Discrete Alterations in the BZLF1 Promoter in Tumor and Non-Tumor-Associated Epstein-Barr Virus

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Background: Although the Epstein-Barr virus (EBV) is associated with malignant and nonmalignant diseases, its lytic replication is predominately associated with nonmalignant diseases such as acute infectious mononucleosis (IM) or chronic active EBV infection. Lytic replication is also associated with type B EBV more than with type A EBV. Sustained lytic replication, however, is not compatible with tumor growth. We investigated whether control of an EBV lytic regulatory gene, BZLF1, differed in these diseases.

Methods: Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) and direct DNA sequence analyses were used to characterize the promoter sequence of BZLF1 (Zp) in 52 tumors (34 non-Hodgkin’s lymphomas, 13 post-transplant lymphoproliferative disease samples, and five nasopharyngeal carcinomas), and in peripheral blood lymphocytes from seven patients with chronic active EBV, six with IM, and 40 healthy, EBV-seropositive individuals. All sequences were compared with the prototype EBV strain B95.8 sequence. All statistical tests were two-sided.

Results: Three polymorphic Zp sequences were detected. Among the malignant samples, sequence Zp-P, associated with 84% of type A EBV, was identical to that of EBV strain B95.8, whereas a second sequence (Zp-V3), associated exclusively with type B EBV (P<.001), contained three base substitutions. Among the nonmalignant samples, a distinct polymorphism, Zp-V4, containing the substitutions detected in Zp-V3 and an additional base change, was identified in all samples from chronic active EBV, IM, and healthy individuals, but in none of the malignant samples (P<.001). Zp-V4 was independent of the EBV type. Conclusions: Polymorphisms in the regulatory sequences of BZLF1 are differentially distributed among malignant and nonmalignant cells and may identify EBV subtypes with various lytic activities, including those not associated with malignancies. [J Natl Cancer Inst 2002;94:1757–63]

Epstein-Barr virus (EBV), a human herpesvirus, has maintained a successful and long-term association with the human population (1). This coexistence has relied on the ability of the virus to sustain lifelong, and usually benign, latent infections. Periodic cycles of lytic replication, without any clinical manifestation, are sufficient to maintain the viral infection within the host and permit frequent EBV transmission among the population (2). The major route of transmission is through saliva, and when in the oropharynx, EBV infects B cells. B cells in the oropharynx not only serve as the major latent reservoir for EBV,
but they also, in all likelihood, serve as the primary site where EBV switches to a lytic cycle (3,4).

In the latent cycle, the replication of the viral DNA is coupled to the replication of the host genome without production of infectious viral particles. Reactivation (i.e., a switch from latency to a productive lytic cycle of EBV) is frequently seen in immunocompromised individuals (5), suggesting that the tight control over latency is, at least in part, dictated by host factors, possibly including immune surveillance mechanisms. However, because latently infected B cells grown in vitro do not spontaneously undergo lytic replication, it is likely that the virus itself may exert some control over the switch from latency to lytic replication. Indeed, one potent regulator of the switch from latency to productive infection (i.e., lytic replication) is the protein encoded by the EBV BamHI fragment Z (BZLF1, ZEBRA, or Zta) (6,7). Although the specific in vivo signals that induce the switch from latency to lytic infection are unclear, BZLF1 expression is stringently regulated by several regulatory domains within its promoter (8,9). A wide variety of signals, including phorbol esters, 12-O-tetradecanoylphorbol 13-acetate (TPA), calcium ionophores, transforming growth factor (TGF)-β1, and anti-immunoglobulin, trigger expression of BZLF1 in vitro, leading to the lytic cascade of events (10–13). Many of these signals regulate BZLF1 expression through the 220-base-pair (bp) fragment immediately upstream of the transcription initiation site.

An additional factor known to influence the efficiency of the shift between latency and lytic replication is the EBV type. EBV types, termed A and B, are distinguished by signal nucleotide changes in specific EBV genes, including EBV nuclear antigens (EBNAs) 2, 3A, 3B, 3C, and Epstein-Barr-encoded small RNA (EBER) (14,15). Both EBV types have been detected in immunocompromised and immunocompetent hosts (16,17). Type B EBV enters the lytic cycle more readily than type A EBV (18). Moreover, different EBV isolates respond with different efficiencies to exogenous inducers of reactivation. For example, anti-immunoglobulin treatment more efficiently induces BZLF1 in the EBV strain Akata than in the EBV strain B95.8 (6,19). Although both of these strains are type A viruses, they differ in the DNA sequences of the promoter regulatory and coding regions of BZLF1 (20–22). Because BZLF1 is necessary and sufficient to mediate the switch from latency to the lytic cycle, it is possible that differences in the lytic potential of different EBV isolates may reflect the presence of different BZLF1 regulatory and/or coding sequences. We therefore asked whether the sequences of the major promoter regulatory elements (Zp) of the BZLF1 gene differ among type A and B EBVs in tumors and nontumor tissues.

**MATERIALS AND METHODS**

**Biologic Samples**

We obtained DNA from a total of 52 tumor samples. DNA was available from 15 EBV-associated Burkitt’s lymphomas that were previously characterized for the presence of EBV and c-myc translocations (23). DNA was also available from 17 non-Hodgkin’s lymphomas, five nasopharyngeal carcinomas, two T-cell lymphomas, and 13 specimens from post-transplant lymphoproliferative disorders. These specimens have been described in previous studies (24–27). Thirty-seven of the 52 tumors originated from North and South America, seven from Africa, and eight from Far East Asia (Japan and Hong Kong).

DNA was isolated, by using a standard phenol/chloroform extraction technique after protease K digestion, from peripheral blood lymphocytes of healthy individuals from North and South America (n = 21), Japan (n = 6), and Saudi Arabia (n = 13) and from peripheral blood lymphocytes of patients with acute infectious mononucleosis (IM) (n = 6). DNA was also isolated from peripheral blood lymphocytes of seven patients with chronic active EBV infection. Seventeen samples from the seven patients with chronic active EBV were obtained at different times (1–6 years after diagnosis) during the course of the disease. Research protocols for studies involving the patients with chronic active EBV were approved by the National Institute of Allergy and Infectious Diseases (Bethesda, MD). All research subjects who provided peripheral blood lymphocytes for the study gave informed consent. Because the EBV strain B95.8 is often used as a reference, we included this as the EBV prototype control.

**Polymerase Chain Reaction–Single-Strand Conformation Polymorphism and DNA Sequence Analyses**

Five hundred nanograms of genomic DNA obtained from all tumor and nontumor samples was directly used as a template in polymerase chain reactions (PCRs). PCR was used to amplify the fragment from nucleotides −221 to +12 (with respect to the transcription start site) of the BZLF1 promoter. PCR conditions consisted of 5 minutes at 95 °C, 30 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C, and 1 minute at 72 °C, followed by a long extension of 10 minutes at 72 °C. [32P]dCTP was included in the PCR buffer. PCR products were separated by electrophoresis through 6% nondenaturing acrylamide gels (19:1, acrylamide to bis) containing 10% glycerol at 6 W for 18 hours and visualized by autoradiography. Autoradiograms were exposed for 2–16 hours. Single-strand conformation polymorphisms (SSCPs) were apparent by differences in the patterns of migration of the PCR product. Amplified product obtained from the EBV strain B95.8 served as a reference control.

Five hundred nanograms of genomic DNA from samples that showed distinct SSCP patterns were then amplified in a separate, nonradioactive PCR. These PCR products were directly sequenced either manually (28) or with the automated MegaBACE 1000 Sequencer (Molecular Dynamics, Piscataway, NJ). At least two independent reactions with two different sequencing primers (sense and antisense) were carried out for each PCR product to confirm the sequences. Detected sequence variations are reported at their base position relative to the transcription start site of the BZLF1 gene.

**EBV Typing**

Type A and B EBVs were determined from genomic DNA of tumor and chronic active EBV samples by PCR with primers for the EBNA-3A and EBNA-3C genes as described (29). PCR with EBNA-3A primers yields an amplification product of 276 bp for type A EBV or 237 bp for type B EBV. PCR with EBNA-3C primers yields an amplification product of 153 bp for type A EBV and 246 bp for type B EBV.
Statistical Analysis

The association of each polymorphic Zp (promoter element of BZLF1) with type A and B EBVs and the distribution of the variants in different cell types (i.e., malignant and nonmalignant) were analyzed by using the Fisher’s exact test (SPSS 11.0; Chicago, IL). All statistical tests were two-sided.

RESULTS

Variations in the Regulatory Sequences of BZLF1 Associated With EBV Type

The purpose of this study was to determine whether discriminatory alterations could be identified in the regulation of the lytic pathway in type A and B EBVs and/or EBV associated with tumor and nontumor tissues. PCR–SSCP analyses were initially used to identify whether sequence variations existed within the major regulatory Zp domains (nucleotides –221 to +12, with respect to the initiation of transcription of BZLF1). We first analyzed 37 samples obtained from North and South America; 20 carried type A EBV and 17 carried type B EBV. Of the 37 samples, seven were obtained from cell lines derived from non-Hodgkin’s lymphomas, 17 were obtained from primary non-Hodgkin’s lymphoma biopsy specimens, and 13 were obtained from post-transplant lymphoproliferative disorder biopsy specimens.

PCR–SSCP analyses demonstrated two distinct patterns of migration (Fig. 1, A). Amplified products of EBV Zp from sev-

![Fig. 1. Polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP: A) and DNA sequence analyses (B) of the promoter region of the Epstein-Barr virus BZLF1 gene (Zp) isolated from tumor samples. A) PCR–SSCP was performed on DNA extracted from 52 tumor samples. During the PCR, the products were labeled with 32P, separated by electrophoresis, and visualized by autoradiography. The autoradiogram of 13 representative samples is shown. Two distinct migration patterns are indicated as P (lanes 1, 3, 5, 7, and 9–13) and V3 (lanes 2, 4, 6, and 8). Lane 1 shows the PCR–SSCP pattern from EBV strain B95.8, the prototype control. B) DNA trace data from positions –150 to –91 (with respect to the site of transcription initiation of the BZLF1 gene) were obtained by direct automatic sequencing of PCR products. Seven representative samples were selected corresponding to migration patterns P and V3. Arrows indicate the position of the substitutions at nucleotides –141 (A to G), –106 (A to G), and –100 (T to G). Zp represents the sequence of the BZLF1 promoter.](image-url)
er al tumor samples migrated faster than the amplified Zp product from EBV strain B95.8 (Fig. 1, A; compare lane 1 with lanes 2, 4, 6, and 8). Of the 37 samples analyzed, 20 demonstrated a pattern similar to that of EBV strain B95.8, and 17 showed an alternate pattern of migration. For further sequence analyses, we chose DNA from 12 samples with the EBV strain B95.8 migration pattern and from eight samples with the alternate pattern. Direct sequencing of independently amplified products from these 20 samples confirmed the presence of discrete sequence variations (Fig. 1, B). The sequences of the amplified products with SSCP patterns similar to that of B95.8 were identical to that found in EBV strain B98.5 and, therefore, were designated as the prototype promoter (Zp-P). Sequences of the samples with an altered SSCP pattern differed from that of Zp-P at three positions: T to G at position –100 (T→G), A to G at position –106 (A→G), and A to G at position –141 (A→G). This sequence containing all three substitutions was designated promoter variant 3 (Zp-V3). It is interesting to note that two of the three substitutions occurred within known functional domains of the BZLF1 promoter. The A→G substitution occurred in the ZIIB domain, a region that strongly binds BZLF1 (9), and the A→G substitution occurred in the ZIC domain, a region that binds the nuclear regulatory factor SP-1 (8,10) and contributes to the ability of Zp to respond to TPA.

Each tumor sample showed a homogeneous PCR–SSCP pattern and homogeneous Zp sequence, consistent with the presence of monoclonal EBV genomes. All 20 samples with type A EBV contained the Zp-P sequence, whereas all 17 samples with type B EBV contained the Zp-V3 sequence (P<.001). These data suggest that the major EBV types may also differ in the BZLF1 promoter sequence.

Because geographic variations in EBV genome sequences exist, we analyzed seven EBV-containing lymphoma specimens from Africa and eight lymphoma and carcinoma tissues from Far East Asia (Japan and Hong Kong) by PCR–SSCP. Direct sequencing of five of the specimens identified the same variants as those observed in the specimens from American subjects (data not shown). All samples from Africa (n = 2) and Far East Asia (n = 1) containing type B EBV carried Zp-V3 (Table 1). All samples from Africa containing type A EBV (n = 5) carried the Zp-P sequence, whereas all samples from Far East Asia (n = 7) containing type A EBV carried Zp-P (n = 2) or Zp-V3 (n = 5) (Table 1). The data suggest that, regardless of its geographic origin, type B EBV carries the BZLF1 promoter Zp-V3. However, there is some degree of geographic heterogeneity for type A EBV, which carries Zp-P or Zp-V3.

**BZLF1 Promoter Sequences in Nonmalignant Host Cells**

We next determined whether differences in the BZLF1 promoter sequence could also be detected in EBV isolated from patients with nonmalignant disorders, such as infectious mononucleosis or chronic active EBV infection. All samples were from the United States. The PCR–SSCP Zp migration patterns of EBV isolated from patients with nonmalignant disorders (Fig. 2, A) and of EBV isolated from tumor samples (Fig. 1, A) differed. Twelve DNAs were analyzed by DNA sequencing, which identified a variant promoter with four single-base substitutions. This variant promoter was designated Zp-V4. Three substitutions were identical to those detected in Zp-V3, and the fourth was a T→C substitution at position –196 (Fig. 2, B). This substitution was located in the ZIA domain, which contains SP1 binding elements and is also the domain necessary for anti-immunoglobulin-mediated response (8,10,13). Five samples (four from two patients with chronic active EBV infection and one from a patient with infectious mononucleosis) of the 23 samples analyzed contained both Zp-V4 and Zp-P as evidenced by the mixed pattern of migration in the SSCP gel (see Fig. 2, A, lanes 9, 11, and 15) and confirmed by the presence of heterogeneous sequences (P+V4 in Fig. 2, B).

Because all the samples tested carried the Zp-V4 promoter sequence, we did not expect an association with the EBV type. Nonetheless, among the seven patients with chronic active EBV infection (two of which carried Zp-V4 and Zp-P promoter sequences), PCR analyses demonstrated that five contained type A EBV and two contained type B EBV. Therefore, the presence of the Zp-V4 promoter sequence appears to be independent of the EBV type.

To further determine which of the Zp sequences is present in healthy seropositive individuals, we analyzed peripheral blood lymphocytes from 21 individuals from North and South America, 13 individuals from Saudi Arabia, and six individuals from Japan by PCR–SSCP. Twenty-one of these samples were also sequenced (data not shown). All samples, regardless of their geographic origin, carried the Zp-V4 sequence; two samples (5%) also carried the Zp-P sequence.

Table 2 summarizes the distribution of Zp sequences in all of the samples examined in our study. All of the 53 non-tumor samples carried Zp-V4, but none of the 52 tumor samples from three diverse geographic regions carried the Zp-V4 sequence (P<.001), suggesting that Zp-V4 is not associated with EBV in malignant cells.

**DISCUSSION**

The purpose of this study was to determine whether the regulatory regions of the BZLF1 (ZEBRA or Zta) promoter differ in
type A and B EBVs and whether any such differences are associated with particular EBV-related diseases. It is well documented that the sequences of type A and B EBVs differ in multiple genes (14,15). These sequence variations may directly contribute to subtle differences in the biology of the virus. Indeed, Buck et al. (18) have provided strong evidence that the two viral types differ in their ability to spontaneously enter into the lytic cycle.

Because the lytic transactivator protein BZLF1 is necessary and sufficient to induce the lytic cycle (6,7), differences in this protein could help modulate the responsiveness to auto-reactivation signals or to other inducers of the lytic cycle. However, the reported sequence variations in the BZLF1 gene occur independently of EBV type (20–22) and, hence, did not seem sufficient to account for the efficiency of the lytic cycle in type B EBV compared with type A EBV. Thus, we hypothesized that additional sequence variations in the promoter of BZLF1 could exist. We focused our studies on the 221-bp region designated Zp that encompasses the binding sites for the transcription factors SP-1, AP-1, and ZRE and that is primarily responsible for regulating BZLF1 transcription (8–13). We determined the sequence variations in the promoter region of BZLF1 amplified from samples from patients with chronic active EBV infection. (A) PCR–SSCP was performed on DNA extracted from 53 non-tumor samples. During the PCR, the products were labeled with 32P, separated by electrophoresis, and visualized by autoradiography. The autoradiogram of 15 samples (lanes 3–17) is shown. Lane 1 shows the PCR–SSCP pattern from a sample with the Zp-V3 promoter, and lane 2 shows the pattern Zp-P from EBV strain B95.8, the prototype control. A migration pattern (V4) distinct from those identified from tumor samples (V3 and P) is shown in the remaining lanes. Lanes 9, 11, and 15 show a combination pattern of P and V4.

(B) DNA trace data from positions –203 to –137 (with respect to the site of transcription initiation of the BZLF1 gene) were obtained by direct automatic sequencing of PCR products. Four representative samples were selected corresponding to migration patterns V4 and P+V4. Arrows indicate the position of the substitutions at nucleotides –196 (T→C) and –141 (A→G). Zp represents the sequence of the BZLF1 promoter. The P+V4 sequence shows heterogeneous bases (C+T at –196 and A+G at –141).

Table 2. Distribution of three polymorphisms detected in the promoter region of the Epstein-Barr virus BZLF1 gene between malignant and nonmalignant cells*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Host cells</th>
<th>No. of patients</th>
<th>No. of patients with Zp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zp-P</td>
<td>Zp-V3</td>
</tr>
<tr>
<td>Malignant</td>
<td>NHL, PTLD, NPC</td>
<td>52</td>
<td>27</td>
</tr>
<tr>
<td>Nonmalignant</td>
<td>CAEBV, IM</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>PBL</td>
<td>40</td>
<td>22</td>
</tr>
</tbody>
</table>

*CAEBV = chronic active Epstein-Barr virus infection; IM = infectious mononucleosis; NHL = non-Hodgkin’s lymphomas; NPC = nasopharyngeal carcinomas; PTLD = post-transplant lymphoproliferative disorders; Zp-P = promoter sequence from the prototype EBV strain B95.8; Zp-V3 = variant promoter with three substitutions; Zp-V4 = variant promoter with four substitutions; Zp = 221-base-pair promoter sequence of BZLF1.
†Sequence variations in the promoter region of BZLF1 were determined by polymerase chain reaction–single-strand conformation polymorphism analyses and direct DNA sequencing. The association between the promoter sequence polymorphism and cell type was statistically significant (P<0.001) as determined by Fisher’s exact test.
‡Number of patients with heterogeneous Zp (Zp-P + Zp-V4).
quence of Zp in 52 EBV-positive tumors that were collected from North and South America, Africa, and Far East Asia, because the distribution of polymorphic variants of EBV is influenced by geography. Our results demonstrate that polymorphisms in Zp (Fig. 1 and Table 1) are strongly associated with the EBV type (Table 1). Thus, tumors from North and South America, Africa, and Far East Asia containing type B EBV genomes are associated with Zp-V3. Similarly, tumors from the same geographic regions containing type A EBV genomes are associated with Zp-P, with the exception of type A EBV genomes from Far East Asia. Although some geographic dependence of these polymorphisms is evident, the association between EBV type and promoter polymorphism was statistically significant ($P<.001$).

It was surprising to determine that none of the Zp polymorphisms detected in tumor samples were strongly associated with nonmalignant EBV-associated diseases (chronic active EBV infection and infectious mononucleosis) or with nonsymptomatic EBV carriers. In fact, the most common Zp sequence detected in samples from patients and subjects with no evidence of malignant disease was a distinct variant, Zp-V4 (Fig. 2). Zp-V4 was consistently present, irrespective of the origin (North or South American, Arab, or Asian) or the EBV type. It is important to note that, although we did not detect the Zp-V3 sequence in the nonmalignant samples, it is possible that Zp-V3 could be present in levels below our limit of detection. By contrast, the absence of Zp-V4 in malignant cells, which cannot be attributed to the assay sensitivity because of the abundance of EBV DNA, was statistically significant ($P<.001$). These data suggest that if EBV containing Zp-V4 were more responsive to some physiologic signals, e.g., TGF-$\beta_1$ or engagement of B-cell immunoglobulin receptor, it would be more likely to lyse the host cell and less likely to be found in malignant cells. Indeed, preliminary functional studies suggest that different Zp sequences vary in their ability to respond to known activators (TPA, calcium ionophore, and anti-immunoglobulin) (data not shown). In addition to the regulation conferred by Zp, negative regulatory elements that bind the cellular transcription factor YY1 exist upstream of the 221-bp Zp region (30,31), and may also vary among the EBV types, potentially modifying the responsiveness of each promoter.

Irrespective of the responsiveness of different BZLF1 promoter variants, this study provides evidence that the polymorphic BZLF1 promoter may be a marker for a viral subtype that does not associate with malignant cells.

**References**


NOTES

T. C. Greiner is supported by Translational Research Grant 6605-01 from The Leukemia and Lymphoma Society.

Manuscript received July 1, 2002; revised September 3, 2002; accepted September 13, 2002.