Activity of the LMP1 gene promoter in Epstein–Barr virus-transformed cell lines is modulated by sequence variations in the promoter-proximal CRE site

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The Epstein–Barr virus (EBV)-encoded tumour-associated latent membrane protein 1 (LMP1) gene expression is transactivated by EBV nuclear antigen 2 (EBNA2) in human B cells. We have previously identified a cyclic AMP-responsive element (CRE) in the B95-8 LMP1 promoter that is essential for transcription activation. Sequencing of LMP1 promoter in the P3HR1-derived EREB2.5 cell line revealed 25 single base pair substitutions in comparison to the B95-8 virus, one of them localized in the CRE element. Sequence variations in this element have been identified in several EBV isolates of both African and Asian origins. The effect of the P3HR1 CRE site variation on binding of factors to the LMP1 promoter sequence (LRS) and promoter activation was investigated with electrophoretic mobility-shift assays and reporter gene transfection assays. ATF1 and CREB1 transcription factors bound with reduced efficiency to the P3HR1 variant and below the detection level to the other tested variants. Accordingly, reporter plasmids carrying the P3HR1 CRE sequence in a B95-8 LRS context displayed 50% lower activity in all tested cell lines. The impaired ability to activate transcription caused by the C to A substitution in CRE was not apparent when the mutated site was placed in a P3HR1 LRS context and the reporter transfected into Jijoye cells, most likely as a consequence of the other base pair substitutions in P3HR1 LRS. Overall, our results suggest that the mutations in the LRS CRE site have been conserved to adjust LMP1 expression to levels that favour cell survival in certain cellular and environmental contexts.

INTRODUCTION

The Epstein–Barr virus (EBV) is a human lymphocryptovirus characterized by its tropism for lymphoid cells. EBV is the aetiological cause of infectious mononucleosis (IM), but it is also associated with several malignancies including Burkitt’s lymphoma (BL), Hodgkin’s disease (HD), nasopharyngeal carcinoma (NPC), gastric carcinoma, T-cell lymphoma and lymphoproliferative disorders in immunocompromised individuals. In vitro, EBV infection of human B-lymphocytes immortalizes the infected cell and gives rise to lymphoblastoid cell lines (LCLs). The ability of the virus to induce and maintain proliferation is attributed to the establishment of latent gene expression programmes where only a subset of EBV genes are expressed leading to three different latency types (I, II and III) (Kieff & Rickinson, 2001). The EBV-encoded latent membrane protein 1 (LMP1) is critically involved in the immortalization and proliferation of B-cells latently infected by EBV (Kaye et al., 1993, 1995; Kilger et al., 1998). It also has the ability to transform rodent fibroblast and human cell lines in culture (Baichwal & Sugden, 1988; Fahraeus et al., 1990b; Moorthy & Thorley-Lawson, 1993; Wang et al., 1985). LMP1 is expressed in latency type II and III. LMP1 transcription can be initiated from two promoters in the EBV genome, a proximal promoter referred to as the ED-L1 promoter and a distal promoter mapped to the terminal repeats named LT-R1 (Chang et al., 1997; Fennwald et al., 1984; Hudson et al., 1985; Sadler & Raab-Traub, 1995). The viral EBV nuclear antigen 2 (EBNA2) protein is the most potent transactivator of the LMP1 gene through the ED-L1 promoter, but requires other viral and cellular genes for transactivation (Kieff & Rickinson, 2001). In latency type III, LMP1 expression is EBNA2-dependent, while in latency II it is EBNA2-independent. In latency II, LMP1 expression can be driven by both the LT-R1 and the ED-L1 promoters (Sadler & Raab-Traub, 1995).

Several regulatory elements in the LMP1 ED-L1 promoter region have been shown to mediate both transcription repression and activation. The RBP-Jκ sites, a PU-box and
an AP-2 consensus site confer activation of the promoter in an EBNA2-dependent manner. Other elements such as an Sp factor-binding site, cyclic AMP response element (CRE), E-box element, octamer-binding site and interferon-stimulated response element (ISRE) have been described to be involved in the regulation of the promoter in an EBNA2-independent manner (reviewed by Zetterberg & Rymo, 2005). These studies have been carried out on the prototype B95-8 strain of EBV, which originates from a marmoset cell line infected with EBV from an IM patient (Miller et al., 1972). However, sequence variations in the lmp1 gene have been reported for different EBV strains, both in the coding sequence and in the promoter region (Chen et al., 1992; Hu et al., 1993; Sandvej et al., 2000; Takacs et al., 2001; Zhou et al., 2001). It is thus necessary to consider the effect of sequence variations in promoter regulation.

In this study, the LMP1 promoter in the EREB2.5-derived virus was sequenced to monitor possible differences between this strain and the B95-8 strain that may affect promoter regulation. The EREB2.5 cell line is a useful tool in the study of LMP1 as it is conditional for the activation of EBNA2, with the consequence that the LMP1 promoter can be switched on or off by the addition or removal of β-oestradiol from the medium (Kempkes et al., 1995). The LMP1 sequence in EREB2.5 originates from the P3HR1 viral strain (Kempkes et al., 1995). Sequencing of the LMP1 regulatory sequence (LRS), defined here as positions −634 to +40 relative to transcription start site (+1) in the P3HR1 virus, revealed 25 nt substitutions and one insertion when compared with the B95-8 sequence. One of the substitutions, a C to an A at position −43 relative to the transcription initiation site, was within the CRE element. Notably, sequence variations in this CRE site have also been found in several other EBV strains (Chen et al., 1992; Hu et al., 1993; Sandvej et al., 2000; Takacs et al., 2001; Zhou et al., 2001). We have previously shown that the CRE element plays a significant role in LMP1 activation, both in an EBNA2-dependent and -independent manner (Sjoblom et al., 1998). Here, we investigated the effect of CRE-sequence variants present in different EBV strains on the ability of the CRE-binding transcription factors to interact with the site in order to elucidate the functional consequences for the regulation of the LMP1 promoter.

**METHODS**

**Plasmid constructions.** The EBNA2 expression vector pEAA6 and control vector (pSV2gpt) have been described earlier (Ricksten et al., 1987). The LRS is defined as nt 169 019–169 692 of B95-8 EBV DNA (GenBank accession no. AJ507799), which corresponds to positions −634 to +40 relative to the transcription initiation site. The B95-8 LRS and B95-8 LRS (CREmut) luciferase reporter plasmids were generated by transferring HindIII digested LRS fragments from the corresponding plasmids in the CAT reporter system (Fahraeus et al., 1990a; Sjoblom et al., 1998) into the HindIII site in the pGL3-Basic vector (Promega). The P3HR1 LRS was created by PCR-amplification of DNA purified from EREB2.5 cells, using primers that correspond to positions −634 and +40 relative to the transcription initiation site of the LMP1 promoter, along with a HindIII restriction site. The PCR product was digested and cloned into the HindIII site in the pG3L- Basic vector. The B95-8 LRS (CREP3HR1) was created by site-directed mutagenesis in the B95-8 LRS plasmid, using an oligonucleotide containing the C to A substitution at position −43 in LRS. All constructs were verified by dideoxy sequencing utilizing the ABI Prism 310 automated sequencer (Applied Biosystems) and standard protocols. Sequencing of the LRS in P3HR1 was carried out on DNA prepared from EREB2.5 cells with a GenoM-48 robot (GenoVision AS).

**Cell culture, DNA transfections and reporter gene assays.** EREB2.5 is a transformed lymphoblastoid cell line expressing conditional EBNA2 (ER-EBNA2) and its activity is regulated by oestrogen (Kempkes et al., 1995). Jijoye is an EBV-positive BL cell line (Pulvertaft, 1965). WW1-LCL is an EBV-immortalized LCL cell line (Gregory et al., 1988) and DG75 is an EBV-negative BL cell line (Ben-Bassat et al., 1977). The cells were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies), 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. The EREB2.5 cell line was also supplemented with 1 μM β-oestradiol (β-oestradiol-water soluble; Sigma). Transient transfections were carried out by electroporation using 5 x 10⁶ cells and 10 μg reporter plasmids as described previously (Sjoblom et al., 1998). Co-transfections with 2.7 nmol DNA of the EBNA2 expression vector (pEAA6) or the empty vector (pSV2gpt) were done in DG75 cells. Cells were harvested after 48 h and assayed for luciferase activity using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. A TD 20/20 luminometer (Turner Design Instrument) was used for the detection of luciferase activity. In addition, half the cells harvested after transfection were used for immunoblot analysis in parallel to reporter assay.

**Electrophoretic mobility-shift assays (EMSA).** Nuclear extract preparation and the EMSA-binding reactions were carried out as described previously (Sjoblom et al., 1998). All oligonucleotides were purchased from Invitrogen. EMSAs were carried out using double-stranded synthetic [γ-³²P]ATP-labelled oligonucleotides corresponding to LRS −50 to −19 position from B95-8, as well as the specific sequence variations in the CRE site detected in P3HR1, Rael, Raji or NPC with blunt ends as shown in Fig. 2. A mutation introduced in the Sp1 site of all probes eliminated the binding of Sp1 factors and facilitated the interpretation of the factor-binding patterns (Sjoblom et al., 1998). The competing oligonucleotides were added at an excess before the addition of the labelled probe and samples were incubated for 20 min at room temperature. In supershift experiments, after the 20 min incubation of nuclear extract with probe, 3–4 μl of the antibody was added and incubated for another 60 min at 4°C. The antibodies CREB1 (sc-186 antibodies, 1:2000), CREB1 (sc-2578 antibodies, 1:2000), ATF1 (sc-270 antibodies, 1:2000), ATF2 (sc-187 antibodies, 1:2000), c-Jun (sc-1694 antibodies, 1:2000) and CREB2 (sc-200 antibodies, 1:2000) were purchased from Santa Cruz. The samples were separated by electrophoresis in 5% polyacrylamide gels (acrylamide: bisacrylamide, 29:1) in 0.5 x TBE for 3 h at 300 V. The bands were either visualized by autoradiography or exposed to a phosphoimage screen and scanned by a Typhoon 9200.  

**Immunoblot analysis.** To prepare protein extracts from transfected cells, cell pellets were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer, incubated on ice for 15 min and spun at maximum speed. Protein extracts were added to NuPAGE LDS sample buffer, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was stained with 0.1% Ponceau S (Sigma) in 5% acetic acid to confirm equal loading and transfer of proteins. The membrane was then blocked in 5% milk, incubated with EBNA2 primary antibody (PE2, 1:5000; Dako) and goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:3000). The protein bands were visualized by chemiluminescence reagents (Pierce) and detected using a ChemiDoc instrument (Bio-Rad).
RESULTS AND DISCUSSION

Sequence differences between the promoter-proximal LRS CRE element in B95-8 and P3HR1 virus

As part of a study of transcription regulatory differences between the B95-8 virus and the P3HR1-derived EREB2.5 virus, the nucleotide sequence of the respective LMP1 ED-L1 promoter regions (referred to as the LRS and defined by the positions −634 to +40 relative to the transcription start site in B95-8) were compared. Sequencing revealed 25 bp substitutions and one insertion in P3HR1 LRS relative to the corresponding B95-8 sequence (Fig. 1a). A ‘C’ to ‘A’ substitution was located in the CRE factor-binding site present in the LRS. The rest of the sequence differences between the two viral strains were located outside the previously reported transcription regulatory elements (Fig. 1a). Variations reported in the CRE site of several other EBV strains are depicted in Fig. 1(b). The EBV strains CAO and C15 are NPC-derived virus isolates that contain a 2 bp substitution in the CRE site (Chen et al., 1992; Hu et al., 1993), and they have an Asian origin. This variation has also been reported for virus isolates from several HD, IM and asymptomatic carriers referred to as group D (Sandvej et al., 2000; Zhou et al., 2001). The Rael and Raji viral strains contain a C to G substitution in the same position as the C to A substitution in the CRE site in P3HR1 (Takacs et al., 2001). The P3HR1 cell line is a subline of the Jijoye BL cell line and has an African origin like the Raji and Rael cell lines (Epstein et al., 1966; Hinuma et al., 1967; Klein et al., 1972; Pulvertaft, 1965). Notably, the phylogenetic tree based on the LMP1 sequence constructed by Kanai et al. (2007) showed that the Asian EBV isolates contain large differences compared with the African BL isolates suggesting independent selection for the CRE-site sequence variants. Stable, genetic variations in CRE sites have also been described for several other promoters (Mitchison, 2001). It has been hypothesized that the activity of some cis-elements such as the CRE site can be changed to a lower level by subtle sequence variations that modulate the level of expression of genes important for cell survival (Mitchison, 2001). The present study together with the reports mentioned above support the notion that the CRE element in the LRS is a preferred target for such mutations that in a certain cellular context might confer evolutionary advantages to the EBV-infected cell and are therefore conserved.

Effect of mutation of the CRE site on binding of transcription factors

Using EMSA analysis we have previously demonstrated that the ATF1–CREB1 and c-Jun–ATF2 factors bind preferentially as heterodimers to the B95-8 LRS CRE site (Sjoblom et al., 1998). In the present study, we compared the factor-binding properties of the B95-8 and P3HR1 LRS CRE sites using nuclear extracts from the EBV-negative DG75 line and B95-8 and P3HR1-derived LRS (−50/−19) oligonucleotides as probes. Five specific complexes bound to the P3HR1-derived probe. The complexes represented

Fig. 1. Sequencing of the LRS −634 to +40 relative to the transcription start site in P3HR1. (a) Illustration of base pair substitutions in the promoter between the P3HR1 and B95-8 viral strains. Known regulatory elements are underlined and labelled. (b) Variations in the CRE sequence in the LRS of different EBV strains.
by bands I and II corresponded to factors binding outside the CRE site, since the bands were competed away by a probe with a mutated CRE site (Fig. 2a, lane 5). The three CRE-specific complexes represented by bands III, IV and V were detected with both the B95-8- and the P3HR1-derived probes. The binding efficiencies of the different CRE complexes, however, appeared to differ (Fig. 2a). The CRE-binding complex III dominated in the factor-binding pattern obtained with the P3HR1-derived probe, whereas complexes IV and V dominated in the pattern obtained with the B95-8-derived probe. Supershift experiments with anti-ATF1, anti-CREB1, anti-ATF2 and anti-c-Jun antibodies, respectively, were carried out to confirm the identity of the factors binding to the CRE site in the P3HR1-derived LRS CRE probe (Fig. 2b). ATF1 and CREB1 antibodies shifted complexes IV and V confirming
Fig. 2. Similar binding pattern but differences in binding efficiency to the CRE site in the LRS from P3HR1-derived compared with the B95-8-derived sequences. Sequences of oligonucleotides used in the EMSA experiments are shown at the top right of the EMSAs. The bold capital letters represent the CRE site in B95-8. The bold lower case letters represent sequence variations relative to the B95-8 CRE site. (a) The $^{32}$P-labelled B95-8 LRS and P3HR1 LRS probes were incubated with DG75 nuclear extract and subjected to EMSA. The first and seventh lane in the autoradiogram show the binding pattern obtained with the nuclear extract. Competition experiments were carried out using different oligonucleotides as indicated above the autoradiogram. Solid arrows show the position of the specific complex bands and broken arrows indicate non-specific complexes. (b) The $^{32}$P-labelled B95-8 LRS and P3HR1 LRS probes were incubated with DG75 nuclear extract and subjected to EMSA. Supershift experiments were carried out with specific antibodies as indicated above the figure. The positions of the shifted complexes are shown by arrowheads. (c) Further competitions were carried out with the $^{32}$P-labelled B95-8 LRS and P3HR1 LRS probes, and DG75 nuclear extract. Unlabelled oligonucleotides corresponding to either B95-8 or P3HR1 variant were used as competitors with each probe at a molar excess of 13-, 25- and 50-fold. The relative intensity of the ATF1–CREB1 complexes was then determined by the ImageQuant software and the relative quantities obtained are shown beneath the autoradiogram. The intensity of the ATF1–CREB1 complexes in the absence of competitors was set to 100. (d) EMSA was carried out using the $^{32}$P-labelled B95-8 LRS and P3HR1 LRS probes with either WW1-LCL or DG75 nuclear extracts. (e) EMSA analysis was performed with $^{32}$P-labelled B95-8 LRS and P3HR1 LRS as well as different variants of the CRE site in LRS context with WW1-LCL nuclear extract. Antibody supershift analyses were performed with anti-CREB1 (reactive with both CREB1 and ATF1) and anti-CREB2 antibodies for each probe.

Our previous study (Sjoblom et al., 1998). However, antibodies against ATF2 and c-Jun did not shift complex III in the P3HR1-derived probe and only weakly diminished this complex in the B95-8-derived probe (Fig. 2b). Thus, our conclusions in the previous study were not verified on this point (Sjoblom et al., 1998). Furthermore, EMSA competition experiments using CRE sequences known to bind the different members of the CRE-binding transcription factor family with different affinities, confirmed that complex III did not represent binding of c-Jun–ATF2 (unpublished data). Thus, the identity of the CRE-binding factors in complex III remains to be elucidated. It was also noted that this complex was weak or not detected with WW1-LCL nuclear extract (Fig. 2d, e). To assess the difference in protein-binding efficiency of the different transcription factor complexes with the CRE site in a more quantitative way, EMSA competition experiments were performed with DG75 nuclear extracts and a 13-, 25- or 50-fold excess of unlabelled LRS CRE probe from B95-8 and P3HR1, respectively (Fig. 2c). The relative efficiency of binding was calculated as the ratio between the intensity of given EMSA bands obtained with one competitor and that of the corresponding bands without competitor. The C to A substitution in the P3HR1 CRE site led to an approximately twofold higher-binding efficiency of the factors in complex III and an approximately twofold lower-binding efficiency of the ATF1–CREB1 heterodimer. To confirm the binding of the same factors to both the B95-8 and P3HR1 CRE site in an EBV-positive context, EMSA was performed with WW1-LCL nuclear extract (Fig. 2d). A similar binding pattern was observed between DG75 and WW1-LCL extracts for both probes, although complex III appeared to be weaker in WW1-LCL in both cases (Fig. 2d). In addition, EMSAs were performed with WW1-LCL nuclear extract and probes encompassing the corresponding LRS CRE sequences of B95-8, P3HR1, Raji/Rael and NPC viral strains, respectively (Fig. 2e). The EMSA bands corresponding to the positions of the ATF1–CREB1 complexes were weak or not detectable with the LRS CRE site in the Raji/Rael- and NPC-derived probes (Fig. 2e). EMSA supershift experiments using an antibody reactive against ATF1, CREB1 and CREM (the anti-CREB1 antibody) shifted the ATF1–CREB1 complexes obtained with probes carrying the B95-8- and P3HR1-derived LRS CRE sites and the CRE-consensus sequence as expected confirming the identity of the ATF1–CREB1 complexes when using the WW1-LCL nuclear extract (Fig. 2e, lanes 2 and 5). However, the antibody did not shift any of the weak bands in the ATF1–CREB1 heterodimer positions formed with probes containing the corresponding LRS CRE site representing Raji, Rael or the NPC C15 and CAO. This indicated that no such complex had been formed, presumably because the LRS CRE sequence in these EBV lines has a considerably reduced ability to bind the ATF1–CREB1 factors (Fig. 2e). This notion was strengthened by the results of EMSA experiments with unlabelled oligonucleotides containing LRS CRE-sequence variants representing Raji, Rael, NPC C15 and CAO virus isolates as competitors against the B95-8-derived probe. Even in a large excess, the variant oligonucleotides did not compete out the ATF1–CREB1 heterodimer complexes (data not shown).

ATF1 and CREB1 contain phosphorylation-dependent activation domains and become transcriptional activators when phosphorylated at specific serine or threonine residues (Livingstone et al., 1995; Masson et al., 1993; Shaywitz & Greenberg, 1999; Sheng et al., 1991) and several signalling pathways are responsible for their phosphorylation (Gupta et al., 1995; van Dam et al., 1995). These intracellular pathways are generally activated by inflammatory cytokines and cellular stress (Tibbles & Woodgett, 1999). The stress responses are adaptive processes that include apoptosis, transformation, development, immune activation, inflammation and adaptation to environmental change (Tibbles & Woodgett, 1999). A possible consequence of the decreased affinity of the CREB1–ATF1 factors for the LMP1 CRE site may be that activation of the signalling pathways in response
to the environmental stress signals would not lead to an upregulation of the expression of LMP1. The ability of the latently infected B cells to maintain a low level of LMP1 despite extracellular signalling in some cases might be of advantage in EBV biology.

**P3HR1 CRE-sequence variant reduces LMP1 promoter activity in a B95-8 LRS context**

In a previous study of B95-8 LRS, we found that a 2 bp transverse mutation at positions −40 and −41 in the CRE site reduced EBNA2-dependent activation fivefold in the context of the wild-type +40/−106 part of B95-8 LRS (Sjoblom et al., 1998). It has also been demonstrated that a 2 bp substitution at positions −42 and −43 in the corresponding LRS CRE site of the C33A virus leads to a threefold decrease of EBNA2-induced promoter activation (Chen et al., 1995). To characterize further the consequences of sequence variation of this CRE site on LMP1 promoter transactivation, LRS from the B95-8 and P3HR1 strains cloned in luciferase reporter plasmids were co-transfected with an EBNA2 expression vector or a control vector into DG75 cells. Similar levels of EBNA2 expression in transfected cells were confirmed using immunoblot analysis (Fig. 3a). The P3HR1-derived reporter plasmid displayed approximately 50% less activity in the presence and absence of EBNA2 in comparison to the B95-8-derived reporter plasmid (Fig. 3a). A reporter plasmid containing a B95-8 LRS region, in which the CRE element had been exchanged for the P3HR1-derived sequence [LRS B95-8 (CREP3HR1)], was induced by EBNA2 to approximately the same extent as the reporter plasmid with the P3HR1 LRS region and also had the same reduction in activity in the absence of EBNA2 (Fig. 3a). This suggests that the reduced EBNA2 response of the P3HR1 LRS reporter plasmid was due to the single base pair substitution in the CRE site. Thus, this sequence variation in the LMP1 promoter in EREB2.5 cell line should be taken into consideration in studies of the regulation of LMP1 expression employing this cell line. Overall, the results suggest that reduced ATF1–CREB1 affinity for the CRE site in LRS leads to reduced promoter activity. Given the fact that complex III had higher-binding efficiency to the P3HR1 probe (Fig. 2c), it appears from reporter experiments (Fig. 3a) as though this complex contributes to a lesser extent to EBNA2 inducibility of the LMP1 promoter and that this function is carried out mainly by the ATF1–CREB1 heterodimers. Therefore, it is likely that sequence variations in the LRS CRE site of Raji, Rael and NPC viral strains and other strains with the same substitutions will lead to decreased promoter activity.

To investigate the effect of the intracellular environment and cellular phenotype on the ability of the P3HR1 LRS CRE sequence variation to mediate promoter activation, a number of in latency III cell lines including WW1-LCL, Jijoye and EREB2.5 were transfected with the same series of LRS-Luc reporter plasmids. WW1-LCL is infected with a type A virus, while Jijoye and EREB2.5 were transfected with the same series of LRS-Luc reporter plasmids. WW1-LCL is infected with a type A virus, while Jijoye is a type B virus. EREB2.5 harbours a type B virus and a type A EBNA2. The reporter plasmid with the P3HR1 CRE site in a B95-8 LRS context [LRS B95-8(CREP3HR1)] was activated to approximately the same relative level in the type III cells as in EBNA2-
transfected DG75 cells. Notably, the P3HR1-derived reporter plasmid showed the same level of relative activity as the B95-8-derived reporter when transfected into Jijoye cells. This suggests that differences in cellular context/intracellular conditions between the different B cell lines modulate LMP1 promoter activity through the other base pair substitutions in the P3HR1-derived promoter. Since Jijoye, unlike the other cell lines, contains a type B EBNA2, it is possible that this difference contributes to the high activity of the P3HR1 reporter plasmid in this cell line. However, this experiment alone does not provide enough evidence to draw this conclusion considering the large array of differences between cell lines. Nonetheless, the variation in the CRE site leads to approximately 40% reduced promoter activity independent of the cellular context as indicated by the B95-8 reporter plasmid with a P3HR1 CRE site (Fig. 3b).

Taken together our results suggest that the P3HR1 CRE sequence causes a change of its factor-binding properties as compared with the corresponding B95-8 site, and as a consequence a modulation of its efficiency in activation of the LMP1 promoter. In our model system this is manifested as a reduced affinity for the ATF1–CREB1 factor complexes and a reduction of LMP1 promoter activation in the presence and absence of EBNA2. In our previous study, we showed that ATF1–CREB1 transcription factors could activate the LMP1 promoter independently of EBNA2 (Sjoblom et al., 1998). Thus, the sequence variation in this site also leads to a modulation of LMP1 expression in the absence of EBNA2.

The fact that LMP1 regulation is dependent on the cell type and latency programme is well documented (reviewed by Kieff & Rickinson, 2001). Lam et al. (2004) have reported that the levels of LMP1 vary as much as 100-fold in individual cells from the same clone and showed that the difference was because of different levels of transcript. Our study of LMP1 promoter activity in different cell lines is consistent with the notion that the activation of the LMP1 promoter is modulated both by the individual cellular context and the sequence variations and, hypothetically, epigenetic changes in the promoter. Thus, it is difficult to predict the precise physiological role of CRE-site sequence variations at different stages of EBV biology. Nonetheless, the selection of specific sequence variations in this site indicates an important regulatory function for this element in LMP1 expression. This is conceivable since a high level of LMP1 expression has been shown to induce cytostasis (Floettmann et al., 1996; Sandberg et al., 2000; Kaykas & Sugden, 2000). A high level of expression also inhibits the activity of viral and cellular promoters in the absence of cytostasis (Narbonnet & Mariame, 2006). Selection of regulatory sites with a reduced responsiveness to transcription factors without shutting it down, may regulate LMP1 expression to a steady-state level where cytostasis is prevented and cell survival is promoted. This hypothesis has been proposed by Chen et al. (1995) and is strengthened by our findings.

It is finally interesting to note that sequence variations in the LMP1-encoding region are common among EBV strains and occur independently of EBV type or origin (Jenkins & Farrell, 1996). A 30 bp deletion in the LMP1 coding sequence is so far the only sequence variation that has been functionally linked to enhanced transforming capacity (Hu et al., 1993). Selection-driven evolution of LMP1 in virus isolates from south-east Asia towards a more malignant phenotype has been suggested from phylogenetic studies (Burrows et al., 2004). Sandvej et al. (2000) have shown that the B95-8 CRE-site variant is significantly more frequent in HD virus isolates than in isolates from IM patients and asymptomatic carriers. They hypothesize that a sequence variation leading to lower LMP1 expression levels is advantageous by reducing the frequency of LMP1 driven malignancies. The question whether particular CRE-site variants in the LMP1 promoter are linked to disease remains to be answered.

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