Characterization of Mutations, Including a Novel Regulatory Defect in the First Intron, in Bruton's Tyrosine Kinase Gene from Seven Korean X-Linked Agammaglobulinemia Families

Eun-Kyeong Jo, Hirokazu Kanegane, Shigeaki Nonoyama, Satoshi Tsukada, Jae-Ho Lee, Kyu Lim, Minho Shong, Chang-Hwa Song, Hwa-Jung Kim, Jeong-Kyu Park and Toshio Miyawaki

*J Immunol* 2001; 167:4038-4045; doi: 10.4049/jimmunol.167.7.4038
http://www.jimmunol.org/content/167/7/4038

**References**
This article cites 39 articles, 14 of which you can access for free at: http://www.jimmunol.org/content/167/7/4038.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Characterization of Mutations, Including a Novel Regulatory Defect in the First Intron, in Bruton’s Tyrosine Kinase Gene from Seven Korean X-Linked Agammaglobulinemia Families

Eun-Kyeong Jo, Hirokazu Kanegane, Shigeaki Nonoyama, Satoshi Tsukada, Jae-Ho Lee, Kyu Lim, Minho Shong, Chang-Hwa Song, Hwa-Jung Kim, Jeong-Kyu Park, and Toshio Miyawaki

In this report, we describe seven mutations, including a novel single base pair substitution in intron 1, of the Bruton’s tyrosine kinase (Btk) gene found in 12 Korean patients with X-linked agammaglobulinemia. Various mutations, including three novel genetic alterations, were discovered using single-strand conformation polymorphism analysis and direct DNA sequencing. The effect of the intron 1 point mutation (intron 1 +5G→A) was further evaluated using reporter constructs. Using luciferase assay experiments, we showed that the transcriptional activity of the mutant was significantly lower than in normal counterparts, indicating that the intronic mutation was functional. In addition, DNase I footprinting analysis showed that a single protected region spanning the position +3 to +15 bp hybridized with a mutant-specific probe, but not with a wild-type probe. EMSA indicated that a distinct nuclear protein has the ability to bind the mutant oligonucleotides to produce a new DNA-protein complex. We also observed decreased expression of Btk proteins in monocytes of patients having the point mutation in intron 1. Taken together with the functional analysis, our results strongly suggest the existence of a novel cis-acting element, which might be involved in the down-regulation of Btk gene transcription. Precise definition of the regulatory defect in the Btk intron 1 may provide valuable clues toward elucidating the pathogenesis of X-linked agammaglobulinemia. The Journal of Immunology, 2001, 167: 4038–4045.

X-linked agammaglobulinemia (XLA) is a disease resulting from mutational defects in the Bruton’s tyrosine kinase (Btk) gene (1). It is characterized by the early onset of bacterial infection, profound hypogammaglobulinemia, a marked decrease in the number of B lymphocytes, and an almost total plasma cell deficiency (2, 3). Although most affected males are recognized as having immunodeficiency early in life, diagnosis may be delayed, particularly in atypical or sporadic cases (4–6). Since associated infections, particularly bacterial meningitis and pneumonia, are often life-threatening (7), accurate diagnosis and genetic counseling are critical for the management of XLA. Recently, the measurement of intracellular Btk protein expression levels in monocytes has been proposed as a diagnostic tool to evaluate human XLA patients and carriers (8).

Btk is a signal-transducing protein expressed in all hematopoietic lineages, except T cells and NK cells (7, 9–11). Btk protein is a member of the Tec family of protein tyrosine kinases (Tec kinases), which includes Itk, Tec, Bmx, and Txk. Btk is composed of five distinct structural domains: the pleckstrin homology (PH), Tec homology (TH), Src homology (SH) 3, SH2, and catalytic kinase (SH1) domains (12–16). In vivo and in vitro studies indicate that Btk protein is essential for B cell survival, cell cycle progression, and proliferation in response to B cell Ag receptor stimulation (17).

The human Btk gene is located in the Xq22 region of the X chromosome, encompassing 37.5 kb and containing 19 exons, 18 of which encode the protein. The first exon and 30 bp of the second exon constitute the 5′ untranslated region of the Btk mRNA (18), and a cluster of transcriptional start sites have been identified upstream of exon 1. DNA-based mutation scanning techniques, such as single-strand conformation polymorphism (SSCP), have been used to identify Btk mutations. A database of Btk mutations lists 600 mutation entries from 518 unrelated families, comprising 378 unique molecular events (http://protein.uta.fi/BTKbase/tables.html). Mutations have been identified throughout all five domains of Btk and are associated with reductions in Btk mRNA, protein, and kinase activity (2, 6, 9, 19–21).

Nevertheless, certain cases have been reported in which reduced Btk mRNA levels were not correlated with mutations in any of the Btk coding regions (20). It is possible that these patients have some transcriptional regulation defect; however, the precise mechanisms of Btk gene regulation in XLA patients that lack Btk mRNA remain to be elucidated. A previous study (22) identified a single
base pair substitution (intron 1 +6T→G) in the first intron of \( Btk \) in an XLA patient, resulting in decreases in both \( Btk \) mRNA expression and luciferase production. Two transcriptional control elements in intron 1 and a strong positive regulator, active in both pre-B cells and B cells, were identified within the first 43 bp of the intron (22).

This report is the first study of Btk deficiency in Korea. We clinically and genetically characterized 12 patients with XLA, identifying seven mutations including three novel genetic alterations (a point mutation in intron 1 and two deletions) of the \( Btk \) gene in seven unrelated families. In addition, we used flow cytometric assays to demonstrate deficient \( Btk \) expression in patients and cellular mosaicism in carriers. We further analyzed the functional activities of the point mutation in intron 1 and concluded that this mutation is probably involved in the defective regulation of \( Btk \) through a pathway dissimilar to that reported previously (22).

**Materials and Methods**

**Subjects**

Seven different Korean XLA families (12 patients) were included in the study (Table I). All of the XLA patients were males, ranging in age from 1 to 23 years. Diagnosis was based on 1) an absence of, or severe deficiency in, circulating B cells; 2) a very low level of serum Igs, in particular \( \text{IgG} \) and 3) a history of recurrent bacterial infections. The male siblings of 11 of the 12 patients had familial disease histories. Informed consent was obtained from all patients and their family members before inclusion in this study.

Infections requiring hospitalization developed in eight patients at a mean age of 9.3 mo (range, 3–28 mo), requiring i.v. Ig replacement therapy. Recurrent ear, nose, and throat infections and pneumonia developed in all eight patients, and \( \text{Pneumocystis carinii} \) pneumonia was documented in one patient (P1). Chronic sinusitis and otitis medium were documented in two children (P3-1 and P6), and chronic arthritis and epididymitis were diagnosed in patient P4-1. Patient P5 died from liver cirrhosis as a result of chronic viral hepatitis, despite receiving regular i.v. Ig therapy. Currently, the six surviving patients (P2-2 also died) receive regular i.v. Ig infusions. Two apparently healthy children (P3-2, P4-2) were diagnosed as XLA patients based solely on their family history, in the absence of any clinical symptoms.

**Flow cytometric analysis of cellular Btk expression**

Heparinized venous blood samples from patients and family members were fractionated on a Ficoll-Hypaque gradient to isolate PBMC. PBMC were washed three times in PBS (pH 7.4) and analyzed by flow cytometry (EPICS XL-MCL; Corixa, Hialeah, FL).

**RT-PCR and DNA sequencing**

Total RNA was prepared from PBMC using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized from RNA using the reverse transcription reaction described previously (20). PCR amplification of the \( Btk \) cDNA was performed using seven overlapping PCR primers as described elsewhere (20). The resulting PCR products were purified using a PCR DNA purification system (Promega, Madison, WI). Direct sequencing of the amplified RT-PCR products by the dideoxy nucleotide-chain termination method (23) was performed using the Sequenase 2.0 kit (USB, Cleveland, OH) and the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) along with an automated Applied Biosystems PRISM 310 genetic analyzer (PE Applied Biosystems). Forward and reverse primers for use in manual DNA sequencing were labeled with \( \alpha \)-35S-dATP, and the reaction products were electrophoresed on denaturing 6% polyacrylamide gels. Mutated sequences were confirmed by opposite-strand sequencing or by sequencing genomic DNA fragments covering intron-exon boundaries.

**SSCP and RFLP analyses**

For SSCP analysis, 5 μl of PCR product was mixed with 9 μl of sample loading buffer containing 95% formamide, 10 mM NaOH, 0.25% bromophenol blue, and 0.25% xylene cyanol. The samples were denatured for 5 min at 100°C in 1× sample buffer (33 mM Tris-sulfate and 7% glycerol, pH 8.3). The DNA was then resolved by 12% PAGE and stained using a Silver Stain Plus kit (Bio-Rad, Hercules, CA).

In family 1, the G to A substitution predicted the creation of a new MboI restriction endonuclease site. We performed PCR-RFLP in this family and 50 Korean normal controls. The exon 1/intron 1 border covering the –67- to +297-bp region was amplified and purified as described for DNA sequencing, treated with MboI, and analyzed by 2% agarose gel electrophoresis.

**Gene reporter constructs**

The following reporter constructs were kindly provided by Dr. J. Rohrer and Dr. M. E. Conley (St. Jude Children’s Research Hospital, Memphis, TN): 1) \( \text{pBtkpro}+1029 \), containing the luciferase reporter gene and pGL2 carrying 1029 bp of intron 1 and the acceptor sequence; 2) \( \text{pBtkpro}+43 \), having a deletion of \( \text{pBtkpro} \); 3) \( \text{pBtkpro}+1029-\text{mt6} \) containing the intron1-5 exons into \( \text{pBtkpro} \); and 4) \( \text{pBtkpro}+43-\text{mt6} \), a deletion derivative. The construction of the reporter constructs has been described previously (22). A PCR-based strategy (22) was used to introduce patient DNA mutations into \( \text{pBtkpro}+1029 \) (\( \text{pBtkpro}+1029-\text{mt5} \)), Briefly, genomic DNA of patient (P1-1) was amplified with the sense primer 5‘-CAGACTGTCTTCCTCTTC-3’ and the antisense primer 5‘-AGCCAGCTCTGAC

<table>
<thead>
<tr>
<th>Table I. Patient laboratory data and family history</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient</strong></td>
</tr>
<tr>
<td>P1-1</td>
</tr>
<tr>
<td>P1-2</td>
</tr>
<tr>
<td>P2-1</td>
</tr>
<tr>
<td>P2-2</td>
</tr>
<tr>
<td>P3-1</td>
</tr>
<tr>
<td>P3-2</td>
</tr>
<tr>
<td>P3-3</td>
</tr>
<tr>
<td>P4-1</td>
</tr>
<tr>
<td>P4-2</td>
</tr>
<tr>
<td>P5</td>
</tr>
<tr>
<td>P6</td>
</tr>
<tr>
<td>P7</td>
</tr>
</tbody>
</table>

*a Reference values: serum IgG at various ages: 5–12 mo, 172–1069; 1–5 years, 345–1236; 6–10 years, 608–1572; and adult, 639–1349 (mg/dl). Serum IgM: 5–9 mo, 33–126; 10–12 mo, 41–173; 2–8 years, 43–207; 9–10 years, 52–242; and adult, 56–352 (mg/dl). Serum IgA: 7–12 mo, 11–106; 2–5 years, 14–159; 6–10 years, 33–236; and adult, 70–312 (mg/dl).
in 100 hours after electroporation, the cells were washed three times in PBS, lysed for Daudi cells, with the capacitance extender set at 960 fi ng/ml gentamicin.


double-stranded oligonucleotide probes that covered the Nuclear extracts and EMSA DNase I footprinting assay (25). Nuclear extracts for the DNase I footprinting assay were coincubated for 15 min and treated with freshly diluted T4 polynucleotide kinase and used as either wild-type or mutant constructs. Excision of BamHI-HindIII DNA fragments from these constructs was a carrier. Since the G to A substitution predicted the creation of a 3'-TCA GGA CTG AGG TGG GTC TGG GGT ATG 19 probe including the patient region at the exon 1/intron 1 border were made. Probe sequences were as follows: −10+19, 5'-TCA GGA CTG AGG TGG GTC TGG GGT ATG GCA-3'. An additional −10+19 probe including the patient’s G to A mutation at position +5 was also made (−10+19 M). Gel-purified oligonucleotides were labeled with [γ-32P]ATP using T4 polynucleotide kinase and purified on an 8% nondenaturing polyacrylamide gel. EMSA was performed as previously described (26). Binding reactions in high salt with detergent were conducted in a solution of 1.5 fmol of 32 P-labeled DNA, 2 μg of nuclear extract, and 0.5 μg of poly(de-ic) in 10 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, and 12.5% glycerol. Where indicated, unlabeled double-stranded oligonucleotides were also added as competitor to the binding reaction and incubated for 20 min before the addition of labeled DNA. After incubation, reaction mixtures were analyzed by electrophoresis on 4 or 4.5% native polyacrylamide gels at 160 V in 0.5X Tris-borate-EDTA buffer. Gels were dried and autoradiographed.

Results

Identification of Btk mutations

Patient cDNAs were successfully amplified and the PCR products were directly sequenced. A point mutation in intron 1, a missense mutation, three deletions, and two splicing errors were found. The Btk mutations and their expression in XLA patients are summarized in Table II.

The pedigree of family 1 is shown in Fig. 1A. A point mutation was observed in the intron 1 region (Fig. 1B). The entire coding region and the 5' and 3' untranslated regions of the cDNA were directly sequenced; however, no abnormalities were identified. To determine whether any cryptic splice sites were activated because of the +5 alteration, PCR primers were designed to amplify the cDNA region surrounding the exon 1/exon 2 cDNA border, as previously described (22). When cDNA from freshly isolated PBMC was used as the template, both PCRs showed the expected products. No PCR products using cryptic splice sites were identified (data not shown). Both patient P1-1 and his younger brother (P1-2) showed the same mutation at position 5 in intron 1. Their mother was heterozygous for G/A at this site, implying that she was a carrier. Since the G to A substitution predicted the creation of a new MboI restriction endonuclease site, the sequencing result was further confirmed by RFLP analysis of this family (Fig. 1C). As expected, the normal control and DNA of another XLA patient (P3-1) were resistant to MboI. The DNA of each brother was cleaved into identical smaller fragments indicating G to A substitution. Their mother and sister possessed the resistant band seen in the controls as well as two smaller bands identical to the ones in lanes 1 and 2, implying heterozygosity. The characterization of this mutation is further described in the next section.

Table II. Btk mutations and expression status in XLA patients and carriers as evaluated by a flow cytometric assay

<table>
<thead>
<tr>
<th>Subject (Patient)</th>
<th>Intron/exon</th>
<th>Domain</th>
<th>Change</th>
<th>Effect</th>
<th>Pattern of Btk expression</th>
<th>Btk expression in monocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-1</td>
<td>Intron 1</td>
<td>5' Untranslated</td>
<td>G-&gt;A</td>
<td>Regulatory defect (+5)</td>
<td>Deficient</td>
<td>18.5</td>
</tr>
<tr>
<td>P1-2</td>
<td>Intron 1</td>
<td>5' Untranslated</td>
<td>G-&gt;A</td>
<td>Regulatory defect (+5)</td>
<td>NT*</td>
<td>NT</td>
</tr>
<tr>
<td>P2-1</td>
<td>Intron 18</td>
<td>Kinase</td>
<td>G-&gt;T</td>
<td>Splice-donor defect (+1)</td>
<td>Deficient</td>
<td>0.9</td>
</tr>
<tr>
<td>P2-2</td>
<td>Intron 18</td>
<td>Kinase</td>
<td>G-&gt;T</td>
<td>Splice-donor defect (+1)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>P3-1</td>
<td>Exon 3</td>
<td>PH</td>
<td>ATC-&gt;ACC</td>
<td>I61T</td>
<td>Deficient</td>
<td>2.3</td>
</tr>
<tr>
<td>P3-2</td>
<td>Exon 3</td>
<td>PH</td>
<td>ATC-&gt;ACC</td>
<td>I61T</td>
<td>Deficient</td>
<td>2.7</td>
</tr>
<tr>
<td>P3-3</td>
<td>Exon 3</td>
<td>PH</td>
<td>ATC-&gt;ACC</td>
<td>I61T</td>
<td>Deficient</td>
<td>0.9</td>
</tr>
<tr>
<td>P4-1</td>
<td>Intron 6</td>
<td>TH</td>
<td>G-&gt;A</td>
<td>Splice-donor defect (+5)</td>
<td>Deficient</td>
<td>1.4</td>
</tr>
<tr>
<td>P5</td>
<td>Exon 8</td>
<td>SH3</td>
<td>+710delAGGG</td>
<td>K237delX275</td>
<td>Deficient</td>
<td>1.4</td>
</tr>
<tr>
<td>P6</td>
<td>Exon 3</td>
<td>PH</td>
<td>+160delG</td>
<td>G54deX56</td>
<td>Deficient</td>
<td>2.3</td>
</tr>
<tr>
<td>P7</td>
<td>From intron 9+191T</td>
<td>SH3</td>
<td>980 bp del</td>
<td>exon 10 skipping</td>
<td>Deficient</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Data represent the percentages of anti-Btk stainable cells exceeding the upper limit of control staining with an isotype-matched irrelevant moAb.

* del, Deletion; NT, not tested.
Two novel deletion mutations were detected in families 5 and 7. Fig. 2A illustrates the pedigree of family 5. SSCP and DNA sequencing analyses of patient P5 showed a 4-bp deletion in exon 8 (Fig. 2), which results in a frameshift producing a premature stop codon (K237delX275). In family 5, two maternal aunts did not display any apparent conformational polymorphism in PCR-SSCP, whereas the mother and a third maternal aunt showed heterozygosity at positions 842–845, implying that both were carriers. However, the mutation was absent in the maternal grandmother (Fig. 2B). Another novel deletion was found in family 7. PCR amplification of genomic DNA from a patient in this family, using a 5′ primer of exon 9 and a 3′ primer of exon 11, revealed a PCR product of ~700 bp. This PCR product was correlated with a 980-bp deletion from intron 9 to intron 10-215C, which results in an amino acid change at codon 61 (I61T).

Two splicing errors were found in the genetic material of families 2 and 4. Point mutations in introns 18 (+1G→T) and 6 (+1G→C) resulted in alternative splicing, involving 33 bp in the 3′-terminal of exon 18 in family 2 and skipping exon 6 in family 4 (Table II). The only missense mutation found was in family 3: three patients (P3-1, P3-2, and P3-3) with significant clinical heterogeneity had the same genetic alteration (314T→C) at exon 3, resulting in an amino acid change at codon 61 (I61T).

**Functional analysis of intron 1 mutants**

**Reporter gene analysis.** To evaluate the effects of a G to A transversion at position +5 in intron 1 of Btk, the mutant constructs (pBtkpro+1029-mt5 and pBtkpro+43-mt5) were made (Fig. 3A). The transcriptional activity of several constructs was measured in luciferase assay experiments (Fig. 3B). A 20- to 50-fold increase in transcriptional activity was observed in Raji and Daudi cells transfected with pBtkpro+1029, the construct containing intron 1, when compared with the corresponding pGL2-basic transfectants. The transfected pre-B cell line, Reh, displayed an almost 3-fold increase in reporter gene expression. The transcriptional activity of the construct containing the mutation at position 5 (pBtkpro+1029-mt5) was dramatically reduced (ranging from 0.01- to 0.004-fold lower) in transiently transfected Raji and Daudi B cell lines, compared with the wild-type construct (pBtkpro+1029). A similar, but less dramatic, decrease (0.07-fold) was seen in pBtkpro+1029-mt5 transfected Reh. However, pBtkpro+1029-mt6 showed ~7- to 48-fold more transcriptional activity than pBtkpro+1029-mt5 in B cell lines. All of the pBtkpro+1029-mt5 luciferase levels were above background, and the pBtkpro+1029 and pBtkpro+1029-mt6 luciferase values obtained broadly agreed with those seen in previous studies (22).

Progressive deletions were made from the 3′ end of pBtkpro+1029 to localize potential regulatory elements. A positive regulatory element was identified within the first 43 bp of the intron (22) in the pBtkpro+1029 construct. We analyzed the transcriptional activity of pBtkpro+43 and mutant derivatives in both pre-B and B cells. pBtkpro+43 elicited significant, but smaller, amounts of luciferase in reporter gene assays with Raji and Daudi cells than did pBtkpro+1029. However, pBtkpro+43-mt5 expressed >15.1-fold more transcriptional activity than pBtkpro+1029-mt5 in B cells, whereas pBtkpro+43-mt6 gave far lower (1.5- to 3.2-fold) increases in transcriptional activity than did pBtkpro+1029-mt6. These results suggest that different mechanisms controlling Btk gene transcription operate in the two mutant constructs. In addition, greater increases in transcriptional activity were obtained in pre-B-cells when pBtkpro+43-mt6 (17.5-fold) and pBtkpro+43-mt5 (32.6-fold) were used, corroborating a previous study that suggested the presence of a negative regulator within this sequence (22).

**DNase I footprinting.** We performed DNase I footprinting experiments using a 300-bp DNA fragment probe containing 324 bp of intron 1 to define cis-acting transcriptional modulation elements present at mutation sites. The addition of up to 100 μg of nuclear proteins from Raji cells yielded no DNase I protection in the Btk intron 1 region when the wild-type probe was used (Fig. 3C). However, when a probe containing the mutation at position +5 in intron 1 (mut-5) was used, the addition of as little as 50 μg of nuclear protein revealed a protected region spanning positions +3 to +15 bp, with a strongly hypersensitive site in the vicinity of the protected region of the gene. We found the same hypersensitivity site with the bottom strand (data not shown).

In addition, when the probe containing the mutation at position +6 in intron 1 (mut-6) was used, we detected a protected site spanning the +4- to +15-bp region, which was distinct from that found using the mut-5 probe. These results suggested differences in the nuclear factor-binding properties of the mut-5 and mut-6 sequences. Moreover, no additional differences were detected in binding patterns using footprinting analysis with any of the three probes mentioned above. Identical binding patterns were observed when similar amounts of Reh nuclear proteins were used (data not shown).
EMSA. We used EMSA to determine whether the mutant DNA sequences could bind nuclear extracts, as shown in footprinting analysis. Using a sequence spanning nts −10 to +19 at the exon/intron border (probe −10+19) to identify nuclear DNA-binding factors, a single gel shift was observed (Fig. 3D, left). When the mutant probe (−10+19 M) was used as a target for DNA, the single band observed for the normal probe was again seen, along with an additional band (Fig. 3D, left, band 2), suggesting that the mutation created an additional binding site in this region. In the competition assays, the additional shift with the lower mobility (band 2) was inhibited by the addition of cold −10+19 M oligonucleotide, not by cold −10+19 probe (Fig. 3D, right top). Furthermore, this shift was not inhibited by oligonucleotides containing unrelated binding sites for the well-known transcription factors AP-1, OCT-1, and NF-κB, even when used at a 1000-fold molar excess, indicating the involvement of a novel transcription factor (Fig. 3D, right bottom).

Flow cytometric analysis of family 1 and other XLA families

The detection of monocyte-expressed Btk protein by flow cytometric analysis has been previously demonstrated (8). Using this approach, we examined the Btk expression of 10 males and their relatives and found that 9 had coding region mutations leading to defective (mean, 1.6; range, 0.9–2.7%) Btk protein expression in monocytes (Fig. 4). In addition, the flow cytometric analysis of PBMC from P1-1 showed diminished Btk expression (18.5%). The flow cytometric assay showed a mosaic pattern of monocyte Btk expression in 12 of 22 sisters or maternal relatives (~55%) of subjects in this study, indicating that they are obligate XLA carriers. A bimodal pattern of monocyte Btk expression was evident in all carriers.

Discussion

This is the first Korean study describing mutations in the Btk gene in patients with XLA; 7 mutations were found in 12 patients. Six of the seven mutations were localized in the Btk coding region, and one point mutation was present in intron 1. Btk protein analysis using flow cytometry clearly showed cellular mosaicism in monocytes from obligate carriers, findings consistent with those obtained using SSCP. There are >600 mutation entries in the database of XLA mutations (http://protein.uta.fi/BTKbase/tables.html) scattered throughout the Btk gene. Mutation distribution in the five structural domains is roughly proportional to the domain length (27). Although no insertion mutations were discovered, three deletions were detected in this study. A novel 4-bp deletion (nts 842–845) in family 5 was absent in the maternal grandmother, although both the mother and a maternal aunt were obligate carriers. Due to the absence of samples from the patient’s maternal grandfather or great-grandparents, the source of the mutant X chromosome could not be determined in this family. Past studies suggest de novo mutation in oocytes or gonadal mosaicism in certain X-linked hereditary diseases (28). Parolini et al. (29) reported the first demonstration of a family with XLA and gonadal mosaicism. Further studies, such as haplotype analysis, are needed to clarify the origin of the P5 mutation in either male or female germlines. A large deletion (980 bp) encompassing exon 10 was found in the genomic DNA of the only sporadic case in this study (P7). The large deletions in Btk observed in this study, and four large deletions ranging from

FIGURE 2. Pedigrees and novel deletion mutations in exon 8 in family 5. A, Pedigree: Male (■, ◆) and female (○, ◊) family members are shown. The filled symbols indicate patients with XLA. Dotted circles indicate that the individual is a carrier. Subject numbers are written below their respective symbols. B, SSCP analysis of the Btk exon 8: DNA was amplified and analyzed as described in the text. Lane 1, P5; lane 2, mother; lane 3, aunt 1; lane 4, aunt 2; lane 5, aunt 3; lane 6, father; lane 7, grandmother; lane 8, maternal grandmother; and lane 9, normal control. *, A carrier. C, Exon 8 DNA sequence from a normal control, carriers (mother and aunt), a maternal grandmother, and P5. *, A 4-bp deletion.
FIGURE 3. Relative luciferase activities and DNase I footprinting analysis in intron 1 of the Btk gene. A, A schematic diagram of the reporter constructs containing the 5′ portion of Btk intron 1, as defined by Rohrer et al. (22). The mutant constructs show the relative locations of previously identified mutation sites (position +6) and those identified in family 1 (position +5). Also shown is the intron 1 acceptor sequence 5′ to the start of the luciferase gene (Luc). B, Relative luciferase activities of the normal constructs (wild type) compared with the mutant constructs (Mut6 and Mut5). The increases are relative to pGL2-basic vector. Construct numbers (43, pBtkpro+43; 1029, pBtkpro+1029) are given below the respective bars. Cell lines are indicated in the top left-hand corner of each series. C, DNase I footprinting of the 5′ end of the first intron of the Btk gene. A 500-bp BamHI/HindIII DNA fragment spanning the 5′ region of intron 1 of the Btk gene and 5′ end-labeled on the top strand was incubated with increasing amounts (50, 75, and 100 μg) of nuclear protein prepared from Raji cells and further digested with DNase I, as detailed in the text. Digested DNA was separated by gel electrophoresis and analyzed via autoradiography. The DNA region protected from DNase I digestion is indicated. G, Maxam and Gilbert “G” sequencing ladder; C, control lane containing the labeled probe digested with DNase I, but without added proteins. D, EMSA of the sequence at the exon 1/intron 1 border. Left, A double-stranded oligonucleotide containing the DNA sequence-spanning nts −10 to +19 at the exon/intron border (probe −10+19) and its mutant form (probe −10+19M) were 5′ end-labeled and used as a probe in EMSA. Each end-labeled oligomer was incubated with increasing amounts (5, 10, and 20 μg) of crude nuclear proteins from Raji cells and the formation of DNA-protein complexes was evaluated by EMSA. Right, The −10+19M-labeled probe, indicated above the lanes, was incubated in the absence or presence of 200× excess cold probe, as shown below. Arrow, Observed gel shift; P, free probe.
and family 1 evaluated in each gated population. Flow cytometric data are presented for the dashed line indicates the control Ab. Five thousand cells were evaluated by gating on the CD14+ population. The example shows the control Ab. Five thousand cells were evaluated in each gated population. Flow cytometric data are presented for family 1 (A) and 5 (B).

2.8 to 38 kb observed in another study (30), emphasize the utility of large-scale sequencing in elucidating disease-causing mutations.

We observed clinical heterogeneity in all of the siblings of families 1, 2, 3, and 4. The most extreme cases were in family 3 and were characterized by missense mutations in the PH domain. Patient P3-1 presented with episodes of severe primary infections, but his brother (P3-2) was healthy. Serum IgG levels were correlated with the clinical condition of the affected members of family 3 (P3-1, 14.0 mg/dl; P3-2, 463.0 mg/dl; P3-3, 281.0 mg/dl). However, similar phenotypic correlations with serum Igs were not revealed in other families. Phenotypic variation is increasingly recognized in XLA and in other primary immunodeficiencies (27, 31–33). Other factors may compensate for Btk function in less severely affected individuals (32).

In family 1, we detected a novel point mutation in intron 1 (+1G→A). The patient’s cDNA was normal by direct sequencing, although he had lower amounts of protein. Although the +5 position in the splice donor site is highly conserved and is found in 84% of splice donor sites, we could not find any deleted or inserted cDNA sequences by RT-PCR and direct sequencing from the patient’s cDNA. Additionally, MboI-RFLP revealed no cleavage patterns in 50 normal controls, indicating that this is not a polymorphic site (data not shown). Since there was no splicing defect in this cDNA, we studied the functional activity of reporter constructs containing this mutation. Previous studies showed that the positive regulatory element in intron 1, identified in the pBtkpro+1029 construct, was active in both B cells and pre-B cells. This suggested that it was not a “classical” enhancer element, since a 79-bp fragment containing the first 43 bp of intron 1 was active only in its original position and orientation (22). We found that pBtkpro+1029-mt5 transcriptional activity production was significantly lower in B cell lines compared with wild-type constructs or another mutant construct, pBtkpro+1029-mt6. Therefore, we hypothesize that the nucleotide at mutation 5 is involved in a critical enhancer element of the first intron and that a mutation at this site affects Btk promoter activity. In addition, there appear to be differences in the mechanisms of transcriptional regulation between the mutant constructs at positions +5 and +6, based on the differential effects of mutant constructs in B cells.

The DNase I footprinting assay identified a strong hypersensitivity site associated with Btk transcriptional regulation, which hybridized with the probe derived from mutation position 5, but not with a wild-type probe, and spanned the region from +3 to +15 bp. Flow cytometric analysis of PBMC from P1-1 showed diminished Btk expression, suggesting that the point mutation in intron 1 decreased Btk protein expression through down-regulation of transcription, whereas mutations in the Btk coding region led to complete shutdown of Btk protein expression. It is interesting that the DNase I-protected region of intron 1 differs from the corresponding sequences in mut-5 and mut-6. Using EMSA, an additional band with lower mobility (band 2) was observed and this binding complex competed for binding with the cold mutant oligonucleotide (−10+19 M), but not with the cold wild-type oligonucleotide, AP-1, OCT-1, or NF-kB. Combined, these data strongly suggest that the effect of this mutation on transcriptional regulation in B cells might be the result of binding of a novel transcription factor.

A previous study showed that a T to G alteration at position +6 created a new Sp1 binding site (22). Although most Sp1 sites are found within promoter sequences, these functional sites are reported in intronic sequences (34–38). Similarly, a functional GC-rich sequence (GC box), essential for the activation of human CD23 by EBV, was identified within intron 1 of type CD23 and was shown to bind a subset of GC-rich proteins that were apparently distinct from Sp1 (39).

A search for sequence-specific transcription factors in the region between +3 to +15 bp of intron 1 and other target elements was conducted using the SITES table of TFD, a database of transcription factors maintained by Dr. D. Ghosh at the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health, Bethesda, MD) (40). The search revealed no significant similarities with any of the potential sites listed in the data bank. Although further studies are clearly indicated, we speculate that an unknown protein factor might be responsible, via binding to the cis-acting element with a point mutation, for the defective regulation of Btk transcription. Our results suggest that several regulatory elements or mechanisms mediate transcriptional regulation of Btk and underline the importance of the first intron in Btk promoter activity. Future studies should clarify the molecular nature of this unknown protein factor and its role in XLA pathogenesis.

Acknowledgments

We thank the many patients and their families for their cooperation; the medical doctors, Drs. Jeong-Soo Kim, Dong-So Kim, Soo-Jong Hong, and Hoon-Kook for provision of the blood samples and the clinical data; and Dullee Min and Youngja Song for technical assistance. We also thank Dr. Jurg Rohrer and Dr. Mary Ellen Conley for provision of reporter constructs and critical reading of this manuscript.

References


