



Transcriptional analysis of Epstein–Barr virus gene expression in EBV-positive gastric carcinoma: unique viral latency in the tumour cells

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Summary Although case-oriented evidence for an association of Epstein–Barr virus (EBV) with gastric carcinoma has been accumulating recently, the interaction(s) between EBV and gastric epithelial cells is/are largely unknown. In this study, we examined seven EBV-positive gastric carcinoma tissues for viral gene expression at the mRNA level, from which studies on the EBV oncogenicity in human epithelial cells will benefit. Reverse transcription–PCR analysis showed that all seven EBV-positive tumour tissues constitutively expressed EBV nuclear antigen (EBNA) 1 mRNA, but not EBNA2 mRNA. The EBNA transcription was initiated from one of three EBNA promoters, Qp; by contrast, both Cp and Wp were silent, thus resulting in the lack of EBNA2 mRNA. Latent membrane protein (LMP) 2A mRNA was detected in three of seven cases; however, neither LMP1 nor LMP2B mRNA was detected in any of the tumours tested. Transcripts from the *Bam*HI-A region of the viral genome were detectable in all cases. BZLF1 mRNA and the product, an immediate-early gene for EBV replication, was not expressed in any of them, thereby suggesting that the tumour cells carried EBV genomes in a tightly latent form. These findings further extended our previous data regarding EBV latency in gastric carcinoma cells at the protein level, and have affirmed that the programme of viral gene expression in the tumour more closely resembles ‘latency I’ represented by Burkitt’s lymphoma than ‘latency II’ represented by the majority of nasopharyngeal carcinomas.

Keywords: Epstein–Barr virus; gastric carcinoma; Epstein–Barr virus-specific mRNA; reverse transcription (RT)–polymerase chain reaction analysis

Epstein–Barr virus (EBV) is a ubiquitous human herpes virus, closely associated with the genesis of Burkitt’s lymphoma (BL), undifferentiated nasopharyngeal carcinoma (NPC) and opportunistic lymphomas in immunocompromised hosts (Liebowitz and Kieff, 1993). Recent studies demonstrated that EBV is also implicated in Hodgkin’s disease (HD) (Herbst *et al.*, 1991; Pallesen *et al.*, 1991), certain T-cell lymphomas (Harabuchi *et al.*, 1990; Su *et al.*, 1991; Korbjuhn *et al.*, 1993) and thymic carcinoma (Leyvraz *et al.*, 1985; Dimery *et al.*, 1988; Patton *et al.*, 1994). All or a significant proportion of neoplastic cells of the tumours harbour EBV DNA and express virus-coded latent infection (immortalisation-associated) proteins. Some of these EBV-related malignancies have been precisely analysed for viral latent gene expression and show characteristic patterns for each individual tumour (Liebowitz and Kieff, 1993).

Over ten major EBV genes are known to be potentially transcribed in cells latently infected with the virus: EBV nuclear antigens (EBNA) 1, -2, -3A, -3B, -3C and leader protein (LP); latent membrane proteins (LMP) 1, -2A, and -2B; untranslated EBV-encoded small RNAs (EBER)-1 and -2 (Liebowitz and Kieff, 1993); and a recently found transcript containing the *Bam*HI-A rightward open reading frame (BARF0) (Gilligan *et al.*, 1990). All the latent transcripts are regularly expressed in *in vitro* EBV-immortalised B-lymphoblastoid cell lines (LCL) and some B lymphoproliferative disorders occurring in immunosuppressed patients (latency III) (Young *et al.*, 1989; Kerr *et al.*, 1992). In contrast, only EBERs, EBNA1 and *Bam*HI-A transcripts are expressed in most *in vivo* BL cells and several BL cell lines (latency I), which are referred to as group I BL cells (Rowe *et al.*, 1987; Brooks *et al.*, 1993); and three LMP genes are additionally expressed along with EBERs, EBNA1 and *Bam*HI-A RNAs in the majority of NPC and HD (latency II) cases (Kerr *et al.*, 1992; Deacon *et al.*, 1993).

Furthermore, the combination of EBNA mRNA expressed depends on which of three mutually exclusive EBNA promoters, *Bam*HI-C, -W and -Q promoters (Cp, Wp and Qp respectively), is active. The Cp- or Wp-initiated large primary transcript is differentially spliced into all six EBNA mRNAs as is seen in cells with latency III (e.g. LCL) (Rogers *et al.*, 1990), whereas Qp mediates the selective expression of only EBNA1 mRNA through bypassing coding regions of the other EBNA genes, consequently leading to the complete lack of EBNA2, -3A, -3B, -3C and -LP mRNAs represented by group I BL and NPC cells (Schaefer *et al.*, 1995a; Nonkwelo *et al.*, 1996).

Gastric carcinoma, the most common malignancy in Japan, is being recognised as one of the EBV-related tumours (Shibata *et al.*, 1991; Imai *et al.*, 1994). Although our previous work revealed that about 7% of Japanese gastric carcinomas were EBV genome-positive and that, in addition to EBERs, EBNA1, but not the other EBNAs or LMP1, protein was expressed in the tumour cells (Imai *et al.*, 1994), details about expression of the other latent genes, LMP2A, -2B and BARF0, and about utilisation of an EBNA promoter, which is also necessary to understand the EBV-induced oncogenic process, are still unknown. In this study, we analysed seven EBV-positive gastric carcinomas more extensively for virus latent and replication cycle gene expression together with EBNA promoter usage at the mRNA level, using the reverse transcription–polymerase chain reaction (RT–PCR) technique.

Materials and methods

Subjects

Tumour tissues used in this study were resected or endoscopically biopsied from the stomachs of seven patients with EBV-positive gastric carcinoma and six patients with EBV-negative gastric carcinoma. The clinical and histological data of the patients with EBV-positive gastric carcinoma are shown in Table I. The six EBV-negative tumours consisted of two well differentiated, two moderately differentiated and two

poorly differentiated histological types. EBV positivity of the cases was screened by the PCR assay for EBV DNA and *in situ* hybridisation for EBER1 as previously described (Imai *et al.*, 1994). All tumour specimens were snap frozen and stored at -80°C until use. They were obtained after informed consent of the patients and used according to the guidelines of the Committee for Experimentation and Protection of Human Subjects, Cancer Institute, Hokkaido University School of Medicine.

Cell lines

All cell lines used were maintained in the exponential growth phase in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. An EBV-transformed LCL was used as a positive control for detection of EBNA1, -2, LMP1, -2A, -2B mRNAs; Cp- and Wp-initiated EBNA mRNAs; and BamHI-A transcripts. A group I BL cell line, Akata (Takada and Ono, 1989), served as a positive control for Qp-initiated EBNA mRNA. For detection of the EBV replication-associated BZLF1 gene mRNA (Countryman *et*

al., 1987), B95-8 cells (Miller and Lipman, 1973) or Akata cells, in which the virus replication cycle was induced by cross-linking of the surface immunoglobulin (Ig)G by treatment with goat antibodies to human IgG (Cappel Research Products, Durham, NC, USA) (Takada and Ono, 1989), was used as a control. A B-lymphoma cell line, BJAB, was used as an EBV-negative control (Klein *et al.*, 1975).

RNA extraction and cDNA synthesis

RNA extraction was carried out within one month of the storage period. After pulverisation of the frozen tissues in a micro-homogeniser, total RNA was extracted by using Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol, precipitated with isopropanol in the presence of 1 μg of glycogen (Boehringer, Mannheim, Germany). The precipitate was pelleted by centrifugation at 15 000 r.p.m. for 15 min, washed twice with ice-cold 75% ethanol, dried and dissolved in diethyl pyrocarbonate (DEPC)-treated distilled water. After DNAase I (Gibco BRL) treatment at 37°C for 15 min followed by 10 min

Table I EBV gene expression in EBV-positive gastric carcinomas

Case no	Age	Sex	Histological type	EBNA1 (U/K)	EBNA2	Active Cp	EBNA promoter Wp	Qp	I	LMP 2A	2B	BamHI-A transcripts	BZAL1
1	81	F	Undifferentiated (lymphoepithelioma-like)	+	-	-	-	+	-	-	-	+	-
2	55	M	Poorly differentiated	+	-	-	-	+	-	+	-	+	-
3	67	M	Poorly differentiated	+	-	-	-	+	-	-	-	+	-
4	71	M	Moderately differentiated	+	-	-	-	+	-	-	-	+	-
5	72	M	Moderately differentiated	+	-	-	-	+	-	-	-	+	-
6	55	M	Moderately differentiated	+	-	-	-	+	-	+	-	+	-
7	53	M	Well differentiated	+	-	-	-	+	-	+	-	+	-

Table II The sequences and coordinates of primers and probes used in this study

Transcript	Product size		Sequence (5'-3')	B95-8 genomic coordinates
EBNA1	273 bp	5' primer	GATGAGCGTTTGGGAGAGCTGATTCTGCA	67510-67539
		3' primer	TCCTCGTCCATGGTTATCAC	108075-108056
		Probe	AGACCTGGGAGCAGATTAC	67608-67627
EBNA2	339 bp	5' primer	GCTGCTACGCATTAGAGACC	47892-47911
		3' primer	TCCTGGTAGGGATTTCGAGGG	48616-48597
		Probe	CAGCACTGGCGTGTGACGTGGTGTAAGTT	48391-48420
Qp initiated	339 bp	5' primer	AGGCGCGGATAGCGTGCCTACCGGA	62426-62452
		3' primer	TCCTCGTCCATGGTTATCAC	108075-108056
		Probe	AGACCTGGGAGCAGATTAC	67608-67627
Cp initiated	297 bp	5' primer	CACTACAAGACCTACGCCTCTCCATCCATC	11425-11454
		3' primer	TCTCCCCTAGGCCCTGAAGGTGAACCGCTT	14832-14813, 17636-17626
		Probe	GCGACCGGTGCCTTCTTAGGAGCTGTCCGA	14708-14737
Wp initiated	235 bp	5' primer	TCAGAGCGCCAGGAGTCCACACAAAT	14384-14410
		3' primer	TCTCCCCTAGGCCCTGAAGGTGAACCGCTT	14832-14813, 17636-17626
		Probe	GCGACCGGTGCCTTCTTAGGAGCTGTCCGA	14708-14737
LMP1	490 bp	5' primer	TCCTCCTCTTGCGCTACTG	169383-169364
		3' primer	TCATCACTGTGTCGTTGTCC	168740-168759
		Probe	GAACAGCACAAATCCAAGGAACAATGCCTG	169061-169090
LMP2A ^a	280 bp	5' primer	ATGACTCATCTCAACACATA	166874-166893
		3' primer	CATGTTAGGCAAATTGCAA	380-361
		Probe	ATCCAGTATGCCTGCCTGTA	62-81
LMP2B ^a	325 bp	5' primer	CAGTGTAATCTGCACAAAGA	169819-169838
		3' primer	CATGTTAGGCAAATTGCAA	380-361
		Probe	ATCCAGTATGCCTGCCTGTA	62-81
BamHI-A ^b	232 bp	5' primer	AGAGACCAGGCTGCTAAACA	157154-157174
		3' primer	AACCAGCTTTCCTTCCGAG	159194-159175
		Probe	AAGACGTTGGAGGCACGCTG	157359-157378
BZLF1	453 bp	5' primer	CATGTTTCAACCGCTCCGACTGG	102963-102941
		3' primer	GCGCAGCCTGTCAATTTTCAGATG	102303-102325
		Probe	GCACGACGCACACGAAACCACAACAGCCA	102661-102690

^aBrooks *et al.*, 1992; ^bBrooks *et al.*, 1993.

heating at 95°C, RNA was ethanol-precipitated again, then resuspended in DEPC-treated water and dispensed into small aliquots. To test the sensitivity of our RT-PCR system, RNA was prepared from 10⁶ appropriate control cells, serially 10-fold diluted from a quantity equivalent to 10⁵ to 10⁰ cells, and the total RNA amount of each diluted sample was adjusted to that of 10⁵ cells by addition of RNA extracted from BJAB cells. For cDNA synthesis, 10 pmol of a 3'-primer specific for each transcript (Table II) was added to the RNA sample, followed by heating at 94°C for 5 min and rapid chilling on ice. Reagents were added to the RNA-primer mixture to give a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM potassium chloride, 3 mM magnesium chloride, 10 mM dithiothreitol, 0.5 mM for each dNTP, 10 U of RNasin (Promega, Madison, WI, USA) and 200 U of Molony murine leukaemia virus reverse transcriptase (Gibco BRL). Reverse transcription was performed at 37°C for 60 min in a total volume of 20 µl and then heated at 94°C for 3 min to stop the reaction.

PCR and detection of amplified products

Full details of the sequences and genome coordinates of primers and probes used to detect EBV transcripts are given in Table II. Our original primer pairs were designed in different exons for individual EBV transcripts so that occasional amplification of contaminated genomic DNA, if any, could be easily discriminated from the relevant RNA amplification by product size. The PCR reaction mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01% gelatin, 200 µM of each dNTP, 20 pmol of each primer and synthesised

cDNA, in a total volume of 100 µl. After it was overlaid with mineral oil, the reaction mixture was heated to 94°C for 3 min, slowly cooled to 70°C, and then 2.5 U of *Taq* polymerase (Gibco BRL) was added to the tubes. Samples were then subjected to 35 cycles of amplification by a Thermal Cycler 480 (Perkin-Elmer, Foster City, CA, USA), each cycle consisting of 94°C for 1 min (denaturation), 45°C for 2 min (annealing) and 72°C for 3 min (DNA extension). The extension time was prolonged to 6 min in the last cycle. The quality of RNA preparations was simultaneously checked by 30 cycles of amplification of cytoplasmic β -actin mRNA with the antisense primer (5'-GGAGCAATGATCTTGATCTTC-3') and the sense primer (5'-CCTTCCTGGGCATGGAGTCCT-3') (Busson *et al.*, 1992). The PCR products were electrophoresed in 1.5% agarose gels (SeaKem; Takara, Otsu, Japan), stained with ethidium bromide (Et-Br), and photographed under a UV-transilluminator. They were denatured in an alkali solution for 20 min with gentle rocking and directly blotted onto nylon membranes (Biodyne-B; Pall, Glen Cove, NY, USA) by vacuum transfer. The blotted membranes were fixed with a UV-crosslinker (Bio Rad, Richmond, CA, USA) and then subjected to hybridisation with [γ -³²P]ATP-5'-end-labelled internal oligonucleotide probes. The exposure time of autoradiograph was 6–12 h for viral transcripts and 2 h for β -actin mRNA.

Immunofluorescence assay

Frozen tissue sections of EBV-positive gastric carcinomas were examined for expression of the BZLF1 protein by streptavidin-biotin immunofluorescence, using mouse BZ.1

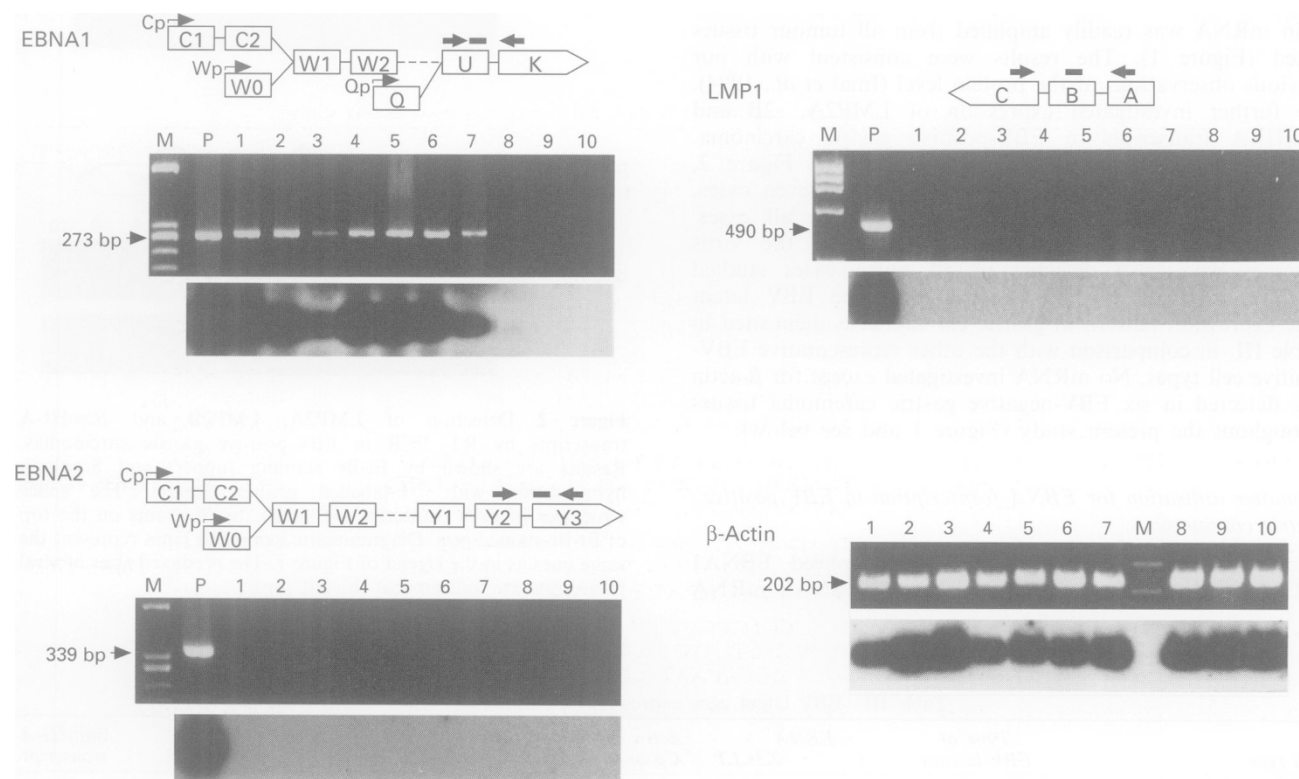


Figure 1 Detection of EBNA1, EBNA2 and LMP1 mRNAs by RT-PCR in EBV-positive and -negative gastric carcinoma tissues. Results are shown by ethidium bromide (Et-Br) staining (upper) and Southern hybridisation with ³²P-labelled probes (lower). Lane M, Φ X174-*Hae*III digest as a size marker; lane P, PCR product of RNA extracted from LCL as an EBV-positive control; lanes 1–7, PCR products of RNAs from EBV-positive gastric carcinoma tissues of case nos. 1–7 (Table I) respectively; lanes 8–10, PCR products of RNAs extracted from three EBV-negative gastric carcinoma tissues (well-differentiated, moderately differentiated and poorly differentiated types respectively). The splice structures of each transcript analysed are shown by diagrams on the top of Et-Br-stained gels. Open boxes and small arrows represent exons and the sites of relevant transcriptional EBNA promoters respectively. The primer-probe combinations used are indicated as large arrows and bars. Simultaneous amplification of cytoplasmic β -actin mRNA was carried out to check for pertinent RNA extraction. The predicted sizes of each viral transcript are indicated at the left (bp).

monoclonal antibody (Young *et al.*, 1991; purchased from Dakopatts). In this assay, B95-8 cells or surface IgG-cross-linked Akata cells served as a BZLF1-positive control, and non-treated Akata cells and EBV-negative gastric carcinoma tissues served as the negative controls.

Results

Sensitivity of RT-PCR

In preliminary experiments using diluted RNA samples from a reference LCL, the RT-PCR-Southern hybridisation system with our original primer-probe combinations could detect EBNA1 (with U/K exons), EBNA2, LMP1, BARF0 and Cp-initiated EBNA transcripts as well as LMP2A and -2B mRNAs amplified with the primers previously reported (Brooks *et al.*, 1992), even at the single-cell level (data not shown). Wp- and Qp-initiated EBNA and BZLF1 mRNAs were detectable at RNA amounts equivalent to 10 LCL, untreated and slg-crosslinked Akata cells respectively (data not shown). Thus, our RT-PCR systems were considered to have sensitivities similar to or higher than those described in other RNA studies on NPC and HD (Brooks *et al.*, 1992; Deacon *et al.*, 1993).

RT-PCR analysis of EBV latent gene expression in EBV-positive gastric carcinomas

RT-PCR analyses revealed that EBNA1 mRNA with BamHI-U and -K exons, which are commonly contained in every EBNA1 transcript generated from any of the three EBNA promoters, could be clearly detected in all seven EBV-positive gastric carcinoma tissues (Figure 1). By contrast, none of the tumour tissues expressed either detectable EBNA2 or LMP1 mRNA, whereas the RNA preparation from control LCL yielded strong signals and β -actin mRNA was readily amplified from all tumour tissues tested (Figure 1). The results were consistent with our previous observations at the protein level (Imai *et al.*, 1994). We further investigated expression of LMP2A, -2B and BamHI-A transcripts in EBV-positive gastric carcinoma, which remain unknown so far. As shown in Figure 2, LMP2A mRNA was detected in three of the seven cases, although LMP2B mRNA was undetectable in all cases. Transcripts from the BamHI-A fragment of the virus genomes were also detected in all seven cases studied (Figure 2). Based on the above results, the EBV latent gene expression pattern in gastric carcinoma is delineated in Table III, in comparison with the other representative EBV-positive cell types. No mRNA investigated except for β -actin was detected in six EBV-negative gastric carcinoma tissues throughout the present study (Figure 1 and see below).

Promoter utilisation for EBNA transcription in EBV-positive gastric carcinomas

Since the unequivocal detection of U/K-spliced EBNA1 mRNA with the concomitant absence of EBNA2 mRNA

suggested Qp-driven EBNA transcription, we next investigated which of the three EBNA promoters actually mediated EBNA gene expression in EBV-positive gastric carcinomas. As expected, EBNA mRNA with the Q/U/K-spliced structure was clearly detected in all cases tested, whereas mRNA with C1/W2 or W0/W2 exons, diagnostic of Cp and Wp usage, respectively, was not detected at all (Figure 3). It is unlikely that the Q/U/K exon-containing EBNA1 transcript detected here was initiated from the BamHI-F promoter (Fp), which was initially reported to be a third EBNA promoter (Schaefer *et al.*, 1991; Sample *et al.*, 1991) and redefined thereafter as an early lytic promoter (Lear *et al.*, 1992; Schaefer *et al.*,

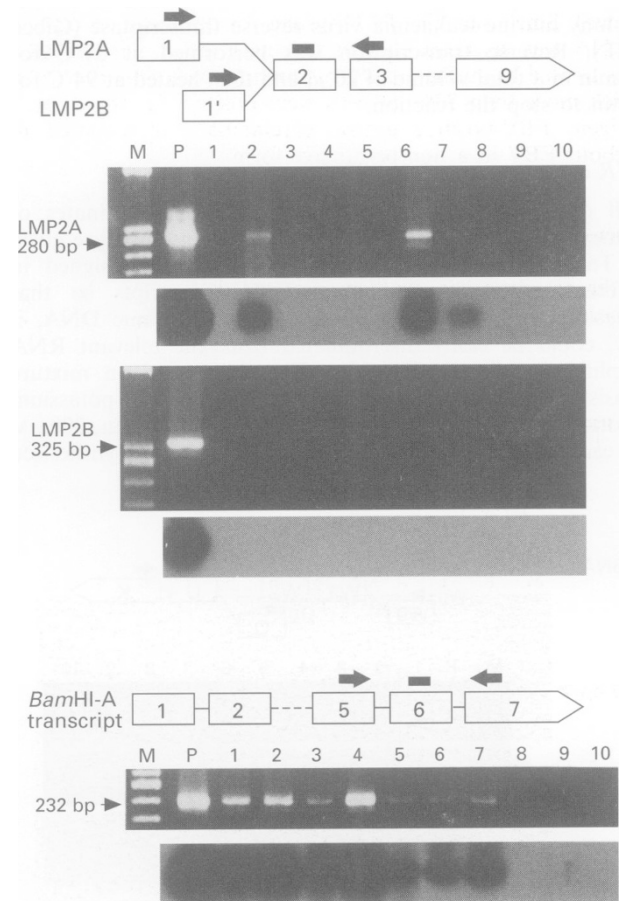


Figure 2 Detection of LMP2A, LMP2B and BamHI-A transcripts by RT-PCR in EBV-positive gastric carcinomas. Results are shown by Et-Br staining (upper) and Southern hybridisation with ³²P-labelled probes (lower). The splice structures of each transcript are shown by diagrams on the top of Et-Br-stained gels. Diagrammatic items and lanes represent the same ones as in the legend of Figure 1. The predicted sizes of viral transcripts are indicated at the left (bp).

Table III EBV latent gene expression in various cell types

Cell type	Type of EBV latency	EBNA		Active EBNA promoter		LMP 2A	2B	BamHI-A transcript
		1	2,3s,LP	Cp or Wp	Qp			
Burkitt's lymphoma	I	+	-	-	+	-	-	+
Nasopharyngeal carcinoma	II	+	-	-	+	+	+	+
Hodgkin's disease	II	+	-	-	+	+	+	+
T-cell lymphoma	II	+	-	-	+	+	+	+
EBV-transformed B cells	III	+	+	+	-	+	+	+
B-lymphoproliferation in immunodeficiency	III	+	+	+	-	+	+	+
Gastric carcinoma		+	-	-	+	± ^a	-	+

^aPositive in 3/7 (43%) cases.

1995b), because the vast majority of Fp-initiated lytic transcripts appeared not to contain the K exon (Schaefer *et al.*, 1995a and b) and, in addition, all tumour tissues examined showed no evidence of lytic infection (see below). Thus, the results indicated that Qp, not Cp or Wp, mediated EBNA transcription in gastric carcinoma.

Analyses of BZLF1 gene expression in EBV-positive gastric carcinomas

Figure 4 shows the results of RT-PCR analysis demonstrating that none of the tumours showed evidence for expression of BZLF1 mRNA. Similar negative results were also obtained by immunofluorescent staining with the BZ.1 monoclonal antibody in frozen tissue sections (data not shown). Since our RT-PCR system for detection of BZLF1 mRNA was sensitive enough to detect ten or fewer Akata cells treated by surface IgG cross-linking (data not shown), in which more than 80% of cells were positive for the BZLF1 antigen, EBV-positive gastric carcinoma was assumed to harbour EBV in a non-permissive form.

Discussion

In the present study, we demonstrated that one of the three hitherto identified EBNA promoters, Qp, was used in EBV-

positive gastric carcinomas, thus resulting in the selective expression of EBNA1 mRNA, but not EBNA2 mRNA, as is seen in BL and NPC (Liebowitz and Kieff, 1993). In addition, neither LMP1 nor LMP2B mRNA was detectable in any case examined. These findings were consistent with our previous results showing by immunoblotting that EBV-positive gastric carcinoma cells lacked EBNA2, -3A, -3B, -3C, -LP and LMP1 proteins (Imai *et al.*, 1994). All seven EBV-positive tumours were also found to have BamHI-A transcripts together with the EBNA1 message, presumably corresponding to latency I of EBV infection typified by group I BL cells (Kerr *et al.*, 1992). However, LMP2A mRNA was detected in three of seven cases tested, which may represent a novel latency of EBV-related tumours distinct from conventional latency I or II (Kerr *et al.*, 1992). Interestingly, this uniquely restricted pattern of latent gene expression in a subgroup of EBV-positive gastric carcinomas mostly resembled that observed in peripheral blood B lymphocytes of healthy EBV-seropositive individuals (Qu and Rowe, 1992; Tierney *et al.*, 1994), which would be advantageous for maintaining the life-long virus carrier state under effective immunity.

It was shown by using an isogenic system of group I BL cells that EBV indeed contributes to the malignant phenotypes (Shimizu *et al.*, 1994). Accordingly, since EBNA1, the putative BamHI-A product and EBERs are commonly expressed both in group I BL and in gastric carcinoma cells, they may also be responsible, to some extent, for the malignant features of gastric carcinoma. Recently a possible role for EBNA1 in the EBV-induced oncogenic process has been proposed in that its Gly-Ala repetitive sequence inhibited the specific cytotoxic T-cell recognition of immunodominant EBNA3B through a cis-acting mode (Levitskaya *et al.*, 1995). Several *in vitro* studies, however, demonstrated that LMP1 potentially confers tumorigenicity on non-lymphoid cells via transformation of established rodent fibroblasts (Wang *et al.*, 1985) and human keratinocytes (Fähræus *et al.*, 1990), inhibition of human epithelial cell differentiation (Dawson *et al.*, 1990), and induction of the functional epidermal growth factor receptor (Miller *et al.*, 1995). Such important findings strongly support crucial roles for LMP1 in the development of undifferentiated NPC, where the LMP1 positivity exceeds 80% of the cases at the transcriptional level (Brooks *et al.*, 1992; Chen *et al.*, 1995). The exact lack of LMP1 expression in EBV-positive gastric carcinomas, irrespective of their histological phenotypes, implies that LMP1 may not be necessary for the tumour, at least to sustain its already established malignant state. Rather, LMP1 might participate in an earlier stage of the tumour development and be down-regulated thereafter. Alternatively, the lack of LMP1 may reflect the result of

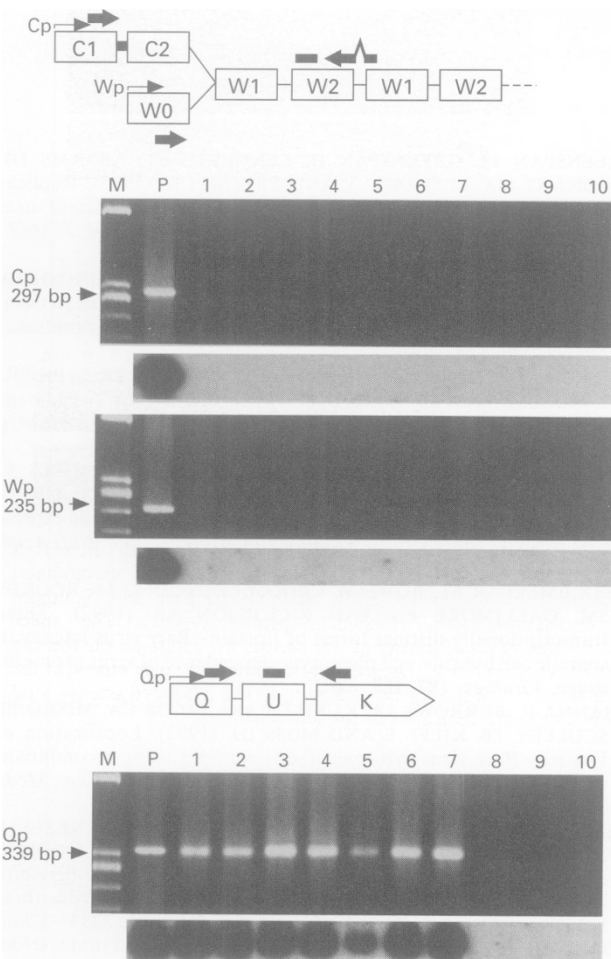


Figure 3 RT-PCR analysis of promoter usage for EBNA gene transcription in EBV-positive gastric carcinomas. Results are shown by Et-Br staining (upper) and Southern hybridisation with ³²P-labelled probes (lower). The structures of EBNA mRNA transcribed from the three different promoters Cp, Wp and Qp are shown by diagrams. Diagrammatic items and lanes represent the same ones as in the legend of Figure 1. The predicted sizes of each transcript are indicated at the left (bp).

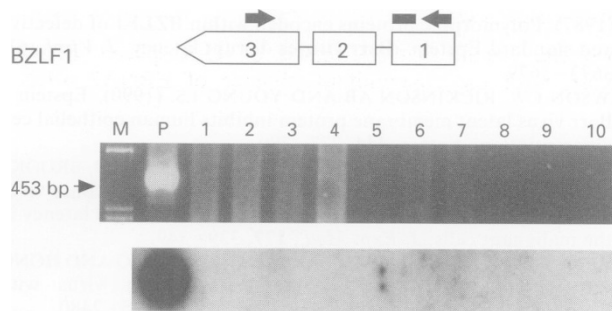


Figure 4 RT-PCR analysis of immediate-early BZLF1 gene expression in EBV-positive gastric carcinomas. Results are shown by Et-Br staining (upper) and Southern hybridisation with a ³²P-labelled probe (lower). The splice structure of BZLF1 mRNA is shown by a diagram on the top of an Et-Br-stained gel. Diagrammatic items and lanes represent the same ones as in the legend of Figure 1. The predicted size of BZLF1 mRNA is indicated at the left (bp).

clonal selection of LMP1-negative tumour cells by immunological pressure because EBV-specific cytotoxic T cells are potentially directed against the viral latent proteins other than EBNA1 (Khanna *et al.*, 1992; Murray *et al.*, 1992). In fact, patients with EBV-positive gastric carcinoma have normally retained virus-specific immune T-cell responses (Imai *et al.*, 1994) as is the case with BL (Rooney *et al.*, 1985), in contrast to NPC patients (Moss *et al.*, 1983).

The EBV replication can be switched on in malignant or non-malignant epithelial proliferative lesions *in vivo*; the immediate-early BZLF1, the early and/or late antigens were detected in NPC (Cochet *et al.*, 1993), thymic carcinoma (Patton *et al.*, 1994) and AIDS-associated oral hairy leukoplakia (OHL) (Greenspan *et al.*, 1985). Similar viral cycle events were also observed *in vitro* by experimental EBV infection of cells of epithelial origin (Li *et al.*, 1992; Sixbey and Yao, 1992). There is a report of a patient with EBV-positive undifferentiated gastric carcinoma, in which a very small proportion of tumour cells expressed the BZLF1 protein, indicating that rare gastric carcinoma cells can potentially enter the lytic cycle (Niedobitek *et al.*, 1992). The present study in contrast, showed that neither the BZLF1 mRNA nor protein was found in gastric carcinoma tissues, results compatible with the absence of the early and viral capsid antigens as previously reported (Imai *et al.*, 1994). In addition, although EBV latency can be disrupted as epithelial cells differentiate, as shown by observations in OHL (Young *et al.*, 1991), none of the gastric carcinomas with various differentiations showed any evidence of virus production. LMP2A, detectably expressed in three of our seven cases, was

shown to block calcium mobilisation in B cells, thereby suppressing EBV replication (Miller *et al.*, 1993, 1994). It is unknown whether the finding can be extended to epithelial cells; however, at least in certain EBV-positive gastric carcinomas, the absence of tumour cells entering the lytic cycle may be associated with LMP2A expression. It was inferred from our series of data that, unlike in NPC or OHL cells, virus replication was uncommon or abortive in gastric carcinoma, and that the vast majority of tumour cells carried EBV in a tightly latent fashion.

The strict localisation of EBV genomes and viral gene expression only in tumour cells and their monoclonality (Oda *et al.*, 1993; Imai *et al.*, 1994) strongly suggest that EBV may be indeed involved, not a passenger, in the development of virus-positive gastric carcinoma, although several questions remain to be resolved. Further studies are necessary to understand more direct oncogenic activities of EBV on normal gastric mucosal epithelia and a factor(s) enhancing them.

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