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Parental Somatic and Germ-Line Mosaicism for a Multiexon Deletion with Unusual Endpoints in a Type III Collagen (COL3A1) Allele Produces Ehlers-Danlos Syndrome Type IV in the Heterozygous Offspring

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Summary

Ehlers-Danlos syndrome (EDS) type IV is a dominantly inherited disorder that results from mutations in the type III collagen gene (COL3A1). We studied the structure of the COL3A1 gene of an individual with EDS type IV and that of her phenotypically normal parents. The proband was heterozygous for a 2-kb deletion in COL3A1, while her father was mosaic for the same deletion in somatic and germ cells. In fibroblasts from the father, approximately two-fifths of the COL3A1 alleles carried the deletion, but only 10% of the COL3A1 alleles in white blood cells were of the mutant species. The deletion in the mutant allele extended from intron 7 into intron 11. There was a 12-bp direct repeat in intron 7 and intron 11, the latter about 60 bp 5′ to the junction. At the breakpoint there was a duplication of 10 bp from intron 11 separated by an insertion of 4 bp contained within the duplicated sequence. The father was mosaic for the deletion so that the gene rearrangement occurred during his early embryonic development prior to lineage allocation. These findings suggest that at least some of the deletions seen in human genes may occur during replication, rather than as a consequence of meiotic crossing-over, and that they thus have a risk for recurrence when observed de novo.

Introduction

Ehlers-Danlos syndrome type IV (EDS type IV; the arterial or ecchymotic form of Ehlers-Danlos syndrome) is a dominantly inherited connective tissue disease characterized by severe tissue fragility that leads to premature death as a result of arterial, bowel wall, or uterine rupture (Beighton 1970; Rudd et al. 1983; Pope et al. 1988; Byers 1989), the consequences of mutations in the COL3A1 gene that encodes the chains of type III procollagen (Tsipouras et al. 1986; Byers 1989; Superti-Furga et al. 1989; Kuivaniemi et al. 1991). The mutations include multiexon deletions in the COL3A1 allele (Lee et al. 1991*a;* Vissing et al. 1991), small in-frame deletions (Richards et al. 1992*a*), missense mutations that result in substitution for glycine in the triple-helical domain by other residues (Tromp et al. 1989*a,* 1989*b;* Kontusaari et al. 1990*b;* Johnson et al. 1992; Richards et al. 1991, 1992*b*), and point mutations that result in splicing errors (Cole et al. 1990; Kontusaari et al. 1990*a;* Kuivaniemi et al. 1990; Lee et al. 1991*b*).

We recently identified a 4-exon (2-kb) deletion in one COL3A1 allele from a 13-year-old girl with EDS type IV whose asymptomatic father was mosaic for the mutation. At the breakpoint in the mutant COL3A1 allele, there was a unique gene rearrangement, a pattern that may help to distinguish deletions that arise during DNA replication from those that occur as a result of homologous recombination during meiosis. These find-

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ings suggest that de novo deletions due to nonhomologous recombination have a risk for recurrence in siblings, because the deletions occurred during mitotic DNA replication and prior to meiosis.

Subject and Methods

Clinical Summary

The proband was the product of a normal-term gestation and was the first child born to a nonconsanguineous couple. Delivery was induced because of amniotic fluid that leaked for the 2 d prior to birth. There were no postnatal difficulties, and the infant went home at 3 d of age. Easy bruising was noted as she began to crawl. Healing of cuts occurred with keloids, a trait she shared with her father. She was well, until 13 years of age when she presented to the emergency room with periumbilical and suprapubic pain of about 8 h duration. Abdominal ultrasound demonstrated a possible pelvic mass. On laparotomy a "probably" perforated fallopian tube was identified and removed. She remained hospitalized for 3 wk, because of large fluid requirements. Free air under the diaphragm prompted reoperation, and 2 liters of purulent fluid was identified, but no perforation of the gastrointestinal (GI) tract could be found. Multiple adhesions and friable bowel were noted. Four days later, an upper-GI series was obtained that suggested perforation of the upper jejunum. At surgery, the region of the jejunum was externalized to create a jejunostomy. Two weeks later, a lower-GI series was suggestive of leaking either from the sigmoid or from the distal ileum. She subsequently improved without further surgery and was released. She has had no further complications. At the time of hospitalization, physical examination documented height of 5'2" and weight of 110 pounds. She had translucent skin with visible vessels over the chest and with discrete hypopigmented keloid scars of the lower extremities and the upper arms. There were scattered bruises. Her fingers were long and thin, and hyperextensibility was limited to the midphalangeal joints. There were no other affected family members.

Radiolabeling of Fibroblast Cultures and Analysis of Collagens

Skin biopsies were taken from the proband and her parents, with appropriate consent. Growth and maintenance of dermal fibroblasts, labeling of collagenous proteins, and their analysis were performed as described elsewhere (Bonadio et al. 1985).

Table 1

Sequence of Oligonucleotide Primers

Primer	Sequence
E6D	5'-AAGAATTCTCCCCAGTATGATTCATATG-3'
E7D	5'-GGGCATGCCCCCCTGGTACATCTGGT-3'
E8U	5'-GGGGATCCTTGGTATCCTGGAGATCC-3'
E11D	5'-AGGTCGACCTGGGATACCTGGATTCC-3'
E12U	5'-TTGGATCCAGGAGCACCTGTTTCACC-3'
E25U	5'-CTGGTCGACCACTTTCTCCTTGACT-3'

NOTE.—The underlined nucleotides represent the restriction sites used to clone the amplified fragments, when necessary.

Restriction-Endonuclease Analysis of Genomic DNA

DNA was prepared from fibroblasts, white blood cells, or sperm by standard methods (Maniatis et al. 1982), except that 10 mM DTT was added to the sperm sample during proteinase K digestion. Very little DNA was isolated from the father's semen samples (<1 μg), consistent with a low sperm count (<2 × 10^6 sperm/ml) thought to be secondary to mumps orchitis in adolescence. Aliquots of genomic DNA from cultured dermal fibroblasts, lymphocytes, and semen were digested with *Hin*dIII or *Bam*HI (New England Biolabs) according to the manufacturer's specifications. The fragments were separated by gel electrophoresis in 0.8% agarose and then were transferred to a Nytran membrane (Schleicher and Schuell) (Maniatis et al. 1982). The filter was hybridized separately with the partial proα1(III) cDNA probe, HpL14 (Superti-Furga et al. 1989), and with a 3.5-kb internal *Hin*dIII fragment from the COL3A1 genomic fragment, IDF-1 (Benson-Chanda et al. 1989). The hybridization probes were labeled with α-[^{32}P]dCTP (New England Nuclear) by using a random-primer labeling kit (Biorad).

DNA Sequence Determination

Total RNA was prepared from dermal fibroblasts of the proband (Chomczynski and Sacchi 1987). The sequence of oligonucleotide primers used for amplification (table 1) was derived from the published cDNA sequence for the COL3A1 gene (Ala-Kokko et al. 1989; Benson-Chanda et al. 1989). The cDNA was prepared by using an oligonucleotide complementary to exon 25 (E25U) of the COL3A1 gene, as a primer for reverse transcription (Willing et al. 1990). The cDNA was used as a substrate for amplification by PCR (annealing at 56°C, extension at 72°C, denaturation at 96°C, for 1, 2, and 1 min, respectively, for 28 cycles) with GeneAmp reagents (Perkin Elmer Cetus) by using primers located

in exon 6 (E6D) and exon 12 (E12U). The amplified DNA was purified, and the double-stranded DNA was sequenced by the dideoxy-chain termination method by using T7 polymerase (Sequenase; U.S. Biochemicals) as described elsewhere (Sanger et al. 1977).

The region of the mutant COL3A1 allele between exon 7 and exon 12 was amplified from the proband's DNA by using primers E7D and E12U. The amplified fragment was purified and was cloned into M13 phage, and single-stranded DNA was sequenced by the dideoxy-chain termination technique (Sanger et al. 1977). Intron 7 from the normal COL3A1 allele was amplified by using primers in exon 7 (E7D) and exon 8 (E8U), and intron 11 was amplified by using primers in exon 11 (E11D) and exon 12 (E12U). The amplified products were purified by both electrophoresis through low-melting-temperature agarose and passage through Chroma Spin™-100 column (Clon Tech, Palo Alto, CA), and the DNA sequence was determined, as above, with the primers used for amplification.

Results

Dermal Fibroblasts from the Proband and Her Father Secrete an Abnormal Type III Collagen

Dermal fibroblasts from the proband and from her mother and father synthesized and secreted normal amounts of type I procollagen (fig. 1*A*). In contrast, cells from the proband secreted very little type III procollagen. While the mother's cells secreted normal amounts of type III procollagen, those from the father secreted approximately half the amount of the control cell strain. Following partial proteolysis with pepsin of the secreted and intracellular collagens, the type III collagen synthesized and secreted by the proband's cells was heterogeneous. There was a small amount of type III collagen with a normal electrophoretic mobility and a similar amount with a faster than normal mobility (fig. 1*B*). The father's cells secreted approximately 30% of the normal amount of electrophoretically normal type III collagen, as well as a lesser amount of an abnormally migrating type III collagen that comigrated with the abnormal form secreted by the proband's cells. The mother's cells secreted normal amounts of a normally migrating type III collagen.

Deletion in One COL3A1 Allele in the Proband and the Father

Southern analysis of fibroblast DNA by using a partial proα1(III) cDNA probe (HpL14) identified novel *Hin*dIII and *Bam*HI fragments in the DNA from the proband's cells and the father's cells (fig. 2*A*). The normal *Bam*HI fragments were 14 kb, 9 kb, and 7 kb in size. The novel *Bam*HI fragment present in the proband's DNA was approximately 5 kb, and there was a decrease in intensity of the 7-kb *Bam*HI fragment, compatible with deletion of about 2 kb from within the 7-kb fragment (see figs. 2*C* and *D*). The novel *Hin*dIII fragment present in proband DNA was larger than the 7-kb *Hin*dIII fragment normally present, compatible with deletion of a *Hin*dIII restriction site (see fig. 2*C*). The deletion mapped to the region of the gene that encodes the amino-terminal end of the triple-helical domain. The same novel bands were present in DNA from the father's fibroblasts. The proportion of the mutant and normal alleles in the fibroblast DNA from the father and the proband was similar.

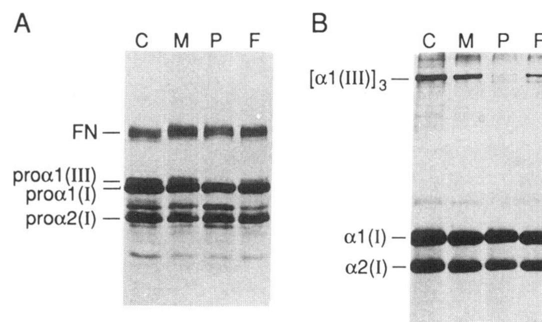

Figure 1 Proα and α chains synthesized by cultured fibroblasts. *A*, Proα chains of type I and type III procollagen separated under reducing conditions. Cells from the proband (P) secrete much less type III procollagen than do cells from the control (C) or from either the mother (M) or the father (F). *B*, Chains of type I and type III collagen separated under nonreducing conditions. Type III collagen migrates as a disulfide-bonded trimer. Cells from the proband secrete both a small amount of type III collagen with a normal mobility and an equal amount of type III collagen with a faster-than-normal mobility. There is a small amount of material that migrates between the two bands and that probably represents heterotrimers that contain both normal and abnormal chains. Cells from the father secrete less normal type III collagen and a small amount of the abnormally migrating protein.

The location of the deletion was confirmed by using the 3.5-kb *Hin*dIII genomic probe, IDF-1 (fig. 2*B* and *C*). DNA from the proband and her father contained novel fragments present on both digests, corresponding to the deleted allele. Single *Hin*dIII and *Bam*HI fragments were identified in fibroblast DNA from the mother (and from a control; data not shown) when probed with IDF-1.

Frequency of the Deleted COL3A1 Allele in Different Tissues from the Father

DNA from white blood cells of the proband contained the mutant allele and the normal allele, in equal

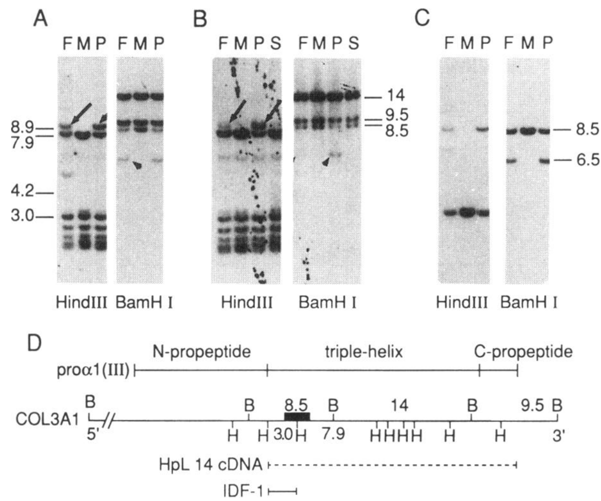

Figure 2 Location and quantitation, in different tissue sources, of the COL3A1 gene deletion. *A*, Fibroblast DNA cleaved with *Hin*dIII or *Bam*HI and probed with a partial cDNA sequence. The presence of a new *Hin*dIII band at 8.9 kb (*arrow*) and a new *Bam*HI band at 5.7 kb (*arrowhead*) is compatible with deletion of a *Hin*dIII site within the 7.9-kb *Bam*HI fragment near the 5′ end of the domain that encodes the triple helix of the proα1(III) chain. The two alleles are of equal proportion in the DNA from proband (P) and are of near equal proportion in the DNA from the father (F). *B*, Genomic DNA from white blood cells, cleaved with *Hin*dIII or *Bam*HI and probed with a partial cDNA sequence. The mutant and normal alleles are present in equal amounts in DNA from the proband, but only a small amount of the mutant allele is present in DNA from the father's white blood cells. The mutant allele is not present in DNA from the mother's (M) white blood cells or in that from the unaffected sister (S). *C*, Fibroblast genomic DNA cleaved with *Hin*dIII or *Bam*HI and probed with the 3-kb genomic *Hin*dIII-*Hin*dIII fragment (IDF-I). *D*, Partial restriction map of the COL3A1 gene, the approximate location of the sequences that encode the major regions of the proα1(III) chain, the probes used, and the location of the genomic deletion (*thick black bar*).

proportion. The deletion allele represented about 10% of the COL3A1 alleles (fig. 2). DNA derived from the white blood cells of both the mother and the unaffected sibling contained only normal COL3A1 alleles. The mutant allele was represented in sperm DNA from the father, but precise quantitation was difficult because of the small amount of DNA available (data not shown).

Sequence Determination of the Deleted Message and Gene

To determine the extent of the deletion, cDNA was synthesized from RNA obtained from the proband's fibroblasts, and primers in exon 6 and exon 12 were used to amplify the cDNA. Two fragments were amplified, one of approximately 315 bp and the other of approximately 95 bp. The 95-bp fragment contained the sequences of exon 7 and exon 12 but lacked those of exons 8–11 (fig. 3*A*). The appropriate Gly-X-Y reading frame was maintained in the shortened fragment. The larger fragment contained only the normal COL3A1 cDNA sequence.

Primers in exon 7 (E7D) and exon 12 (E12U) were used to amplify a 660-bp fragment from genomic DNA of the proband. The normal allele of approximately 3 kb did not amplify efficiently under the amplification conditions. The deletion junctions were approximately 50 bp from the 3′ end of exon 7 in intron 7 and 250 bp from the 5′ end of exon 12 in intron 11. At the breakpoint in intron 7 there was a 12-bp sequence that was identical to a sequence approximately 60 bp 5′ to the breakpoint in intron 11; a 5-bp motif (CAAAA) contained in the repeat was also contained in DNA just 3′ to the breakpoint in intron 11 (see fig. 4). At the breakpoint itself, there were both a duplication of the sequence from intron 11 and a 4-bp insertion.

Discussion

During the cell divisions required to go from a single-cell embryo to an adult, a number of mutations must

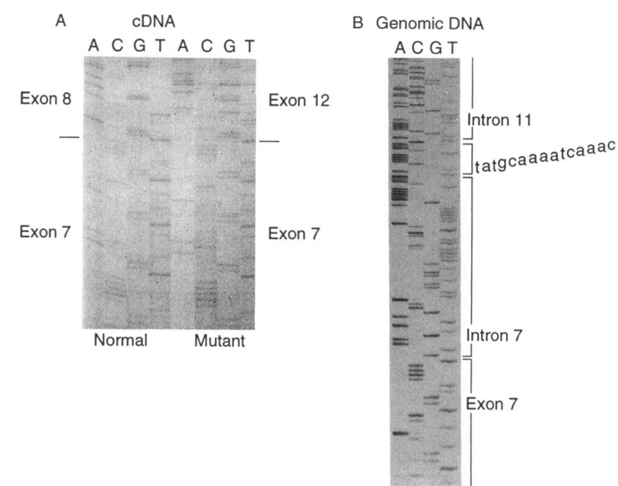

Figure 3 DNA sequence of cDNA of the normal and mutant alleles and of genomic DNA derived from the mutant COL3A1 allele, through the deletion junctions. *A*, cDNA sequence. The cDNA was amplified by using primers in exon 6 and exon 12, and the double-stranded product was sequenced by using the exon 6 primer. The sequence of exon 7 is directly adjacent to that of exon 12 in the mutant allele. *B*, Sequence of the mutant COL3A1 allele in exon 7 and intron 7/11.

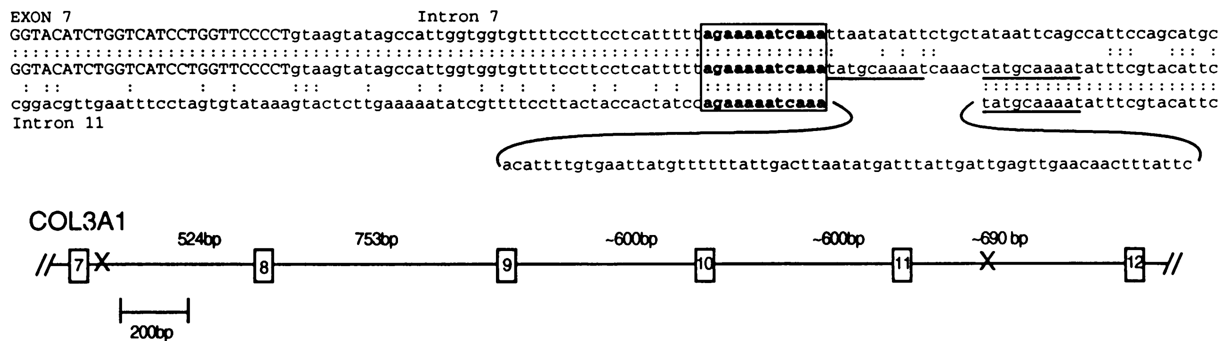

Figure 4 *Top,* Sequence of the normal COL3A1 allele (*top* and *bottom*) and of the mutant allele (*center*). The 12-bp identical domains in intron 7 and intron 11 are boxed, the duplication at the junction is underlined, and the inserted sequence (caaa) is left unmarked. The DNA sequence deleted between the 12-bp duplication and the duplicated region in intron 11 is placed below the bottom line. Exon sequence is in uppercase letters, and intron sequence is in lowercase letters. *Bottom,* Schematic arrangement of the segment of the COL3A1 gene in which the rearrangement occurred. The exons appear as boxes, and the introns appear as lines. The X in intron 7 and intron 11 marks the position of both the duplicated 12-bp sequence and the boundaries of the deletion. Exons 7–12 contain 54 bp each. The size (in bp) of each intron is indicated.

occur within all normal individuals. Clonal expansion of these mutation-bearing cells gives rise to somatic and/or germ-line mosaicism. Germ-line mosaicism has been used to explain the birth to unaffected parents of more than one child affected with a dominantly inherited or X-linked recessive disorder (David 1972; Fryns et al. 1983; Edwards 1986; Byers et al. 1988). With molecular probes, germ-line and somatic mosaicism for mutations has been demonstrated in several disorders, including Duchenne muscular dystrophy (Bakker et al. 1987; Darras and Francke 1987; Wood and McGillivray 1988; Lebo et al. 1990), different forms of osteogenesis imperfecta (Constantinou et al. 1989, 1990; Cohn et al. 1990; Wallis et al. 1990), ornithine transcarbamylase deficiency (Maddalena et al. 1988; Legius et al. 1990), hemophilia A (Higuchi et al. 1988; Gitschier et al. 1989; Bröcker-Vriends et al. 1990), factor IX deficiency (Taylor et al. 1991; Solera et al. 1992), von Willebrand disease (Murray et al. 1992), and EDS type IV (Kontusaari et al. 1992; Richards et al. 1992*b*). In McCune-Albright syndrome, mosaicism for a dominant lethal mutation appears to result in the condition (Weinstein et al. 1991). In other dominantly inherited single-gene disorders in which recurrence among siblings can be explained best by germ-line mosaicism for the mutation in one parent (e.g., achondroplasia [Fryns et al. 1983], pseudoachondroplasia [Hall et al. 1987], Crouzon syndrome [Rollnick 1988; Navarrete et al. 1991], hemoglobin Köln disease [Bradley et al. 1980], and Apert syndrome [Allanson 1986], among others), there is substantial evidence that this mechanism is surprisingly prevalent (Hall 1988).

The risk of recurrence for a dominantly inherited disorder among siblings in a family in which one parent is mosaic for the deleterious mutation depends on the proportion of gametes that carry the mutation, which, in turn, reflects the time at which the mutation occurred during embryogenesis and the proportion of cells carrying the mutation that were allocated to the germ line (Hartl 1971; Cohn et al. 1990; Wijsman 1991). We sampled three tissues in the mosaic father in this family—skin, germ cells (sperm), and leukocytes in blood. Because the deletion allele was represented in all three, the mutation occurred prior to segregation of cells to the germ line and different somatic cell lineages. About 10% of the COL3A1 alleles in leukocytes and 40% of the COL3A1 alleles in the fibroblasts we studied were the mutant species. We could not estimate the proportion of the mutant allele in sperm, because of the very small amount of DNA available. The difference in proportion of normal and mutant alleles in white cells and skin fibroblasts could result from a difference in allocation of cells early in embryogenesis or, alternatively, reflects sampling from a clonally derived region in skin enriched for cells that contained the mutant allele. If progenitor cells that contained the mutant COL3A1 allele were not selected against, and if they divide at the same rate as cells that contained only the normal allele, then the proportion of mutant alleles in blood indicates that the mutation occurred when at least five founder cells were available to form the embryo.

Despite the relatively high proportion of the mutant COL3A1 allele in the DNA from the father's fibro-

Table 2

Effect of Mosaicism on Production of Type III Procollagen Molecules That Contain All Normal or All Mutant Chains

Normal Allele/ Mutant Allele	Proportion of Cells		Proportion of Molecules[a]		Ratio of Molecules[a]	Normal Procollagen (%)
	Normal:Normal	Normal:Mutant	N_3	A_3	(N_3:A_3)	
.5/.5	0	1	.12	.12	1:1	12.5
.55/.45	.1	.9	.21	.11	2:1	21
.6/.4	.2	.8	.30	.10	3:1	30
.7/.3	.4	.6	.48	.08	6:1	48
.8/.2	.6	.4	.65	.05	13:1	65
.9/.1	.8	.2	.83	.02	33:1	83
1.0/0	1	0	1	0		100

[a] N_3 = molecule that contains three normal chains; and A_3 = molecule that contains three mutant chains.

blasts, his cells produced considerably more normal type III collagen than did his daughter's cells. The cells from the father were a combination of those that contained only the normal allele and those that contained the normal allele and the mutant allele. As the proportion of cells that contain the mutant allele decreases from 100% (in the heterozygote), there is a rapid increase in the ratio of the normal type III collagen molecules to trimers that contain only the product of the mutant allele, assembled by the different cell populations (see table 2). In light of these findings, it is conceivable that some individuals with multiple aneurysms but without evidence of skin abnormalities of EDS type IV could be mosaic for a deleterious COL3A1 mutation, reflecting the presence of populations of cells with the mutant allele.

Multiexon deletions that produce disease vary in frequency among different genes. For example, X-linked ichthyosis, due to steroid sulfatase deficiency, results from deletions in >80% of affected individuals (Ballabio et al. 1989; Shapiro et al. 1989), Duchenne muscular dystrophy results from deletions within the dystrophin gene in about 60% of individuals (Kunkel et al. 1986; den Dunnen et al. 1987), and 5%–10% of individuals with EDS type IV may have deletions (Superti-Furga et al. 1989), but deletions in type I collagen genes are rare causes of osteogenesis imperfecta (Barsh et al. 1985; Chu et al. 1985; Byers et al. 1988; Willing et al. 1988; Wenstrup et al. 1990).

DNA sequences at deletion breakpoints differ substantially among mutations. There may be extensive homology for up to 200 bp (e.g., Alu-Alu–mediated deletion in the low-density lipoprotein receptor gene [Lehrman et al. 1985, 1986]), short regions of homology as little as 14 bp (e.g., factor IX deletion [Chen and Scott 1990]), very short direct repeats of 4–7 bp in the retinoblastoma gene (Canning and Dryja 1989), and short inverted repeats at deletion junctions (e.g., COL1A1 deletion in lethal osteogenesis imperfecta [Barsh et al. 1985; Chu et al. 1985] and globin deletions in β-thalassemias [Henthorn et al. 1990]). In addition, deletion endpoints may occur in A-T–rich domains that have little or no sequence homology (Willing et al. 1988; Weinreb et al. 1990). The time at which deletions occur has not been determined in most instances. Deletions could occur during DNA replication prior to lineage determination (as occurred in the father of our proband), during expansion of the germ-line pool, or during meiosis in the final stages of gametogenesis. It may be possible to identify the time at which recombinational events occur by characteristic features of the rearranged DNA sequence. For example, the types of deletion we identified, in which (1) there are sequences distant to the breakpoints that provide some homology and (2) new sequence is generated at the breakpoint, are most likely to arise as replication events with templates derived from a single chromosome. In contrast, deletions that involve homopurine or homopyrimidine tracts, causing either unequal sister-chromatid exchange (Weinreb et al. 1990) or extensive homology at the breakpoints, could derive from either meiotic or mitotic recombination and are more likely to involve both chromosomes.

If DNA deletions occur during replication, then the risk of recurrence of the disease in siblings of the proband will depend on the timing of the mutation. In any case, the recurrence risk for the disorder in sibships is unlikely to be zero, although it may be low. Search for

evidence of the deletion in somatic and, when available, germ cells would help to define recurrence risk and to clarify the time at which these mutations occur.

Acknowledgments

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