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Detection of Epstein–Barr Virus (EBV) in Hepatocellular Carcinoma Tissue: A Novel EBV Latency Characterized by the Absence of EBV-Encoded Small RNA Expression

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In this study, we investigated the presence of Epstein–Barr virus (EBV) in liver tissue from 35 patients with hepatocellular carcinoma (HCC). EBV DNA was detected in 13 patients (37%) by Southern blot hybridization. In 10 of these patients, EBV DNA was present in tumor tissue only, whereas in the other 3, it was detected in both tumor and nontumor tissues. The quantity of EBV DNA detected was equivalent to 1–10 viral DNA molecules/100 cells. EBV-determined nuclear antigen was detected in 7–13% of the carcinoma cells in three tumor tissue samples that contained approximately one copy of the EBV genome/10 cells. A single terminal fragment of EBV DNA was identified in these tissues, suggesting that the EBV-infected cells in HCC represent clonal proliferation. Western blotting and reverse transcription–polymerase chain reaction analyses demonstrated that these three tumor tissue specimens were positive for EBV-determined nuclear antigen 1 and *Bam*HI A transcripts but were negative for the other latent EBV products, including EBV-encoded small RNA. The results indicated that there is a high EBV load in HCC tissue and that all of the HCC tissue examined showed a novel pattern of EBV latency characterized by absence of EBV-encoded small RNA expression.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that infects the majority of the human population. EBV is commonly transmitted by saliva and establishes latent infection in B lymphocytes, where it persists for the lifetime of the host. The virus has been associated with human malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, opportunistic lymphoma in immunocompromised hosts, and some cases of Hodgkin's disease, which can occur after prolonged persistence and reactivation of latent EBV (Rickinson and Kieff, 1996). EBV persists in a latent form in such tumor cells, and three distinct forms of EBV latent gene expression have been described (Kerr et al., 1992). B lymphoblastoid cell lines (LCL) transformed by EBV *in vitro* display the full pattern of latent gene expression (latency III) encompassing six nuclear antigens [EBNAs 1, 2, 3A, 3B, 3C, and leader protein (Lp)], three latent membrane proteins (LMPs 1, 2A, and 2B), transcripts from the *Bam*HI-A region of the virus genome (BARF0), and EBV-encoded nuclear RNAs (EBER1 and EBER2). This pattern is seen in opportunistic lymphoma cells. EBV-positive Burkitt's lymphoma cells express EBNA1 but not other EBNAs and LMPs (latency I). Latency II is characterized by expression of EBNA1 and all the LMPs and is recognized in non-B cell tumors, such as nasopharyngeal carcinoma, T cell tumors, and Hodgkin's disease. BARF0 and EBERs are commonly expressed in all latent forms mentioned above.

The EBER sequence is usually detectable in the nucleus of latently EBV-infected cells. Its high levels of expression (up to 10^7 copies/cell) facilitate detection by *in situ* hybridization (ISH) (Chang *et al.,* 1992; Howe and Shu, 1989). The absence of EBER expression has been described previously in the case of lytic EBV infection (Gilligan *et al.,* 1990) but not of latent infection. EBER ISH has served as something of a diagnostic gold standard of EBV existence in tissue samples.

This study was aimed to clarify the association of EBV with hepatocellular carcinoma (HCC). The importance of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in the development of primary HCC has been established in epidemiological studies (Beasley *et al.,* 1981; Kiyosawa *et al.,* 1990; Obayashi *et al.,* 1972; Resnick *et al.,* 1983). However, evidence for a direct role of hepatitis viruses in liver carcinogenesis remains tentative. Carcinogenesis is a multistep process, and although these viruses are likely to be essential contributing factors, alone they are insufficient for hepatocarcinogenesis. EBV can be associated with liver disease during EBV-induced infectious mononucleosis and post-transplantation lymphoprolif-

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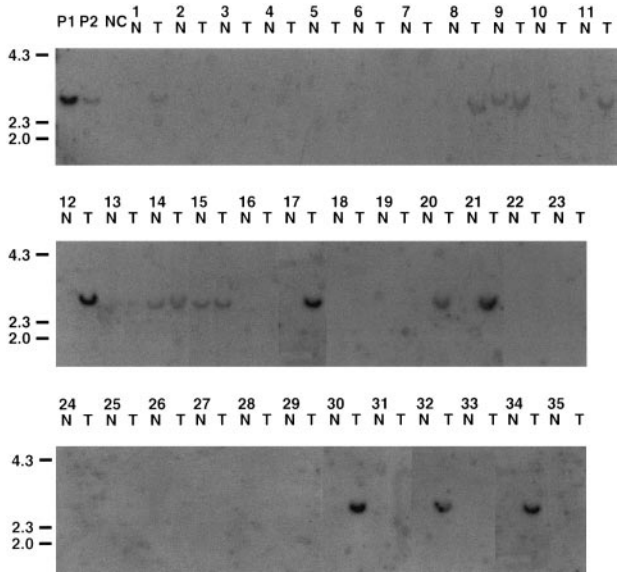


FIG. 1. Southern blot hybridization of EBV DNA in HCC tissues. The number on each lane indicates the patient from whom the DNA was extracted (N, nontumor tissue; T, HCC tumor tissue). The patients were stratified according to their hepatitis status: patient 1, both hepatitis B antigen (HBVAg) and hepatitis C antibody (HCVAb) positive; patients 2–23, HCVAb positive only; patients 24–31, HBVAg positive only; patients 32–35, hepatitis virus negative. P1, 42 ng of Raji cell DNA, corresponding to one EBV copy/10 cells; P2, 4.2 ng of Raji cell DNA, corresponding to one EBV copy/100 cells; NC, 20 μg of BJAB cell DNA. λ-DNA digested with *Hin*dIII was used as the size marker and is indicated on the left (in kb pairs).

erative disorders (Rickinson and Kieff, 1996). We therefore addressed whether EBV infection may be a cofactor for hepatocarcinogenesis. The results indicated that there was a high EBV load in HCC tissue and that all of the HCC tissue examined showed a novel pattern of EBV latency characterized by absence of EBER expression.

RESULTS

Detection of EBV DNA by Southern blot hybridization

EBV DNA was detected in 13 (37%) of the 35 patients with HCC. The virus was present in only the tumor tissues from 10 patients and in both tumor and nontumor tissues from 3 patients (Fig. 1). The assay could detect a single copy of EBV DNA/100 cells. Tumor tissues from six patients (patients 12, 17, 21, 30, 32, and 34) contained about one copy of EBV DNA/10 cells. In comparison with serum HBV and HCV markers, EBV DNA was detected in 9 of the 22 HCV-positive HCCs (patients 2–23), 1 of 8 HBV-positive HCCs (patients 24–31), and 2 of 4 hepatitis virus-negative HCCs (patients 32–35). Patient 1, who was positive for HBV and HCV, was also positive for EBV DNA. All control samples from normal liver tissues were negative for EBV DNA.

Identification of EBV-infected cells by immunofluorescence

To localize the EBV-infected cells, three tumor tissue specimens (from patients 12, 21, and 30) that contained about one copy of EBV DNA/10 cells and were available as frozen tissues, were examined by immunofluorescence using multiple EBNA-positive and -negative human sera. Clinical details of the three patients are shown in Table 1. EBNA was detected in a fraction (7–13%) of the cells from all three tissue samples tested (Fig. 2A). Positive cells were detected by EBNA-positive sera only and not by EBNA-negative sera. By two-color immunofluorescence for simultaneous detection of EBNA and cytokeratin, EBNA-positive cells were also positive for cytokeratin (Fig. 2B). They distributed diffusely and did not proliferate in discrete foci. LMP1, and the antigens associating with virus replication such as BZLF1 and gp350/220, were not detected. These results suggest that EBV-infected carcinoma cells are in a latent state of EBV infection.

Detection of EBER1 by ISH

EBER1 was not detected in any of the three tumor tissue samples (Fig. 3A). Conversely, very intense signals were obtained in the EBV-positive gastric carcinoma tissues used as a positive control (Fig. 3B).

To ensure that the EBER1 negativity was transcriptional silencing and not deletion, we checked detectability of the EBER DNA sequences by Southern blotting. As shown in Fig. 4, all three HCC samples were positive for EBER DNA.

Determination of EBV latent gene expression by Western blotting and RT-PCR

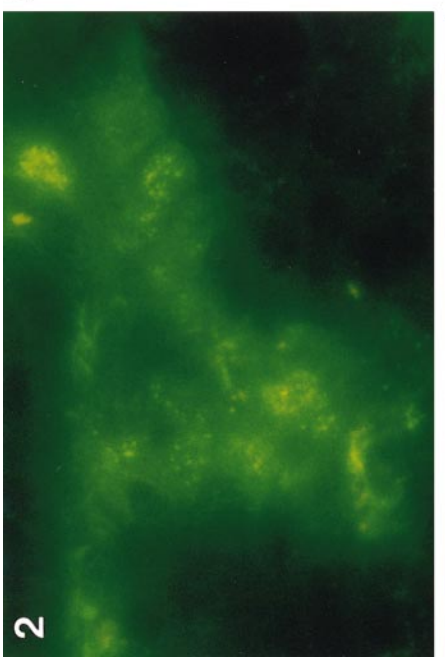
Western blot analysis (Fig. 5) showed that the three liver tissue samples were positive for EBNA1 but negative for EBNA2, -3A, -3B, -3C, and -Lp; LMP1; and BZLF1. RT-PCR analysis (Fig. 6) detected Qp- but not Wp- or Cp-initiated EBNA mRNA. These results indicate that EBV-infected cells in HCC use the *Bam*HI Q promoter to transcribe the EBNA1 gene. RT-PCR analysis showed

TABLE 1

Patient Profile

Case	Age/Sex	HCVAb[a]	HBsAg[b]	Pathological findings (N/T[c])
12	49/M	+	−	Mild fibrosis/moderate[d]
21	52/M	+	−	Mild fibrosis/moderate
30	63/M	−	−	Liver cirrhosis/moderate

[a] Hepatitis C virus antibody.
[b] Hepatitis B virus surface antigen.
[c] Nontumorous area/tumorous area.
[d] Moderately differentiated carcinoma.



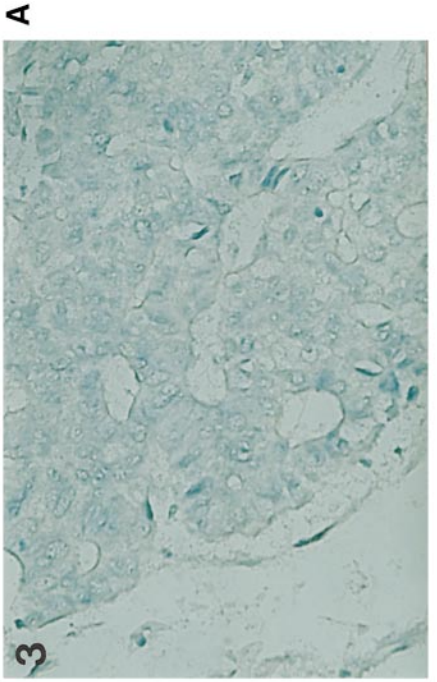


FIG. 2. Two-color immunofluorescence. Simultaneous detection of EBNA (A) and cytokeratin (B) in a tumorous area of HCC (patient 12). EBNA-positive carcinoma cells are also positive for cytokeratin. Original magnification, 800×.

FIG. 3. ISH for EBER1. (A) Tumorous area of HCC (patient 14) that is positive for EBV DNA by Southern blot hybridization. The tissue contains no lymphoid stroma and is completely negative for EBER1. (B) EBV-positive gastric carcinoma. Dark blue, nuclear staining indicates EBER1 expression. Positive staining is observed in all carcinoma cells. Methyl green counterstaining; original magnification, 400× (A) and 200× (B).

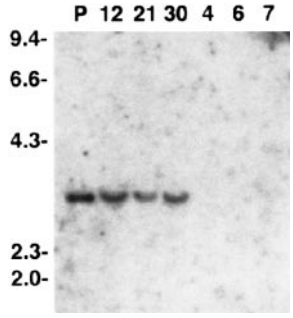


FIG. 4. Detection of EBER DNA sequences by Southern blotting. The probe DNA is the 1.0-kb *Sac*I–*Eco*RI fragment from the *Eco*RI K fragment of EBV DNA containing the EBER DNA sequences. P, 80 ng of Raji cell DNA. The number on each lane indicates the patient from whom the DNA was extracted. λ-DNA digested with *Hin*dIII was used as a size marker and is indicated on the left (in kb pairs).

that the *Bam*HI A transcripts were expressed, but LMP1, -2A, and -2B; BZLF1; or EBER1 could not be detected.

Monoclonality of EBV-infected cells in HCC

Southern blot hybridization of *Bam*HI-digested DNA from each EBV-positive patient (patients 12, 21, and 30) detected a single terminal fragment band between 9.0 and 14 kb but not a ladder of smaller bands (<6.0 kb) representing the linear EBV genome (Fig. 7). Rehybridization of the same blots with the *Eco*RI-I probe again yielded a single band identical to that detected with the *Xho*I-a probe (data not shown), suggesting that each EBV-positive case contained EBV DNA in a plasmid form.

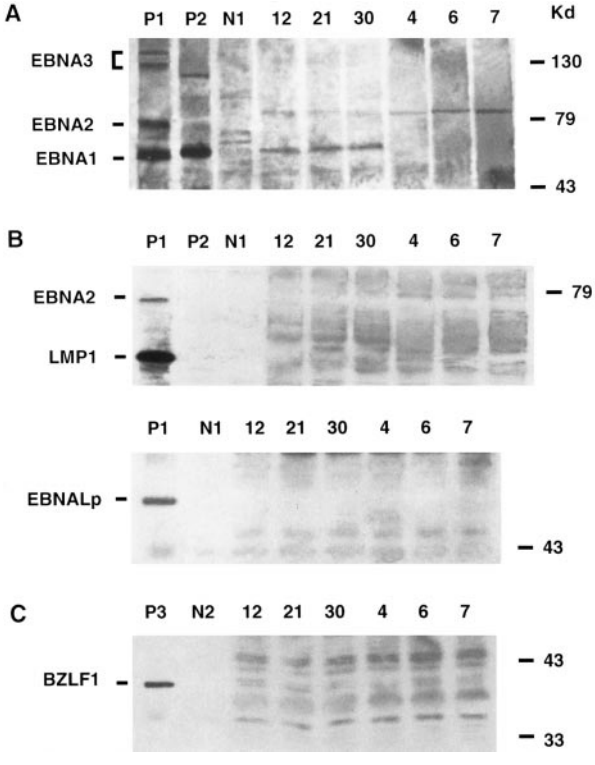


FIG. 5. EBV-related protein detection by Western blotting with human serum containing EBNA1, -2, and -3 (A), monoclonal antibodies to EBNA2 and LMP1 (B), EBNA Lp (C), and BZLF1 (D). P1, B lymphoblastoid cell lines immortalized by Akata EBV; P2, EBV-positive Akata cells; N1, EBV-negative Akata cells; P3, Akata cells in which virus replication was induced by cross-linking of surface immunoglobulin G; N2, Akata cells not treated with surface immunoglobulin G. The number on each lane indicates the patient from whom the tumor tissue was obtained.

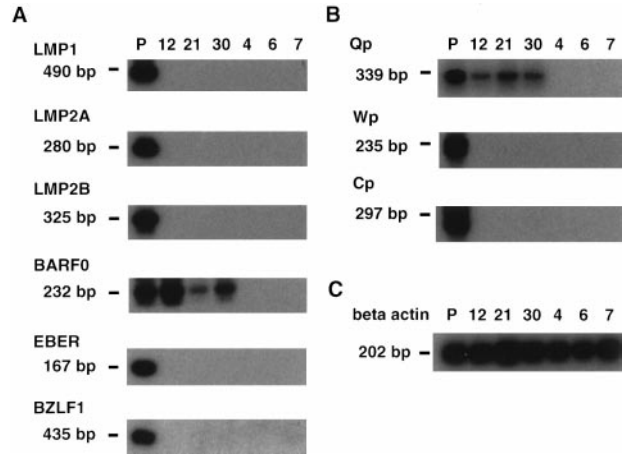


FIG. 6. Detection of EBV-related gene expression (A) and the promoter use for EBNA expression (B) and β-actin (C) by RT-PCR and Southern hybridization. P, PCR products of RNAs extracted from EBV-positive Akata cells (for detection of Qp) or lymphoblastoid cells (for detection of all the transcripts other than Qp). The number on each lane indicates the patient number.

DISCUSSION

In the present study, we demonstrated that there is a high load of EBV in the liver tissues of patients with HCC. Previous estimates of EBV, based on quantitative analyses of DNA content by PCR, indicated that most EBV-seropositive healthy individuals have less than one copy

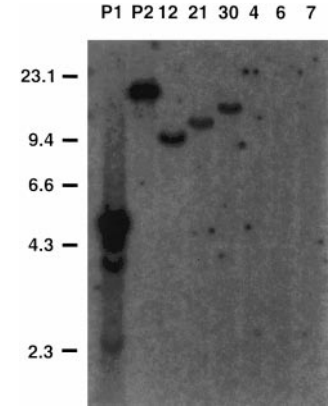


FIG. 7. Clonotypic EBV DNA analysis in HCC with the *Xho*I-a as a probe. P1, 200 ng of B95–8 cell DNA; P2, 200 ng of Raji cell DNA. The number on each lane indicates the patient from whom the DNA was extracted. λ-DNA digested with *Hin*dIII was used as a size marker and is indicated on the left (in kb pairs).

of EBV/10^5 peripheral blood mononuclear cells (Saito *et al.,* 1989; Wagner *et al.,* 1992). The present estimate by Southern blotting indicates that samples from HCC tissues contain >1000 times this level of EBV DNA. We used the large internal repeats as hybridization target, and Raji cell DNA that we used as a standard for estimation of EBV copy number contained seven repeats. Because the number of 3.1-kb repeat units varies considerably among naturally occurring isolates around a mean of six repeats (Allan *et al.,* 1989), there may be some variation in the number of EBV copies estimated in this assay.

Southern blot hybridization with the *Xho*I-a as a probe demonstrated that EBV-positive cells in tumor tissues from HCC were monoclonal in origin and were derived from a single EBV-infected cell, as in the case of Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (Imai *et al.,* 1994; Rickinson and Kieff, 1996). However, the immunofluorescence study indicated that only a fraction of the carcinoma cells were infected with EBV. This is in contrast to other EBV-associated malignancies in which all carcinoma cells were infected with EBV. This difference could be due to one of two reasons. First, HCC cells were originally 100% EBV positive, but during proliferation of HCC, some of them lost the EBV genome. Alternatively, EBV infection could have occurred after carcinogenesis. The diffuse distribution and monoclonal nature of the EBV-positive cells suggest that the former hypothesis is more likely.

The growth-promoting properties of EBV are known well. It is also possible that EBV-positive HCC cells promote the proliferation of EBV-negative HCC cells through the secretion of as-yet-unrecognized growth factors. Moreover, EBV-infected cells are targets of EBV-specific cytotoxic T cells (Rickinson and Kieff, 1996). Thus EBV infection could contribute directly to tumorigenic potential, or it could contribute to HCC by exacerbating the inflammatory process in liver tissue. When considering the role of EBV in hepatocarcinogenesis, it is noteworthy that about 40% of HCV-positive patients had a high EBV load in tumor tissues. Patients 32 and 34 are also interesting because they are not infected with HBV or HCV, and the causative event leading to hepatocarcinogenesis is not clear. EBV might be particularly important in the development of HCV-related HCC and hepatitis virus-negative HCC. However, this study does not provide a direct evidence for a causal role of EBV in hepatocarcinogenesis. Further study should clarify the significance of EBV existence in a small fraction of carcinoma cells.

The most interesting and unique finding is a novel pattern of latent gene expression in HCC tissues. Western blot and RT-PCR analyses indicated that EBV expression in HCC could be characterized as EBNA1 restricted (Kerr *et al.,* 1992), which is similar to those in Burkitt's lymphoma (Rowe *et al.,* 1987) and gastric carcinoma (Imai *et al.,* 1994), except for the absence of EBER. RNA ISH showed that EBER was not expressed in EBV-positive HCC tissues under the condition where intense signals were detected in tissues from EBV-positive gastric carcinoma. RT-PCR analysis also failed to detect EBER expression in these tissues, although amplification of β-actin secured the intact RNA in each HCC specimen. These results indicate that EBER RNA is not expressed in EBV-infected HCC tissues, which implies that the present gene expression pattern is a novel one. The importance of this finding is that the EBER has been accepted as a marker of latent EBV infection (Chang *et al.,* 1992) and as such is routinely used as a diagnostic tool to detect EBV within tissue samples. The data presented here suggest that by using the EBER ISH, a number of EBV-infected cells could remain undetected.

MATERIALS AND METHODS

Patients and tissue specimens

The HCC tumor tissue samples and adjacent nontumorous liver tissues were obtained by surgical resection from 35 patients who underwent hepatectomy in the Second Department of Surgery, Tokyo University Hospital, between December 1996 and June 1997. The specimens were each divided into two portions: one was fixed in 10% formalin and then embedded in paraffin, and the other was frozen rapidly and stored at −80°C until use.

The patient population consisted of 25 men and 10 women, ranging in age from 42 to 73 years (average, 60.9). Pathologically, the nontumorous area included chronic active hepatitis in 3 patients, fibrosis in 25 patients, and cirrhosis in 7 patients. The tumor tissues consisted of well differentiated HCC in 6 patients, moderately differentiated HCC in 24 patients, poorly differentiated HCC in 4 patients, and undifferentiated HCC in 1 patient. Twenty-two patients were HCV antibody positive, 8 were HBV surface antigen positive, 1 was positive to both, and 4 were negative to both. The disease stage was classified as I in 8 patients, II in 15 patients, and III in 12 patients according to the staging system of the American Joint Committee on Cancer/International Union against Cancer. Normal liver tissues from 35 metastatic liver carcinomas served as controls.

Southern blot hybridization

High-molecular-mass DNA (20 μg) was extracted from each of the frozen tissue specimens, digested with *Bam*HI, separated in a 0.8% agarose gel, and transferred to a nylon membrane (Hybond N+; Amersham, Arlington Heights, IL). Serially diluted DNA from Raji cells (50 copies of EBV DNA/cell) was used for quantitative estimation of positive bands. The probe DNA was the *Bam*HI W fragment of EBV DNA. For detection of the EBER DNA sequences, the cellular DNA was digested with *Eco*RI

endonuclease, and the 1.0-kb *Sac*I–*Eco*RI fragment from the *Eco*RI K fragment of EBV DNA was used as a probe. They were labeled with [^{32}P]dCTP (3000 Ci/mmol) by the random primer-based method (Multiprime DNA labeling system; Amersham) according to the manufacturer's instructions. After hybridization, the membrane was washed and exposed to x-ray film.

RNA ISH

Sections (5 μm) were cut from the formalin-fixed, paraffin-embedded tissue specimens and used for RNA ISH. EBER1 was detected with a digoxigenin-labeled oligonucleotide probe complementary to the EBER1 sequence, 5′-AGACACCGTCCTCACCACCCGGGACTTGTA-3′, using the procedure described by Chang *et al.* (1992). A sense probe for EBER1 was used as a negative control. EBV-positive gastric carcinoma tissue samples were used to prepare positive control sections.

Protein expression

The expression of EBNAs 1, -2, -3A, -3B, -3C, and -Lp; LMP1; and BZLF1 was examined by Western blotting (Imai *et al.,* 1998), with reference human serum containing antibodies to EBNAs and monoclonal antibodies to EBNA2 (PE2), EBNA Lp (JF186), LMP1 (CS1–4), and BZLF1 (BZ1). The expression of EBNAs, LMP1, BZLF1, and gp350/220 was also assessed by immunofluorescence as described previously (Imai *et al.,* 1998). Cytokeratin was stained by a mixture of type I and II keratin monoclonal antibodies (AE1 and AE3; ICN ImmunoBiologicals).

Reverse transcription-polymerase chain reaction

Details of these procedures have been described previously (Sugiura *et al.,* 1996). Briefly, total RNA was extracted from the frozen tissues using Trizol reagent (GIBCO BRL, Gaithersburg, MD), and the cDNA was synthesized from 1 μg of RNA using 10 pmol of each 3′ primer for *Bam*HI-C, -W, and -Q promoters (Cp, Wp, and Qp, respectively); LMP1,-2A, and -2B; BZLF1; *Bam*HI-A transcripts; and EBER1 (Sugiura *et al.,* 1996; Tierney *et al.,* 1994). The cDNA synthesized from 100 ng of total RNA was directly subjected to 40 cycles of PCR amplification, electrophoresed, blotted onto nylon membranes, and hybridized with [^{32}P]ATP-5′-end-labeled internal oligonucleotide probes. Each reverse transcription-polymerase chain reaction (RT-PCR) assay used RNA equivalent to 5000 cells and could detect a single EBV-infected cell for LMP1, -2A, and -2B; BARF0; Cp-initiated EBNA1 mRNAs; and 10 cells for BZLF1-, Wp-, and Qp-initiated EBNA1 mRNAs (Sugiura *et al.,* 1996). On the basis that Raji cells contain 10^7 copies of EBER/cell (which was the maximum estimate of EBER copies in EBV-infected cells) (Howe *et al.,* 1989), our RT-PCR system could detect 10 copies of EBER in a reaction. The quality of the RNA was checked by 30 cycles of amplification of cytoplasmic β-actin.

Cell clonality

The three samples were examined by Southern blot hybridization with the 1.9-kb *Xho*I-a probe from the right-end *Bam*HI–Nhet fragment of EBV DNA. Assessment of cell clonality was performed by counting the number of fused terminal fragment bands of >8.0 kb, which represent latent infection (Raab-Traub *et al.,* 1986). Hybridization with the *Eco*RI-I probe from the left end of the genome was also carried out to investigate EBV integration into the cellular DNA, a rare event reported to occur at the termini (Matsuo *et al.,* 1984). Blots of *Bam*HI-digested DNA were first hybridized with the *Xho*I-a probe and then rehybridized with the *Eco*RI-I probe.

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