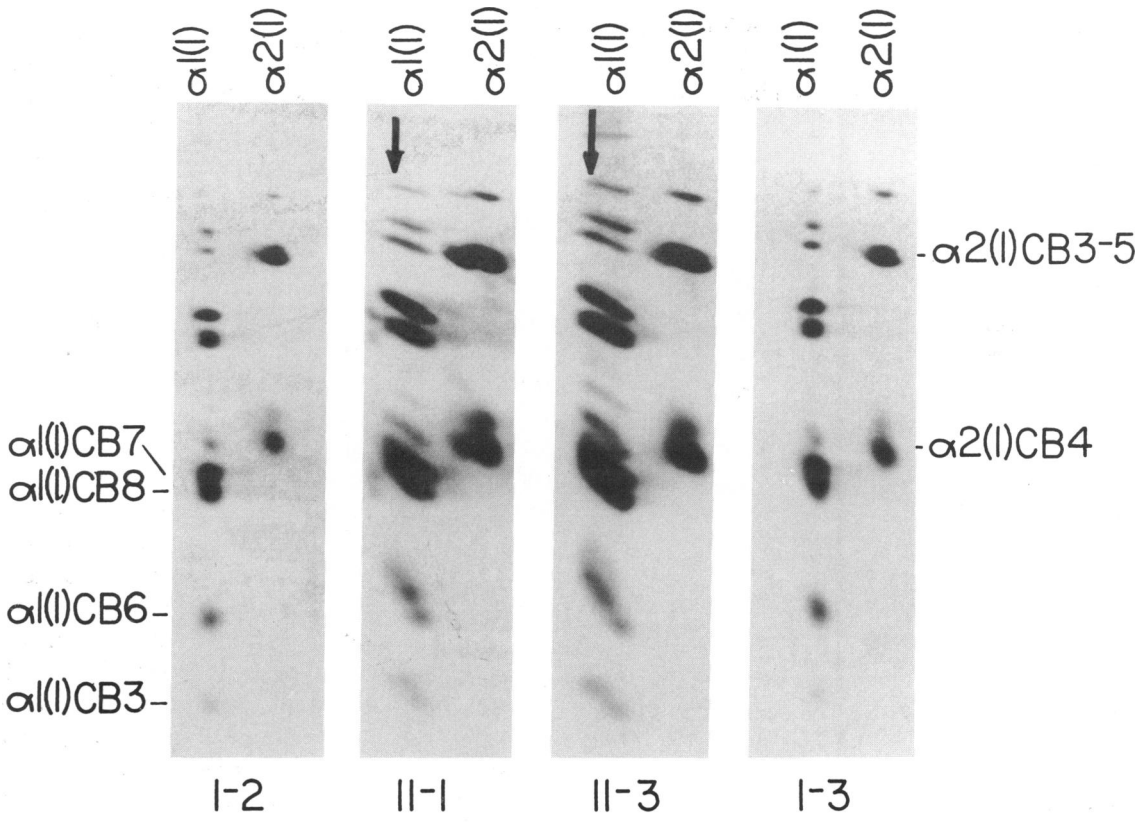
Estimation of Primordial Germ-Cell Number

If subsequent to the time the mutation occurred all primordial germ cells contributed equally to the final composition of gametes, then the proportion of abnormal sperm indicates that one-quarter of the primordial cells that formed the germ line carried the mutant allele. This establishes a minimum number of four germ-line precursor cells in human males, but sets no maximum. Because we know little about early events in germ-cell formation, there are several assumptions inherent in our conclusion: (1) there is no cell selection that results from the mutation; (2) all cells segregated to form the germ line divide at equal rates; (3) loss of germ-cell primordia during development is random; and (4) the fraction of cells lost is low, so that stochastic processes do not significantly affect the final composition of the germ line. Although the apparent level of mosaicism in sperm may change over time due to cycling of sperm stem-cell populations, the similarity in the level of mosaicism in the two sperm samples argues that they are representative of the entire germ-cell population in the mosaic individual and that the mutant allele is a stable component of the father's sperm.

Studies of mice have suggested that a small number of cells is allocated to form the germ line early in embryonic development. Studies of allophenic mice have led to the estimate that there are two to nine primordial germ cells (Mintz 1974). Quantitation of germ-line involvement in animals mosaic in somatic cells for mutations in coat-color genes has suggested that very few cells (Searle 1978), perhaps as few as five (Russell 1964), form the germ line. A minimum number of three primordial germ cells—determined after retroviral infection of mouse blastomeres destined to give rise to the germ-line primordia (Soriano and Jaenisch 1986)—is also compatible with estimates from the other sources. Measurements of the ratio of the X-linked phosphoglycerate kinase isozymes among cell types, including germ-line precursors, has suggested that all three germ layers and the germ line in the mouse are derived from a common precursor pool estimated to be about 200 cells (McMahon et al. 1983). In humans, analysis of proportions of cells that express the X-chromosomal A and B isozymes of glucose-6 phosphate dehydrogenase has suggested that cells from several tissues of mesodermal origin are derived from a common pool of about 16 cells (Fialkow 1973), but no such estimate is available for germ-cell precursors.

Somatic Mosaicism

In transgenic mice that develop after infection of some blastomeres with a retrovirus, the extent of somatic mosaicism for the presence of individual integrated viruses is the same in all somatic tissues, which implies that there is free mixture and equal contribution of a small pool of somatic cell progenitors to all tissues (Soriano and Jaenisch 1986). Thus if the segregation of somatic cell lineages is similar in mice and humans, we would expect to detect mosaicism at a uniform level in all somatic tissues (Soriano and Jaenisch 1986). While the similarity in the level of mosaicism in hair root and lymphocyte DNA samples supports this concept, the absence of detectable mosaicism in fibroblast DNA suggests that if the hypothesis holds in humans, the mutation occurred in a precursor that contributed to the germ line but not all somatic tissues, there was selection against cells that carried the mutant allele in the fibro-



blast lineage, or the fibroblast culture we analyzed was of clonal origin.

Frequency of Germ-Line Mosaicism Differs among Diseases

We are aware of eleven families in which recurrent OI type II among siblings is explained best by parental germ-line mosaicism for a mutation in one of the genes of type I collagen (Byers et al. 1988*b*; P. H. Byers, unpublished data). The frequency of parental germ-line mosaicism for a mutation that produces a recognizable OI type II phenotype in children (11 families of approximately 140 studied) is comparable to that estimated for pseudoachondroplasia (Hall et al. 1987) and Duchenne muscular dystrophy (Bakker et al. 1987), but this rate appears to be considerably higher than that for many other dominant disorders, in which sibship recurrence is rarely recognized. The apparent differences in recurrence rate among sibs due to parental germ-line mosaicism for a mutation could have several explanations. First, germ-line mosaicism would be ascertainable with highest frequency when the mutation rate for a gene was high early in germ-cell differentiation. Second, germ-line mosaicism as a cause for recurrence of a disorder among sibs may be overlooked in favor of recessive inheritance. Third, if a lethal phenotype can be produced by somatic mosaicism for a mutation in a single gene, then such conditions would not be expected to recur among sibs, yielding a very low recurrence rate in a sibship.

Clinical Implications of Germ-Line Mosaicism

Germ-line mosaicism for a dominant mutation rather than recessive inheritance may explain recurrence of other disorders in children of unaffected parents. A consideration of germ-line mosaicism for a dominant mutation may be particularly appropriate where there is not a defined biochemical or genetic marker for the disease. In light of molecular evidence of germ-line mosaicism in Duchenne muscular dystrophy (Bakker et al. 1987; Darras and Franke 1987; Monaco et al. 1987) and in a hemoglobinopathy (Bradley et al. 1980) and clinical evidence consistent with germ-line mosaicism in a number of other disorders (pseudoachondroplasia [Hall et al. 1987], ectrodactyly [David 1972], Apert syndrome [Allanson 1986], achondroplasia

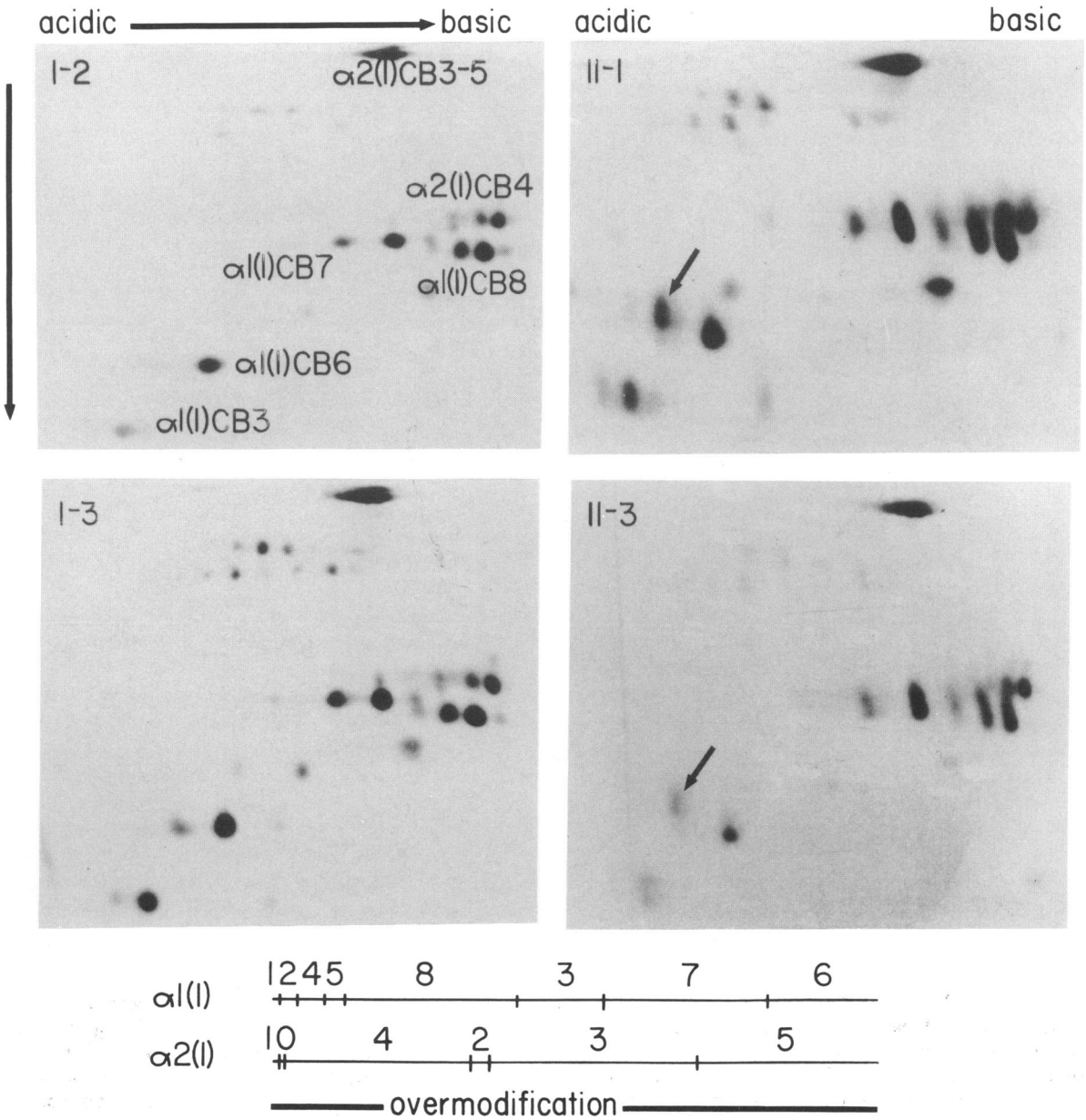


Figure 3 Localization of a defect in protein structure. Left panel (this page), two-dimensional analysis of labeled, CNBr-cleaved collagens. Individuals are identified as in the pedigree (fig. 1). The position of the slowly migrating α chain in the first-dimension separation is identified by the vertical arrow. Each CNBr peptide from the slowly migrating α1(I) chain migrates slower in the second-dimension gel than its homologue from the normal chain, a finding best explained by excessive posttranslational modification along the full length of the triple helix. Right panel (facing page), labeled collagens from the medium, cleaved with CNBr, separated by isoelectric focusing in a first dimension, and separated by size in the second dimension. The acidic and basic ends of the first-dimension gel are indicated with the direction of migration indicated by a horizontal arrow. The vertical arrow indicates the direction of separation for the second dimension gel. The identities of the CNBr peptides from the α1 and α2 chains of type I collagen are indicated, with their locations within the chains and the extent of overmodification shown on the line diagram below. For collagens from the two affected infants, an arrow identifies the α1(I)CB6 peptide with the acidic shift.

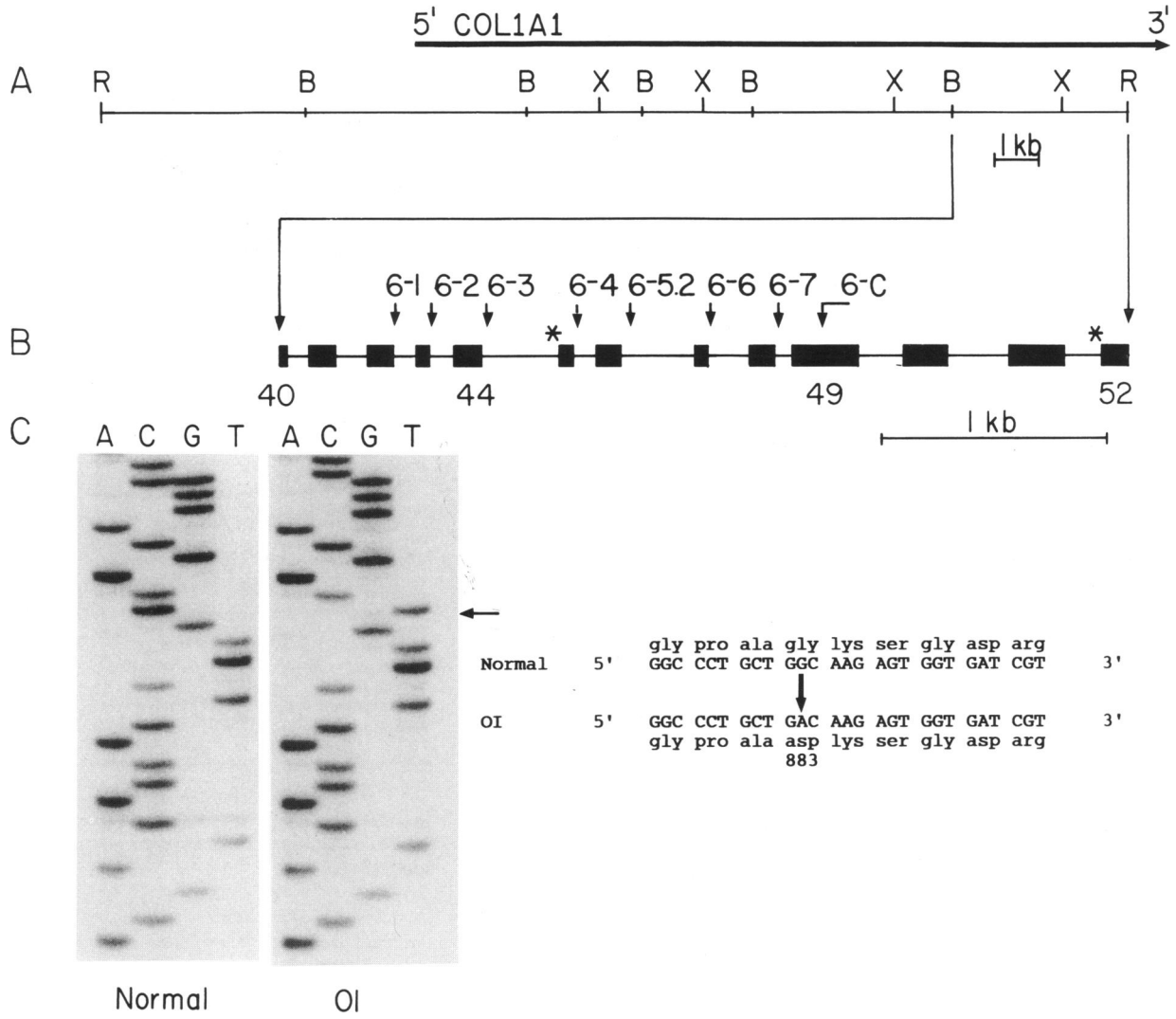


Figure 4 Isolation and DNA sequence analysis of the COL1A1 alleles. *A*, Restriction map of the 23-kb *Eco*RI fragment isolated from cells derived from II-1 and its context with respect to the COL1A1 transcription unit (arrow). *B*, Gene structure of the region of COL1A1 encoding α1(I)CB6. Exons are shown as solid boxes and selected exons are numbered below. Eight synthetic oligonucleotide primers were used for analysis of the nucleotide sequences of the exons in the region. The sites at which the primers anneal and their orientations are indicated with arrows. Oligonucleotide 6-C is complementary to exon sequences, while the remainder were designed to be complementary to intron sequences 3′ to the exon from which sequence was to be derived. Asterisks mark the locations of the two nucleotide polymorphisms used to distinguish the alleles. One was a nucleotide polymorphism (T or A) 126 nucleotides 5′ to the *Eco*RI site used in the subcloning. The other was a single nucleotide change in intron 44, which disrupted a *BalI* site. *C*, Nucleotide sequence using primer 6–3. The mutation was a single base change in the codon for the glycine at position 883 of the α1(I) chain. The nucleotide sequences of the normal allele and the mutant allele (OI) and their implied protein sequences are shown. The sequence of the message complementary strand is shown.

[Dodinval and Le Marec 1987], and periodic hypokalemic paralysis [Ropers and Szliwowski 1979]), we suspect that parental germ-line mosaicism is more common than previously appreciated and may account for apparent genetic heterogeneity in these and additional disorders. For OI type II, our best estimate of recurrence risk is the clinically observed recurrence rate of 6%–7% (Byers et al. 1988*b*). However, this figure implies an underlying frequency of parental mosaicism that could be significantly higher. Where the mutation is known, our studies provide a way to analyze the frequency and level of mosaicism in sperm from fathers

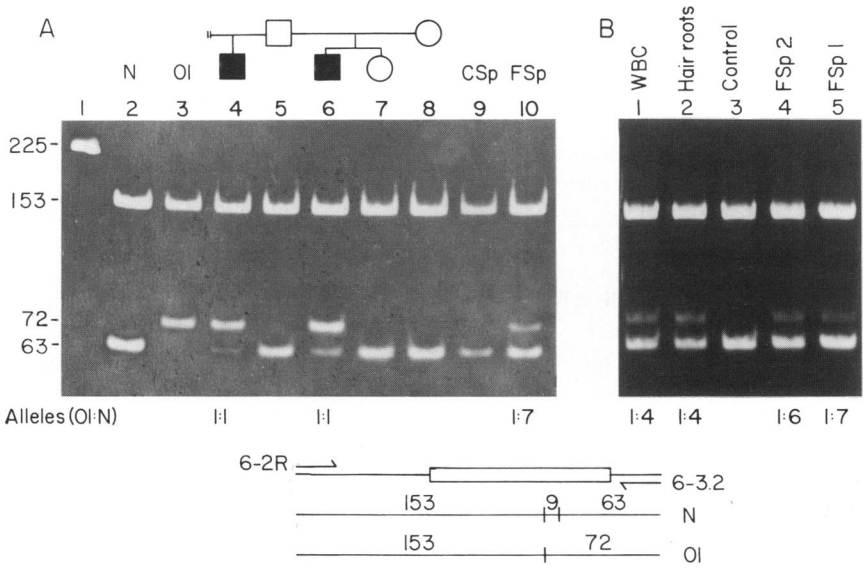


Figure 5 Amplification of the genomic region of COL1A1 carrying the mutation. The structure of the amplified fragment and the orientations of the primers, 6-3.2 and 6-2R, are shown on the line diagram below. The exon carrying the mutation is boxed. Locations of the *BglI* sites in the fragment are indicated by vertical lines, and the sizes of the restriction fragments from the normal allele (N) and the mutant allele (OI) are indicated. *A,* Family study. Lane 1 contains an undigested amplified fragment. Lanes 2 and 3 contain amplified, restricted products derived from the isolated normal (N) and mutant (OI) alleles, respectively, from the infant in lane 4. The pedigree is shown above the lanes (4–8) containing amplified somatic DNA from the family. Lanes 9 and 10 contain amplified products of sperm DNA from control (CSp) and the father (FSp). *B,* Analysis and quantitation of mosaicism in sperm and somatic tissues from the father. DNA derived from the tissues indicated above the lanes was amplified and digested with *BglI*, and the resulting fragments were separated by gel electrophoresis. The ratios of the two fragments are shown below each of the lanes in which the abnormal allele was detected. The 1:3 ratio of cleaved to uncleaved fragments in amplified DNA from the two affected infants reflects an artifact of the amplification process in that, during the last few rounds of amplification, the high concentration of the amplified fragments drives annealing of amplified fragments to each other (D. H. Cohn and P. H. Byers, unpublished data). The two heteroduplexes formed with one strand from each allele cannot be cleaved at the 3′ *BglI* site (because of the mismatch within the site) which results in apparent enrichment of the abnormal allele. Near the end of the amplification, when the annealing of amplified strands with each other is the dominant reaction and the initial ratio of normal to abnormal alleles is 1:1, the predicted ratio of uncleaved to cleaved products is 3:1, which is precisely the ratio we observe. By mixing amplified products from the two alleles, heat denaturing and reannealing, we have demonstrated the predicted denaturation-dependent shift in the ratio of the restriction fragments that is governed by the relative concentrations of the alleles (data not shown).

and somatic tissues from mothers of OI type II infants, regardless of whether there has been recurrence in a family.

In transgenic mice that carried a transgene in which a point mutation produced substitution for a triple-helical glycine residue, a phenotype analogous to OI type II resulted even when the transgene accounted for as little as 10% of the COL1A1 mRNA (Stacey et al. 1988). In the mosaic father described here, even though as many as 40% of the cells in some of his tissues carry a mutation that is lethal in his heterozygous offspring, he has no clinical phenotype. This observation suggests that, in addition to the qualitative elements of a defect, phenotypic expression of genetic diseases produced by structural changes in proteins may depend on the abundance of the cells that carry the mutation and their distribution in tissues.

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