J. Microbiol. Biotechnol. (2000), **10**(5), 620–627

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Analysis of Genetic Polymorphisms of Epstein-Barr Virus Isolates from Cancer Patients and Healthy Carriers

CHO, SUNG-GYU AND WON-KEUN LEE*

Department of Biological Science, The Institute of Bioscience and Biotechnology, Myongji University, Yongin, Kyonggi-Do 449-728, Korea

Received: April 26, 2000
Accepted: August 5, 2000

Abstract To determine the prevalence of genetic polymorphisms in Epstein-Barr virus (EBV) strains in the Korean population, the restriction site polymorphisms for *Bam*HI and *Xho*I enzymes were analyzed with 16 EBV isolates from cancer patients and 7 EBV isolates from healthy carriers, using polymerase chain reaction techniques. None of the 23 isolates were found to carry an extra *Bam*HI site in the *Bam*HI F-fragment (f-variant). Of the 12 type-1 isolates from the cancer patients, 10 lost both the LMP1 *Xho*I site and the *Bam*HI site between the *Bam*HI W1* and I1* fragments (a W1*I1* fusion variant or type C). The latter W1*I1* fusion variant was due to a mutation of thymidine to adenine, as evidenced by a sequence analysis. The remaining two type-1 isolates showed either no variation at both sites or the loss of only the *Xho*I site. In contrast, two type-2 isolates and two intertypic recombinants with a type-1 allele at the EBNA2 locus and type-2 alleles at all or some of the EBNA3 loci retained both enzyme sites. In similar analyses of the 7 isolates from the healthy carriers, five of six type-1 isolates lost these two sites, however, one type-2 isolate did not. These results clearly indicate a strong association of both the LMP1 *Xho*I site loss and the W1*I1* fusion variant with the type-1 rather than the type-2 EBV strains circulating in the immunocompetent Korean carriers.

Key words: EBV isolates, f-variant, W1*I1* fusion variant, LMP1 *Xho*I site polymorphism

The Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, persists for life as a latent infection in the memory B cells of all infected individuals. EBV is the etiological agent of infectious mononucleosis (IM) and is also associated with a number of benign and malignant tumors in humans, including posttransplant lymphoproliferative diseases (PTLD), Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), and nasal NK/T-cell lymphoma. *In vitro* EBV efficiently transforms resting B-lymphocytes into continuously proliferating lymphoblastoid cell lines (LCLs).

Most EBV strains can be classified into two different types, type-1 (or EBV-1) and type-2 (or EBV-2), on the basis of their genetic polymorphisms in the EBNA2 and EBNA3 family genes [8, 27]. The two major types are present in all human populations, although there are some differences in the distribution of one type over the other among different geographic locations. Type-1 viruses are predominant in the populations of European and Asian countries, while types-1 and 2 are almost equally prevalent in the populations of Africa and Papua New Guinea. In addition to these two major types, it has been shown that there are also rare hybrid types or intertypic recombinants carrying both type-1 and type-2 EBNA alleles in the viral genome [4, 24, 34]. However, their prevalence in the general human population remains to be determined.

Further differentiation of EBV strains is possible due to the presence of various repeated sequences in the viral genome, which are readily detectable as restriction fragment-length polymorphisms (RFLPs) in Southern blottings [15, 22]. Some of such repeats occur within the open reading frames of EBV latent genes that are commonly expressed in all EBV-transformed B-cells and give rise to the polymorphisms in the size of respective latent proteins. Therefore, the polymorphic patterns of EBV nuclear antigens, known as Ebnotypes, encoded by EBV strains have been used as useful strain-specific markers for the epidemiological investigation of EBV transmission within families and EBV carriage [9, 10].

EBV strains can also be differentiated by RFLP due to point-mutation using Southern blotting and polymerase chain reaction (PCR) techniques [20, 22]. The principal aim of

*Corresponding author
Phone: 82-31-330-6194; Fax: 82-31-335-8249;
E-mail: wklee@bio.myongji.ac.kr

such RFLP studies is to determine whether certain EBV variants may be associated with EBV-related diseases through displaying a marked geographic prevalence in their incidence. As a result, *Bam*HI and *Xho*I site polymorphisms have been intensively studied with EBV strains present in NPC tumors occurring in the areas of Southern China and the Mediterranean, where this type of tumor is endemic [1, 3, 14, 16, 19, 20, 23]. Similar EBV RFLP analyses have also been performed with EBV isolates derived from IM [16, 22] and BL tumors [1, 22], T/NK-cell lymphoma [5, 32], and gastric cancers [30]. Although these studies failed to link any particular genetic markers with certain EBV-related diseases, several RFLP markers have emerged, which are highly prevalent among the EBV strains from China yet not in those from Europe and Africa. These markers include the loss of a *Bam*HI site separating the *Bam*HI W1* and I1* regions, resulting in a fused *Bam*HI W1*I1* fragment (previously termed as type-C, as opposed to wild-type version, type-D), and the loss of an *Xho*I site in the exon 1 of the LMP1 gene, leading to a disappearance of the 1.9 kb *Xho*I fragment from the right end of the genome, and presence of an extra *Bam*HI site in the *Bam*HI F fragment, thereby yielding a smaller *Bam*HI fragment (an "f" variant). Among these, the f-variant is particularly interesting in that it has been detected in both NPC tumors and healthy carriers in China, yet rarely detected in other areas including North Africa, Europe, and Japan [1, 16, 20, 30]. The exception to this was a recent report of detection of this variation in some HD cases of European origin [16]. Thus, it would appear that EBV strains with the f-variant are almost exclusively present in the Chinese population. Another interesting finding is the almost unique association of both *Bam*HI W1*I1* fusion and LMP1 *Xho*I RFLPs with type-1 strains found in Asia and yet not with those of European origins [1, 16, 30].

Only a few molecular epidemiological studies have been performed on EBV strains circulating in the Korean population. Therefore, the present study was undertaken to analyze *Bam*HI and *Xho*I site polymorphisms using 16 EBV isolates in spontaneous LCLs derived from Korean patients [18, 28] and 7 isolates rescued by a B cell transformation assay from the throat washings of healthy virus carriers. The results indicate a high prevalence of type-1 EBV strains carrying both *Bam*HI W1*/I1* and LMP1 *Xho*I polymorphisms in the Korean population.

MATERIALS AND METHODS

EBV Isolates and Cell Culture

The B-lymphoblastoid cells (SNU-9, -20, -99, -247, -265, -285, -291, -299, -315, SNU-321, SNU-445, SNU-447, SNU-538, SNU-817, SNU-889, and SNU-1103) were all spontaneous LCLs derived from different Korean cancer patients, as described previously [17]. The SNU-20 and SNU-99 cells harbored type-2 isolates, whereas the SNU-817 and SNU-889 cells included intertypic recombinants carrying a type-1 EBNA2 gene plus either type-2 EBNA3A, -3B, and C genes (SNU-817) or a type-1 EBNA3A gene, type-2 EBNA3B, and -3C genes (SNU-889) [S.-M. Kim *et al.*, manuscript in preparation]. The remaining LCLs harbored type-1 viruses [18, 28]. B95-8 was a marmoset B-LCL carrying the prototype B95-8 type-1 EBV strain and AG876 was African Burkitt's lymphoma cells carrying the A876 type-2 EBV strain. All cells were maintained in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 μg/ml of gentamycin and were subcultured every 3–4 days at an initial cell density of 3.0×10^5 cells/ml.

Polymerase Chain Reaction

The DNA used for the PCR was prepared as described previously [28]. Briefly, cells (1.0×10^5) in 70 ml of 0.2× PBS (phosphate buffered saline) were heated at 94°C for 10 min and then incubated at 55°C for 1 h with proteinase K followed by the heat inactivation of the enzyme at 94°C for 30 min. A 50-μl PCR reaction was set up with a 10 μl aliquot of DNA preparation, 50 pmoles of each primer, 0.2 mM dATP, dCTP, dGTP, and TTP, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.5 unit *Taq* polymerase (Promega). An amplification by 35 thermocyles was performed in a Perkin-Elmer 480 Thermal Cycler. The primers, annealing temperatures, and expected sizes of the PCR products are all listed in Table 1.

Analysis of *Bam*HI and *Xho*I Site Polymorphisms

To detect the *Bam*HI site polymorphisms for the W1*I1* fusion and f-variants plus the LMP1 *Xho*I site polymorphism, the EBV DNA was amplified across an expected polymorphic enzyme site, as described above. For the analysis of the *Bam*HI W1*I1* and *Xho*I site variations, 10 μl aliquots of the resulting PCR products were digested with *Bam*HI or *Xho*I, respectively, and then resolved on a 1% agarose gel containing ethidium bromide. For an analysis of the f-variant, the amplified DNAs were purified by agarose gel separation and elution prior to *Bam*HI digestion. The expected sizes of the amplified DNAs and their digested derivatives are summarized in Table 1.

Isolation of Virus

The virus isolation from the throat washings of randomly chosen healthy Korean carriers was carried out essentially as described by Yao *et al.* [33]. The throat washings were performed by gargling 10 ml phosphate-buffered saline (PBS), pH 7.4, followed by clarification on bench centrifugation and filtration through a 0.45 μm-pore-size membrane. Two ml of the throat filtrate was supplemented with 10% FBS, and incubated for 2 h at 37°C with mononuclear cells (1–

Table 1. PCR primers and probes used in this study.

PCR	Primers sequences (5'-3')	Coordinates*	Annealing temp. (°C)	Sizes (bp)
F/f	AGCGGTGCTTCACGCTCTTC	55291-55310	58	Uncut: 541
	GGCAATGGGACGTCTTGTAA	55832-55813		Cut: 415/126
Probe	CAACTGCCACAGACCCCATT	55611-55620		
W1*/I1*	ATGCTGCAGTAGTAGGGATC	9062-9081**	57	Uncut: 499
	TGACGGTCCGGAACATTTCC	9560-9541**		Cut: 330/169
Probe	TAAAGCTGTCCTTCTGTCAC	9309-9290**		
LMP1 *Xho*I	AGAAACACGACTTACTCT	169577-169560	57	Uncut: 497
	ACAATGCCTGTCCGTGC	169081-169097		Cut: 347/150
Probe	AAGATGAGAGCAAAGG	169304-169288		
EBNA2	AGGCTGCCCACCCTGAGGAT	48170-48189	60	Type 1: 168
	GCCACCTGGCAGCCCTAAAG	48339-48320		Type 2: 186
EBNA3A	GAAACCAAGACCAGAGGTCC	93596-93615	56	Type 1: 276
	TCCCAGGGCCGGACAATAGG	93871-93857		Type 2: 237
EBNA3C	AGAAGGGGAGCGTGTGTTGT	99939-99958	57	Type 1: 153
	GGCTCGTTTTTGACGTCGGC	100091-100072		Type 2: 246

*Nucleotide positions of B95-8 EBV genome [2].
**Nucleotide positions of Raji EBV DNA covering large deletion in B85-8 relative to Raji [25].

2×10^7) prepared from umbilical cord blood by Ficoll Hyphae-based centrifugation. Thereafter, the cells were seeded at 10^6 per well in 0.3 ml-well microplates using 0.2 ml of an RPMI 1640 medium supplemented with 15% FBS and 3 μg/ml gentamycin. The culture was refed with 0.1 ml of the medium after 2 weeks and then maintained up to 5 weeks until transformed cell clumps occurred. The transformed cells were expanded to LCLs, from which DNA samples for PCR analysis were made as described above.

EBV Genotype Analysis

The genotypes for the EBNA2, -3A, and 3C genes of the EBV isolates derived from the throat washings were determined by PCR techniques [27]. The techniques were designed to amplify those EBNA sequences containing type-specific deletions by the use of primers targeting by flanking sequences conserved in both type-1 and type-2 alleles. The PCR amplifications were performed as described above. The resulting PCR products were then analyzed on a 2% agarose gel containing ethidium bromide. The primers used for the amplification of the EBNA2 sequence are listed in Table 1 and the primers for the EBNA3A and 3C sequences were previously described by Sample *et al.* [27].

Southern Blot and Sequence Analysis

The DNAs in the agarose gel were blotted onto a Hybond-N^+ membrane (Amersham Life Science) and hybridized to internal oligonucleotide probes specific for each of the amplified regions. The probe DNAs (Table 1) were labeled with fluorescein-11-dUTP using terminal deoxyribonucleotide transferase (Amersham Life Science). The hybridized probes were then visualized by incubation with an anti-fluorescein antibody conjugated to horseradish peroxidase and by an ECL method according to the manufacturer's instructions (Amersham Life Science). To confirm and identify the mutation responsible for the W1*I1* fusion variation, the PCR products were purified and cloned in a pT7Blue T-vector (Novagen), and their nucleotide sequences were determined using an automatic DNA sequencer or T7 Sequenase version 2.0 DNA sequencing kit and ^{35}S-dATP (Amersham Life Science).

RESULTS

Analysis of *Bam*HI W1*/I1* and F/f, and LMP1 *Xho*I Site Polymorphisms in EBV Isolates from Cancer Patients

To detect the EBV strains carrying a *Bam*HI W1*I1* fusion variant due to the loss of the *Bam*HI site separating *Bam*HI W1* and I1* fragments, 499-bp DNA fragments including the *Bam*HI site were amplified from 16 EBV isolates (Fig. 1A). There was no amplification in the B95-8 EBV sample, reflecting a substantial deletion covering the W1* and I1* regions compared to the Raji EBV [25]. As shown in Fig. 1B, after the *Bam*HI digestion of the amplified DNAs, six of the 16 isolates yielded two smaller fragments of 330-bp and 169-bp, indicative of the presence of a *Bam*HI site between W1* and I1* as in Raji EBV, whereas the remaining 10 isolates exhibited single bands of 499-bp, indicative of the presence of a mutated *Bam*HI site. The sequence specificity of these PCR analyses

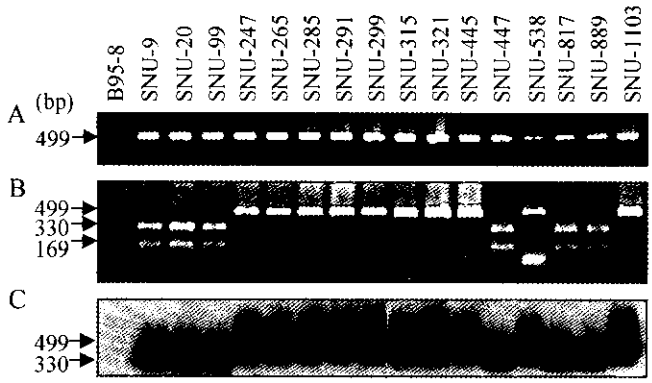

Fig. 1. Analysis of *Bam*HI W1*/I1* polymorphism in EBV isolates derived from cancer patients.
EBV DNA fragments of 499 bp encompassing the *Bam*HI site separating *Bam*HI W1* and I1* fragments were amplified from EBV isolates and resolved directly (A) or after *Bam*HI digestion (B) on a 1% agarose gel containing EtBr. (C) Southern blot analysis of DNAs in a gel of panel B with a probe complementary to the *Bam*HI I* sequences. The sizes of the DNA fragments in the base pairs are indicated on the left side of each panel.

Table 2. Summary of genomic variations of EBV isolates.

EBV isolates	Type	*Bam*HI F/f	*Bam*HI W1*/I1*	LMP1 *Xho*I
SNU-9	1	F	P[c]	P
SNU-247	1	F	L[d]	L
SNU-265	1	F	L	L
SNU-285	1	F	L	L
SNU-291	1	F	L	L
SNU-299	1	F	L	L
SNU-315	1	F	L	L
SNU-321	1	F	L	L
SNU-445	1	F	L	L
SNU-447	1	F	L	P
SNU-538	1	F	L	L
SNU-1103	1	F	L	L
SNU-20	2	F	P	P
SNU-99	2	F	P	P
SNU-817	IR[a]	F	P	P
SNU-889	IR[b]	F	P	P
MJU-1	1	F	L	L
MJU-2	1	F	L	L
MJU-3	1	F	L	L
MJU-4	1	F	L	L
MJU-5	1	F	P	L
MJU-6	1	F	L	L
MJU-7	2	F	P	P

[a]Intertypic recombinant with genotype of type-1 EBNA2 and EBNA3A/ type-2 EBNA3B and 3C.
[b]Intertypic recombinant with genotype of type-1 EBNA2/type-2 EBNA3A, -3B, and 3C.
[c]P-present.
[d]L-lost.

was confirmed by Southern blotting (Fig. 1C), in which both full-length 499-bp and digestion-derived 330-bp bands

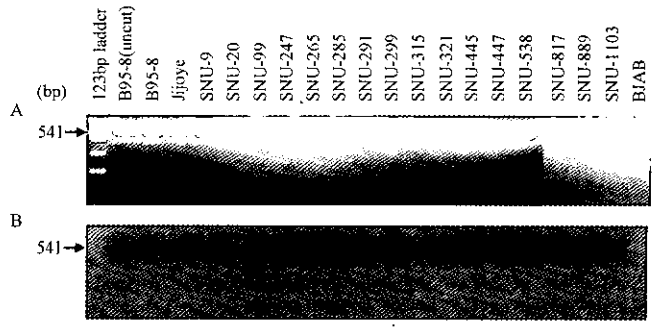

Fig. 2. Analysis of *Bam*HI F/f polymorphism in EBV isolates derived from cancer patients.
Amplified EBV DNA fragments (541 bp) encompassing an extra *Bam*HI site expected in an f-variant were purified, resolved on a 1% agarose gel with EtBr after *Bam*HI digestion (A), and analyzed by Southern blotting using a *Bam*HI F-specific probe (B). The sizes of the DNA fragments in the base pairs are indicated on the left side of each panel.

hybridized to a probe complementary to the Raji *Bam*HI I1* sequences. These results are summarized in Table 2. Ten type-1 isolates showed the loss of the *Bam*HI site, while two type-1 (SNU-9 and SNU-447) and two type-2 isolates (SNU-20 and SNU-99) retained it. Interestingly, two intertypic recombinants (SNU-817 and SNU-889), which carry a type-1 EBNA2, no type-2 alleles, and some of the EBNA3 loci (Kim *et al.*, manuscript in preparation), also retained the wild-type *Bam*HI site.

Similar PCR analyses were carried out to detect those EBV strains carrying a small f-variant, due to the presence of an extra *Bam*HI site within the *Bam*HI F-fragment. The PCR products of 514 bp containing the expected extra *Bam*HI site were purified and subjected to *Bam*HI digestion. All the purified DNAs including that from B95-8 EBV, which is the wild-type for the F-fragment, were not cleaved into the 415 bp and 126 bp sub-fragments, which would be expected from the f-variant (Fig. 2A). The sequence specificity of the analysis was confirmed by a Southern blot analysis using a probe targeting the F-fragment (Fig. 2B). Thus, the results indicate that all 16 EBV isolates included the wild-type F-fragment like the B98-5 strain (Table 2).

Previous cloning and sequencing studies of the LMP1 genes of Chinese NPC EBV strains [6, 12] revealed LMP1 variants that had lost the *Xho*I site in the exon 1 of the gene due to a G-to-A mutation at position 169,426 compared with the B95-8 LMP1 gene. To detect a polymorphism at this *Xho*I site, LMP1 DNAs including the *Xho*I site were amplified from the 16 EBV isolates and B95-8 (Fig. 3A). After the *Xho*I digestion, the 497 bp PCR products derived from five isolates (SNU-9, SNU-20, SNU-99, SNU-817, and SNU-889) as well as B95-8 were cleaved into the two sub-fragments of 347 bp and 150 bp, whereas those from the remaining 11 isolates yielded a single band, indicative of the loss of the *Xho*I site (Fig. 3B). Southern blotting

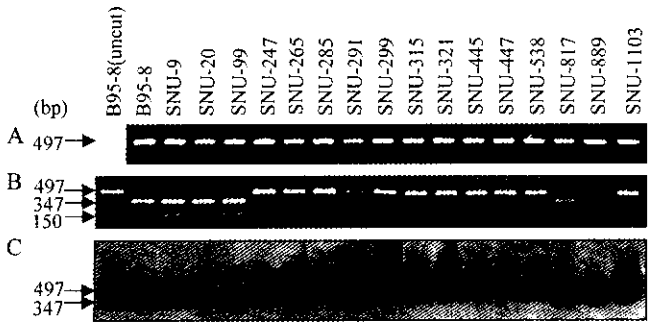

Fig. 3. Analysis of LMP1 *Xho*I polymorphism in EBV isolates derived from cancer patients.

EBV DNA fragments of 497 bp encompassing the LMP1 *Xho*I site were amplified from EBV isolates and resolved directly (A) or after *Xho*I digestion (B) on a 1% agarose gel containing EtBr. (C) A Southern blot analysis of the DNAs in a gel of panel B with a probe complementary to the LMP1 sequences. The sizes of the DNA fragments in the base pairs are indicated on the left side of each panel.

using a probe specific for the LMP1 gene confirmed the specificity of the PCR analysis (Fig. 3C). As summarized in Table 2, the results indicate that 11 of the 12 type-1 isolates lost the *Xho*I site, whereas one type-1 (SNU-9), two type-2 isolates, plus two intertypic recombinants included the wild-type *Xho*I site. Like the *Bam*HI W1*/I1* polymorphism, the LMP1 *Xho*I polymorphism also appears to be strongly associated with type-1 rather than type-2 EBV strains.

Sequencing Analysis of the *Bam*HI W1*/I1* Polymorphism

Previous cloning and sequencing experiments [7], carried out in parallel with this study, showed that the LMP1 genes of SNU-321, -538, and -1103, yet not that of SNU-20, had a point mutation of guanine to thymine at position 169,426, leading to the loss of the *Xho*I site [6, 12]. Therefore, it would appear likely that the same G-to-T mutation is also responsible for the *Xho*I polymorphism in the other isolates used in this study. To reveal the mutation(s) responsible for the *Bam*HI W1*/I1* polymorphism, the 499 bp DNA fragments obtained from 9 isolates were cloned and sequenced with the W1*I* variant (SNU-247, -265, -285, -291, -299, -315, -321, -445, and -1103) and one isolate with no variation (SNU-20). In agreement with the above PCR analyses, all but the SNU-20 isolate had a T-to-C mutation at 9,233 relative to the Raji sequence [25], that abolished the *Bam*HI site between the W1* and I1* fragments. In addition to this mutation, all isolates except for SNU-1103 had a conserved mutation of T-to-C at 9,216 and, in the case of SNU-291, there was an additional change of A-to-G at 9,241 relative to the Raji sequence. Therefore, the sequence analyses confirmed that the T-to-C mutation within the *Bam*HI recognition sequence was responsible for the *Bam*HI W1*/I1* polymorphism.

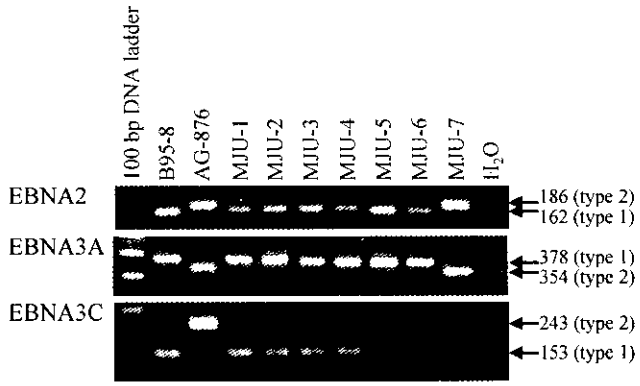

Fig. 4. Typing analysis of EBV isolates derived from healthy virus carriers.

EBV isolates rescued from the throat washings of virus carriers were amplified for EBNA2, -3A, or 3C sequences along with B95-8 type-1 and AG876 type-2 controls using primers specific for each EBNA gene to determine their types. The PCR products were then analyzed by 2% agarose gels containing EtBr. The types and sizes of the DNA fragments are indicated on the left side of each panel.

Analyses of *Bam*HI and *Xho*I Site Polymorphisms of EBV Isolates from Healthy Virus Carriers

The above polymorphism analyses involved EBV isolates derived from patients with non-EBV-related cancers. To determine whether the results obtained were applicable to healthy virus carriers, B cell transformation experiments were set up to rescue EBV strains as LCLs from the throat washings of 12 healthy virus carriers, using umbilical mononuclear cells as the source of the target B-cells. From 7 of the 12 carriers, EBV-transformed lymphoblastoid cell lines (LCLs) were established, and designated MJUs-1 to -7. To determine the EBV types of these isolates, PCR-mediated genotyping analyses for the EBNA2, -3A, and 3C genes were carried out. As shown in Fig. 4, six isolates, MJBs-1 to 6, gave 168 bp type-1 bands for the EBNA2 gene, 276 bp type-1 bands for the EBNA3A gene, and 153 bp type-1 bands for the EBNA3C gene, as did the B95-8 type-1 control, whereas only one isolate (MJB-7) and the AG876 type-2 control gave 186 bp type-2 bands, 237 bp type-2 bands for the EBNA3A gene, and 246 bp type 2-bands for the EBNA3C gene. Therefore, these results clearly indicate that MJB-1 to 5 were type-1 isolates and MJB-6 was a type-2 isolate.

Using these 7 isolates, PCR analyses were then carried out for *Bam*HI W1*/I1* and *Bam*HI F/f, and LMP1 *Xho*I site polymorphisms. In an analysis for the f-variant, none of the 541 bp DNA fragments amplified from the 7 isolates could be cleaved into two sub-fragments (415 bp and 126 bp) upon *Bam*HI digestion that would be expected from the f-variant, thereby indicating that the 7 isolates were wild-types of F-fragments like B95-8, SNU-1103, and AG976 (Fig. 5A). Analyses of the *Bam*HI W1*/I1* and *Xho*I site polymorphisms revealed that five type-1 isolates (MJB-1, -2, -3, -4, and -6) lost both enzyme sites,

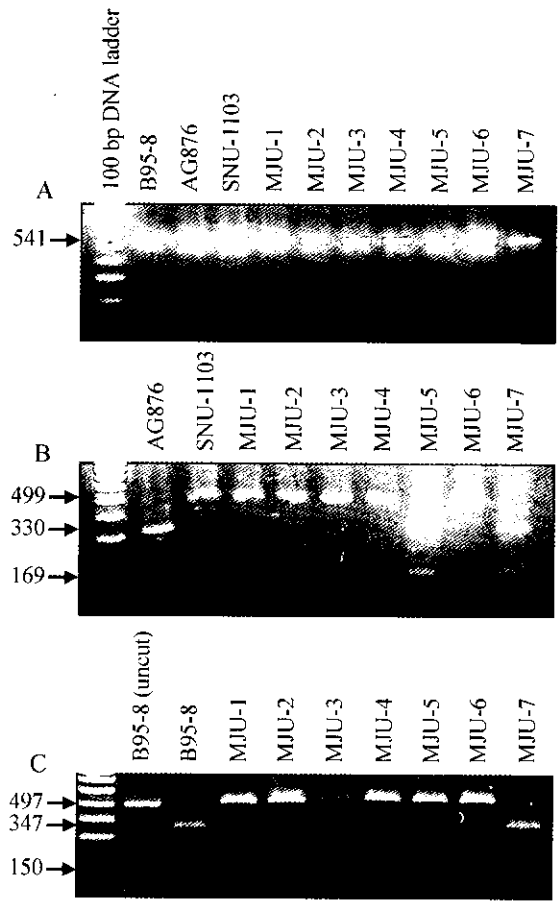

Fig. 5. *Bam*HI and *Xho*I site polymorphism analysis of EBV isolates derived from healthy virus carriers.
The EBV isolates were analyzed for *Bam*HI F/f (A), *Bam*HI W1*/I1* (B), and *Xho*I (C) polymorphisms as described in the legends of Figs. 1 to 3, respectively.

whereas the type-2 isolate, MJB-7, retained these sites and the type-1 isolate, MJB-5, lost only the *Bam*HI site (Figs. 5B and 5C). These results were thus consistent with the notion that the *Bam*HI W1*/I1* and LMP1 *Xho*I site variations are highly associated with type-1 rather than with type-2 EBV strains in normal Korean carriers.

DISCUSSION

There have been several reports describing the EBV-related tumors in Korea [13, 17, 29]. However, few studies have been conducted on the molecular epidemiological features of EBV strains prevalent in the Korean population. In this study, 16 EBV isolates derived from cancer patients and 7 EBV isolates derived from healthy carriers were analyzed to possibly reveal the prevalence of *Bam*HI F/f, *Bam*HI W1*/I1*, and LMP1 *Xho*I polymorphisms among the EBV strains found in Korea. None of the EBV isolates analyzed showed the f-variant. This result was expected, as viruses carrying the f-variant have been almost exclusively detected in the Chinese population and rarely in other geographic locations [1, 16, 20, 30]. The current results thus were consistent with the previous notion that EBV strains with the f-variant may be specific to China. Interestingly, in the Chinese population, the f-variant was detected at a significantly higher frequency in NPC patients than in healthy virus carriers [21, 16, 23], suggesting a possible association of this variant with the development of NPC. However, the variant was also detected with a significant frequency (10/23) in European HD biopsies [16], suggesting its related role in the pathogenesis of other associated tumors.

For the W1*/I1* and *Xho*I polymorphisms, 10 of the 12 type-1 isolates from the cancer patients and five of the six type-1 isolates from the healthy carriers lost both the LMP1 *Xho*I site and the *Bam*HI site at the junction of the *Bam*HI W1* and I1* fragments. The ladder *Bam*HI polymorphism was also confirmed by a sequencing analysis and was shown to be due to a T-to-C mutation within the *Bam*HI recognition sequence. However, three type 2 isolates (two from cancer patients and one from a healthy carrier) and one type-1 isolate from a cancer patient retained both enzyme sites, whereas the remaining type-1 isolates showed a polymorphism only at either the LMP1 *Xho*I site (SNU-447) or the *Bam*HI site between W1* and I1* (MJU-5). Accordingly, these results indicate that both *Bam*HI W1*/I1* and LMP1 *Xho*I polymorphisms are strongly associated with type-1 rather than type-2 viruses in Korea, although only a limited number of type-2 isolates were analyzed. In this respect, it was interesting to note that two intertypic recombinants (SNU-817 and SNU-889) did not show any polymorphisms at either site, like type-2 isolates. According to the genotyping analyses, these viruses carried a type-1 EBNA2 gene plus either type-2 EBNA3A, -3B, and -3C genes (SNU-889) or a type-1 EBNA3A gene and type-2 EBNA3B and C genes (SNU-817) (Kim *et al.*, manuscript in preparation), indicating that they were intertypic recombinants generated through the recombination of type-1 and type-2 viruses. Therefore, the finding that these recombinants were not polymorphic at either site, like type-2 isolates, provides further support for the notion that these polymorphisms are strongly associated with type-1 strains in Korea. Given the relative genomic positions of these variations and the EBNA3 genes, the results also strongly infer that the right ends of the recombinants are genomes derived from the current type-2 viruses circulating in Korea.

Such a type-1-related linkage of the W1*/I1* and *Xho*I polymorphisms was first noted by Abdel-Hamid *et al.* [1] in their analyses of EBV genomes found in EBV-associated tumors derived from various geographic locations that were typed only for the EBNA2 gene. Both RFLPs were detected in all type-1 viruses in Chinese and

Malaysian NPC tumors examined, however, they were rarely detected in type-1 and type-2 viruses in the NPC and BL tumors derived from other areas including the Mediterranean, the United States of America, and Africa. The exception was type-2 viruses from Alaska that showed both variations. Based on these observations, an interesting proposal was made that the W1*/I1* and *Xho*I polymorphisms only mark type-1 viruses prevailing in Asians but not in Caucasians. Consistent with this, similar type-1-related associations were also observed in the analyses of EBV DNA in the throat washings and gastric tumors of the Japanese [30]. However, definitive testing of such a type-related association with a polymorphic marker will require the analysis of a large panel of EBV isolates in LCLs. More importantly, the interpretation of a type-related association should follow the genotypes determined by an analysis of multiple EBNA loci and not just the EBNA2 gene alone. In fact, Khanim *et al.* [16] analyzed the LMP1 *Xho*I RFLP using many tumor biopsies plus a large panel of EBV isolates in spontaneous LCLs derived from various geographic locations. They typed viruses based on analyses of both EBNA2 and 3C genes and found the *Xho*I site polymorphism almost exclusively in the type-1 EBV strains from China and not in those from Europe, Africa, and Papua New Guinea, giving strong support to the possible association of the *Xho*I RFLP with type-1 viruses in China. The present results obtained from the EBV isolates, whose types were confirmed by analyses of at least 3 EBNA genes, give further support to the type-1-related linkage of the W1*/I1* and *Xho*I polymorphisms in Asia. It is intriguing to propose that the type-1 EBV strains in the wild comprise of at least two genetically distinct subtypes, which are characterized by the presence or absence of both W1*/I1* and *Xho*I variations. Accordingly, intratypic or intertypic recombinations between viruses with and without polymorphisms can then give rise to various rare variants including a type-1 without either variation (SNU-9) or a type-1 with a variation at only one site (SNU-447 and MJU-5), or type-2 viruses including both variations such as those in the NPC biopsies from Alaska [1]. The significance of the W1*/I1* polymorphism in any EBV-associated diseases remains unknown. This polymorphic *Bam*HI site lies in a region which has been naturally or experimentally deleted without affecting the *in vitro* B cell transforming activity of EBV [25, 26]. Interestingly, many multiple spliced rightward transcripts derived from this region have been found in all types of EBV-latently infected cells [31]. However, none of the transcripts have been shown to include an exon containing this *Bam*HI site.

In summary, EBV isolates derived from 23 immunocompetent virus carriers, i.e. 15 patients with non-EBV-related cancers and 7 healthy donors, were analyzed for their genetic polymorphisms. The present results indicated little association of the f-variant with both the type-1 and type-2 strains, yet a high prevalence of type-1 strains carrying both *Bam*HI W1*/I1* and LMP1 *Xho*I polymorphisms in the immunocompetent Korean carriers. This work and the EBV isolates analyzed will provide a framework for future molecular epidemiological studies of EBV strains in the Korean population.

Acknowledgments

The authors are grateful to Drs J.-G. Park and S. K. Lee for providing LCLs derived from cancer patients and AG876 cells, respectively. This work was supported by a grant from The Ministry of Education (1996) and in part by a grant from the Ministry of Health & Welfare (HMP-98-M-1-0005), Republic of Korea.

REFERENCES

1. Abdel-Hamid, M., J.-J. Chen, N. Constantine, M. Massoud, and N. Raab-Traub. 1992. EBV strain variation: Geographical distribution and relation to disease state. *Virology* **190:** 168–175.
2. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310:** 207–211.
3. Bouzid, M., D. Djennaoui, J. Dubreuil, A. Bouguermouth, D. Ellouz, J. Abdelwahab, and G. Decaussin. 1994. Epstein-Barr virus genotypes in NPC biopsies from North Africa. *Int. J. Cancer* **56:** 468–473.
4. Burrows, J. M., R. Khanna, T. B. Sculley, M. P. Alpers, D. J. Moss, and S. R. Burrows. 1996. Identification of a naturally occurring recombinant Epstein-Barr virus isolate from New Guinea that encodes both type 1 and type 2 nuclear antigen sequences. *J. Virol.* **70:** 4829–4833.
5. Chang, Y.-S., I.-J. Su, P.-J. Chung, C.-H. Shu, C.-K. Ng, S.-J. Wu, and S. T. Liu. 1995. Detection of an Epstein-Barr virus variant in T-cell-lymphoma tissues identical to the distinct strain observed in nasopharyngeal carcinoma in the Taiwanese population. *Int. J. Cancer* **62:** 673–677.
6. Chen, M. L., C. N. Tsai, C. L. Liang, C. H. Shu, C. R. Huang, D. Sulitzeanu, S. T. Liu, and Y. S. Chang. 1992. Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein-Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. *Oncogene* **7:** 2131–2140.
7. Cho, S., S.-G. Cho, Y.-S. Shim, and W. K. Lee. 1998. Sequence analysis of the latent membrane protein 1 genes of Epstein-Barr virus isolates in Korea. *J. Microbiol.* **36:** 130–138.
8. Dambaugh, T., K. Hennessy, L. Chamnankit, and E. Kieff. 1984. U2 region of Epstein-Barr virus DNA may encode Epstein-Barr nuclear antigen 2. *Proc. Natl. Acad. Sci. USA* **81:** 7632–7636.

9. Gratama, J. W., M. A. Oosterveer, G. Klein, and I. Ernberg. 1990. EBNA size polymorphism can be used to trace Epstein-Barr virus spread within families. *J. Virol.* **64:** 4703–4708
10. Gratama, J. W., M. A. Oosterveer, W. Weimar, K. Sintnicolaas, W. Sizoo, R. L. Bolhuis, and I. Ernberg. 1994. Detection of multiple 'Ebnotypes' in individual Epstein-Barr virus carriers following lymphocyte transformation by virus derived from peripheral blood and oropharynx. *J. Gen. Virol.* **75:** 85–94.
11. Guo, H.-Y. and M. H. Ng. 1991. Detection and prevalence of the f variant of Epstein-Barr virus in Southern China. *Virology* **185:** 67–71.
12. Hu, L. F., E. R. Zabarovsky, F. Chen, S. L. Cao, I. Ernberg, G. Klein, and G. Winberg. 1991. Isolation and sequencing of the Epstein-Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. *J. Gen. Virol.* **72:** 2399–2409.
13. Huh, J., K. Cho, D. S. Heo, J. E. Kim, and C. W. Kim. 1999. Detection of Epstein-Barr virus in Korean peripheral T-cell lymphoma. *Am. J. Hematol.* **60:** 205–214.
14. Jeng, K.-C. G., C.-Y. Hsu, M. T. Liu, T. T. Chung, and S. H. Liu. 1994. Prevalence of Taiwan variant of Epstein-Barr virus in throat washings from patients with head and neck tumors in Taiwan. *J. Clin. Microbiol.* **32:** 28–31.
15. Katz, B. Z., J. C. Niederman, B. A. Olson, and G. Miller. 1988. Fragment length polymorphisms among independent isolates of Epstein-Barr virus from immunocompromised and normal hosts. *J. Infect. Dis.* **157:** 299–308.
16. Khanim, F., Q.-Y. Yao, G. Niedobitek, S. Sihota, A. B. Rickinson, and L. S. Young. 1996. Analysis of Epstein-Barr virus gene polymorphisms in normal donors and in virus-associated tumors from different geographical locations. *Blood* **88:** 3491–3501.
17. Lee, S. S., J. J. Jang, K. J. Cho, S. K. Khang, and C. W. Kim. 1997. Epstein-Barr virus-associated primary gastrointestinal lymphoma in non-immunocompromised patients in Korea. *Histopathology* **30:** 234–242.
18. Lee, W. K., S. M. Kim, Y. S. Sim, S. G. Cho, S. H. Park, C. W. Kim, and J. G. Park. 1998. B-lymphoblastoid cell lines from cancer patients. *In Vitro Cell Dev. Biol. Anim.* **34:** 97–100.
19. Lung, M. L. and G. C. Chang. 1992. Detection of distinct Epstein-Barr virus genotypes in NPC biopsies from Southern Chinese and Caucasians. *Int. J. Cancer* **52:** 34–37.
20. Lung, M. L., R. S. Chang, M. L. Huang, H.-Y. Guo, D. Choy, J. Sham, S. Y. Tsao, P. Cheng, and M. H. Ng. 1990. Epstein-Barr virus genotypes associated with nasopharyngeal carcinoma in Southern China. *Virology* **177:** 44–53.
21. Lung, M. L., G. C. Chang, T. R. Miller, W. M. Wara, and T. L. Phillip. 1994. Genotype analysis of Epstein-Barr virus isolates associated with nasopharyngeal carcinoma in Chinese immigrants to the United States. *Int. J. Cancer* **59:** 743–746.
22. Lung, M. L., R. S. Chang, and J. H. Jones. 1988. Genetic polymorphism of natural Epstein-Barr virus isolates from infectious mononucleosis and healthy carriers. *J. Virol.* **62:** 3862–3866.
23. Lung, M. L., W. P. Lam, J. Sham, D. Choy, and Z. Yong-Sheng. 1992. Analysis of Epstein-Barr virus in localized nasopharyngeal carcinoma tumors. *Cancer* **71:** 1190–1192.
24. Midgley, R. S., N. W. Blake, Q. Y. Yao, D. Croom-Carter, S. T. Cheung, S. F. Leung, A. T. Chan, P. J. Johnson, D. Huang, A. B. Rickinson, and S. P. Lee. 2000. Novel intertypic recombinants of Epstein-Barr virus in the Chinese population. *J. Virol.* **74:** 1544–1548.
25. Parker B. D., A. Bankier, S. Satchwell, B. Barrell, and P. J. Farrell. 1990. Sequence and transcription of Raji Epstein-Barr virus DNA spanning the B95-8 deletion region. *Virology* **179:** 339–346.
26. Robertson, E. and E. Kieff. 1995. Reducing the complexity of the transforming Epstein-Barr virus genome to 64 kilobase pairs. *J. Virol.* **69:** 983–993.
27. Sample, J., L. Young, B. Martin, T. Chatman, E. Kieff, A. Rickinson, and E. Kieff. 1990. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. *J. Virol.* **64:** 4084–4092.
28. Shim, Y. S., C. W. Kim, and W. K. Lee. 1998. Sequence variation of EBNA2 of Epstein-Barr virus isolates from Korea. *Mol. Cells* **8:** 226–232.
29. Shin, W. S., M. W. Kang, J. H. Kang, M. K. Choi, B. M. Ahn, J. K. Kim, H. S. Sun, and K. W. Min. 1996. Epstein-Barr virus-associated gastric adenocarcinomas among Koreans. *Am. J. Clin. Pathol.* **105:** 174–181.
30. Sidagis, J., K. Ueno, M. Tokunaga, M. Ohyama, and Y. Eizuru. 1997. Molecular epidemiology of Epstein-Barr virus (EBV) in EBV-related malignancies. *Int. J. Cancer* **72:** 72–76.
31. Smith, P. R., O. de Jesus, D. Turner, M. Hollyoake, C. E. Karstegl, B. E. Griffin, L. Karran, Y. Wang, S. D. Hayward, and P. J. Farrell. 2000. Structure and coding content of CST (BART) family RNAs of Epstein-Barr virus. *J. Virol.* **74:** 3082–3092.
32. Wu, S.-J., J.-D. Lay, C.-L. Chen, J.-Y. Chen, M.-Y. Liu, and I.-J. Su. 1996. Genomic analysis of Epstein-Barr virus in nasal and peripheral T-cell lymphoma: A comparison with nasopharyngeal carcinoma in an endemic area. *J. Med. Virol.* **50:** 314–321.
33. Yao, Q. Y., R. J. Tierney, D. Croom-Carter, D. Dukers, G. M. Cooper, C. J. Ellis, M. Rowe, and A. B. Rickinson. 1996. Frequency of multiple Epstein-Barr virus infections in T-cell-immunocompromised individuals. *J. Virol.* **70:** 4884–4894.
34. Yao, Q. Y., R. J. Tierney, D. Croom-Carter, G. M. Cooper, C. J. Ellis, M. Rowe, and A. B. Rickinson. 1996. Isolation of intertypic recombinants of Epstein-Barr virus from T-cell-immunocompromised individuals. *J. Virol.* **70:** 4895–4903.