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A Recurrent RNA-Splicing Mutation in the SEDL Gene Causes X-Linked Spondyloepiphyseal Dysplasia Tarda

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Spondyloepiphyseal dysplasia tarda (SEDL) is a genetically heterogeneous disorder characterized by mild-to-moderate short stature and early-onset osteoarthritis. Both autosomal and X-linked forms have been described. Elsewhere, we have reported the identification of the gene for the X-linked recessive form, which maps to Xp22.2. We now report characterization of an exon-skipping mutation (IVS3+5G→A at the intron 3 splice-donor site) in two unrelated families with SEDL. Using reverse transcriptase (RT)–PCR, we demonstrated that the mutation resulted in elimination of the first 31 codons of the open reading frame. The mutation was not detected in 120 control X chromosomes. Articular cartilage from an adult who had SEDL and carried this mutation contained chondrocytes with abundant Golgi complexes and dilated rough endoplasmic reticulum (ER). RT-PCR experiments using mouse/human cell hybrids revealed that the SEDL gene escapes X inactivation. Homologues of the SEDL gene include a transcribed retropseudogene on chromosome 19, as well as expressed genes in mouse, rat, *Drosophila melanogaster Caenorhabditis elegans*, and *Saccharomyces cerevisiae*. The latter homologue, p20, has a putative role in vesicular transport from ER to Golgi complex. These data suggest that SEDL mutations may perturb an intracellular pathway that is important for cartilage homeostasis.

Introduction

Spondyloepiphyseal dysplasia tarda (SEDL) is a rare osteochondrodysplasia that is first evident in affected individuals at 3–12 years of age. Clinical features include short trunk, barrel-shaped chest, and mild-to-moderate short stature. Premature osteoarthritis is common, especially in weight-bearing joints (Taybi and Lachman 1990). The X-linked recessive form (SEDL [MIM 313400]) was first reported by Jacobsen (1939), and its estimated prevalence is 1.7 per million (Wynne-Davies and Gormley 1985). Rare families exhibiting autosomal inheritance patterns have also been described (Pfeiffer et al. 1992; Schantz et al. 1988). Localization of the SEDL gene to Xp was originally reported by Szpiro-Tapia et al. (1988) and was further refined to the ~2-Mb interval at Xp22, between DXS16 and DXS987, by Heuertz et al. (1995). The critical region was refined to <170 kb, by critical recombination events at DXS16 and AFMa124wc1, in two Australian families with SEDL (Gedeon et al. 1999); subsequently, a highly conserved gene was cloned *in silico* from a publicly available genomic sequence. In three unrelated Australian families with SEDL, different dinucleotide deletions in this gene were identified that result in frameshifts and generate premature stop codons.

In the present study, we describe a large North American family with SEDL and confirm the location of the disease gene to Xp22.2 in this family by two-point linkage analysis. We demonstrate a mutation at the intron 3 splice-donor site of the SEDL gene in affected individuals and obligate carriers, and we show evidence for abnormal splicing of exon 3 in cell lines from an affected man. We also document the presence of the same mutation in another unrelated patient with SEDL, suggesting that it is a hot spot for mutation within the SEDL gene. Ultrastructural analysis of articular cartilage from an adult SEDL patient carrying this mutation demonstrates unusual chondrocyte histomorphology. Finally, we show that the SEDL gene escapes X inactivation. Its homology to the yeast gene that encodes protein p20 (Sacher et al. 1998), a component of the transport protein particle (TRAPP), leads us to hypothesize that the SEDL gene product has a key role in trafficking chondro-

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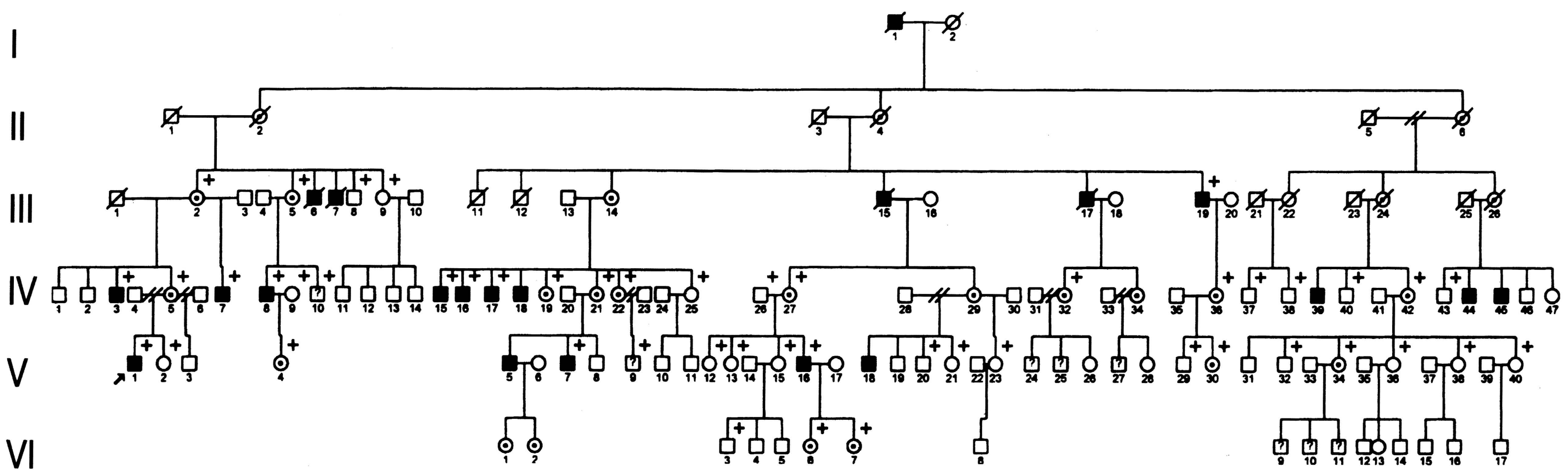

Figure 1 Abbreviated pedigree of a North American family with SEDL. Plus signs (+) indicate genotyped individuals; arrow indicates proband (V:1); question marks (?) indicate questionable clinical status.

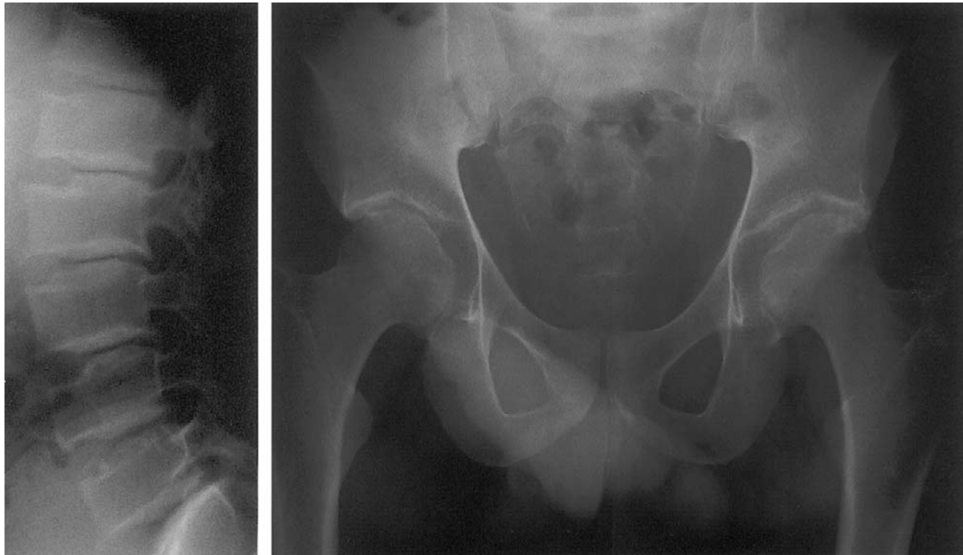

Figure 2 Radiographs of the lateral spine (*left*) and pelvis (*right*) of a 31-year-old man (IV:8 in fig. 1) with SEDL. Note platyspondyly and superior and inferior humping of vertebrae, as well as irregular hip-joint surfaces and short femoral necks.

cyte proteins from endoplasmic reticulum (ER) to Golgi complexes and therefore is essential for maintaining homeostasis of the cartilage extracellular matrix.

Subjects and Methods

Clinical Summary

The present study was approved by the Institutional Review Board of Vanderbilt University Medical Center, and informed consent was obtained from all participants. The proband of family 1 (V:1 in fig. 1) was a 19-year-old man who sought medical attention because of chronic lower back pain. The proband's height was 173 cm (25th percentile), and his arm span was 187 cm. His trunk was disproportionately short, and his chest was barrel-shaped. Facial features were unremarkable, and results of the neurologic examination were within normal limits. Radiographs revealed platyspondyly with superior and inferior humping of the vertebral bodies; the pelvis was somewhat narrow, and the femoral necks were short. Family history revealed 21 affected males spread over five generations, a group that constitutes the largest SEDL pedigree ascertained to date (see fig. 1). The surnames in the first generation suggest that the family is of British descent. A maternal first cousin once removed (IV:8 in fig. 1) underwent replacement of the right hip joint at age 31 years, as well as replacement of the left shoulder joint at age 34 years, because of osteoarthritis. Another maternal male relative (IV:15 in fig. 1) underwent hip joint replacement at age 50 years. Five affected men, ages 19–43 years, were examined by one of us (G.E.T.). Heights were 147–173 cm (mean ± SD 159 ± 10 cm), and arm span exceeded height by a mean ± SD of 20 ± 12 cm. Radiographs of an affected man (IV:8 in fig. 1) are shown in figure 2.

Family 2 was ascertained at the Universitatskinderklinik in Mainz, Germany. The proband displayed normal linear growth until he was 6 years of age but, since that time, has gradually demonstrated disproportionate short stature. His height at 11.5 years of age was 129 cm (>2 SD below the mean for his age; 50th percentile for 8.5 years), and his arm span exceeded his height by 12 cm. Changes typical of SEDL were also evident on radiographs. He has an unaffected brother and no other affected relatives.

Cell Culture, RNA, and DNA Isolation

Patient lymphoblastoid cell lines were grown and maintained under standard culture conditions. Genomic DNA was extracted from whole blood, cultured cells, or dried buccal swabs, using either the Qiagen blood kit or QIAamp kit (Qiagen). Total RNA was isolated from cultured cells using method of Chomczynski and Sacci (1987) or RNeasy kit (Qiagen).

Linkage Analysis

Fifty-two members of family 1, including 10 of 16 living affected males and 12 obligate female carriers, were genotyped at 18 microsatellite markers spanning the region from DXS1223 to DXS989 at Xp22.2 (Dib et al. 1996; GenBank). Two-point analysis was performed using the MLINK program of LINKAGE, version 5.2 (Lathrop and Lalouel 1984), assuming X-linked

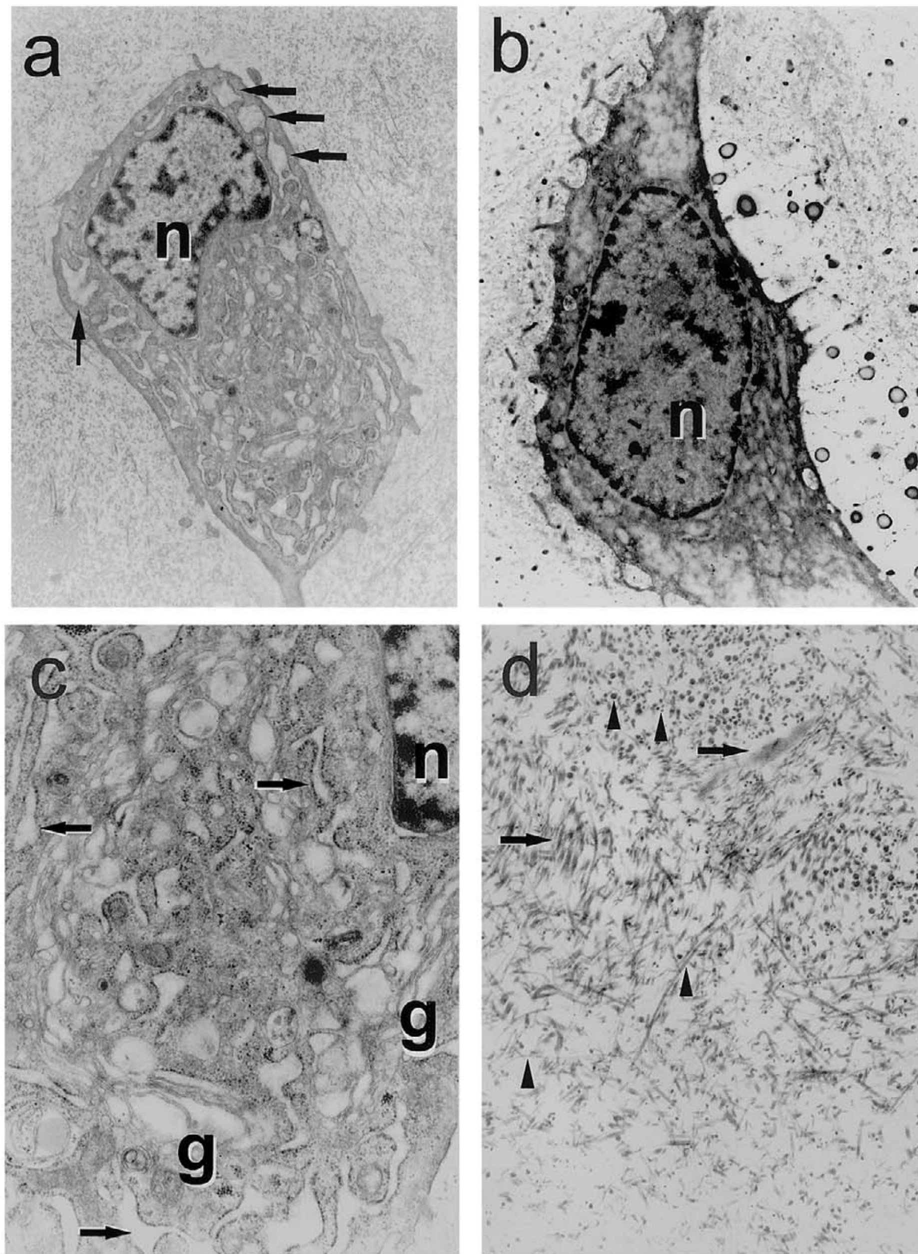

Figure 3 Articular cartilage ultrastructure from the femoral head of a 50-year-old man with SEDL (IV:15 in fig. 1) and a 50-year-old patient with idiopathic osteoarthritis. *a,* SEDL chondrocyte with dilated rER. Arrows indicate rER, and n indicates nucleus (original magnification, 8,140 ×). *b,* Chondrocyte from a 50-year-old man with osteoarthritis (original magnification, 8,140 ×). Note normal nuclear:cytoplasmic ratio and absence of dilated rER. *c,* SEDL chondrocyte cytoplasm. The abundant Golgi complexes are indicated by g, and dilated loops of rER are indicated by arrows (original magnification, 23,830 ×). *d,* SEDL extracellular matrix. Arrows indicate collagen fibrils that are short and frayed, and arrowheads indicate fibrils of variable diameter (original magnification, 23,830 ×).

recessive inheritance of SEDL with full penetrance and allele frequency of .00001.

Histologic Analysis

Residual articular cartilage was obtained during hip-joint replacement in family member IV:15 (fig. 1). Samples were either frozen or fixed in formalin or buffered glutaraldehyde and were prepared for histologic analysis by routine methods (Brodie et al. 1998).

PCR, RT-PCR, and Sequence Determination

PCR was performed on genomic DNA as described elsewhere (Gedeon et al. 1999), using oligonucleotide primer pairs designed from the unique X-specific se-

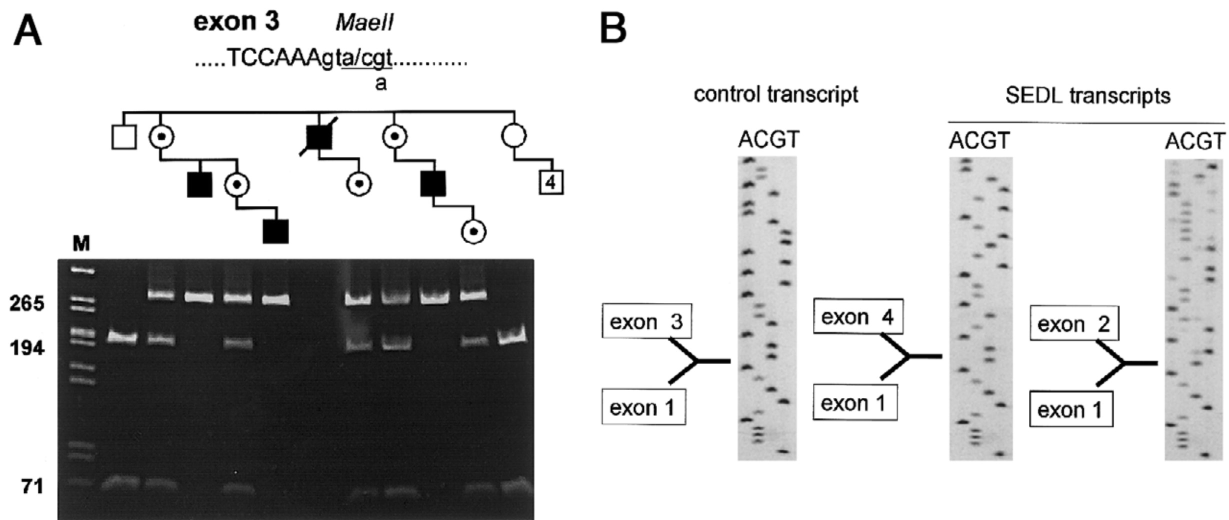

Figure 4 Splice mutation in SEDL. *a,* PCR amplification of a fragment of the SEDL gene spanning the exon 3/intron 3 junction. The mutant allele contains a +5G→A transition at the intron 3 splice-donor site, which disrupts a *Mae*II restriction site. The proband of family 1 is the affected man in the third generation of the pedigree as drawn. *b,* Sequencing of cDNA from a lymphoblastoid cell line from the proband of family 1. Sequencing reveals two transcripts (each missing exon 3, which contains the translational start site).

quence that flanked each of the four coding exons on the SEDL gene. The RT-PCR experiments were also performed as described by Gedeon et al. (1999). Genomic DNA was sequenced from both strands, using PCR primers and ThermoSequenase cycle sequencing kit (USB).

X-Inactivation Studies

Mouse/human somatic-cell hybrids that retain either an active or inactive human X chromosome have been described elsewhere (Brown et al. 1997; Carrel et al. 1999). The SEDL primers used were 5′-AGGAGC-CATATATTGAAGACCATG-3′ (forward, designated “5′21F”) and 5′-TCCTGAGTATACACCATTGTGG-3′ (reverse, designated “3′52R”). Possible amplification of the pseudogene SEDLP (Gedeon et al. 1999; Gecz et al. 2000) was assessed by digesting RT-PCR products with *Bsa*J1, which cleaves only the pseudogene amplimer. Conditions for RT-PCR and oligonucleotide primers for MIC2 and A007K03 are as described elsewhere (Carrel et al. 1999).

Linkage of SEDL Phenotype to Xp22 Confirmed

Before the discovery of the SEDL gene, we performed two-point linkage analysis between the SEDL phenotype and 18 microsatellite markers at Xp22.2. Peak LOD scores were obtained at DXS16 and DXS8022 ($Z =$ 5.71 and 8.22, respectively, at $\theta = 0$), which were later found to flank the disease locus. However, despite the large size of the North American pedigree, no recombinants were identified that could narrow the candidate region within the 18-cM span from DXS7109 to DXS1226. The critical region was subsequently limited to <170 kb by identifying recombinations in two small Australian pedigrees (Gedeon et al. 1999).

Histologic Analysis of SEDL Cartilage

Histologic analysis revealed pathologic changes that are typical of osteoarthritis (Pritzker 1998), including horizontal fibrillation near the articular surface and occasional clustering of chondrocytes in the resting zone (data not shown). Cellularity was unremarkable, and staining with toluidine blue revealed a normal distribution of glycosaminoglycan. Figure 3 shows the ultrastructure of articular cartilage from the hip joint of a family 1 member (individual IV:15). The extracellular matrix contained a normal number of collagen fibrils, some of which appeared short, frayed, or of variable diameter. Chondrocytes contained excessive cytoplasm with abundant Golgi complexes and dilated, rough ER. In contrast, chondrocytes from an age- and sex-matched patient with idiopathic osteoarthritis were mostly apoptotic, with those remaining viable cells demonstrating a normal nucleus:cytoplasm ratio and no organelles of unusual appearance.

Splice-Donor–Site Mutation in Intron 3

Direct sequencing of gDNA from the proband of each family revealed a G→A transition in the fifth base of the intron 3 splice-donor site. The mutation disrupts a *Mae*II

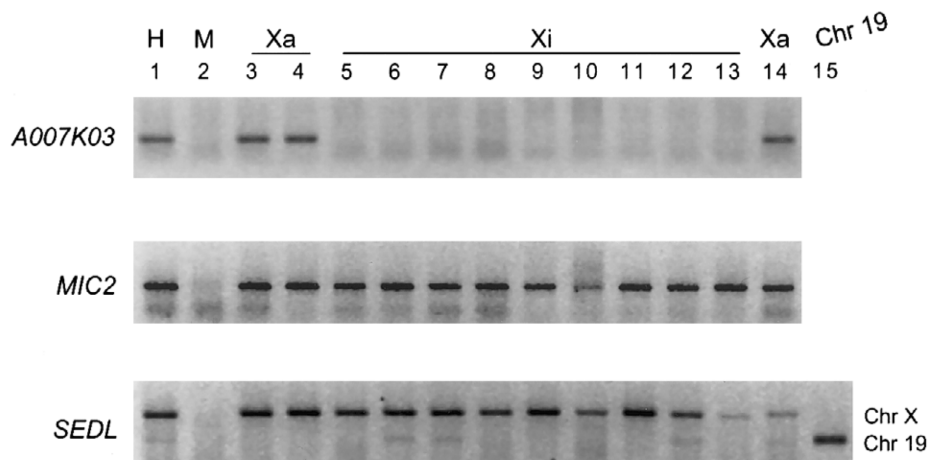

Figure 5 Expression of genes from active or inactive X-containing hybrids. cDNA from mouse/human somatic-cell hybrids that retained either the human active (Xa) or inactive (Xi) X chromosome was amplified with gene-specific primers. A negative image of ethidium bromide–stained products separated by agarose gel electrophoresis is shown. Lanes contain human female cell line (*lane* 1); mouse tsA1S9az31b cell line (*lane* 2); t60-12 (*lane* 3); Aha11aB1 (*lane* 4); t86-B1maz1b-3a (*lane* 5); t11-4Aaz5 (*lane* 6); t75-2maz34-4a (*lane* 7); t48-1a-Daz4a (*lane* 8); LT23-1E2Buv5Cl26-7A2 (*lane* 9); L23-4B (*lane* 10); t51-S2 (*lane* 11); tHM-1a (*lane* 12); tHM-1d (*lane* 13); A23-1aCl5 (*lane* 14); genomic DNA from a somatic-cell hybrid cell line retaining human chromosome 19 (*lane* 15). PCR products in all lanes have been digested with *Bsa*J1 to distinguish SEDL product from pseudogene SEDLP product, since only the latter is cleaved by *Bsa*J1. Control transcripts A007K03 (which is subject to inactivation) and MIC2 (which escapes inactivation) demonstrate the inactivation status for each hybrid shown.

restriction site, which allowed us to efficiently screen all family members (fig. 4*a*). The mutation cosegregated with the affected phenotype and in known carriers, with two exceptions. One was a phenotypically normal 10-year-old boy (V:9 in fig. 1), and the other was a man (IV:10 in fig. 1) of normal stature who denied symptoms of osteoarthritis. Despite repeated inquiries, we were unable to obtain radiographs of either individual, and individual IV:10 was unavailable for physical examination. The mutation was not seen in 120 control X chromosomes (CEPH panel) after complete *Mae*II digestion of gDNA PCR amplimers. The identical mutation was seen in the affected individual and his mother in family 2 (data not shown). No other sequence changes were seen in the coding region or flanking intron sequences in either proband. No mutations in the SEDL gene were detected by genomic sequencing of the control patient with osteoarthritis.

cDNA Pattern in SEDL Lymphoblastoid Cells

The predominant SEDL transcript from several normal tissues lacks exon 2 (Gedeon et al. 1999; Gecz et al. 2000). When RT-PCR was performed, using total lymphoblastoid cell RNA from the proband of family 1, two SEDL transcripts were seen (fig. 4*b*). The longer transcript lacked exon 3 but retained exon 2; the shorter transcript lacked both exon 2 and exon 3. There was no evidence for the use of alternative splice sites (data not shown), and no other consensus sequences for translation initiation (Kozak 1986) are seen in exons 4–6. Because exon 3 contains the translation start site, we hypothesize that no SEDL protein is produced from this mutant allele.

X-Inactivation Studies

Figure 5 illustrates the expression of SEDL in a series of somatic-cell hybrids that retain either an active or an inactive human X chromosome. Amplification of EST A007K03 (which is subject to inactivation [Carrel et al. 1999]) and MIC2 (which escapes inactivation [Brown et al. 1997]) is shown for comparison. SEDL is expressed in all cell lines tested, indicating that SEDL escapes X inactivation. Identity of the RT-PCR product as the SEDL gene product is further demonstrated by absence of amplification within the parent mouse cell line (lane 2) and insensitivity to digestion by *Bsa*J1.

Discussion

Effect of IVS3+5G→A Mutation on Splicing

The RT-PCR experiments using lymphoblastoid RNA from the proband in family 1 failed to generate any transcripts with a normal splicing pattern. Some transcripts retained exon 2, which is usually spliced out (Gedeon et al. 1999), but the majority of the transcripts lacked both exon 2 and exon 3. Exon 3 contains the translation start site, and, although there are five additional methionine residues in the open reading frame,

Table 1

IVS+5G→A Mutations Causing Human Diseases

Gene	Mutated Sequence	Intron	No. of bp Matched with Consensus[a]	Reference
SEDL	AAgtacat	3	5/8	Present study
β-globin	TTgtgaat	1	5/8	Lapoumeroulie et al. 1986
COL3A1	CTgtaaat	27	5/8	Thakker-Varia et al. 1995
COL1A1	CTgtaaat	14	5/8	Bonadio et al. 1990
COL1A2	GTgtgaat	33	5/8	Ganguly et al. 1991
HPRT	CTgtaaat	7	5/8	Gibbs et al. 1989
CYBB	CGgtaaaa	3	5/8	deBoer et al. 1992
PROS1	TGgtacat	10	5/8	Reitsma et al. 1994
WT1	AAgtgcat	9	5/8	Bruening et al. 1992
BTK	TGgtaaat	2	6/8	Duriez et al. 1994
MYBPC3	ACgtgaat	7	6/8	Carrier et al. 1997
CYP17	TGgtgaat	7	6/8	Yamaguchi et al. 1997
HPRT	ATgtaaat	8	6/8	Gibbs et al. 1989

[a] Splice-donor consensus sequence AGgt(a/g)agt derived from 3,274 exon/intron junctions (Zhang 1998), where capital letters denote last 2 bp of exon.

none has an associated Kozak consensus sequence for initiation of translation. Therefore, we would not expect any protein to be translated from this mutant allele. In addition to the two families reported here, three additional unrelated families with SEDL have been ascertained that carry this same mutation (Gedeon et al. 2001 [in this issue]). This suggests that the IVS3+5G residue may represent a hypermutable site in the SEDL gene.

The thermodynamic data in the literature are inadequate for direct calculation of the effect of the IVS3+5G→A transition on RNA splicing. However, a rough estimate is attained by using the consensus sequence data compiled by Shapiro and Senapathy (1987). By use of their method, a score of 76.1 is obtained for the wild-type allele, whereas a score of 61.7 is obtained for the mutant allele. For comparison, the human consensus sequence for splice-donor sites (AGgt[a/g]agt) (Zhang 1998) yields a score of 96–100, depending on whether adenine or guanine lies in the third position of the intron. A more convenient way to assess the significance of splice-donor site substitutions is based on the analysis of factor IX splice mutations by Ketterling et al. (1999), where one considers bases −2 to +6 of the splice site. They propose a "5–6" hypothesis, whereby normal splicing is expected to be severely compromised if a donor site matches the consensus sequence at fewer than seven positions. This is exemplified by the IVS3+5G→A transition in SEDL, as well as 12 other such mutations extracted from OMIM, which are listed in table 1. Thus, all thirteen mutations lend further support to Ketterling's "5–6" hypothesis.

Two clinically normal male subjects in family 1 (V:9 and IV:10 in fig. 1) were shown to harbor the mutant allele by both haplotype analysis and *Mae*II restriction analysis of PCR amplimers. Although V:9 may be presymptomatic, IV:10 is a mature adult who states that his height is 183 cm and that he has no skeletal complaints. We were unable to obtain radiographs or a cell sample of either individual and therefore cannot determine whether IV:10 is mildly affected or has normal SEDL transcripts in his tissue.

Significance of SEDL Escaping X Inactivation

The data in figure 5 clearly indicate that the SEDL gene escapes X inactivation. The closest flanking genes identified at Xp22.2 (Rab9 and Cxorf5) also escape X inactivation (Carrel et al. 1999). This clustering further supports a model in which regional mechanisms may control the expression of genes that escape X inactivation (Miller and Willard 1998; Carrel et al. 1999). The majority of the mutations in SEDL patients characterized thus far (Gedeon et al. 1999 and 2001; Mumm et al. 2000; and the present study) are predicted to severely truncate or eliminate the protein product. The observation that SEDL escapes X inactivation suggests that haploinsufficiency at the locus is inadequate to produce any phenotypic changes in female SEDL carriers. Although Whyte et al. (1999) observed subtle radiographic changes in older SEDL carriers, no signs or symptoms of premature osteoarthritis were noted in the women of the family reported here or in those described in the report by Gedeon et al. (1999).

Putative Role for SEDL Gene Product, Sedlin

The SEDL gene is highly conserved, and homologues are found in mouse, rat, *Caenorhabditis elegans, Drosophila melanogaster,* and *Saccharomyces cerevisiae*

(Gedeon et al. 1999). The yeast homologue, designated p20 or YBR254c, shares 41% identity and 57% amino acid similarity with human SEDL (Sacher et al. 1998). Yeast p20 is a 20-kD hydrophilic protein which is part of a complex of ⩾10 proteins constituting TRAPP. The TRAPP complex has been shown to play a key role in transport of yeast proteins from the ER to the Golgi apparatus (Barrowman et al. 2000). A precedent for human disease in such a transport process has been established by combined deficiency of factors V and VII (Nichols and Ginsburg 1999). This autosomal recessive coagulopathy results from mutations in the ERGIC-53 gene, which encodes a transmembrane glycoprotein–binding component of the ER-Golgi intermediate compartment (ERGIC). Mutations in ERGIC-53 result in impaired secretion of clotting factors V and VIII from megakaryocytes and hepatocytes.

Transient transfection experiments with FLAG- or GFP-tagged SEDL (Gecz et al. 2000) suggested localization of the SEDL protein to perinuclear structures, whereas transfection with truncating mutant transcripts disrupted this localization. Furthermore, the appearance of SEDL chondrocytes from articular cartilage (fig. 3) suggests that there may be disruption in cellular trafficking between rER and Golgi. Since the histopathology of SEDL cartilage has not been described elsewhere, it is uncertain whether the cellular architecture depicted in figure 3 is representative of this genetic disorder. The cellular appearance is dissimilar to the gross dilation of rER that is seen as a result of any of the following: retention of abnormal type II collagen in patients with spondyloepiphyseal dysplasia congenita (Tiller et al. 1995), abnormal type IX collagen in some patients with multiple epiphyseal dysplasia (Spayde et al. 2000), or abnormal COMP in patients with pseudoachondroplasia (Rimoin et al. 1994). Such ultrastructural abnormalities are not seen in fibroblasts of patients with these three disorders, since the abnormal protein is not produced appreciably in fibroblasts. Although the human SEDL gene appears to be expressed ubiquitously, we propose that the tissue-specific nature of the SEDL phenotype may lie in the protein cargo that is transported by the TRAPP system in chondrocytes. Delineation and further study of additional SEDL mutations (Gedeon et al. 2001 [in this issue]) may reveal critical motifs within the SEDL protein structure and afford us a better understanding of its function.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for SEDL [MIM 313400] and sedlin [MIM 300202])

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