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Identification of *pol* Gene Mutation among BLV Proviruses Found in the Southern Province of Korea

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=Abstract=

Bovine leukemia virus (BLV) is an etiological agent of chronic diseases in cows worldwide. The BLV is one of retroviruses that contain a multi-functional enzyme, reverse transcriptase produced from the *pol* gene in its genome. We have sequenced some regions in the *pol* gene of BLV proviruses found in the Southern province of Korea from samples that turned out to be BLV positives by a PCR analysis. On the 5' side of the BLV *pol* gene (polymerase region), it was found that there were four leucines located at every 7 amino acids. They can form a leucine zipper motif that was not same as the *pol* gene of Japanese BLV isolate. The sequencing result of the proviral *pol* gene in Korean-type BLV also revealed some mutations leading to amino acid changes such as CCT(Pro)→CTC (Leu), AAT(Asn)→AAA(Lys), and non-sensible variations i.e., TCT(Ser)→TCC(Ser), ATT(Ile)→ATC(Ile) and ACG(Thr)→ACA(Thr). On the 3' side of the *pol* gene (integrase region), some nucleotide sequences were mutated and led to amino acid changes. Among them, a mutation, GAA(Glu)→GAC(Asp) occurred in many Korean-type BLV proviruses was very interesting because the amino acid was regarded as one of the most conserved amino acids in the retroviral integrase. It was also notable that the mutation on any leucine residue did not occur, in spite of its frequent appearance.

Key Words: BLV, Integrase, Leucine zipper, *pol* gene, RT

INTRODUCTION

Bovine leukemia virus (BLV) is an exogenous retrovirus grouped as a type C oncornavirus in *Retroviridae* (1) and is closely related to HTLV-1 and HTLV-2 in human (11, 12). The BLV is a causative agent of bovine leukosis and produces clonal tumors of B cell in cows after a long latent period (11, 16). However, its significance was not well understood although more than 30% of dairy cattle in Korea were infected with BLV (6, 9). The figure is almost triple compared with the rate of BLV infection in the United States (4).

Since BLV is a retrovirus, its genome consists of three major genes such as *gag, pol* and *env*. Among them, the *pol* gene produced a well-known reverse transcriptase (RT) that is a key enzyme during viral replication and reverse transcription and is a multi-functional enzyme conducting RNA-/DNA-dependent DNA polymerization, Ribonuclease H (RNase H) activity and tRNA binding activity (15). Also at the 3'

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side, one-third of the BLV *pol* gene can encode an integrase (IN) gene (10) that is necessary for BLV proviral DNA to integrate into the host chromosomes. The whole genome of BLV was identified by Sagata *et al.* (12) and Coulston *et al.* (2), and it was demonstrated that the Japanese BLV isolate is significantly different from the Australian BLV isolates with various mutations in their genomes. Also the *pol* gene sequences of a Belgian BLV isolate (11) and an American BLV isolate (GeneBank data accession no. AF033818) revealed that there was significant mutation being occurred.

Hence we report here the mutated sequences of the *pol* gene among Korean-type BLV proviruses, that were located in the 5' side of the *pol* gene (polymerase region) and the 3' side of the *pol* gene (integrase region). Subsequently, its significance was discussed in aspects of appearance of a leucine zipper motif and a critical mutation in a catalytic domain of the BLV integrase only found in the Korean-type BLV.

MATERIALS AND METHODS

Isolation of cow PBMC

For BLV detection, peripheral blood mononuclear cells (PBMC) were isolated from blood of 25 cows that were ramdomly bred near the Daegu/Kyungpook area, the Southern province of Korea. The blood samples from cows were rotated for two hours in a gyrorotator in order to improve PBMC separation and the blood was diluted with 1X HBSS (1:1). Seven ml of diluted blood was carefully layered over 3 ml of Histopaque 1077 (Sigma) in 15 ml of sterile centrifuge tubes (Corning). After the first centrifugation at 1,000 xg for 25 minutes, the buffy coat layer in the middle of the tube was removed into another tube with a 2 ml pipette, washed with 1X HBSS and filled up to 15 ml. After repeated washing with the HBSS and centrifugation, the pellet (PBMC) was collected and subjected to DNA extraction.

Genomic DNA extraction

PBMC were mixed with 3 ml of cell lysis buffer [0.1 M NaCl, 0.2 M sucrose, 0.02 M EDTA, 0.3 M Tris-HCl (pH 8.0), 0.5% SDS and 200 μg/ml Proteinase K] and the mixed solution was incubated in a 25℃ water bath overnight. Genomic DNA was extracted in sufficient quantities as described in Sambrook *et al.* (13) for BLV detection.

PCR analysis

Polymerase chain reaction (PCR) was employed to detect BLV among cow blood samples with BLV primers (BLV04/05, Table 1). These primers were successfully applied to detect BLV for past few years by us (6). The primers can cover the 5' side of *pol* gene (positions marked as 2464-2621, Figure 1) and they produced 158 base pairs (bp) DNA if the cows were infected with BLV and the cow PBMC had BLV proviruses. For amplification of the 3' side of *pol* gene (positions marked as 4441-4623), the primer set BLV08/09 was used. The PCR was performed as described in Kwon and Sninsky (7).

Southern blot analysis

Ten μl of the PCR reaction was analyzed on the composite agarose gel. This gel contained wide range/standard agarose (3:1, Sigma) in Tris-Borate buffer and was stained with an ethidium bromide solution (10 mg/l). The gel was subjected to Southern blot transfer with a DIG-nucleic acid detection kit (Boeringer Mannheim). First, it was submerged in solution containing 1.5 M NaCl and 0.5 M NaOH, with agitation for 15 to 20 minutes. The gel was neutralized with solution containing 0.5 M Tris-HCl (pH 7.0) and 1.5 M NaCl. After washing step was done, DNA was transferred from the gel to a nylon filter with 20X SSC (3 M NaCl, 0.3 M Na-Citrate, pH 7.0) buffer overnight. After 2 hour pre-hybridization, the filter was hybridized with the BLV probe (BLV06, Table 1) which

Table 1. List of oligonucleotide sequences used in this study

Primers	Nucleotide sequences	Positions
BLV04	5'-TTTGTGCATGACCTACGAGCTACA-3'	2464-2487
BLV05	5'-AAGCGGTCTTCGACTGGAATCT-3'	2600-2621
BLV06	5'-GAGATCTAGGCAAATGATATGTGGAGGG-3'	2556-2583
BLV08	5'-CCCTACAACCCCACAAGTTCGG-3'	4441-4462
BLV09	5'-ATGGTGTAGCTCCCATCTGGTCTT-3'	4600-4623

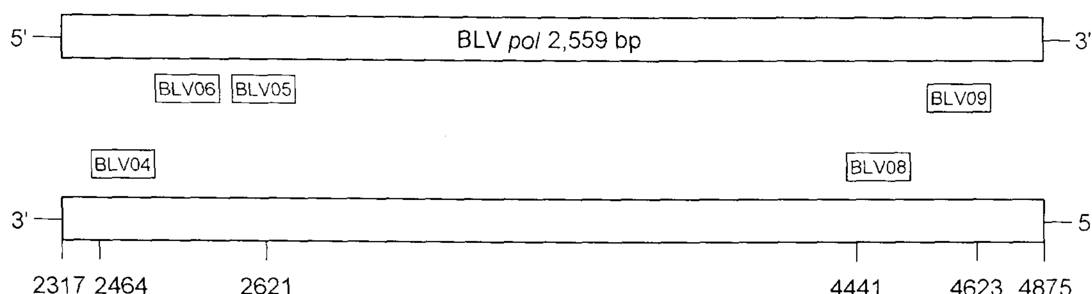

Figure 1. Map of the BLV *pol* gene for sequencing and the site of PCR for detection of Korean-type BLV. The diagram was made on a basis of the complete BLV nucleotide sequences reported by Sagata *et al.* (12).

was end-labelled with DIG-ddUTP (Boeringer Mannheim). Hybridization and detection was carried out according to a manufacturer's recommendation.

Purification of PCR product

The DNA bands at 158 bp (with BLV04/05) and 183 bp (with BLV08/09) were purified for the purpose of DNA sequencing by a QIAEX II gel extraction kit (Qiagen). The procedure was done according to a manual provided by Qiagen. DNA was again subjected to phenol: chloroform (50:50) treatment and ethanol precipitation for further purification.

DNA sequencing

The partial *pol* gene fragments were sequenced in the Cooperative Laboratory Center of Pukyung National University by a ABI PRISM 377 with a dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). Briefly, 40 μg/μL of prepared DNA (purified PCR DNA) was mixed with 8 μl of terminator mix, 1 μl of primers (3.2 pmol) and 8 μl of dH_2O was added to make total volume of 20 μl in a reaction tube. Each sample was subjected to PCR with 25 cycles of a designated thermal profile (96℃/10 seconds; 50℃/5 seconds; 60℃/4 minutes). After ethanol precipitation process, 2 μl of DNA in a tube was mixed with the prepared loading buffer (8 μl) by vortexing. The mixtures were heated to 90℃ for 2 minutes to denature in a heatblock. As soon as vortexing and spinning, the samples were placed on ice for 10 minutes and loaded into wells of a gel plate for sequencing.

RESULTS

Infection rate of BLV in the Southern province of Korea

We tested 25 blood samples of cows that were raised near the Daegu/Kyungpook area. For a fast BLV detection, PCR was done with BLV primers (BLV04/05) of which regions on the BLV *pol* gene were showed in Figure 1

and the primer sequences were listed in Table 1. As a result, 15 out of 25 cows PBMC were turned out to be BLV positives and the rate of BLV infection was averaged out to 60.0% (Figure 2). Since the rate of BLV infection was higher than expectation, a Southern blot analysis of the PCR results was carried out and the averaged value of Southern blot results was 32.0% that was almost half of the value found with PCR results. In brief, on the sample lot 5C composed of 15 cows (Figure 2A), eleven out of 15 cows PBMC showed BLV positive signals with the expected DNA size, 158 bp (→) but only four samples (lanes 8, 10, 12 and 13) revealed BLV positive signals in the Southern blot. Interestingly, the Southern blot gave positive results only from the PCR bands appeared strongly. However, the sample lot 6C showed four BLV positives (lanes 3, 5, 6 and 7) that were exactly identical to results of the Southern blot (Figure 2B). Subsequently, the eight BLV positive DNA bands (named as BLV5C8, BLV5C10, BLV5C12, BLV5C13, BLV6C3, BLV6C5, BLV6C6 and BLV6C7) were purified and the DNA fragments were subjected to do sequencing.

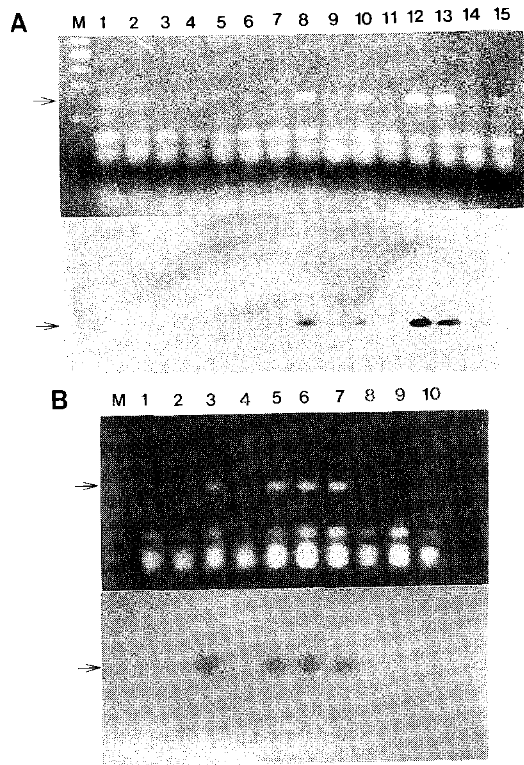

Figure 2. PCR results and Southern blot results for detecting the Korean-type BLV. The used primers were BLV04 and BLV05. For Southern blotting, BLV06 used as a probe. **(A)** Sample lot 5C consists of 15 cows; **(B)** Sample lot 6C consists of 10 cows. Arrows (→) indicate 158 bp. Lane description: M, marker DNA ΦX174 RFI *Hae*III digested (0.25 μg). Numbers represent different cow PBMC samples.

Sequencing of 5' side of the *pol* gene (polymerase region) of the BLV

The 8 sequences were showed in Figure 3 and were compared to other published *pol* gene sequences of BLV because the used primer sequences were based on and derived from the *pol* gene sequence reported by Sagata *et al.* (12). And we had reasoned that BLV found in the Southern province of Korea would differ from the Japanese BLV isolate because there was considerable geographical distance between two nations. The sequences of the BLV proviruses were also compared to that of other countries such as a Belgian isolate (11), an Australian isolate (2), and an American isolate (Gene-Bank data accession no. AF033818).

The analysis of partial sequences in the *pol* gene among the BLV proviruses revealed some mutations leading to amino acid changes such as CCT(Pro)→CTC(Leu), AAT(Asn)→AAA (Lys), and no variations such as TCT(Ser)→ TCC(Ser), ATT(Ile)→ATC(Ile), ACG(Thr)→ ACA(Thr) and many others especially in samples of BLV6C. The mutation, CCT(Pro)→ CTC(Leu) gave one additional leucine to the Korean-type BLV *pol* gene compared with the Japanese BLV *pol* gene. We found that there were four leucines appeared at every 7 amino acid in the polymerase region. They can form a leucine zipper motif that is different from the *pol* gene of Japanese BLV. Other isolates of BLV also showed non-sense mutations, for examples, TCT(Ser)→TCC(Ser), ACG(Thr)→ACA

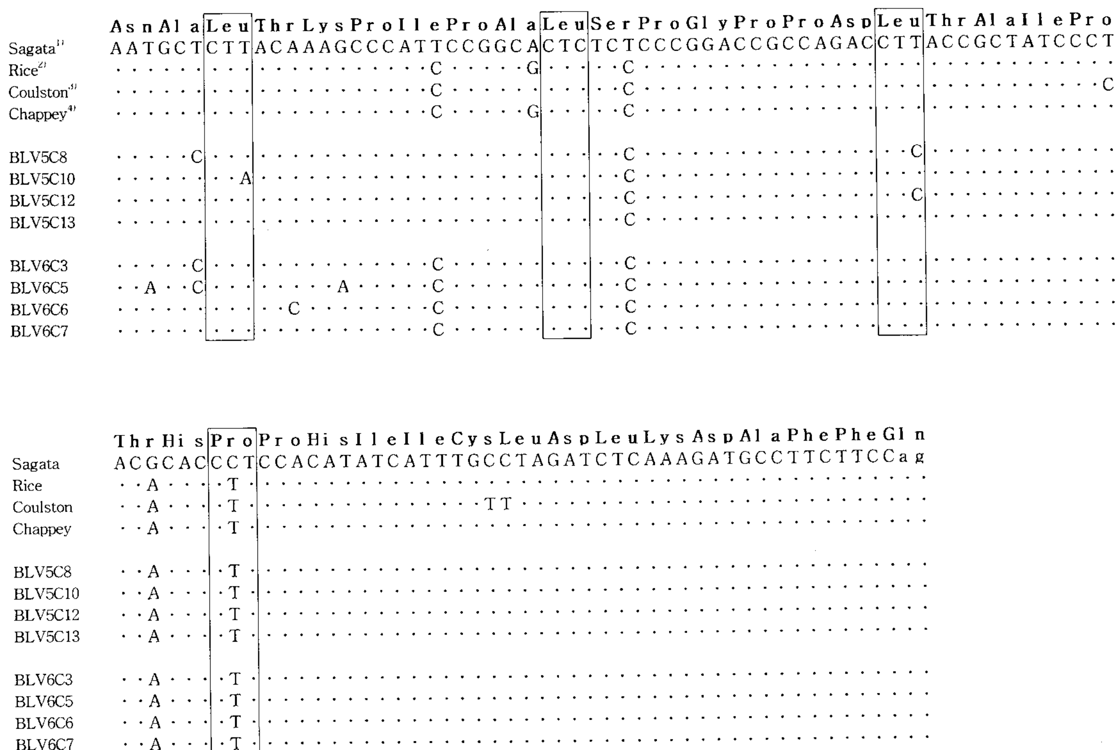

Figure 3. A partial DNA sequence of 5' side of the BLV *pol* gene (polymerase region of RT) produced by primers BLV04/05. A motif of leucine zipper was marked with open boxes. The fourth leucine position in the *pol* gene of Korean-type BLV was marked as Pro instead of Leu because the *pol* gene sequence of Japanese BLV used as a reference sequence. The length of sequenced region was 158 bp and analyzed sequences were only the region between the primers because the sequences of the primer region gave only one sequence due to PCR. The lower case letters are parts of primer sequences. [1] The sequence was according to Sagata *et al.* (12) published as a Japanese BLV isolate. [2] The sequence was according to Rice *et al.* (11) published as a Belgian BLV isolate. [3] The sequence was according to Coulston *et al.* (2) published as an Australian BLV isolate. [4] The sequence was according to Chappey (GeneBank data accession no. AF033818) as an American isolate.

(Thr), GCA(Ala)→GCG(Ala), ATT(Ile)→ATC (Ile), CCT(Pro)→CCC(Pro), TGC(Cys)→TGT (Cys), CTA(Leu)→TTA(Leu) and TGC(Cys)→ TGT(Cys). It was notable that the appearance of proline codon in this region was highly frequent with 21.1%, compared to that in the whole *pol* gene with 9.3%.

Sequencing of the 3' side of the *pol* gene (integrase region) of the BLV

With the BLV positive samples by a Southern blot analysis, we performed another DNA sequencing after PCR using a new primer set (BLV08/09) which are derived from an important integrase domain in the *pol* gene. Its region and the primer sequences were presented in Figure 1 and Table 1. The DNA sequences were showed in Figure 4. In general, some significant mutations in the *pol* gene of Korean-type BLV were found such as GAT(Asp)→ GTA(Val) in all samples. This mutation was also found in the *pol* gene of other countries' BLV. Other mutation found in the *pol* gene of Korean-type BLV was GAA(Glu)→GAC(Asp) in some samples of BLV5C (BLV5C12 and BLV5C13) and BLV6C (BLV6C3 and BLV6C 7) although this kind of mutation did not lead to a dramatic change because of their acidity.

The frequent appearance of leucine (13/46 AA, 28.3%) in this integrase region is intere-

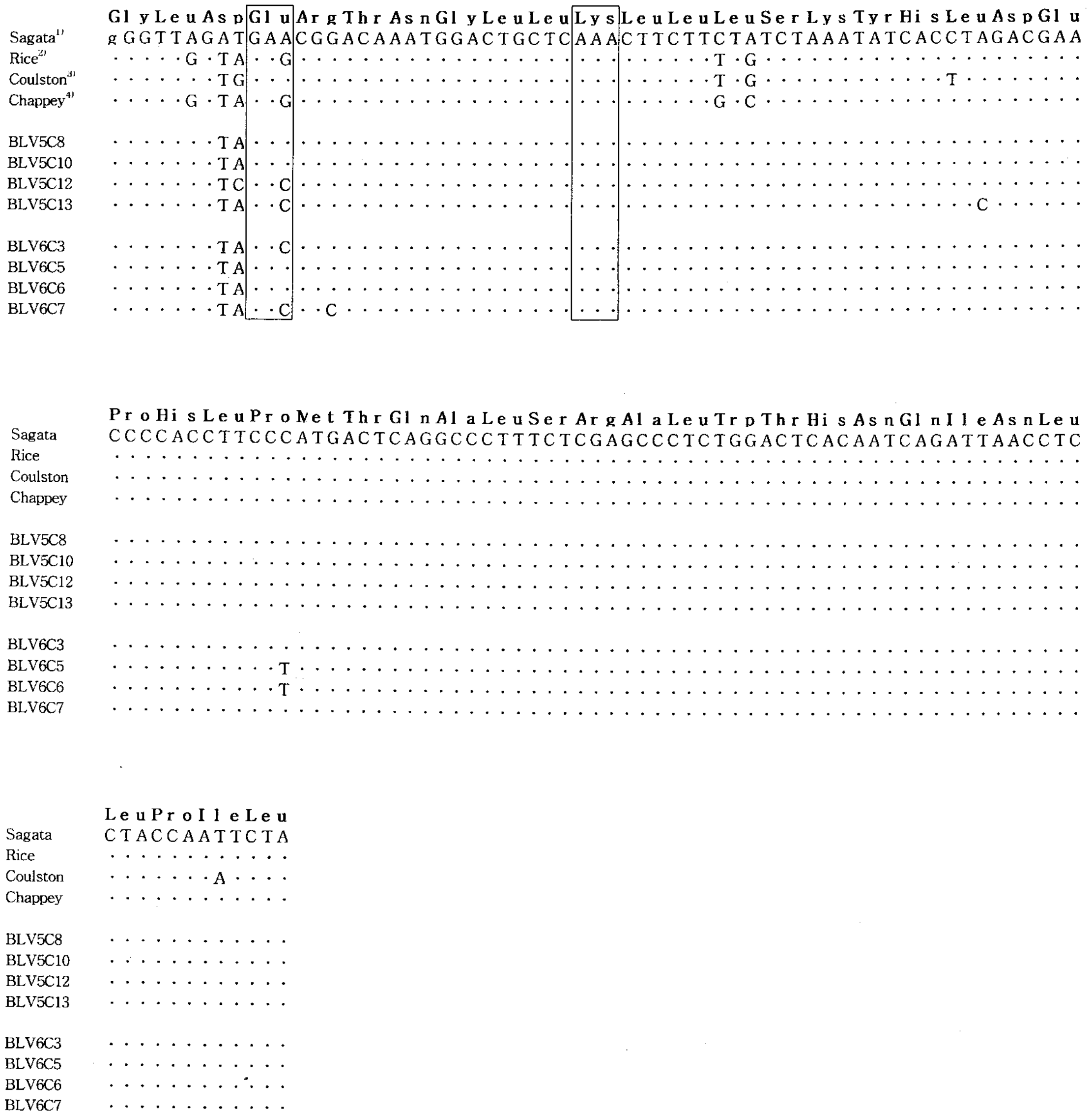

Figure 4. A partial DNA sequence of 3' side of the BLV *pol* gene (IN region) produced by primers BLV08/09. The amino acids of two large open boxes are regarded as the most conserved amino acids in the catalytic domain of IN according to the evolutionary relationship of retroviral IN (5). Total 13 leucines appeared among 43 amino acids. The length of sequenced region was 183 bp and analyzed sequences were only the region between the primers. The lower case letter is a part of primer sequences. All footnotes are same as described in the figure 3.

sting. Its frequency was exactly two times higher than a value found in the total BLV *pol* gene (121/852 AA, 14.2%). Surprisingly, nothing was changed at all among the many leucine codons in the *pol* gene of Korean-type BLV, unlike CTA(Leu)→TTG(Leu) in the Belgian/Australian isolates and TTA(Leu) in the Australian isolate. Only the American BLV isolate had a mutation from CTA(Leu) to GTC (Val).

DISCUSSION

We previously reported that the BLV infection was very serious in the Daegu/Kyungpook area of Korea (6) and by a PCR analysis, the rate of BLV infection was more than 60.0% which was two times higher than the rate reported ten years ago. However, a Southern blot analysis showed only 32.0% of the cow PBMC being infected with BLV in this study. Its value was very close to the average BLV infection rate in Korea (29.7%; ref. 9) that was surveyed by an antibody test. This agrees that the PCR method is more sensitive to detect BLV in cow PBMC than any other methods developed so far (14). If there was a considerable increase of BLV infection in the Southern province of Korea for last ten years, the PCR result that we have had cannot be ignored. Thus, in order to find out the reasons of discrepancy between the PCR result and the Southern blot result, sequencing the eight PCR DNA fragments (Southern blot positives) was performed.

In the first sequencing result of the *pol* gene of Korean-type BLV (polymerase region, Figure 3), some mutations can lead to amino acid changes in a N-terminal region of RT compared to the result of Sagata *et al.* (12). One base substitution in the codon of CCT to CTT leads an amino acid change from proline to leucine. Consequently, it could play a role of forming a leucine zipper motif (Leu-X_6-Leu-X_6-Leu-X_6-Leu; ref. 8) as marked by open boxes. This change was also found in the *pol* gene of other countries' BLV but no authors mentioned about it. The motif was confirmed by a computer analysis and it was the only leucine zipper motif in the whole 852 amino acids encoded in the *pol* gene of BLV. However, it is intriguing that prolines frequently appear in this region at 21.1%. This fact may contribute to nullify the leucine zipper motif by generating a random coil instead of α-helix in the region of BLV RT. Anyway, considering that this region of the RT contained DNA polymerization activity (5, 15), abolishing the leucine zipper motif by many prolines would lead to the weak activity. Additionally, it is possible that the leucine zipper motif generated by the *pol* gene mutation in the Korean-type BLV may contribute to the higher BLV infection rate in the Southern province of Korea.

The sequencing result of 3' side of the BLV *pol* gene was presented in the Figure 4 and the region was regarded as an IN activity (10). In general, there were a few mutations found as expected. A typical change, GAT(Asp)→GTA (Val) that was also known in the BLV *pol* gene of other countries, was found in Korean-type. However, a mutation GAA(Glu)→GAC (Asp) was specifically found only in the Korean-type BLV so far while it did not affect its acidic amino acid property. By a comparative analysis for evolutionary relationship of the retroviral IN, the glutamic acid and lysine (open boxes, Figure 4) must be the most highly conserved amino acids in a catalytic domain of IN (5), but in the Korean-type BLV *pol* gene, the glutamic acid codon was replaced with the aspartic acid codon by a single nucleotide change. Some other Korean-type BLV still maintained its sequence integrity such as BLV5C8, BLV-5C10, BLV6C5 and BLV6C6. Thus, it suggests that the sequenced region is a part of BLV IN and the BLV IN would come from the 3' side of the BLV *pol* gene. Since the *pol* gene of BLV was poorly studied until now, it is worthwhile to investigate more intensively on the Korean-type BLV that gave a critical mutation in the area of BLV IN domain. We presumed that the change GAA(Glu)→GAC(Asp) may affect on the BLV infection either positively or negatively.

Another interesting point was that no mutation was occurred at all among the whole leucine codons that represented 28.3% of the sequenced region in the *pol* gene of Korean-type BLV. It suggests that the leucines may

play an important role in the region of the 3' side of the BLV *pol* gene. We can explain the reasons of no mutation on whole leucine codons, such as 1) the region (3' side of *pol* gene) is a part of a BLV integrase. Thus, this region cannot be permitted to be mutated for BLV IN ensuring its enzymatic activity; 2) leucine is one of important hydrophobic amino acids which can form a hydrophobic core in enzymes (3); 3) in a retroviral gene expression system, retroviruses including BLV can use a set of overlapping reading frames for encoding extra proteins besides Gag, Pol and Env (15). This idea can be supported by a reason why mutations in the *pol* gene of a Belgian isolate BLV and an Australian isolate BLV were limited to only CTA(Leu)→TTG(Leu) or TTA(Leu) as shown in the Figure 4.

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