

Mutations in the Human $\lambda 5/14.1$ Gene Result in B Cell Deficiency and Agammaglobulinemia

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Summary

B cell precursors transiently express a pre-B cell receptor complex consisting of a rearranged mu heavy chain, a surrogate light chain composed of $\lambda 5/14.1$ and VpreB, and the immunoglobulin (Ig)-associated signal transducing chains, Ig α and Ig β . Mutations in the mu heavy chain are associated with a complete failure of B cell development in both humans and mice, whereas mutations in murine $\lambda 5$ result in a leaky phenotype with detectable humoral responses. In evaluating patients with agammaglobulinemia and markedly reduced numbers of B cells, we identified a boy with mutations on both alleles of the gene for $\lambda 5/14.1$. The maternal allele carried a premature stop codon in the first exon of $\lambda 5/14.1$ and the paternal allele demonstrated three base-pair substitutions in a 33-basepair sequence in exon 3. The three substitutions correspond to the sequence in the $\lambda 5/14.1$ pseudogene *16.1* and result in an amino acid substitution at an invariant proline. When expressed in COS cells, the allele carrying the pseudogene sequence resulted in defective folding and secretion of mutant $\lambda 5/14.1$. These findings indicate that expression of the functional $\lambda 5/14.1$ is critical for B cell development in the human.

Early B cell development is dependent on the orchestrated control of sequential immunoglobulin gene rearrangements and selective expansion of cells that have successfully passed checkpoint controls that evaluate rearrangements (1, 2). The pre-B cell receptor, which consists of the membrane form of a rearranged mu heavy chain, a surrogate light chain composed of VpreB and $\lambda 5/14.1$, and the immunoglobulin-associated signal transducing chains, Ig α and Ig β , play a pivotal role in this process (3, 4). Failure to express the membrane form of mu heavy chain in both humans and mice results in a complete block in B cell differentiation (5–7). In the mouse, experimentally introduced defects in $\lambda 5$ cause a block in B cell development at the transition between the pro-B cell and the pre-B cell stage (8). However, the block is not absolute, and by 4 mo of age, affected mice have ~20% of the normal number of B cells and they are able to make antibodies to both T cell-dependent and -independent antigens (8). The effects of mutations in VpreB have not been evaluated in the mouse, perhaps because there are at least two genes for VpreB (9), both of which are transcribed and expressed in early B cell development (10).

The genes for the surrogate light chains are expressed exclusively in pro-B cells and pre-B cells (9, 11), and can act as markers for these stages of differentiation. The proteins

encoded by these genes can escort the mu chain to the cell surface (12, 13), and they may assess the ability of the rearranged mu chain to bind effectively to light chains (13). Whether the surrogate light chain combines with the mu chain to form an extracellular ligand-binding motif is less clear (13). The NH₂-terminal portion of VpreB has high homology to the variable region (9), and the COOH-terminal portion of $\lambda 5/14.1$ has homology to J region and lambda constant regions (11, 14). VpreB and $\lambda 5/14.1$ are noncovalently linked to one another (15), and, $\lambda 5/14.1$ is covalently linked to the mu chain via a COOH-terminal cysteine (13, 15, 16).

The organization of the surrogate light chain genes is somewhat different in the human compared to the mouse. In the human, only a single VpreB gene has been reported; however, there are three $\lambda 5$ -like genes that have been named based on their size in EcoRI-digested genomic DNA (14, 17). The functional $\lambda 5$ -like gene is on a 14-kb EcoRI fragment. There are also two $\lambda 5$ -like pseudogenes, *16.1* and *16.2* (also called *F λ -1* [17, 18]), that have over 95% homology to $\lambda 5/14.1$ in exons 2 and 3, but lack exon 1 and associated regulatory elements (17). In addition, there are 7 to 10 lambda constant region genes, the most 5' of which has high homology to $\lambda 5/14.1$. It has been postulated that this gene, *G λ 1*, may be expressed in an unrearranged form

like $\lambda 5/14.1$ (19). Within the human lambda light chain locus on chromosome 22q11.22, the gene for *VpreB* is within the lambda variable region cluster of genes (20, 21), whereas the genes for $\lambda 5/14.1$ and *16.2* are 800–1,000 kb distal to the lambda constant region genes. The pseudogene *16.1* is 1,500 kb distal or telomeric to $\lambda 5/14.1$ (21).

In humans, mutations in Bruton's tyrosine kinase (*Btk*)¹, a cytoplasmic tyrosine kinase that is defective in X-linked agammaglobulinemia (XLA; references 22, 23), as well as mutations in mu heavy chain (5), result in a block in B cell maturation that is first manifest at the transition from the pro-B cell to pre-B cell stage of differentiation (4, 24, 25). However, mutations in these two genes do not account for all of the patients with isolated defects in B cell development (5, 26–32); therefore, we examined the possibility that some patients might have mutations in other components of the pre-B cell receptor complex.

Materials and Methods

Patients. DNA from eight unrelated patients with sporadic agammaglobulinemia was screened for mutations in $\lambda 5/14.1$, *VpreB*, *Ig α* , and *Ig β* . The patient with mutations in $\lambda 5/14.1$ had the onset of recurrent otitis at 2 mo of age and was recognized to have hypogammaglobulinemia and absent B cells at 3 yr of age when he developed hemophilus meningitis complicated by arthritis. At that time he was seronegative for the T cell-dependent antigens tetanus toxoid, diphtheria toxoid, and conjugated hemophilus influenza, despite previous immunization. He also failed to make antibody to the T cell-independent antigens in blood group substances. On four evaluations since that time, the patient has never had detectable CD19⁺ B cells by routine clinical testing. At 5 yr of age, while receiving gammaglobulin replacement therapy, he had no measurable serum IgM and IgA (<8 mg/dl). He had normal numbers of T cells and normal proliferative responses to mitogens.

Mutation Detection. Genomic DNA was analyzed by single-strand conformation polymorphism (SSCP; reference 26) using the following primers to amplify $\lambda 5/14.1$: exon 1, 5'-ACC-AGGGCACCCTCTCTA-3' and 5'-GTCATCCTTTC-CCGCCTCT-3'; exon 2, 5'-TGGGTCACAGCCTACACACT-3' and 5'-CAGAAGAGGGTGGGACAGC-3'; exon 3, 5'-TCTCACCCCTCTCTGTCC-3' and 5'-TGCAGAG-AGAGAGACCCTTCC-3'. PCR conditions were 5 min at 95°C followed by 30 cycles of 95°C for 45 s, 62°C for 30 s, and 72°C for 30 s with a final extension of 5 min at 72°C. Before electrophoresis, PCR products from exons 1 and 3 were digested with PvuII and BstUI, respectively. BstUI would be expected to cleave the functional $\lambda 5/14.1$ exon 3 but not exon 3 from the pseudogenes. Sequences demonstrating altered mobility in SSCP were cloned into TA vector (Invitrogen, Carlsbad, CA) and sequenced using M13 primers. The mutations were confirmed using a second independent PCR reaction.

Analysis of $\lambda 5/14.1$ cDNA. Reverse transcriptase PCR (RT-PCR) was used to amplify $\lambda 5/14.1$ cDNA from the patient's mononuclear bone marrow cells using the primers 5'-ACGCAT-GTGTGGCAGC-3' and 5'-GGCGTCAGGCTCAGGTA-

3'. The PCR products were cloned and sequenced as above. PCR products were digested with MspI (New England Biolabs, Beverly, MA) for 2 h at 37°C and visualized by ethidium bromide staining in a 1.8% agarose gel.

Immunofluorescence Staining. Peripheral blood lymphocytes and bone marrow cells were stained using previously described techniques and reagents (5). In addition, fixed cells were stained with an IgG1 murine monoclonal anti-VpreB antibody produced in mice immunized with a recombinant human VpreB protein (Wang, Y.-H., J. Nomura, O.M. Faye-Petersen, and M.D. Cooper, manuscript in preparation).

Vector Construction. The pre-B cell line Nalm6 was used as the source of cDNA to amplify the genes for $\lambda 5/14.1$ and *VpreB* by RT-PCR. PCR primers for $\lambda 5/14.1$ were 5'-AGGGCACCCTCTCTAGGGA-3' and 5'-TGCAGAGAGAGACCCTTCC-3', and for *VpreB* were 5'-GGCCACAGGAGTCA-GAGCT-3' and 5'-GATGCGTGCCTCTGTCTT-3'. PCR products were cloned into the TA vector and the Pro 142Leu mutant was generated by replacing the endogenous SacI-BamHI fragment of exon 3 with that of the patient. After the sequence had been verified, the three cDNA products were cloned into the pcDNA3 expression vector (Invitrogen) that contains a CMV promoter and SV40 origin of replication.

Transfection and Immunoprecipitation. COS7 cells were transfected with expression vectors for the normal or mutant $\lambda 5/14.1$, with or without *VpreB* using lipofectamine (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's protocol. The cells were metabolically labeled with 0.3 mCi of ³⁵S-Translabel (ICN, Irvine, CA) for 2 h, 44 h after transfection. Labeled cells were lysed in 1% Triton X-100, 10 mM Tris (pH 8.0), 140 mM NaCl, 1% bovine hemoglobin, 0.2 U/ml aprotinin, and 1 mM PMSF. After preclearing, lysates were incubated with murine anti-VpreB or goat antilambda chain antibodies (Sigma Chemical Co., St. Louis, MO). Specific proteins were immunoprecipitated with protein G Sepharose (Pharmacia, Uppsala, Sweden) and eluted with a sample buffer containing 50 mM Tris (pH 6.8), 10% glycerol, 1% SDS, 0.001% bromophenol blue, with or without 5% 2-mercaptoethanol. Proteins were separated by 12.5% SDS-PAGE and detected by fluorography.

Results

Identification of Mutations in $\lambda 5/14.1$. Genomic DNA samples from eight patients with defects in B cell development but without mutations in *Btk* or mu heavy chain were screened for mutations in genes that encode components of the pre-B cell receptor complex. PCR was used to amplify individual exons by flanking splice sites and the products were analyzed with SSCP. A 5-yr-old boy with <1% of the normal number of B cells and severe hypogammaglobulinemia was found to have alterations in exons 1 and 3 of the surrogate light chain gene, $\lambda 5/14.1$. Although both the patient's mother and father had normal percentages of circulating CD19⁺ B cells, 6 and 10% respectively, SSCP analysis of genomic DNA from the patient's parents indicated that the abnormality in exon 1 was derived from the maternal allele and the abnormality in exon 3 was derived from the paternal allele. Both exons were cloned and sequenced.

The patient's exon 1 sequence revealed a C to T transition at codon 22, resulting in the substitution of a premature stop codon for the wild-type glutamine (Fig. 1). In

¹Abbreviations used in this paper: *Btk*, Bruton's tyrosine kinase; RT-PCR, reverse transcriptase PCR; SSCP, single strand conformation polymorphism; TdT, terminal deoxythymidyl transferase.

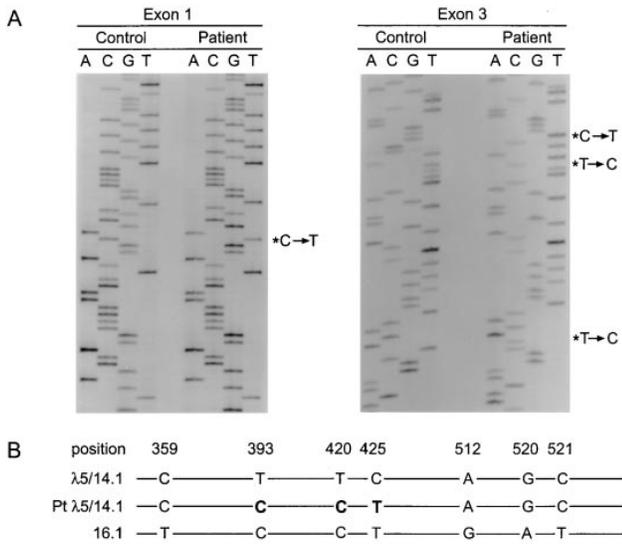


Figure 1. Comparison of the normal and mutant $\lambda 5/14.1$ genes. (A) Partial DNA sequence of the first (left) and third (right) exon of $\lambda 5/14.1$ in the patient and in a normal control. The basepair substitutions are indicated by an asterisk. (B) Sequence alignment of the third exon of the functional $\lambda 5/14.1$ gene, the patient's $\lambda 5/14.1$ gene, and the 16.1 pseudogene. Only the basepairs at which $\lambda 5/14.1$ and the pseudogene 16.1 differ are indicated. The basepairs at which the patient's $\lambda 5/14.1$ gene correspond to the 16.1 gene are shown in bold type.

exon 3, there were three basepair substitutions: T to C at nucleotide 393 (codon 131), T to C at nucleotide 420 (codon 140), and C to T at nucleotide 425 (codon 142). The first two substitutions do not change the coding sequence; however, the third results in the replacement of the wild-type proline with leucine. The proline at this site, which occurs in the loop linking the second and third strands of one of the two beta pleated sheets that compose the immunoglobulin domain (33), is conserved not only in lambda constant region domains in all species evaluated, but also in most immunoglobulin domains (11). SSCP analysis of exon 3 of $\lambda 5/14.1$ in DNA from 100 individuals did not demonstrate the pattern seen in the patient's paternal allele in any other samples.

The three basepair substitutions in the paternal allele of $\lambda 5/14.1$ are the same as those found at the corresponding site in exon 3 of the $\lambda 5/14.1$ pseudogene, 16.1 (17; Fig. 1). To determine whether the altered sequence could be attributed to gross rearrangements within the lambda locus, DNA from the patient was digested with EcoRI and HindIII and examined by Southern blot using an exon 3 $\lambda 5/14.1$ probe. The results demonstrated the expected fragments representing the $\lambda 5$ -like genes and the lambda constant region genes, and did not reveal any altered fragments (Fig. 2).

The mutation in the paternal allele was confirmed by analyzing cDNA from the patient's bone marrow. RT-PCR, using primers from within exons 2 and 3, allowed the amplification of the 3' portion of the patient's $\lambda 5/14.1$ gene. The C to T basepair substitution at nucleotide 425 would be expected to destroy an MspI site at this locus. To deter-

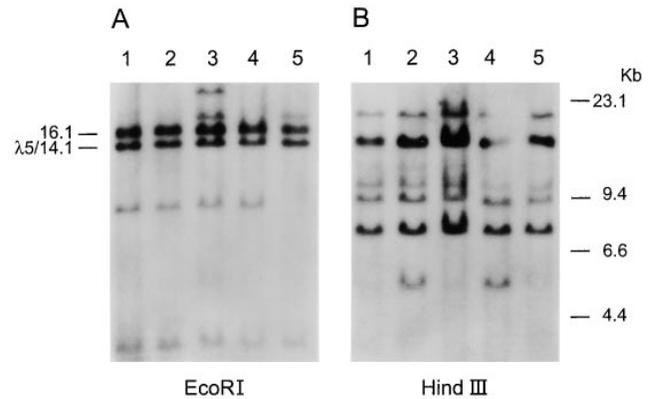


Figure 2. Southern blot analysis of the lambda and lambda-like genes. Genomic DNA from the patient (lane 1) and four controls (lanes 2–5) was digested with EcoRI (A) or HindIII (B) and analyzed with a probe from the third exon of $\lambda 5/14.1$. The molecular size standards are shown at the right side of the figure. In the EcoRI digest, the genes for $\lambda 5/14.1$ and the pseudogene 16.1 are indicated at the left side of the figure. The bands at 8, 18, and 23 kb in the EcoRI digest represent previously reported polymorphic variants in the number of lambda constant region genes (49).

mine the proportion of the transcripts derived from the maternal and paternal alleles, the RT-PCR products from a control and the patient were digested with MspI and examined in an agarose gel. Although all of the product from the normal control was digested, none of the product from the patient was cleaved, indicating that all of the transcripts were derived from the paternal allele (Fig. 3). The patient's PCR product was sequenced and revealed the three basepair substitutions specific to the 16.1 pseudogene sequence flanked by sequence characteristic of the functional $\lambda 5/14.1$ gene (Fig. 1). These results provide strong support for the occurrence of a gene conversion event in the lambda locus.

In Vivo Consequences of Mutations in $\lambda 5/14.1$. The effects of the $\lambda 5/14.1$ gene mutations on B cell development

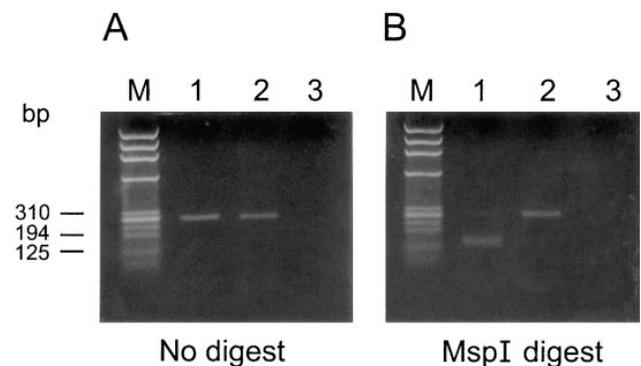


Figure 3. Analysis of $\lambda 5/14.1$ cDNA from the patient's bone marrow. RT-PCR was used to amplify the 3' portion of the $\lambda 5/14.1$ transcripts from an age-matched control (lane 1), the patient (lane 2), or a negative control (lane 3). Molecular size standards (lane M), the undigested PCR products (A), and the MspI-digested products (B) were separated by electrophoresis. All of the 266-basepair product from the control, but none from the patient, was digested to yield 143- and 123-basepair fragments.

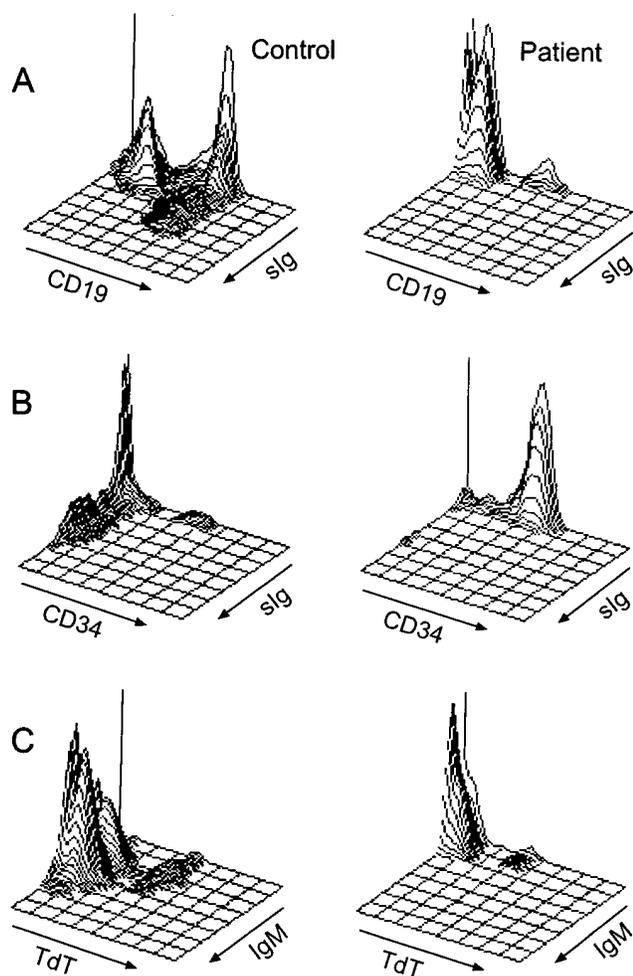


Figure 4. Flow cytometric analysis of bone marrow B cell precursors from a normal age-matched control (*left*) and the patient with mutations in $\lambda 5/14.1$ (*right*). (A) Isometric contour plots of lymphoid-gated cells stained with antibodies to CD19 and surface immunoglobulin (kappa + lambda light chains). In the control, 46% of cells were CD19⁺, of which 29% were surface Ig (sIg)⁺. In the patient, 6% of cells were CD19⁺, with only 0.7% of which were sIg⁺. (B) Three-color immunofluorescence was used to stain cells for CD19, CD34, and sIg and CD19⁺ cells were analyzed. In the control sample, 11% of CD19⁺ cells were CD34⁺ and 60% were CD34⁻, sIg⁻. In the patient, 87% of CD19⁺ cells were CD34⁺. (C) Permeabilized cells were stained for TdT and surface or cytoplasmic IgM. In the control, 5% of cells were TdT⁺, IgM⁻, 1% were TdT⁺, IgM⁺, and 40% were TdT⁻, IgM⁺. In the patient, 5% of cells were TdT⁺, IgM⁻, 0.2% were TdT⁺, IgM⁺, and 0.4% were TdT⁻, IgM⁺.

were examined in the patient's peripheral blood and bone marrow. In normal individuals, between 5 and 18% of the peripheral blood lymphocytes are B cells as defined by expression of CD19. Our past studies have shown that in patients with X-linked agammaglobulinemia, who have mutations in *Btk*, B cells represent between 0.01 and 0.10% of lymphocytes (34, 35), whereas in patients with mutations in μ heavy chain, B cells cannot be detected (5). In the patient with mutations in $\lambda 5/14.1$, 0.06% of the peripheral blood lymphocytes expressed CD19. Like normal circulating B cells, these cells had low intensity expression of sur-

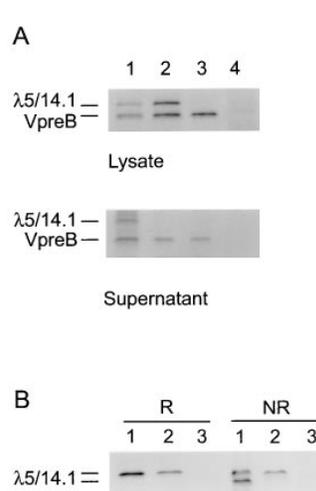


Figure 5. Transfection of COS7 cells with expression vectors for normal or mutant $\lambda 5/14.1$ and normal *VpreB*. (A) Cells were transfected with *VpreB* and normal $\lambda 5/14.1$ (lane 1), *VpreB* and mutant $\lambda 5/14.1$ (lane 2), *VpreB* alone (lane 3), or an empty vector (lane 4). After metabolic labeling, both lysates and supernatants were immunoprecipitated with antibody to *VpreB*. (B) COS7 cells were transfected with normal $\lambda 5/14.1$ (lane 1), mutant $\lambda 5/14.1$ (lane 2), or an empty vector (lane 3), immunoprecipitated using a polyclonal antilambda antibody, and electrophoresed under reducing (R) or nonreducing (NR) conditions.

face IgM and dim expression of CD38. By contrast, B cells seen in patients with mutations in *Btk* expressed an immature phenotype as defined by high intensity expression of both surface IgM and CD38 (34, 35).

Bone marrow cells from the patient and a normal age-matched control were stained in suspension with antibodies to surface immunoglobulin and CD19. Although a small number of CD19⁺ cells could be detected, there were almost no mature B cells as defined by coexpression of CD19 and surface immunoglobulin (Fig. 4 A). Over 85% of the CD19⁺ cells were positive for CD34 (Fig. 4 B), indicating a block at the transition between the pro-B cell and pre-B cell stage of differentiation. This block was also seen in permeabilized cells stained for terminal deoxynucleotidyl transferase (TdT) and surface or cytoplasmic IgM (Fig. 4 C). Although the number of TdT⁺ cells in the patient was comparable to the control, there were very few cells at the next stage of differentiation, cells which express cytoplasmic μ heavy chain. Bone marrow from $\lambda 5$ knockout mice has markedly decreased expression of *VpreB* (36), suggesting that in the absence of $\lambda 5$, *VpreB* is unstable. In bone marrow of controls, a mean of 82% of TdT⁺ cells was dimly positive for cytoplasmic *VpreB*, whereas, in the patient, 14% of the TdT⁺ cells were *VpreB*⁺.

Functional Consequences of Mutations in $\lambda 5/14.1$. To determine the functional consequences of the amino acid substitution in exon 3 of $\lambda 5/14.1$, expression vectors for *VpreB* and the normal and mutant $\lambda 5/14.1$ genes were introduced into COS7 cells by lipofection. In metabolically labeled cells transfected with *VpreB* and the normal or mutant $\lambda 5/14.1$, both *VpreB* and $\lambda 5/14.1$ could be immunoprecipitated from cell lysates using a monoclonal anti-*VpreB* antibody, demonstrating that the mutant as well as the normal $\lambda 5/14.1$ could bind to *VpreB*. However, analysis of supernatants from cells transfected with the same vectors indicated that normal $\lambda 5/14.1$ was secreted into the supernatant with *VpreB*, as previously described (13); whereas the mutant $\lambda 5/14.1$ was not secreted (Fig. 5 A).

Because appropriate protein folding is required for protein secretion, and the amino acid substitution in the mu-

tant $\lambda 5/14.1$ was at a site that would be expected to influence tertiary structure, the capacity of the normal and mutant $\lambda 5/14.1$ proteins to fold was compared in reducing and nonreducing conditions. As shown in Fig. 5 B, under nonreducing conditions, $\sim 50\%$ of the normal $\lambda 5/14.1$ moved through the gel more rapidly because of protein folding, but none of the mutant $\lambda 5/14.1$ demonstrated altered migration. When coupled with the decreased expression of V_{preB} in the patient's bone marrow, these studies suggest that the mutant $\lambda 5/14.1$ was improperly folded and degraded within the endoplasmic reticulum (37).

Discussion

The results of these studies demonstrate that mutations in $\lambda 5/14.1$ can result in profound B cell deficiency in humans. Transcription of the $\lambda 5/14.1$ pseudogenes, *16.2* and *G λ 1*, has been described (18, 19), but it is not clear whether these transcripts are needed for normal development. The severe block in B cell differentiation in our patient indicates that the surrogate light chain pseudogenes in the patient could not compensate for the mutations in the $\lambda 5/14.1$ gene. The fact that the patient had two different mutations in $\lambda 5/14.1$ and that he was not a member of an isolated or inbred population, suggests that there may be other similar patients.

The mutation derived from the maternal allele in the patient was a C to T transition in the first exon resulting in the replacement of a wild-type glutamine with a premature stop codon. Premature stop codons in many genes, including *Btk*, β globin, and dihydrofolate reductase, are associated with faulty processing of the pre-messenger RNA and poor accumulation of the transcript in the cytoplasm (26, 38, 39), as was seen in this case. Thus, this mutation is functionally a null mutation.

The mutation in the paternal allele most likely occurred by gene conversion rather than by two unequal crossover events or three separate basepair substitutions. None of the three basepair substitutions seen in the patient's paternal allele were found individually in 100 normal people. Gene conversion is an uncommon mechanism of mutation; however, it has been suggested that gene conversion between the two highly homologous human fetal globin genes, *A γ* and *G γ* , explains the genetic variation within these tandemly duplicated genes (40). Immediate proximity of the donor and recipient DNA does not appear to be necessary for gene conversion. Although the functional von Willebrand factor gene is on chromosome 12, a portion of the

gene is duplicated as a pseudogene on chromosome 22. In two families with von Willebrand disease, two different segments of the functional gene were replaced by portions of the pseudogene resulting in three basepair substitutions (41), as in our patient. The fact that gene conversion is used to generate antibody diversity in some species (42, 43) suggests that immunoglobulin domains may be unusually susceptible to this type of mutational event.

It is intriguing that the defects in $\lambda 5/14.1$ seen in the patient described in this paper resulted in a disease more severe than that seen in mice lacking $\lambda 5$ due to homologous recombination. One could postulate that the mutant $\lambda 5/14.1$ protein produced in the patient's bone marrow might have a more deleterious effect than the absence of $\lambda 5$ in the knockout mice. However, based on the observation that the patient's father, who was heterozygous for this mutation, had normal B cell numbers, the possibility of a dominant negative mutation is unlikely. The immune function in the $\lambda 5$ knockout mice improved with time (8); it is possible that older patients with defects in $\lambda 5/14.1$ may repair their B cell defects and antibody-forming capacity. Other explanations can be considered. The leaky phenotype in $\lambda 5$ -deficient mice has been attributed to B cell precursors in which light chain gene rearrangement preceded heavy chain gene rearrangement, thus negating the requirement for $\lambda 5$ expression (8, 44). Although studies using human EBV-transformed B cell lines first suggested that rearrangement of light chain genes could occur before that of the heavy chain genes (45), the frequency or efficiency of this event may differ in the two species. There may be other factors that compensate for the lack of expression of $\lambda 5$, and these factors may differ in the human compared to the mouse. Finally, the recovery of $\lambda 5$ -deficient mice is dependent on the expansion of early B cell precursors that have successfully rearranged both heavy and light chain genes. There may be differences in the regulation of this expansion in the human compared to the mouse.

Early B cell development in the mouse, but not the human, is highly dependent on signaling through the IL-7R (46, 47). By contrast, defects in *Btk* disrupt the pro-B cell to pre-B cell transition in the human, but not in the mouse (4, 25, 48). Our studies demonstrating the greater consequences of mutations in $\lambda 5/14.1$ in the human compared to the mouse further emphasize the use of studying multiple models of B cell development. Comparison of the effects of mutations in the same gene in different species highlights the aspects of B cell signaling that are invariant and those that may be influenced by other genetic or environmental factors.

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