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Characterization of the BcLF1 promoter in Epstein–Barr virus

Pey-Jium Chang,[1,2] Yu-Sun Chang[2] and Shih-Tung Liu[2]

[1] Institute of Microbiology and Immunology, National Yang-Ming University, Shih-Pai, Taipei 112, Taiwan

[2] Molecular Genetics Laboratory, Department of Microbiology and Immunology, Chang-Gung University, Kwei-Shan, Taoyuan 333, Taiwan

The location of the BcLF1 promoter of Epstein–Barr virus (EBV) has been identified by primer extension, which indicates that the 1 site of the BcLF1 mRNA is located at nucleotide 137676 of the EBV genome. According to deletion analysis, the region upstream from nucleotide 38 is not essential for transcription of BcLF1. A 23 bp region in the promoter, from nucleotide 38 to 16, was identified as necessary for regulating the expression of BcLF1, i.e. the promoter activity is activated by 12-*O*-tetradecanoylphorbol 13-acetate but is repressed by phosphonoacetic acid. The results presented also demonstrate that the oriLyt sequence in *cis* is essential for enhancing the expression of BcLF1.

Epstein–Barr virus (EBV) is a human herpesvirus which infects lymphoid and epithelial cells. This virus is the aetiological agent of infectious mononucleosis and is implicated in nasopharyngeal carcinoma, Hodgkin's disease and Burkitt's lymphoma (Rickinson & Kieff, 1996). After infection of B lymphocytes, EBV immortalizes the cells and is maintained in a latent condition. Latency can be disrupted by treating the infected cells with phorbol esters, sodium butyrate or anti-immunoglobulin (Faggioni *et al.*, 1986; Luka *et al.*, 1979; Takada & Ono, 1989; zur Hausen *et al.*, 1978). Such treatment causes cells to express two viral immediate-early genes, BRLF1 and BZLF1. Expression of these two is necessary for the activation of EBV early genes and virus lytic replication (Biggin *et al.*, 1987; Hardwick *et al.*, 1988; Sinclair & Farrell, 1992). At the latter stage of EBV lytic development, the late genes, including BcLF1 which encodes viral capsid antigen (VCA), are expressed (Baer *et al.*, 1984). Previous studies have shown that expression of BcLF1 is prevented by inhibitors of EBV DNA polymerase, including phosphonoacetic acid (PAA), phosphonoformic acid and acyclovir (Datta & Hood, 1981; Summers & Klein, 1976), suggesting that expression of BcLF1 may rely on EBV DNA replication. A recent study suggests that transcription of BcLF1 may require a *trans* factor (Serio *et al.*, 1997). In this study, we analyse the BcLF1 promoter and define the region which is necessary to regulate transcription of BcLF1.

Author for correspondence: Shih-Tung Liu.
Fax +886 3 328 0292. e-mail cgliu@cguaplo.cgu.edu.tw

We first determined the transcription start site of BcLF1 mRNA by primer extension. This was done with the primer 5′ GTCTCGAGGGCTTCTCTTCACAGCTG, which is complementary to the 5′ region of BcLF1 (nt 137496–137521 of the EBV genome). Primer extension was performed with 100 μg of total RNA isolated from P3HR1 cells which had been treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (30 ng/ml) and sodium butyrate (3 mM) for 2 days. Next, the primer (100 ng) was end-labelled (Sambrook *et al.*, 1989) with 5 μCi [γ-^{32}P]ATP (5000 Ci/mmol) (Amersham) and 10 U of T4 polynucleotide kinase (Promega). The radioactively labelled primer was then annealed with RNA template at 65 °C for 20 min and then incubated at 37 °C for 2 h. Reverse transcription was performed with 1 U of AMV reverse transcriptase (Promega) at 42 °C for 30 min. The cDNA synthesized was precipitated by ethanol and was then analysed in an 8% (w/v) acrylamide sequencing gel (Sambrook *et al.*, 1989). DNA sequencing was performed with the same primer by the dideoxy chain

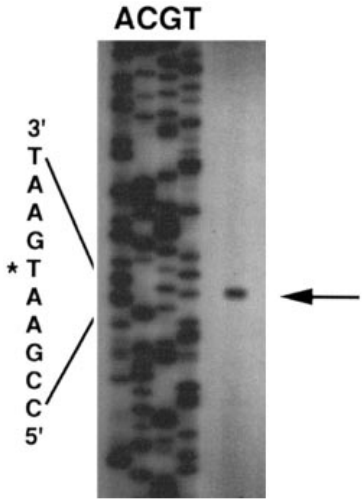

Fig. 1. Mapping of the transcription start site of BcLF1. The letters above each lane denote the dideoxynucleotide used to terminate each reaction. The asterisk indicates the 5′ terminus of RNA; the arrow indicates the cDNA product of primer extension.

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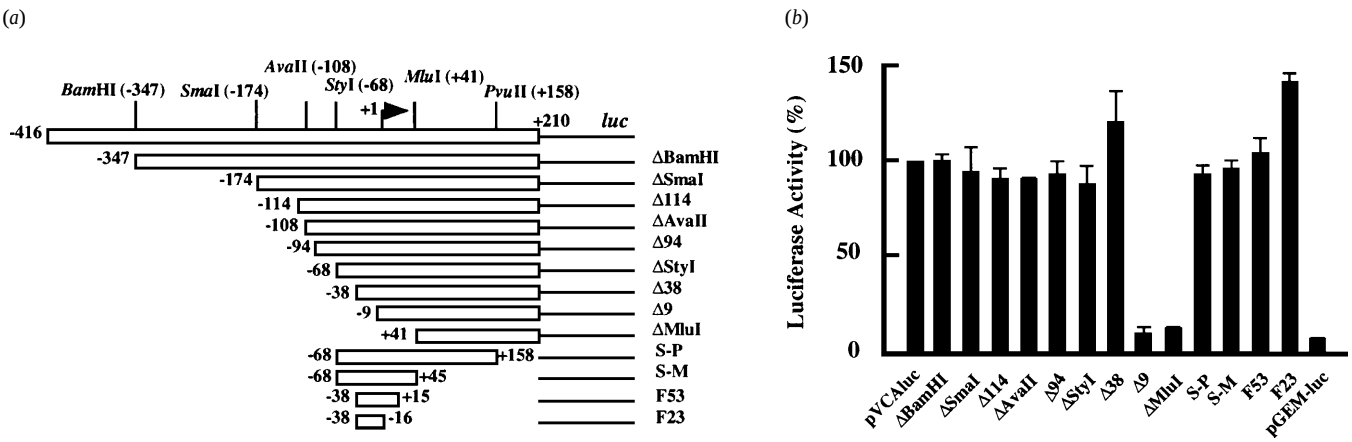

Fig. 2. Deletions in the promoter region of BcLF1 (*a*) and the luciferase activity exhibited by the deletion plasmids (*b*). The arrow and +1 denote the transcription start site. Numbers represent the nucleotide positions relative to the +1 site. The initiation codon of BcLF1 starts from +211.

termination method (Sanger *et al*., 1977), using pVCAluc (see below) as a template. According to our results, the +1 site is located at nucleotide position 137676 (Fig. 1). This was confirmed with a second primer, 5′ GGTCTGTTTTCCAC-ACCCTCATTTGAGGC, complementary to the region between nt 137435–137463. Sequence analysis revealed that a TATA sequence is located between nt −34 and −28 and that a YY1-like sequence, GGACCATATTTC (Mills *et al*., 1994; Shi *et al*., 1991), is located between nt −109 and −98. The initiation codon of BcLF1 is located at position +211.

We constructed a reporter plasmid, pVCAluc, to analyse the BcLF1 promoter. A *luc* fragment, isolated from pGL2-Basic (Promega) by *Hin*dIII/*Bam*HI digestion, was filled-in with Klenow fragment of DNA polymerase (Promega) and inserted into the T4 DNA polymerase-treated *Kpn*I site of pGEM-7Zf(−) (Promega) to generate plasmid pGEM-luc. Next, the promoter and the untranslated region of BcLF1, from nt −416 to +210, was amplified by PCR with primers 5′ GAATG-TGCTCCAGGAGAAGAAGTGG and 5′ GACACAAGGT-AAGAGGGAGATGG, and was inserted upstream from the *luc* gene at the *Sma*I site of pGEM-luc. The subsequent plasmid, pVCAluc (10 μg), was transfected into P3HR1 cells, and the cells were treated with TPA and sodium butyrate immediately after transfection. The luciferase activity exhibited by the plasmid was examined 2 days after transfection. Cell lysate was prepared 48 h after transfection and luciferase activity was monitored for 10 s with a luminometer (Berthod model LB953) according to a method described by de Wet *et al*. (1987). Each transfection experiment was repeated at least three times, and each sample was prepared in duplicate. Under lytic conditions, pVCAluc exhibited approximately 10-fold higher luciferase activity than control plasmid pGEM-luc (Fig. 2*b*). Under latent conditions, the activity exhibited by pVCAluc was approximately 20-fold lower (Fig. 3), showing that the BcLF1 promoter is active only under lytic conditions. Next, we generated mutations to determine the region essential for the regulation of BcLF1 expression. These deletions (Fig. 2*a*) included Δ*Bam*HI, Δ*Sma*I, Δ*Ava*II, Δ*Sty*I and Δ*Mlu*I, which were generated by restriction digestion of pVCAluc; Δ114, Δ94, Δ38 and Δ9, which were constructed by inserting PCR fragments into the *Sma*I site of pGEM-luc; S-P and S-M, which were generated by *Pvu*II and *Mlu*I digestion of Δ*Sty*I, respectively; and F53 and F23, which were generated by inserting synthetic oligonucleotides into the *Sma*I site of pGEM-luc. Under latent conditions the plasmids described above exhibited luciferase activity at the background level (data not shown), in a similar way to pVCAluc. This implies that the deleted regions do not contain any sequence that would negatively regulate the expression of BcLF1 under latent conditions. On the other hand, these plasmids displayed different patterns of expression under lytic conditions. According to our results, deleting the region between nt −416 and −38, including Δ*Bam*HI, Δ*Sma*I, Δ114, Δ*Ava*II, Δ94, Δ*Sty*I and Δ38, did not influence *luc* expression (Fig. 2*b*).

It seems, therefore, that the sequence upstream from nucleotide −38, including the YY1 sequence, is not essential for the transcription of BcLF1. However, the luciferase activity decreased to background level (Fig. 2*b*) if the regions upstream from −9 and from the *Mlu*I site (Δ*Mlu*I) (Fig. 2*a*) were deleted. In addition, deleting the region from nt +210 to +45 (Fig. 2*a*) did not decrease *luc* expression (Fig. 2*b*). We also inserted the *Bam*HI-b fragment of EBV, which covers the region between −1681 and −348, into the *Bam*HI site (nt −347) of Δ*Bam*HI. This insertion did not influence pVCAluc expression, indicating that the *Bam*HI-b fragment is also not essential for transcription of BcLF1.

We deleted the sequence in the untranslated region and the 3′ region of the promoter to determine whether this influenced *luc* expression. Oligonucleotides covering the regions between nt −38 and +15 (F53) and nt −38 and −16 (F23) were synthesized and cloned into the *Sma*I site of pGEM-luc (Fig. 2*a*). Both plasmids expressed luciferase activity under lytic

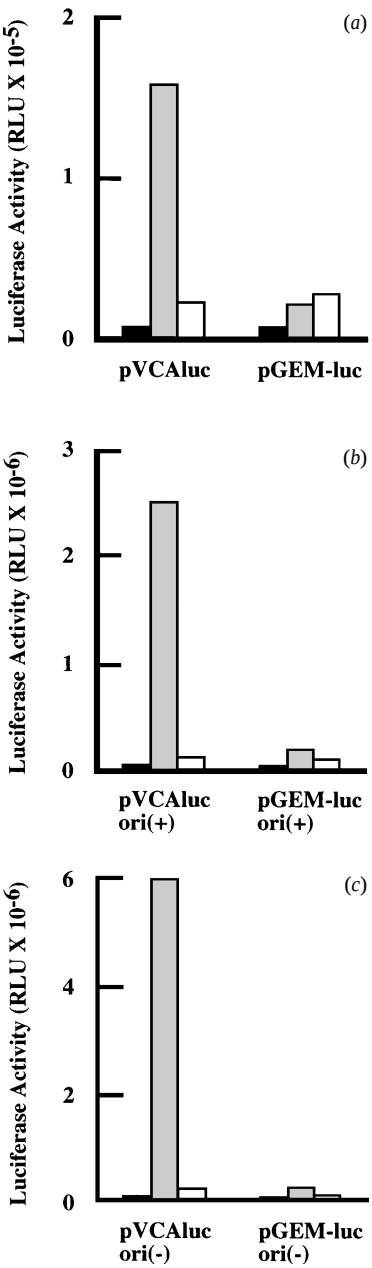

Fig. 3. Effect of oriLyt on transcription of the BcLF1 promoter. P3HR1 cells were transfected with pVCAluc (*a*) or with pVCAluc containing oriLyt (*b*, *c*). The oriLyt fragment was inserted in opposite orientations in (*b*) and (*c*). Black column, control (cells not treated with TPA and sodium butyrate); grey column, cells treated with TPA and sodium butyrate; white column, cells treated with TPA, sodium butyrate and PAA. pVCAluc ori(+), plasmid pVCAluc consisting of an oriLyt sequence with an orientation identical to that of *luc*; pVCAluc ori(−), the same as pVCAluc except that the oriLyt is in the opposite orientation. pGEM-luc ori(+) and pGEM-luc ori(−), identical to pVCAluc ori(+) and pVCAluc ori(−), respectively, but without BcLF1 promoter.

conditions (Fig. 2*b*), but not under latent conditions. This suggests that the 23 bp fragment in F23 contains the sequence necessary to regulate BcLF1 transcription. Our results further verified that PAA treatment (200 μg/ml) could inhibit the transcription activity of F23 (data not shown), implying that *luc* expression by F23 is dependent on EBV lytic replication. Sequence analysis indicated that this 23 bp region contains a TATA sequence, but no sequence homologous to any transcription factor binding site. Exactly how transcription from this 23 bp region is regulated remains unknown. One possibility is that the promoter may contain a sequence allowing the binding of a negative regulator, which represses BcLF1 expression or prevents the binding of TFIID to the TATA sequence during latent and early lytic stages. This negative regulator may be removed after EBV lytic replication, allowing binding of TFIID to the TATA sequence to initiate the transcription of the gene. Another possibility is that transcription of BcLF1 by TFIID may require a positive factor, which is expressed only during the lytic cycle. Furthermore, the 23 bp region which regulates BcLF1 transcription closely resembles the regulatory element in the promoter of the late gene of type 1 herpes simplex virus (HSV), which also contains a TATA sequence only (Johnson & Everett, 1986). This finding implies that these two herpesviruses may use a similar mechanism to regulate transcription of their late genes.

As is generally known, the expression of BcLF1 depends on lytic replication of the EBV genome. However, a recent study noted that a transiently transfected plasmid containing BcLF1 promoter (but lacking the oriLyt of EBV) is expressed and regulated by lytic DNA replication (Serio *et al.*, 1997). Our results confirmed this finding, and indicated that without oriLyt the promoter of BcLF1 in plasmid pVCAluc is active under lytic conditions (Fig. 3*a*). However, if an oriLyt sequence (nt 52358–53819 of the EBV genome) was inserted downstream from BcLF1 in either orientation in pVCAluc, the resulting plasmids could transcribe the luciferase activity at a level approximately 17- to 40-fold higher than that of pVCAluc (Fig. 3*b*, *c*), thereby confirming that lytic DNA replication is crucial for enhancing expression of BcLF1.

In conclusion, we have identified the +1 site of BcLF1 mRNA by primer extension. Experimental results indicated that regulation of BcLF1 transcription requires a 23 bp sequence, from nucleotides −38 to −16, containing a TATA sequence only. Although BcLF1 expression can be regulated independently of lytic replication, the presence of an oriLyt can enhance expression of BcLF1.

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