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Mutation Analysis of *LMX1B* Gene in Nail-Patella Syndrome Patients

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Summary

Nail-patella syndrome (NPS), a pleiotropic disorder exhibiting autosomal dominant inheritance, has been studied for >100 years. Recent evidence shows that NPS is the result of mutations in the LIM-homeodomain gene *LMX1B*. To determine whether specific *LMX1B* mutations are associated with different aspects of the NPS phenotype, we screened a cohort of 41 NPS families for *LMX1B* mutations. A total of 25 mutations were identified in 37 families. The nature of the mutations supports the hypothesis that NPS is the result of haploinsufficiency for *LMX1B*. There was no evidence of correlation between aspects of the NPS phenotype and specific mutations.

Introduction

Nail-patella syndrome (NPS [MIM 161200]), or onycho-osteodysplasia, is an autosomal dominant, pleiotropic disorder characterized by dysplastic nails, absent or hypoplastic patellae, exostoses ("horns") of the ilia, dysplasia of the elbows, and, in some cases, nephropathy (Beals and Eckhardt 1969). Recent evidence suggests that glaucoma may also be part of the syndrome (Lichter et al. 1997). NPS was first recognized as an inherited disorder by Little (1897), who reported a four-generation pedigree with 18 affected members who had absent patellae and thumbnails. The independently mapped incidence is estimated at 1 in 50,000 live births.

Genetic linkage between NPS and the ABO blood group was first reported >40 years ago (Renwick and Lawler 1955) and was subsequently localized to the interval between D9S315 and D9S2172 (McIntosh et al. 1997; Eyaid et al., in press). Recently, Dreyer et al. (1998) demonstrated that NPS is the result of mutations within the *LMX1B* gene. At the same time, Chen et al. (1998) showed that mice ablated for *Lmx1b* exhibited skeletal pathology similar to NPS. Iannotti et al. (1997) independently mapped *LMX1B* to 9q34, and Vollrath et al. (1998) mapped *LMX1B* within the NPS candidate interval and identified four mutations that segregate with the NPS phenotype in unrelated families. The available evidence suggests that NPS results from heterozygosity for loss-of-function mutations in *LMX1B* (Dreyer et al. 1998; Vollrath et al. 1998).

LMX1B is a member of a diverse family of regulatory proteins characterized by the presence of two zinc-finger structures and a homeodomain (Curtiss and Heilig 1998). The homologous gene in the chick has been implicated in the dorsoventral patterning of the developing limb bud (Riddle et al. 1995; Vogel et al. 1995). Comparison of the phenotype observed in *Lmx1b*-ablated mice with NPS suggests that the skeletal phenotype is the result of a deficiency in dorsoventral patterning during development. Specifically, *Lmx1b* −/− mice demonstrated ventralization of dorsal-limb characteristics (e.g., absence of claws and patellae and duplication of ventral structures such as foot pads and ventral-specific tendons and musculature). In addition, renal pathology similar to that seen in NPS was observed (Chen et al. 1998). To determine whether specific mutations within *LMX1B* were responsible for certain aspects of NPS, we undertook mutation analysis of a cohort of 41 families.

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Subjects and Methods

Patient Samples

Families 1–5 have been described elsewhere (McIntosh et al. 1997). Additional families and patients with sporadic cases of NPS were identified from clinic records or were referred to us. In each case, at least the proband was diagnosed by a clinical geneticist, orthopedist, or pediatrician. Appropriate informed consent was obtained from each individual. The clinical findings within each family are summarized in table 1. In most cases, information was provided directly by the affected individuals or by their parent(s). Only qualitative results are shown; no quantification of severity has been attempted. Not all family members had been tested for each manifestation of the syndrome at the time they were sampled, and some manifestations (e.g., glaucoma) may not yet be present because of age-related penetrance. In multiplex families, haplotyping with markers around the NPS locus (McIntosh et al. 1997) did not reveal any inconsistencies with linkage of the phenotype to chromosome 9q34 (data not shown).

DNA Analysis

Genomic DNA was extracted from whole blood as described by Bellus et al. (1995*a*). Mutations were identified via a combination of mutation-detection enhancement (MDE) gel analysis and DNA sequencing (Bellus et al. 1995*b;* Dreyer et al. 1998). We used the PCR primers used by Dreyer et al. (1998), except for exons

Table 1

Summary of Phenotypic Information Provided by Study Participants

Family	No. of Affected Members	Nail Hypoplasia	Patella Hypoplasia	Elbow Hypoplasia	Iliac Horns	Nephropathy	Clubfoot
2	14	13/14	9/14	5/14	Unknown	Unknown	Unknown
4	10	10/10	7/9	9/10	1/2	2/3	2/9
5	9	9/9	9/9	7/8	4/5	1/1	2/9
7	6	6/6	4/4	5/6	1/1	0/1	1/6
8	2	2/2	2/2	2/2	Unknown	Unknown	0/2
9	1 (S)	1/1	1/1	1/1	1/1	1/1	1/1
10	2	1/2	2/2	2/2	1/2	1/2	1/2
12	2	2/2	2/2	2/2	2/2	2/2	0/2
14	8	8/8	8/8	6/7	5/5	3/7	1/7
17	2	2/2	1/2	0/2	1/1	0/1	0/2
20	1 (S)	1/1	1/1	1/1	1/1	1/1	0/1
21	5	5/5	4/5	5/5	4/4	1/2	1/5
23	2	0/1	2/2	2/2	2/2	0/2	1/2
25	2	2/2	2/2	2/2	1/1	1/2	0/2
26	2	1/2	2/2	2/2	Unknown	Unknown	0/1
30	1 (S)	1/1	1/1	1/1	Unknown	Unknown	1/1
31	1 (S)	1/1	1/1	1/1	1/1	1/1	1/1
32	1 (S)	1/1	Unknown	1/1	Unknown	Unknown	0/1
34	1 (S)	1/1	1/1	1/1	1/1	1/1	1/1
35	3	3/3	2/3	2/3	2/2	0/2	0/3
42	1	1/1	1/1	1/1	Unknown	1/1	0/1
44	3	3/3	2/2	2/2	1/1	Unknown	0/2
47	1	1/1	1/1	1/1	1/1	1/1	0/1
52	3	3/3	3/3	0/1	3/3	Unknown	1/3
53	1	1/1	1/1	1/1	Unknown	Unknown	0/1
54	3	3/3	2/2	1/2	1/1	Unknown	0/3
55	2	2/2	2/2	2/2	2/2	1/1	1/2
56	5	5/5	5/5	5/5	0/3	1/4	0/5
57	2	2/2	2/2	1/2	Unknown	Unknown	0/2
60	2	2/2	2/2	2/2	Unknown	1/2	0/2
65	2	2/2	2/2	2/2	2/2	1/2	1/2
66	1 (S)	1/1	1/1	1/1	1/1	1/1	1/1
67	1 (S)	1/1	1/1	1/1	Unknown	0/1	0/1
68	1 (S)	1/1	1/1	1/1	Unknown	0/1	1/1
69	1 (S)	1/1	1/1	Unknown	Unknown	1/1	0/1
70	1 (S)	1/1	1/1	1/1	Unknown	0/1	0/1
71	1 (S)	Unknown	1/1	1/1	Unknown	1/1	Unknown
Total	91	100/104	90/100	80/99	39/46	24/46	18/87

NOTE.—S=sporadic; Unknown=unknown, not tested, or no comment.

1 and 2, which we amplified with primers LMX-1F 5′-GGCAGACGGACTGCGCC, LMX-1R 5′-TGTCCACAGCCGGACGAC, LMX-2F 5′-CCCGGTGCGACCGGGAC, and LMX-2R 5′-TGACCGGGCTCGAGTGC.

Segregation of mutations with the NPS phenotype was confirmed by restriction-enzyme digest or allele-specific oligonucleotide hybridization (Bellus et al. 1995*b*). Similarly, a population of 100 people unaffected with NPS was analyzed to show that missense mutations identified in patients were not benign variants.

Homeodomain Expression and DNA Binding

Wild-type and mutant homeodomains containing missense mutations in the N-terminal region, helix 1, and helix 2 were generated by in vitro transcription/translation of the pcDNA-5′ UT FLAG vector (Lefebvre et al. 1997) containing the respective mutant and wild-type cDNAs. The expression constructs were generated by cloning of mutagenized PCR products into the pcDNA-5′UT FLAG vector at the *Bam*HI and *Eco*RI restriction sites, followed by confirmation with DNA sequencing. Mutations were introduced into the homeodomains by the two-step PCR mutagenesis method described by Ausubel et al. (1994). Wild-type homeodomains (amino acids 186–285) were PCR amplified with primers HD5′ and HD3′. For mutant homeodomains, two rounds of amplifications were performed: first, HD5′ with MUT3′ and MUT5′ with HD3′, and then, finally, with HD5′ and HD3′. For the respective mutations, the sequences of primers were as follows (nucleotide mutations are underlined): HD5′, 5′-GCAAGGGATCCGGGGATGACGGG-3′; HD3′, 5′-GCCAGCGAATTCTAGGAAGCCATCAT-3′; MUT5′(A213P), 5′-CAGCAGCGAAGAGCCTTCAAGCCCTCCTTCGAGGTC-3′; MUT3′(A213P), 5′-GACCTCGAAGGAGGGCTTGAAGGCTCTTCGCTGCTG-3′; MUT5′(R200Q), 5′-CGGAGGCCCAAGCGACCCCAGACCATCCTCACCAC-3′; MUT3′(R200Q), 5′-GTGGTGAGGATGGTCTGGGGTCGCTTGGGCCTCCG-3′; MUT5′(R226P), 5′-GCCTTGCCGAAAGGTCCCAGAGACACTGGCAGCTG-3′; and MUT3′(R226P), 5′-CAGCTGCCAGTGTCTCTGGGACCTTTCGGCAAGGC-3′.

Parallel in vitro transcription/translation reactions were performed separately with unlabeled and labeled [^{35}S]methionine. The efficiency of the reaction and quantity of protein were estimated by SDS PAGE. The expected product was 100 amino acids in length, and 3 μl of the in vitro transcribed/translated product and 10 fmol of the [^{32}P]end-labeled FAR/FLAT sequence probe were used in electrophoretic mobility shift assay (EMSA) experiments as described elsewhere (Johnson et al. 1997).

Results

Since mutations within the homeodomain are often the cause of genetic disease (Engelkamp and van Heyningen 1996), we first sequenced this region of *LMX1B* in an affected person from each of the 41 families in the cohort. The remainder of the gene was screened for mutations by performing MDE gel heteroduplex analysis. A total of 25 mutations were identified in 37 of the 41 families (tables 1 and 2).

Four novel frameshift mutations were identified that would result in the incorporporation of a novel polypeptide sequence prior to the occurrence of a premature termination codon (table 2, fig. 1). The single-base deletions, 320delG and 534delG, were identified in sporadic cases and were not present in either parent. The precise extent of the larger deletion/insertion mutations, 485ins8 and 611del7, was determined by cloning and sequencing of each allele. Each of the larger mutations was identified in a small family and was present only in persons with NPS.

All the other mutations identified were single-base substitutions comprising eight nonsense mutations, five putative splice mutations, and six missense mutations. A single nonsense mutation, Y146X, was identified prior to the homeodomain; the others were within the homeodomain. Two of the nonsense mutations, R198X and R208X, were recurrent mutations that have been described elsewhere, in unrelated families (Dreyer et al. 1998; Vollrath et al. 1998). The R198X mutation was observed in two small families, and R223X was found in two unrelated families and a sporadic case.

We identified five putative splice mutations—71−1G→A, 257+2T→C, 688+1G→A, 688+1G→T, and 765+1G→A—that would be predicted to result in exon skipping. Among adult tissues tested, only kidney tissue expressed *LMX1B* (Dreyer et al. 1998). No RNA was available from any person carrying a splice mutation to analyze for confirmation of the predicted effects or for assessment of mRNA stability.

Among the six missense mutations identified, one, C142W, affects a highly conserved cysteine residue within the LIM-B domain. It segregated with the NPS phenotype in one family and was not detected in a sample of 100 unrelated non-NPS control individuals (data not shown). The other five missense mutations were within the homeodomain (fig. 1) and in three instances proved to have variable effects on DNA binding (see below). The N246K mutation in helix 3 of the homeodomain was found to recur in an individual unrelated to the one in whom it had been reported previously (Dreyer et al. 1998). Another mutation, R200Q, was observed in five unrelated families and represents the most common NPS mutation identified to date. Analysis of parents showed that this was a de novo occurrence

Table 2

Mutations Identified in NPS Patients Arranged by Domain and Then Mutation Type

Mutation	Nucleotide[a]	Domain	Detection	Putative Effect	Families[b]
71−1G→A	71−1G→A	LIM-A	+*Alu*I	Loss of exon 2, frameshift, PTC	21
257+2T→C	257+2T→C	LIM-A	+*Cac*8I	Loss of exon 2, frameshift, PTC	4
320delG	320delG	LIM-B	Sequence	Frameshift, PTC	71
C142W	C426G	LIM-B	ASO	2° Structure?	57
Y146X	C438A	LIM-B	+*Dde*I	PTC	56
485ins8	485insACTCCGGT	Linker	+*Rsa*I	Frameshift, PTC	8
534delG	534delG	Linker	Sequence	Frameshift, PTC	31
R198X	C592T	HD	+*Mae*III	PTC	12, 25, 67 (D)
R200Q	G599A	HD	−*Msp*I	DNA binding	10, 26, 32, 34, 47
611del7	611del7	HD	ASO	Frameshift, PTC	44
R208X	C622T	HD	ASO	PTC	554
A213P	G637C	HD	−*Hae*III	DNA binding	20, 55, 66
E216X	G646T	HD	−*Taq*I	PTC	14
S218P	T652C	HD	+*Nla*IV	DNA binding?	30
R223X	C667T	HD	ASO	PTC	7, 9, 52
672+1G→A	672+1G→A	HD	ASO	Loss of exon 4, frameshift, PTC	35
672+1G→T	672+1G→T	HD	ASO	Loss of exon 4, frameshift, PTC	60
R226X	C676T	HD	Sequence	PTC	23
R226P	G677C	HD	Sequence	DNA binding	70
A230V	C689T	HD	−*Pvu*II	DNA binding?	2
697insA	697insA	HD	Sequence	Frameshift, PTC	69 (D)
W243X	G728A	HD	+*Mae*I	PTC	17
Q245X	C733T	HD	Sequence	PTC	53
N246K	C738A	HD	Sequence	DNA binding	42, 68 (D)
750+1G→A	750+1G→A	HD	ASO	Loss of exon 5	65

NOTE.—HD=homeodomain; ASO=allele-specific oligonucleotide hybridization; and PTC=premature termination codon.

[a] Nucleotide numbering follows GenBank AF057135.

[b] Numbers refer to the families in which the mutations were identified (see Table 1). (D) indicates first described by Dreyer et al. (1998) in the families indicated.

in three of five unrelated families. Similarly, the A213P mutation in helix 1 of the homeodomain was observed in three unrelated families. The recurrence of the A213P and N246K mutations cannot be explained by presence of a CpG hotspot.

Missense mutations in the homeotic domain affected DNA binding to the FAR/FLAT sequence element of the rat insulin promoter to various extents. The R200Q and R226P mutations affect highly conserved arginine residues in the N-terminal arm and helix 2 of the homeodomain, respectively. They both reduced DNA binding but did not abolish it in the in vitro assay. In contrast, the A213P mutation abolished DNA binding to the FAR/FLAT element (fig. 2). The in vitro studies suggest that the clinical phenotype arises because of loss of function of the mutated gene product and that different mutations affect DNA binding in different ways. However, the extent of this effect in vivo is unclear.

Discussion

In our screening of 41 NPS families, we have identified 25 mutations in 37 families (fig. 1, table 2). The locations of the remainder remain unknown at this time. In each of these multigenerational families, the results of genotyping with polymorphic markers around the NPS locus were consistent with linkage to 9q34 (data not shown). It is possible that some mutations in exons 3, 7, and 8 were not detected by MDE gel analysis, although this methodology has proved successful previously and detected mutations in exon 3 and neutral polymorphisms upstream of exon 7 (data not shown). Mutations may reside within the promoter sequences; this possibility is attractive, since it appears that NPS is the result of haploinsufficiency (see below), and reduced transcription of one allele could effectively mimic the effect of a nonsense mutation. It is also possible that a substantial portion of *LMX1B* has been deleted in these families. To assess this possibility, we performed Southern blot analysis with multiple restriction-enzyme digests of genomic DNA; no differences were observed between patients and controls (data not shown). Furthermore, analysis of a *Taq*I polymorphism within the coding region (identified during mutation screening and also described by Vollrath et al. [1998]) showed that each family included at least one affected member heterozygous for exon 4—that is, this exon was present within the mutant allele.

We found six point mutations to have recurred in unrelated individuals. In at least one instance for each

mutation, de novo occurrence was documented. In cases where the mutations were segregating within families, haplotype analyses showed that the mutations had recurred on distinct chromosomal backgrounds (data not shown). The recurrence of mutations R198X, R200Q, R208X, and R223X is not unexpected, since each is the result of a transition at a CpG dinucleotide. CpG dinucleotides are believed to be hypermutable because of deamination when they are methylated (Cooper et al. 1997). Conversely, A213P and N246K result from transversions, and their recurrence cannot be explained by this mechanism.

It has been suggested that NPS results from loss-of-function mutations in *LMX1B,* that is, haploinsufficiency (Dreyer et al. 1998; Vollrath et al. 1998). Comparison of the mutations identified here (table 2) with those described elsewhere does not provide evidence to contradict the hypothesis. Since premature termination codons most often result in instability of the mutant mRNA as well as a shortened protein product (Maquat et al. 1995), it is unlikely that the products of frameshift and nonsense mutations can dramatically affect the function of the normal gene product through a dominant-negative mechanism. It is not possible to eliminate the possibility, however, since RNA from *LMX1B*-expressing tissues is not readily available. Furthermore, a balanced chromosomal translocation involving 9q34.1 has been observed to result in NPS in the absence of other anomalies (Duba et al. 1998); a child of the individual analyzed by Duba et al. (1998) exhibited partial trisomy for 9q34.1 associated with various anomalies, but not features of NPS. This suggests that the interruption of *LMX1B* by translocation results in a loss of function rather than a polypeptide with novel function or one that interacts with the product of the normal allele in a dominant-negative manner.

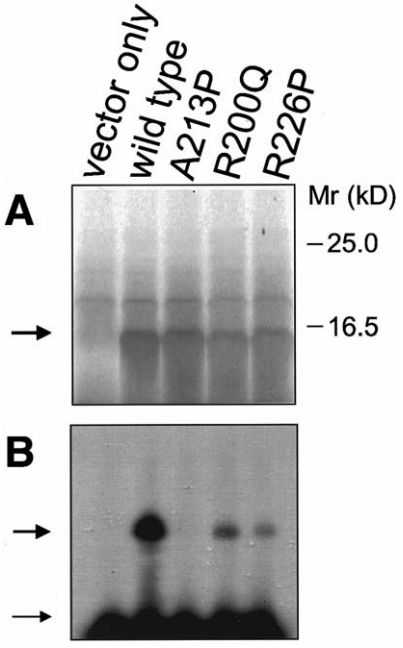

Figure 2 Homeodomain DNA binding. *A,* SDS-PAGE of in vitro transcribed/translated wild-type and mutant homeodomains. The arrow indicates the translated protein. Mr = relative molecular weight. *B,* EMSA of wild-type and mutant homeodomains to FAR/FLAT sequence probe. The thicker arrow indicates DNA-homeodomain complex; the thinner arrow indicates the free DNA probe.

Of the six novel missense mutations, one mutates a cysteine residue preserved in all LIM domains identified to date, possibly disrupting the secondary structure of the domain, and three have been shown to reduce or abolish DNA binding (fig. 2). Homeodomain-DNA interactions have been well studied since the original description of the family of homeotic genes in *Drosophila.*

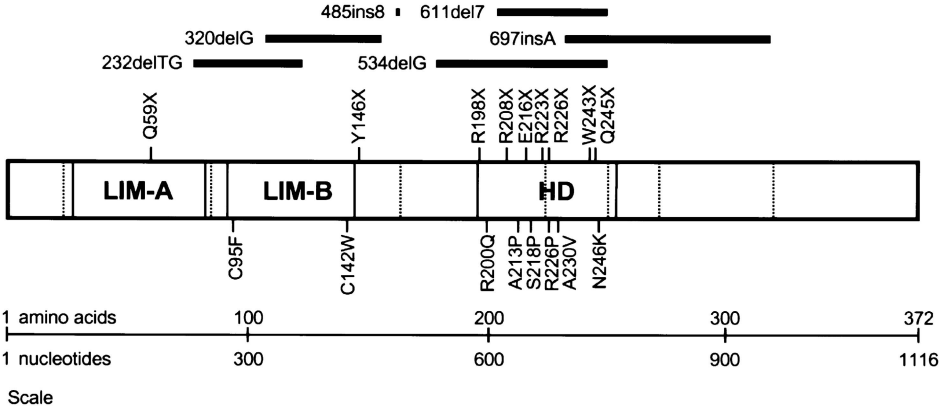

Figure 1 Relative positions of NPS mutations within *LMX1B* gene product. Nonsense mutations are above the gene product, missense mutations below. The portions of the gene product altered by frameshift mutations are indicated at the top. The positions of intron-exon boundaries are indicated by dotted lines, the boundaries between domains by solid lines. For comparison, mutations described elsewhere are included (Dreyer et al. 1998; Vollrath et al. 1998). HD denotes homeodomain.

The studies have included footprinting, transactivation assays, mobility-shift assays, and crystal structure determinations (Gehring et al. 1994). The positions of homeodomain missense mutations described in our cohort of NPS patients correlate well with residues known to be important for secondary structure, binding specificity, and/or direct DNA-backbone interactions. For example, the N-terminal arm of the homeodomain, consisting of six amino acids, is thought to be flexible and interacts with the DNA minor groove to determine sequence specificity for protein binding (Qian et al. 1989). In vitro experiments mutating this region (either by deletion or by swapping) have resulted not only in loss of DNA sequence specificity (Zeng et al. 1993), but also in a 10-fold decrease in DNA binding affinity (Qian et al. 1994). The third recognition helix is the most conserved and directly interacts with the DNA major groove. The N246K substitution, described by Dreyer et al. (1998), affects an asparagine residue important in mediating hydration and internal mobility of the homeodomain-DNA complex (Qian et al. 1993). Similarly, the arginine substitution in helix 2 (R226P) disrupts one of two arginines that directly interact with the phosphate backbone of the α-strand of the core DNA motif (Gehring et al. 1994). Finally, although the remaining residues in helix 1 and helix 2 are not known to interact directly with the DNA backbone, they do maintain the tertiary structure important in the helix-turn-helix configuration, which maintains direct DNA contact with the third helix. According to secondary-structure predictions (data not shown), the A213P substitution disrupts the α-helix in this region by replacing it with a sterically restrained amino acid, and this would be predicted to affect the overall tertiary structure of the complex. At least in vitro, the nature of the mutation correlates with a graded effect on DNA binding. However, since early premature termination of the protein and these missense mutations give similar phenotypes, it may be that these subtle differences are not apparent in vivo in the context of the cellular transcriptional apparatus. At the same time, these data suggest that the mutant *LMX1B* proteins, although unable to bind target DNA, do not act in a dominant-negative manner on the transcription apparatus.

Wilkie (1994) distinguishes between two categories of genes, in disorders inherited in a dominant fashion, in which mutations cause haploinsufficiency. In the first group are genes, expressed in certain cells at high levels, in which the 50% reduction in gene product results in the phenotype (e.g., *COL1A1* [MIM 120150] in osteogenesis imperfecta type I [MIM 166200] and *COL2A1* [MIM 120140] in Stickler syndrome [MIM 108300; Byers 1997]). The second group comprises regulatory genes whose products are required at a certain level at precise developmental stages (e.g., *PAX3* [MIM 193500] in Waardenburg syndrome [MIM 193500; Tassabehji et al. 1993] and *GLI3* [MIM 165240] in cephalopolysyndactyly [MIM 175700; Vortkamp et al. 1991]). *LMX1B* would appear to fit into the second category.

Comparison of phenotype with genotype (tables 1 and 2) did not reveal any correlation between disease severity and type or location of mutation. The vast majority of patients reported the typical orthopedic characteristics of the syndrome, and the prevalence of kidney disease was also close to that reported in earlier studies (Carbonara and Alpert 1964). Both nephropathy and kidney disease, although less common than dysplasia of the nails, patellae, and elbows, were present in families and sporadic cases with nonsense, missense, or frameshift mutations. Furthermore, patients bearing the R200Q and R226P mutations, which had a less severe effect on DNA binding than other missense mutations (fig. 2), exhibited the same degree of phenotypic variability as those in whom complete loss of function was assumed (tables 1 and 2).

A recent study suggested that open-angle glaucoma (OAG) may be part of the NPS phenotype (Lichter et al. 1997). Of the patients in this study, seven reported OAG (members of families 7, 14, 21, and 26), and in two families glaucoma was reported along with NPS in family members not available for testing (families 23 and 44). In addition, OAG was reported in two other families in whom mutations have yet to be identified. Vollrath et al. (1998) reported four loss-of-function mutations that segregated with NPS and OAG, one of which recurred in family 5 in our study. It is of importance that glaucoma was not reported in this family. These data support the hypothesis that OAG is another aspect of the pleiotropic NPS phenotype, and not due to coincidence or a closely linked gene. Furthermore, a role for *Lmx1b* during ocular development derives from the observation that *lmx1b*$-/-$ mice exhibit structural abnormalities of the anterior chamber of the eye (R. L. Johnson, personal communication).

The correlation of *LMX1B* loss-of-function mutations and the nail, patellar, and elbow dysplasia seen in NPS reflects a disruption of normal dorsoventral patterning of both hard and soft tissues in the limb. Similarly, the renal and ocular manifestations in NPS likely result from perturbations of different molecular pathways governing the processes of patterning and cell differentiation in these tissues. However, the specific actions of *LMX1B* and of downstream and upstream effectors in these tissues have yet to be determined.

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Electronic-Database Information

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

GenBank, http://www2.ncbi.nlm.nih.gov/genbank (for nucleotide numbering of NPS mutations)

Online Mendelian Inheritance in Man (OMIM), http://www3.ncbi.nlm.nih.gov/htbin-post/Omim (for NPS linked to *LMX1B*)

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