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Activity of the EBNA1 promoter associated with lytic replication (Fp) in Epstein-Barr virus associated disorders

A A T P Brink, C J L M Meijer, J M Nicholls, J M Middeldorp, A J C van den Brule

Department of Pathology, University Hospital Vrije Universiteit, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
A A T P Brink
C J L M Meijer
J M Middeldorp
A J C van den Brule

Department of Pathology, University of Hong Kong, Queen Mary Hospital, Hong Kong, China
J M Nicholls

Correspondence to:
Dr van den Brule
vandenbrule@azvu.nl

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Abstract

***Background/Aims*—In Epstein-Barr virus (EBV) positive cell lines that are stably infected, three different promoters are known to direct the transcription of EBV nuclear antigen 1 (EBNA1). These are located in the BamHI-C, BamHI-Q, and BamHI-F regions of the viral genome (Cp, Qp, and Fp, respectively). Fp is activated upon induction of the viral lytic cycle. The aim of this study was to investigate the activity of Fp in EBV associated diseases.**

***Methods*—Using reverse transcriptase polymerase chain reaction, a qualitative analysis of EBNA1 promoter usage in various EBV associated diseases was performed.**

***Results*—Fp driven transcription was detected in the context of primary infection and/or lytic replication; at least a portion of the Fp driven transcripts encoded EBNA1. Qp driven EBNA1 transcripts were detected in most samples across the range of disorders tested. Cp driven EBNA1 transcripts were detected in the context of immune suppression and in samples containing EBV positive (non-neoplastic) lymphoid cells.**

***Conclusions*—These results confirm the previously proposed "housekeeping" function of the Qp promoter.**

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Keywords: Epstein-Barr virus; Epstein-Barr virus nuclear antigen 1; BamHI-F region

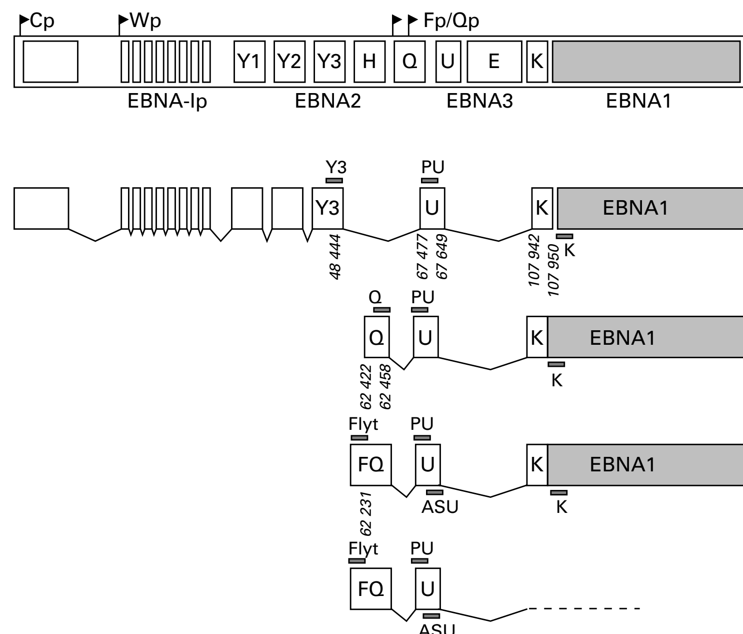

Figure 1 Schematic representation of Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) transcripts derived from the four different promoters. Large open boxes represent exons; small shaded bars represent reverse transcriptase polymerase chain reaction (RT-PCR) primers. Genomic coordinates given are those of the B95-8 prototype strain.[5]

Epstein-Barr virus (EBV) is associated with several lymphoid and epithelial malignancies.[1] These are endemic Burkitt's lymphomas, nasal natural killer (NK)/T cell lymphomas, post-transplant lymphoproliferative disorders, AIDS related lymphomas, nasopharyngeal carcinomas, approximately 40% of cases of Hodgkin's disease, and 8–10% of gastric carcinomas.[2] In these malignancies, viral replication rarely occurs. Instead, the virus is latently present within the neoplastic cells (reviewed in Kieff[3]). The viral episome is maintained by the expression of EBV nuclear antigen 1 (EBNA1),[4] which interacts with the host cell DNA and with the origin of replication (oriP) in the viral genome.

The transcription of EBNA1 can be driven by promoters located in the BamHI-W or BamHI-C fragments of the EBV genome (Wp and Cp, respectively; fig 1). Transcription from Cp/Wp results in long polycistronic mRNAs encoding not only EBNA1 but also one or more of the other EBNAs.[6] This situation results in the extensive gene expression pattern (latency type III) that is found in lymphoblastoid cell lines (LCLs) and lymphomas of the immunocompromised: EBNA1, EBNA2, EBNA3a, EBNA3b, EBNA3c, and EBNA4; together with the latent membrane proteins (LMPs) LMP1, LMP2a, and LMP2b; the small non-coding RNAs, EBER-1 and EBER-2; and rightward transcripts driven from the BamHI-A region of the viral genome (BARTs).

Alternatively, EBNA1 can be transcribed from a promoter in the BamHI Q fragment of the viral genome (Qp; fig 1). This is a TATA-less promoter that resembles housekeeping gene promoters and thus guarantees EBNA1 expression in all EBV positive cells.[7] In Burkitt's lymphomas, Hodgkin's disease, nasopharyngeal carcinomas, gastric carcinomas, and non-Hodgkin lymphomas of immunocompetent patients, Cp and Wp are inactive as a result of methylation[8] and EBNA1 transcription is Qp driven.[7,9] This results in the expression of EBNA1 but none of the other EBNAs. In addition, EBERs and BARTs are detected in Burkitt's lymphomas (latency type I). Hodgkin's disease, nasopharyngeal carcinomas, gastric carcinomas, and non-Hodgkin lymphomas

Table 1 Oligonucleotide primers and probes used in RT-PCR analysis

Target transcript	Oligo	B95.8 genomic coordinates	Sequence
EBNA1*	Y3 (s)	48397–48416	TGGCGTGTGACGTGGTGTAA
	Q (s)	62440–62457	GTGCGCTACCGGATGGCG
	Flyt (s)	62336–62355	GACCACTGAGGGAGTGTTCC
	K (as)	107986–107967	CATTTCCAGGTCCTGTACCT
	ASU (as)	67610–67589	TCTACTGGCGGTGTATCATGCG
	PU (probe)	67544–67563	AGAGAGTAGTCTCAGGGCAT
BARTs	A3 (s)	157154–157173	AGAGACCAGGCTGCTAAACA
	A4 (as)	159194–159175	AACCAGCTTTCCTTTCCGAG
	AP (probe)	157359–157378	AAGACGTTGGAGGCACGCTG
U1A	U1A1 (s)	–	CAGTATGCCAAGACCGACTCAGA
	U1A2 (as)	–	GGCCCGGCATGTGGTGCATAA
	U1A3 (probe)	–	AGAAGAGGAAGCCCAAGAGCCA

*All EBNA1[16] primers except Flyt, and all BART primers[18] were described previously. We also used BLZF1 and BHRF1 specific primers as described previously.[19]
as, antisense; BARTs, BamH1A region driven transcripts; EBNA, Epstein-Barr virus nuclear antigen; RT-PCR, reverse transcription polymerase chain reaction; s, sense.

Table 2 Analysis of EBNA1 promoter usage in Epstein-Barr virus (EBV) associated disorders

Sample type	Qp	Cp	Fp*	BARTs
In vitro systems				
JY cells[23]	+	+	+	+
C15 tumours[22]	+	–	+	+
In vivo disorders				
Infectious mononucleosis (n = 1)	1/1	1/1	1/1	1/1
Reactive node, EBV positive cells (n = 1)	1/1	0/1	ND	1/1
Nasopharyngeal carcinoma (n = 15)	15/15	5/15	0/5	15/15
Lymphomas in patients without overt immunodeficiency				
Hodgkin's disease (n = 19)	14/19	4/19	0/13	19/19
Burkitt's lymphoma (n = 4)	3/4	0/2	1/2	4/4
Anaplastic large cell lymphoma (n = 1)	1/1	0/1	0/1	1/1
Nasal T/NK cell lymphoma (n = 6)	6/6	0/5	0/2	6/6
B non-Hodgkin's lymphoma (n = 4)	2/4	1/4	ND	4/4
Nodal PTCL NOS (n = 3)	2/3	1/3	0/2	3/3
Lymphomas in immunocompromised patients				
AIDS related lymphoma (n = 2)	2/2	1/2	0/2	2/2
Post-transplant LPD (n = 7)	6/7	7/7	3/6	7/7

*Some cases could not be tested owing to lack of material.
BARTs, BamH1-A region driven transcripts; EBNA1, EBV nuclear antigen 1; LPD, lymphoproliferative disease; ND, not done; NK, natural killer; PTCL NOS, peripheral T cell lymphomas not otherwise specified.

express EBNA1, EBERs, BARTs, and LMPs (latency type II).

Recently, another promoter for EBNA1 transcription was discovered. This promoter (Fp; fig 1) is localised 100–200 bp upstream of Qp,[9–11] and was found to be active in cell lines after the induction of the viral lytic cycle.[9 11 12] Originally, Fp was erroneously thought to be the promoter used for EBNA1 transcription during type I latency, but this misinterpretation was based on experiments in which part of the cells underwent spontaneous activation of the lytic cycle.[13 14] Recent studies have shown that Fp and Qp driven transcripts can be distinguished in reverse transcriptase polymerase chain reaction (RT-PCR) assays simply by using specific primers.[7 10 15] However, these studies were all performed on EBV positive cell lines, whereas the presence and importance of Fp driven EBNA1 transcripts in EBV associated malignancies remained unknown. Therefore, we have designed an RT-PCR assay using a forward primer (Flyt) in combination with an EBNA1 specific antisense primer as described previously (table 1; primer K),[16 17] or with an antisense primer located in the U exon (table 1; fig 1). This primer enables the distinction between Fp and Qp driven transcripts using total RNA from clinical material and cell lines without the need for nested PCRs. Transcripts derived from Cp and Qp can be detected using the Y3 and Q sense primers, respectively, in combination with the U or K antisense primers (table 1).[16]

Materials and methods

SELECTION OF CLINICAL MATERIAL

All neoplasms tested in our study (table 2), with the exception of three nodal peripheral T cell lymphomas not otherwise specified, were associated with EBV (EBER1/2 RNA in situ hybridisation performed as described previously[20] showed that most neoplastic cells contained EBV). The three nodal peripheral T cell lymphomas not otherwise specified were considered not "EBV associated"; that is, samples contained EBV positive cells but these were relatively few and non-neoplastic. The definition of EBV associated lymphomas was discussed previously.[21] C15 is an nasopharyngeal carcinoma derived xenotransplant propagated in mice.[22] The JY lymphoblastoid cell line[23] was used as a positive control; the EBV negative Burkitt's lymphoma cell line Ramos was used as a negative control.

PREPARATION OF RNA

RNA was isolated from 5×10^6 cultured EBV positive JY and EBV negative Ramos cells and from 10 cryosections, 5 µm thick, of biopsy samples using 1 ml of RNAzol™ (Biotecx Laboratories, Houston, Texas, USA). RNA was stored as an isopropanol precipitate at −80°C. Because the detectability of EBNA1 transcripts relies strongly upon the quality of the RNA,[17] the integrity of the RNA was checked by gel electrophoresis; the presence of 28S/18S ribosomal bands was used as an indication of high quality RNA. Only samples that showed ribosomal bands on the gel were included in our study. As an additional quality control, RT-PCRs for the housekeeping gene U1A were performed.[24] To check for the presence of amplifiable EBV mRNA in the preparations, we performed RT-PCR for BARTs, which are expressed in all types of latency.[25]

RT-PCR CONDITIONS

Multiprimed reverse transcription followed by single specific RT-PCR was performed as described previously.[17] An amount of RNA equivalent to 1.5 cryosections, 5 µm thick, of the biopsies or to 100 000 cultured cells was reverse transcribed in a 20 µl reaction volume containing 10 pmol each of primer K, ASU, A4, and U1A2; subsequently, 1.5 µl of the generated cDNA was subjected to PCR. PCR products were analysed by gel electrophoresis, transferred to nylon filters (Qiabrane; Qiagen, Chatsworth, California, USA) by alkaline blotting, and hybridised with ^{32}P labelled specific oligonucleotide probes (table 1).[16 18 19] Films were exposed to these blots for four hours or overnight.

Results

ANALYSIS OF EBNA1 TRANSCRIPTION IN EBV POSITIVE CELL LINES

In the JY cell line, Cp/Wp driven EBNA1 transcripts were detected; Qp driven EBNA1 transcripts were detected to a lesser extent (fig 2A,

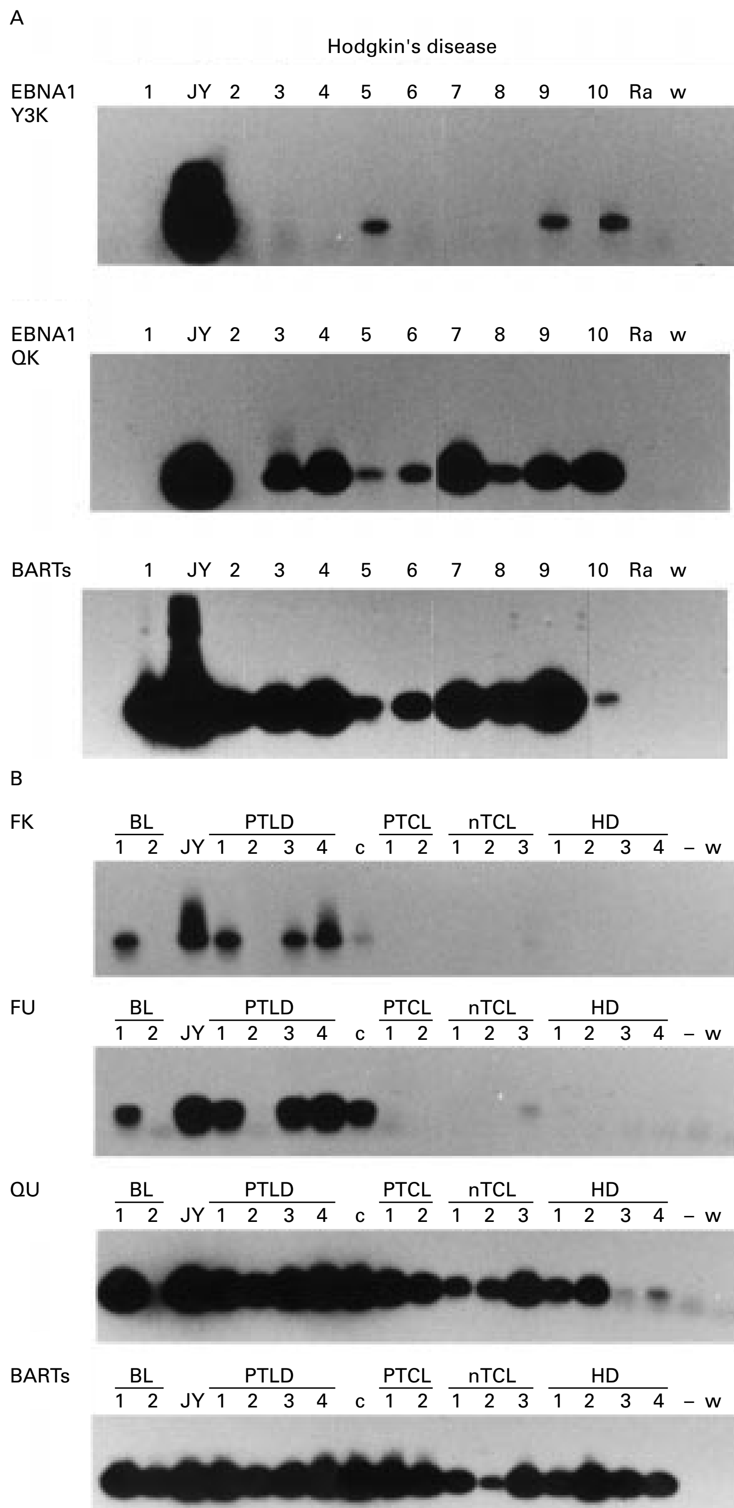

Figure 2 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of EBNA1 promoter usage in Epstein-Barr virus (EBV) associated disorders. (A) Analysis of Cp (top) and Qp driven (middle) EBNA1 transcripts and BamHI-A rightward transcripts (BARTs, bottom) in Hodgkin's disease; (B) Analysis of Fp driven transcripts (Fp-U-K spliced, top; and Fp-U spliced, second row), Qp driven transcripts (Q-U spliced, third row) and BARTs in different EBV associated disorders. BL, Burkitt's lymphoma; c, C15 tumour; HD, Hodgkin's disease; JY, lymphoblastoid cell line; nTCL, nodal T cell lymphoma; PTCL, peripheral T cell lymphoma; PTLD, post-transplant lymphoproliferative disorder; Ra/−, EBV negative Ramos cell line; w, water control.

upper and middle panel, respectively). Fp driven transcripts (Fp-U spliced) were also detected (fig 2B, second panel), indicating that at least in a proportion of cultured JY cells, EBV has entered into the lytic state. Moreover, using the Flyt and K primer pair, a signal was detected (fig 2B, upper panel), indicating that (part of) the detected Fp driven transcripts encode EBNA1 in JY cells.

The C15 tumour showed clear expression of Qp driven but not Cp driven EBNA1 transcripts (not shown). After overnight exposure of the PCR blot, a faint signal for Fp driven EBNA1 transcripts (Fp-U-K spliced; fig 2B, upper panel) was seen in the C15 tumour; as in the JY cells and the clinical samples, Fp-U spliced transcripts were also detected (fig 2B, second panel) and this signal was stronger than the Fp-U-K signal.

ANALYSIS OF EBNA1 TRANSCRIPTION IN BIOPSIES FROM EBV ASSOCIATED DISEASES (TABLE 2)

Fp-U spliced transcripts

Fp-U spliced transcripts were only detected in post-transplant lymphoproliferative disorders (half of the cases tested), the infectious mononucleosis sample (not shown), and one of the two Burkitt's lymphoma samples tested (fig 2B, second panel). We also assessed the presence of Fp-U-K spliced transcripts and found signals in the same samples that were positive for Fp-U spliced transcripts (fig 2B, upper panel).

Q-U-K spliced EBNA1 transcripts

Q-U-K spliced EBNA1 transcripts were detected across the range of diseases tested in most samples, with the exception of five of the 19 Hodgkin's disease samples (in which no EBNA1 transcription was detectable), one of four Burkitt's lymphoma samples, two of four B cell non-Hodgkin's disease samples (of which one expressed Cp driven EBNA1 only and the other showed no detectable EBNA1 transcription), and one nodal peripheral T cell lymphoma not otherwise specified sample that contained < 10 EBV positive cells in each section and showed a weak signal for BARTs. Although the Q primer might also detect Fp driven transcripts (fig 1), the absence of Fp signals in all cases except the abovementioned post-transplant lymphoproliferative disorders, infectious mononucleosis, and Burkitt's lymphoma samples indicates that all signals found with the Q-U and the Q-K primer pairs are truly derived from Qp driven transcripts.

Q-U spliced transcripts

Q-U spliced transcripts were detected in the samples that were also positive for Q-U-K spliced transcripts (fig 2B, third panel). The signals obtained with the Q-U primer pair were stronger than those of the Q-U-K primer pair, which might be because the amplimer generated with the Q-U primer pair is shorter; in fact, one Hodgkin's disease and one Burkitt's lymphoma sample, which were negative for Q-U-K, did show a signal with the Q-U primer pair and this was also true for the nodal peripheral T cell lymphoma that remained negative in the Q-U-K RT-PCR (fig 2B, third panel).

Cp driven EBNA1 transcription was clearly detected in all post-transplant lymphoproliferative disorders, the infectious mononucleosis

sample, and one of the two AIDS related lymphomas (not shown). Moreover, in five of 15 nasopharyngeal carcinoma samples a Cp signal was observed, although these signals were relatively weak compared with the positive controls and the post-transplant lymphoproliferative disorders. One B cell non-Hodgkin's lymphoma clearly expressed Cp driven EBNA1 transcripts. In three of the 19 Hodgkin's disease samples a faint signal was observed after overnight exposure; only one of the 19 Hodgkin's disease samples clearly expressed Cp driven EBNA1 transcripts. A faint Cp signal was also observed in one nodal peripheral T cell lymphoma not otherwise specified.

Discussion

The detection of Fp driven EBNA1 transcription in JY cells disagrees with the findings of Schaefer *et al*,[15] who detected Fp-U spliced transcripts but not Fp-U-K spliced transcripts in JY cells. This discrepancy might result from interlaboratory differences in the JY cell culture. Lytic reactivation in some of the JY cells cultured in our laboratory has been demonstrated in a previous study using immunohistochemistry for the BZLF1 lytic activator.[26] The detection of Fp driven EBNA1 transcription in the C15 tumour probably reflects viral replication that occurs in nude mice in the absence of a cytotoxic T cell response.

The detection of Fp driven EBNA1 transcription in infectious mononucleosis and in post-transplant lymphoproliferative disorders probably reflects the lytic replication of EBV. Infectious mononucleosis is known as the clinical manifestation of a primary EBV infection.[27] Post-transplant lymphoproliferative disorders, like infectious mononucleosis, usually occur in patients not previously exposed to EBV.[28] Moreover, the expression of the lytic replication activator BZLF1 was detected previously in infectious mononucleosis[29] and post-transplant lymphoproliferative disorders.[26] This is also the case for Burkitt's lymphoma[30]; Burkitt's lymphoma may even contain cells entering the lytic phase,[31] which was recently shown to be related to a good response to chemotherapy.[32]

We determined whether BZLF1 transcription and/or protein synthesis coincided with Fp activity, but we could not find a clear correlation: the infectious mononucleosis sample and the three post-transplant lymphoproliferative disorders that showed Fp activity also showed BZLF1 mRNA (using RT-PCR) and protein (by immunohistochemistry using the BZ1 monoclonal antibody (Dako, Glostrup, Denmark). However, the three post-transplant lymphoproliferative disorders that did not show Fp activity also showed BZLF1 mRNA and protein expression. Moreover, all five nasopharyngeal carcinoma samples that were negative for Fp driven transcripts showed BZLF1 transcription, but BZLF1 protein could not be detected immunohistochemically. It is not known whether BZLF1 protein expression is necessary to induce Fp activity, although it was shown previously[11] that the activation of Fp is dependent on de novo protein synthesis after the initiation of the lytic cycle.

The detection of Fp-U-K spliced RT-PCR products indicates that EBNA1 encoding transcripts can be Fp driven in clinical material. The same was shown recently for cell lines.[33] However, the Fp-U RT-PCR signals were clearly stronger than the Fp-U-K RT-PCR signals, indicating that a proportion of Fp driven transcripts may encode proteins other than EBNA1. This was also suggested for cell lines in a previous study.[33] Alternatively, RT-PCRs with the U antisense primer might proceed more efficiently than those with the K antisense primer, because with the U antisense primer a shorter amplimer is generated.

In a previous study,[34] we showed that lymphomas of immunocompromised patients were positive in RT-PCR both with the Y3/K and with the Q/K primer pair, but the latter signals were ascribed to Fp activity because the Q/K primer pair does not enable the distinction between Fp and Qp driven EBNA1 transcripts (fig 1). In our present study, we show that Qp driven EBNA1 transcription in the absence of Fp activity does occur in the lymphomas of immunocompromised patients.

The ubiquitous expression of Qp driven transcripts indicates that Qp can indeed be considered a "housekeeping" promoter,[7] which ensures constant EBNA1 transcription and thus maintenance of the viral episome in the host cells. The absence of detectable Qp transcripts in some of the samples might reflect the number of EBV infected cells, as is probably the case in the sample from the peripheral T cell lymphoma not otherwise specified (hence the weak BART signal found in this sample). Alternatively, specific downregulation of Qp activity by EBNA1 protein might occur.[35] Recently, it was shown that this effect can be overcome by E2F[36] and, therefore, the ratio between EBNA1 and E2F in EBV associated diseases is likely to determine Qp activity.

The detection of Cp driven EBNA1 transcription in post-transplant lymphoproliferative disorders, infectious mononucleosis, and AIDS related lymphomas is in agreement with the literature, Cp being the promoter that is active during latency type III, which is commonly found in these disorders.[37–39] The EBV gene expression pattern that prevails in nasopharangeal carcinoma is latency type II,[16 40] but these tumours are known to contain abundant reactive lymphoid infiltrate. It is not unlikely that EBV infected B cells are present and contribute to the Cp signals observed. The same holds true for nodal peripheral T cell lymphomas not otherwise specified that contain EBV positive cells: we have shown that in these lymphomas the EBV positive cells are mostly (reactive) B cells[41] and, in a previous study, Cp driven BHRF1 transcription was shown for this kind of lymphoma.[19] Therefore, Cp activity is associated not only with a latency type III expression pattern but also with the presence of EBV positive reactive B cells.

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