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SHORT REPORT

Deletion of a branch-point consensus sequence in the *LMX1B* gene causes exon skipping in a family with nail patella syndrome

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Nail patella syndrome (NPS) has been shown to result from loss of function mutations within the transcription factor LMX1B. In a large NPS family a 17bp intronic deletion encompassing a consensus branchpoint sequence was observed to segregate with the NPS phenotype. RNA analysis demonstrated that deletion of the branchpoint sequence resulted in skipping of the downstream exon. A mechanism to explain this phenomenon is presented. *European Journal of Human Genetics* (2000) **8**, 311–314.

Keywords: *LMX1B*; nail patella syndrome; branch-point; exon skipping; splice site mutation

Introduction

Nail patella syndrome (NPS) is a pleiotropic condition characterized by dysplasia of the nails, hypoplasia of the patellae, elbow dysplasia and progressive kidney disease.[1] Recent evidence suggests that open angle glaucoma is also part of the syndrome[2] and that other organ systems may also be affected (I McIntosh, unpublished results). The syndrome is inherited in an autosomal dominant manner and has been shown to result from mutations in the LIM-homeodomain encoding *LMX1B* gene.[3–6] The LMX1B transcription factor plays a role in defining the development of dorsal specific structures during limb development;[7] its role in other organs is unclear. Analysis of over 60 *LMX1B* mutations in NPS families supports the hypothesis that the syndrome results from haploinsufficiency due to loss of function mutation.[3–6]

During the search for *LMX1B* mutations causing NPS, a 17bp deletion was identified upstream of the 3' splice consensus which removed a consensus branchpoint sequence. The effect of this deletion on the splicing of *LMX1B* RNA was studied further.

Materials and methods

Patient samples and DNA amplification

Family 1 has been described previously.[8] Genomic DNA was extracted from peripheral blood lymphocytes, PCR-amplified and sequenced as described.[5] Appropriate informed consent was obtained from all participants.

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RNA analysis

Skin fibroblasts obtained from a member of family 1 with NPS were cultured and total RNA was extracted using a commercial reagent (Trizol; Life Technologies, Gaithersberg, MD, USA). RT-PCR was performed using a commercial kit (Advantage RT-PCR, Clontech, Palo Alto, CA, USA) with primers LMX-1F (5'-GACGGACTGCGCCAAG) and LMX-3anti (5'-GAAGCAGCCCAGGTGGTAC). PCR products were subjected to electrophoresis in 2% (w/v) agarose, 1 × TBE, Southern blotted and hybridized with oligonucleotides specific for exons 2 (LMX-2F: 5'-GTGTCAGCAAGCCCTCAC) and 3 (LMX-A: 5'-GCTCTTCGCGGCCAAGTGCAG). To confirm the exact nature of the splicing event, RT-PCR products were gel purified and sequenced.

Results

Mutation analysis of genomic DNA from affected members of Family 1 failed to detect any changes in coding sequence or canonical splice sites. However, a 17 bp deletion was identified starting 37 bp upstream of exon 2 (Figure 1). The deletion removed a recognition sequence for the restriction enzyme *EagI*; restriction digestion of PCR amplified DNA from 14 affected and 11 unaffected members of Family 1 indicated that the deletion was found only in persons with NPS (data not shown). The deletion was not detected in a further 50 unrelated individuals from an unaffected control population screened by *Eag*I digestion of PCR amplified DNA. Of interest, the deleted region included the sequence CGCTGAC

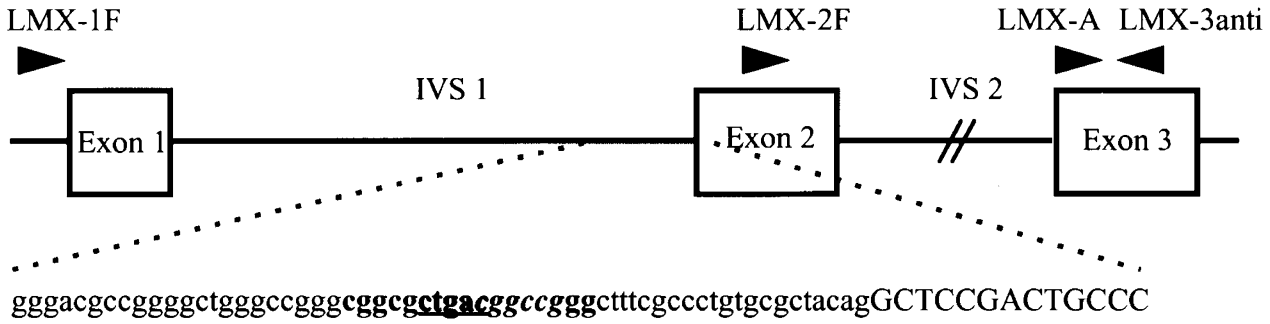

Figure 1 Schematic of the region of *LMX1B* discussed in the text. Exons 1–3 are shown as boxes with intervening sequences as lines. The sequence around the deletion is expanded below: the deletion is in bold, the *Eag*I site in italics, the consensus branchpoint sequence underlined and exonic sequence in upper case. The positions and orientation of primers are shown above as arrowheads.

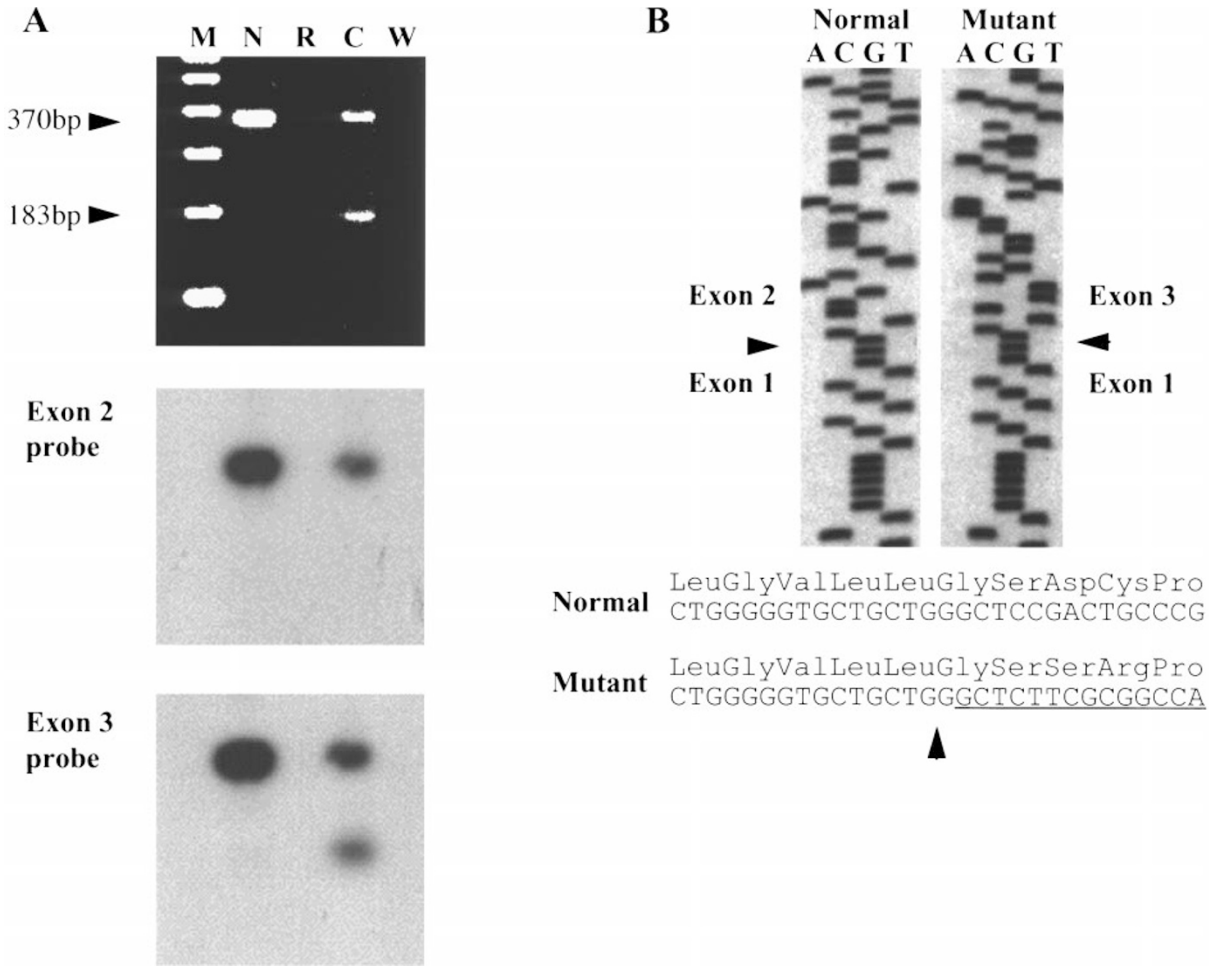

Figure 2 RT-PCR analysis **A** RT-PCR was performed as described in Materials and methods. The products of normal splicing (370bp) and with exon 2 skipped (183bp) are arrowed (upper panel). The gel was blotted and hybridized with oligonucleotides specific for exon 2 (middle panel) and exon 3 (lower panel). M: marker, 100bp ladder; N: normal control template; R: patient RNA template; C: patient cDNA template; W: no template control. **B** Sequence of mutant and normal PCR products. PCR products shown in **A** were extracted from the gel and sequenced. The exon boundary is marked with an arrowhead. The normal and mutant sequences and encoded amino acids are shown below. Exon 3 sequence is underlined. The alteration in reading frame predicts a premature termination coding 57 bp into exon 3.

which not only is a perfect match for the consensus for the branchpoint sequence necessary for lariat formation (YNY-TRAC), but is positioned at a distance to perform such a function (within 15–40 nucleotides of the 3' splice site).

To determine the effect of this deletion on pre-mRNA splicing, total RNA was extracted from cultured skin fibroblasts, reverse transcribed and amplified using primers from exons 1 and 3. Two bands were seen upon gel electrophoresis of the PCR product from the patient's cDNA compared with a single band from control fibroblast cDNA (Figure 2A). The sizes of the PCR products were those expected for a wild type transcript (upper band), and one lacking exon 2 (lower band). This hypothesis was confirmed by Southern blotting and hybridization with probes specific for exon 2 and exon 3 (Figure 2A, lower panels). To show that the splicing of exon 1 to exon 3 was precise, the PCR products were gel purified and sequenced (Figure 2B). The skipping of exon 2 disrupts the reading frame leading to a premature termination codon within exon 3. Any polypeptide synthesized from this transcript would lack all functional domains and would be predicted to be non-functional. To insure that the exon skipping in this subject resulted from deletion of the branchpoint sequence, and not from any other mutation within the intron, intron 1 was amplified from an affected

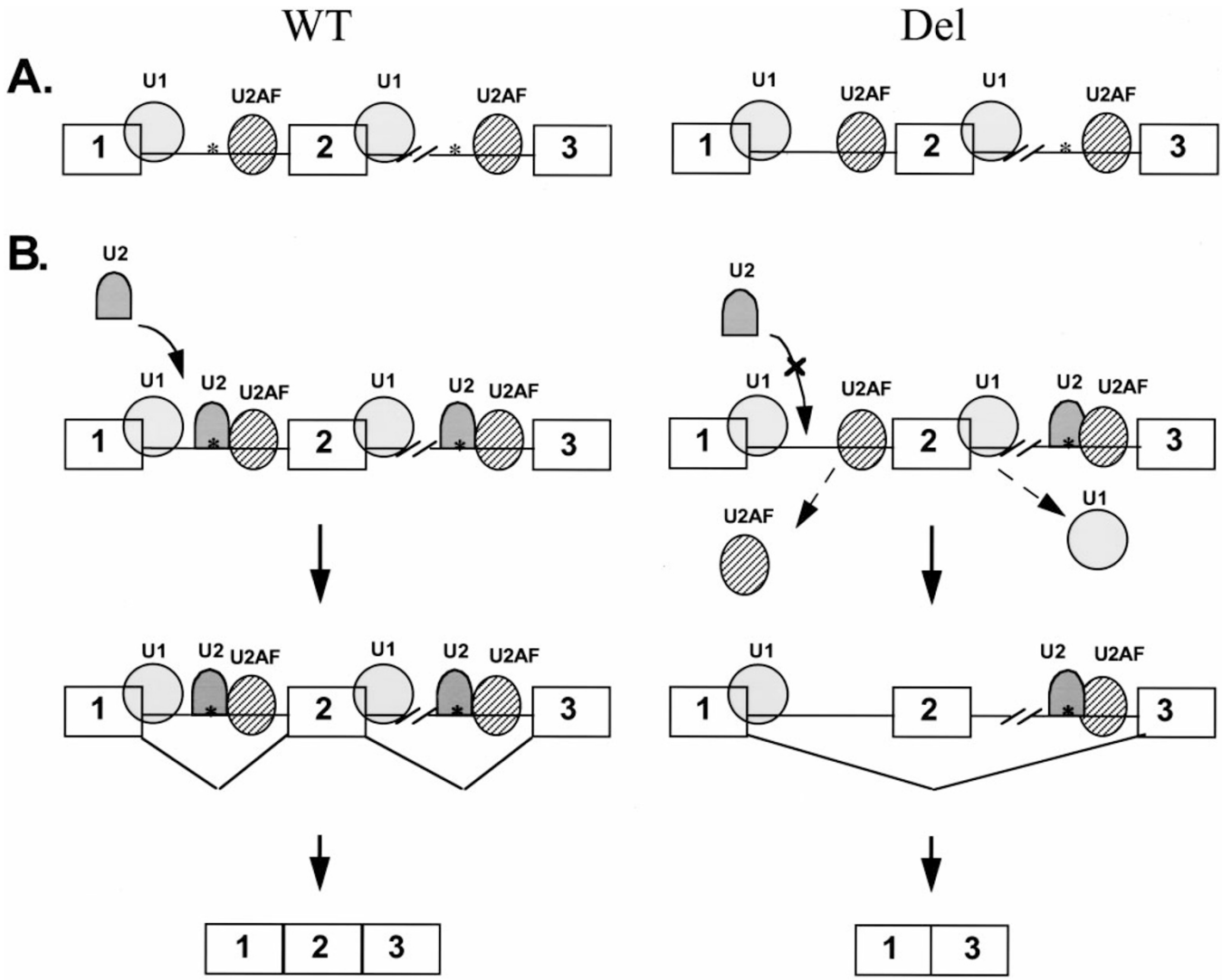

Figure 3 Model of exon skipping resulting from a branch point sequence deletion. **A** In both the WT and deleted variant, exon 2 boundaries are initially defined by U1 snRNP and U2AF binding to the 5' splice site consensus sequence and the polypyrimidine tract, respectively. **B** U2 snRNP is recruited to the spliceosome and binds the BPS. In the deleted variant, U2 snRNP is unable to bind within intron 1 due to the absence of the BPS. Consequently, destabilization of U2AF and U1 snRNP and loss of exon 2 boundary definition may occur. Remaining splicing factors promote the assembly of the active spliceosome (not shown). Splicing reactions in the WT pre-mRNA result in the joining of exons 1, 2 and 3; however, in the deleted variant, splicing reactions ligate exons 1 and 3. *branch point sequence (BPS).

member's DNA and sequenced. No other variants were observed. The NPS phenotype in this large family is comparable to that seen in other NPS families examined.[5,8]

Discussion

In recent years many mutations of canonical splice sites have been reported which have been shown to affect exon definition and the splicing of pre-mRNA, but few mutations of the branchpoint sequence have been identified.[9] Such mutations have been found associated with X-linked hydrocephalus,[10] hepatic lipase deficiency,[11] fish-eye disease,[12] congenital contractual arachnodactyly,[13] Sandhoff disease,[14] and Ehlers-Danlos syndrome.[15] These mutations resulted in cryptic splicing,[10,11,14] intron retention[12] and exon skipping.[13,15] That only a handful of such mutations have been identified may reflect the lack of stringency both within the branchpoint consensus, and the flexibility in distance from branchpoint to splice acceptor, that the splicing machinery can tolerate. In the case presented here, the GC-rich nature of the surrounding sequence may preclude the utilization of an alternate branchpoint.[16]

In vitro experiments have demonstrated the importance of the branchpoint sequence in the stepwise assembly of an active spliceosome complex.[17] Early in spliceosome assembly, U1 snRNP and U2AF bind to the 5' splice site and the polypyrimidine tract, respectively. Binding of these splicing factors and specific SR proteins defines the 5' and 3' boundaries of the exon and is a prerequisite for U2 snRNP binding.[18,19] Only after binding of U2 snRNP can other snRNPs such as U4, U5 and U6, assemble on the pre-mRNA and form an active spliceosome (Figure 3).

In the absence of a branchpoint sequence, stable binding of the U2 snRNP cannot occur. The initial binding of U1 snRNP and U2AF is unaffected; however, without U2 snRNP binding, the U1 snRNP and U2AF interactions with the pre-mRNA become destabilized.[20] If these interactions are destabilized across the downstream exon, the exon boundaries will not be defined. This mechanism may explain the exon skipping observed in the subject studied (Figure 3). Deletion of the branchpoint sequence, in the absence of a suitable cryptic site for U2 snRNP binding, resulted in the skipping of exon 2.

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