Epstein–Barr virus (EBV) EB1/Zta protein provided in trans and competent for the activation of productive cycle genes does not activate the BZLF1 gene in the EBV genome

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The Epstein–Barr Virus (EBV) gene BZLF1 encodes the transcription factor EB1 (also known as Zta) which is essential for the switch from latency to the lytic cycle: EB1 expressed from a plasmid transfected into B cell lines carrying latent EBV episomes, induces a productive viral cycle. Furthermore, EB1-specific DNA-binding sequences (ZREs) have been found in the promoters of many EBV early genes, including the BZLF1 promoter PZ and the PR promoter. At promoter PR, bicistronic mRNAs are initiated which contain, from 5' to 3', the BRLF1 and the BZLF1 open reading frames (ORFs) encoding respectively the R and EB1 proteins. The current model for the activation of the EBV lytic cycle implies that downregulation of the PZ promoter activity is a key element for latency and that a limiting step in the activation of the productive cycle is the translation of EB1. Once made, EB1 autoactivates promoter PZ, activates the PR promoter at which an mRNA coding for the EBV transcription factor R is initiated and activates the EBV early genes and the ORIlys, due to unrestricted accessibility of the EB1-responsive elements in the viral genome. We show here that EB1 expressed from a plasmid activated most if not all of the EBV early genes in the viral genome but not its own gene, BZLF1. Moreover, transfected EB1 induced the transcription of the bicistronic mRNAs from which R is efficiently translated but not EB1. Our results demonstrate that EB1 provided in trans, although competent to activate the productive cycle genes, was not sufficient to overcome the downregulation of the PZ promoter.

Introduction

The Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus that persists latently for the lifetime of the infected host. The persistence of EBV and the fact that EBV-infected B lymphocytes from the peripheral blood can proliferate continuously when cultured in vitro (immortalization) are believed to be predisposing factors for the emergence of EBV-associated human malignancies such as Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin’s disease (HD) and B and T cell lymphomas in immunocompromised individuals (Liebowitz & Kieff, 1993). Little is known about EBV persistence in vivo, and the only models for EBV latency are B cells immortalized in vitro or Burkitt’s lymphoma cell lines.

Various chemical agents, including the tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA), can cause the virus to switch from a latent to a lytic replication cycle in vitro (zur Hausen et al., 1978). The activation seems to be linked to the expression of two EBV-encoded transcription factors: the BZLF1-encoded factor EB1 (also called Z, Zta or ZEBRA; (Chevallier-Gréco et al., 1986; Takada et al., 1986; Countryman et al., 1989; Farrell et al., 1989) and the BRLF1-encoded factor, called R (or Rta) (Hardwick et al., 1988) (Fig. 1).

The current model for the reactivation of EBV in latently infected B cells growing in culture is the following. In such cells, the BZLF1 and BRLF1 genes (Fig. 1) are not detectably transcribed. Activation of the lytic cycle by an initial physiological stimulus is signalled by EB1 being translated from a 1 kb monocistronic mRNA initiated at promoter PZ and/or from 3' and 4 kb bicistronic mRNAs initiated at promoter PR and containing both the BZLF1 ORF and the BRLF1 ORF from which R is translated (Manet et al., 1989) (Fig. 1). A weak transcription of PZ and PR promoters is induced by TPA in the absence of de novo protein synthesis and probably requires the post-transcriptional activation of pre-existing cellular factors (Laux et al., 1988). Interestingly, promoter PZ contains a TRE (TPA responsive element) (Urier et al., 1989; Flemington &
and BRLF1 genes has been enlarged. The BZLF1 gene is transcribed either as a monocistronic mRNA (1.0 kb) initiated at promoter PZ, or as a bicistronic mRNA (3.0 and 4.0 kb) initiated at promoter PR from which R is translated. In promoter PZ the AP-1 site and the two EB1-responsive elements (ZREZ1 and ZREZ2) are shown. TR: Terminal Repeats; ORIP, plasmid origin of replication; ORI1, lytic origin of replication; DR and DL: early promoters overlapping with the ORIlyt.

In the schematic representation of the mRNAs initiated at promoter PR, thick lines represent ORFs, thin lines intron sequences and medium thick lines non-coding sequences.

Speck, 1990a), which binds dimeric AP-1 factors that are subject to post-transcriptional regulation induced by TPA (Fig. 1) (Angel et al., 1987, 1988). However, efficient transcription from promoters PZ and PR seems to be due to their activation by EB1. Indeed, promoter PZ contains two EB1-binding sites called here ZREZ1 and ZREZ2 (Fig. 1) and transient expression assays confirmed that EB1 autoactivates its own expression through these ZREZs (Urier et al., 1989; Flemington & Speck, 1990b). Promoter PR is also activated by EB1 through two ZREs in transient expression assays (Fig. 1) (Sinclair et al., 1991).

The model described above suggests that down-regulation of the PZ promoter activity is a key element for latency and that a limiting step in the activation of the lytic cycle is the translation of EB1. Once made, EB1 activates its own expression from promoters PZ and PR (Urier et al., 1989; Flemington & Speck, 1990b; Sinclair et al., 1991) and activates the EBV early genes (Buisson et al., 1989; Chavrier et al., 1989; Chevallier-Gréco et al., 1989; Farrell et al., 1989; Kenney et al., 1989; Lieberman et al., 1989) and the ORIlyt (Cho & Tran, 1993; Schepers et al., 1993; Fixman et al., 1995) due to unrestricted accessibility of the EB1-responsive elements in the viral genome. Accordingly, transfection of an EB1 expression plasmid in latently EBV-infected B cells, is followed by activation of all the EBV early genes including the BRLF1 gene (Chevallier-Gréco et al., 1986; Crouthyman et al., 1989) and by a productive lytic cycle (Rooney et al., 1989). These observations confirmed that most if not all the ZREs in the viral genome are accessible to transfected EB1. However, it has not been directly demonstrated that transfected EB1 can alleviate the downregulation of promoter PZ in the viral genome and that EB1 and R are translated from the bicistronic mRNAs initiated at promoter PR in the viral genome. In this study, we have directly evaluated these two points.

Our results show that although transfected EB1 can activate most, if not all, of the EBV early genes in the viral genome, it does not detectably activate transcription from promoter PZ. Moreover, in experimental conditions where transfected EB1 induces the transcription of the bicistronic mRNAs from which R is efficiently translated, neither the BZLF1 1 kb mRNA nor the EB1 protein were detectably expressed from the viral genome. The lack of autoactivation was also seen when promoter PZ was carried by a plasmid and transfected into EBV-positive B cells.

Methods

Cell lines. Akata and Raji cells (EBV-positive Burkitt’s lymphoma cell lines) were maintained in RPMI 1640 supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% heat-inactivated fetal calf serum at 37°C.

Chemical stimulation. Exponentially growing cells were treated with 20 ng/ml TPA (Sigma) and/or 3 mM-butyrate (Sigma), or were treated with anti-human IgG (Sigma) at a final concentration of 0.1 mg/ml.

Plasmids

(i) Expression vectors. The expression vectors for EB1 (pAAC-EB1) and for EB1-CAT (pAAC-EB1-CAT) have been described elsewhere (Giot et al., 1991; Mikaelian et al., 1993). Plasmid pAAC contains the cytomegalovirus (CMV) promoter and the SV40 early polyadenylation signal. The expression plasmids for Jun and Fos contain the human c-jun and c-fos cDNAs cloned in pSG5 vector (Stratagene) (a gift from I. Crenon, B. I. P., Marseille, France)

(ii) Reporter gene. Construction of the plasmid containing the pZ-CAT reporter gene (pZ225) has been described elsewhere (Urier et al., 1989).

Transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. One day before transfection, lymphoid B cells were resuspended at a density of 0.5 x 10⁶ cells/ml in fresh medium. Transfections were performed by electroporation using 10–100 µg of DNA and 5 to 10 x 10⁶ cells depending on the experiments. Cells were shocked at 220 V, at 960µF with a Zapper electroporation unit (Bio-Rad) and then incubated in fresh medium with 10% FCS at the concentration of 0.5 x 10⁶ cells/ml.

CAT assays. CAT assays were performed essentially as described previously (Gorman et al., 1982). Sonication of the cells, however, was replaced by lysis in a buffer containing 0.25 M-Tris–HCl pH 8.0 and 0.05% SDS.

Immunoblots. Cells (5 x 10⁶) were washed three times with cold PBS solution and incubated with 100 µl of lysis buffer as already described (Mikaelian et al., 1993). As measured by Bradford assay, equal amounts of protein were loaded and separated on a 10% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. The efficiency of the transfer was assayed by Ponceau Red staining then the membrane was incubated
with the primary antibody. Four antibodies were used: the monoclonal antibody mAbZ125 detects both EB1 and EB1<sup>gen4</sup> proteins; the monoclonal antibody mAbBC12 detects R; the rabbit polyclonal antibody to bacterially synthesized EB1 Leucine Zipper AbLZ<sup>gen4</sup> detects only EB1; and the rabbit polyclonal antibody to bacterially synthesized EB2 AbEB2 detects the BMLF1 protein EB2 (also called Mta). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse (for mAbZ125 and mAbBS12 antibodies) or goat anti-rabbit (for AbLZ<sup>gen1</sup> and AbEB2) immunoglobulins (Amersham). The proteins were visualized by means of the enhanced chemiluminescence kit (Amerham) as instructed by the supplier.

**RNA extraction, poly(A)<sup>+</sup> isolation and Northern blotting.** Total RNA was extracted by denaturation in guanidium isothiocyanate followed by pelleting through a CsCl cushion (Glinis et al., 1974). Poly(A)<sup>+</sup> RNA was isolated by using oligo(dT) Dynabeads (Dynal) as instructed by the supplier. Poly(A)<sup>+</sup> RNAs were then size fractionated by electrophoresis in formaldehyde agarose gels and transferred onto nylon membrane (Hybond N; Amersham). The immobilized RNAs were hybridized for 18 h at 42 °C with labelled DNA fragments in 50% formamide, 1% SDS, 10% dextran sulphate, 1 M-NaCl, 150 μg/ml herring sperm DNA and 100 μg/ml yeast tRNA. The filters were washed twice with 2x SSPE at room temperature, twice with 2x SSPE/0.1%SDS at 65 °C, once with 0.5x SSPE/0.1%SDS at 65 °C.

**Preparation and labelling of probe DNA.** The DNA probe R (see Fig. 4a) was purified after digestion of the plasmid pSP7.1 (Guffat et al., 1990) by XbaI/EcoRI and labelled using a multiprime DNA-labelling kit (Apligene). The DNA probes LZ-EB1 and DBD-EB1 (see Fig. 4) were prepared as follows: 10 ng of the pAAC-EB1 genome was digested with Tpa/I (LZ-EB1) and PstI/HindIII (DBD-EB1) purified DNA fragments were labelled by asymmetric PCR in a 100 μl reaction containing 50 mM-Tris-HCl (pH 8.3), 150 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1 mM dATP, dGTP, dTTP and 50 μCi of [α-<sup>32</sup>P]dCTP (300 μCi/mmol), 1 μM-primer and 2 U Taq polymerase. Forty cycles of PCR were performed following the temperature profile: 94 °C, 30 s; 50 °C, 30 s; 72 °C, 2 min. The sequences of the oligonucleotides used for the amplification were: primer LZ-EB1: 5’ CGACAGGAAGATATT 3’, primer DBD-EB1: 5’ GGGTAATGGCAATCCGTGACCTC 3’.

**Results**

Several reports have convincingly demonstrated that EB1 expressed from a plasmid transfected into B cell lines carrying latent EBV episomes activates transcription of the early mRNAs from the viral genome, including the bicistronic mRNAs expressed from promoter PR (Fig. 1) and the lytic cycle (Countryman et al., 1989; Rooney et al., 1989). However it has not been directly demonstrated that transfected EB1 could activate transcription from the PZ promoter in the viral genome. To clarify this point, we have constructed an EB1<sup>gen4</sup> hybrid protein, in which the dimerization domain of EB1 has been replaced by the dimerization domain of the yeast transcription factor GCN4 (Fig. 2a). Such an hybrid protein can be differentiated from the EB1 protein translated from mRNAs transcribed from the viral genome by the use of two antibodies, mAbZ125 and AbLZ<sup>gen1</sup> (Fig. 2a and Methods).

**EB1 and EB1<sup>gen4</sup> expressed from a transfected plasmid activate the expression of early genes in the EBV viral genome**

In order to compare functionally the EB1 protein and the hybrid protein EB1<sup>gen4</sup>, their relative stability and transcriptional potential were evaluated following transient expression in Raji cells. Upon transfection of the expression vector pAAC-EB1<sup>gen4</sup>, the EB1<sup>gen4</sup> protein was detected using mAbZ125 (Fig. 2b, lane 4) and that of the EB1 protein expressed after transfection of Raji cells with the expression vector pAAC-EB1 (Fig. 2b, lanes 2 and 3), or induced from the viral genome in Raji cells treated by TPA plus butyrate (TPA/BA) (Fig. 2b, lane 1). Comparable amounts of EB1 and EB1<sup>gen4</sup> proteins accumulated, suggesting a similar stability (Fig. 2b, compare lanes 2 and 3 to lane 4). As expected, the antibody AbLZ<sup>gen1</sup> detected the EB1 proteins expressed from pAAC-EB1 (Fig. 2b, lanes 14 and 15), and induced by TPA/BA from the viral genome (Fig. 2b, lane 13), but did not detect the EB1<sup>gen4</sup> protein (Fig. 2b, lane 16). The transfected EB1 and EB1<sup>gen4</sup> proteins both induced expression of the EBV early antigens which were detected using a human antibody (not shown). Both proteins also induced expression of the EBV early protein R (Fig. 2b,
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Fig. 3. Induction of EBV early proteins in Akata cells transfected with pAAC-EB1\textsuperscript{gen4} or treated with TPA/BA or with anti-IgG. (a). Akata cells were mock-transfected or transfected with the indicated quantities of pAAC-EB1\textsuperscript{gen4} (pEB1\textsuperscript{gen4}) or treated for 48 h with TPA/BA or for 12 h with anti-IgG. (b). Visualization of the EBV early protein EB1 (AbLZ\textsuperscript{eb1}), R (mAb8C2) and EB2 (AbEB2) expressed in Akata cells treated with increasing quantities of pAAC-EB1 (lanes 2–4) or with TPA/BA (lane 5) or with anti-IgG (lane 6). mAbZ125 was used to visualize the EB1\textsuperscript{gen4} proteins expressed from transfected plasmid pAAC-EB1\textsuperscript{gen4}.

lanes 5 to 8) and of the BMLF1-encoded EBV early protein EB2 (Fig. 2b, lanes 9 to 12). These results demonstrate that when transiently expressed in Raji cells, EB1\textsuperscript{gen4} and EB1 were functionally equivalent and activated the transcription of EBV early genes including transcription from the promoter controlling the expression of the bicistronic mRNAs from which R is translated.

**EB1\textsuperscript{gen4} expressed from a transfected plasmid does not activate the expression of EB1 from the viral genome**

Surprisingly, in Raji cells transfected with pAAC-EB1\textsuperscript{gen4}, no EB1 protein expressed from the viral genome could be detected using AbLZ\textsuperscript{eb1} (Fig. 2b, lane 16). However, it must be emphasized that the amounts of R (Fig. 2b, lanes 6, 7 and 8) and EB2 (Fig. 2b, lanes 10, 11 and 12) proteins induced by transfected EB1 and EB1\textsuperscript{gen4} were very low compared to the level of these proteins induced by TPA/BA (Fig. 2b, lanes 5 and 9). Transfected EB1\textsuperscript{gen4} might therefore, have induced the expression of EB1 from the viral genome, but in amounts undetectable by our methods. In order to rule out that this observation was specific to Raji cells, the EB1\textsuperscript{gen4} expression plasmid was transfected in increasing amounts in Akata cells. As shown in Fig. 3(b), in Akata cells transfected with plasmid pAAC-EB1\textsuperscript{gen4}, the EB1\textsuperscript{gen4} protein was expressed (mAbZ125, lanes 2 to 4) and migrated slightly faster than the EB1 protein induced from the viral genome by TPA/BA (mAbZ125, lane 5) or anti-IgG (mAbZ125, lane 6). The amount of EB1\textsuperscript{gen4} protein expressed increased with the amount of expression plasmid transfected (Fig. 3b, mAbZ125, lanes 2 to 4).

However, transfected EB1\textsuperscript{gen4} again inefficiently induced the R (Fig. 3b, mAb8C2, lanes 2 to 4) and EB2 proteins (Fig. 3b, lanes 2 to 4) from the viral genome and did not detectably induce EB1 protein from the viral genome (Fig. 3b, AbLZ\textsuperscript{eb1}, lanes 2 to 4). Moreover, the increase in the amount of EB1\textsuperscript{gen4} protein expressed was not followed by an increase in the amount of R and EB2 proteins induced (Fig. 3b, mAb8C2 and AbEB2, lanes 2, 3 and 4), the maximum induction level being obtained after transfection of 25 \( \mu \)g of plasmid pAAC-EB1\textsuperscript{gen4} (lane 2).

In conclusion, these results suggest that transfected EB1\textsuperscript{gen4} could not induce transcription from the PZ promoter of the 1 kb mRNA carrying the BZLF1 ORF, and that only R was detectably translated from the bicistronic mRNAs carrying both the BZLF1 and the BRLF1 ORFs.

**Promoter PZ in the Raji viral genome is not detectably activated by transfected EB1\textsuperscript{gen4}**

To rule out the possibility that the 1 kb mRNA initiated at promoter PZ (see Fig. 1) was induced by EB1\textsuperscript{gen4} in Raji cells but was not detectably translated, we used Northern blotting to analyse the mRNAs transcribed from the BZLF1/BRLF1 region in Raji cells treated by TPA/BA or transfected with the expression vector pAAC-EB1\textsuperscript{gen4}. Three \( ^{32} \)P-labelled DNA fragments were hybridized to the Northern blots (Fig. 4a). Probe DBD-EB1 covers only the third exon of BZLF1, corresponding to the EB1 dimerization domain, and is therefore specific for the mRNAs transcribed from promoters PZ and PR in the viral genome (Fig. 4a). Probe DBD-EB1 covers the BZLF1 second exon and should detect EB1\textsuperscript{gen4} mRNA expressed from the transfected plasmid, the 1 kb mRNA and the bicistronic mRNAs transcribed from the viral genome (Fig. 4a). Probe R covers the BRLF1 ORF and should only detect the bicistronic mRNAs transcribed from the viral genome (Fig. 4a).

As shown in Fig. 4(b), two major RNA species, 3.0 kb and 1.0 kb long, were detected with probe DBD-EB1 in the poly(A)\(^+\) RNAs from Raji cells treated by TPA/BA (lane 2). The 3.0 kb RNA species corresponds to bicistronic mRNAs since it was also detected by the R DNA probe (lane 6) (the 4 kb mRNA species is less abundant). The 1.0 kb RNA species induced by TPA/BA, corresponds to the monocistronic mRNA initiated at promoter PZ, since it was detected by the DBD-EB1 DNA probe (lane 2), by the LZ-EB1 DNA probe (lane 4) but not by the R DNA probe (lane 6).

In Raji cells transfected with plasmid pAAC-EB1\textsuperscript{gen4}, although a low level of expression of R protein was detected (Fig. 2b, mAb8C2, lane 8), the 3.0 kb mRNA species was not detected by the DNA probes DBD-EB1
(a) pAAC pEB1<sup>gen4</sup> 3 mM Butyrate

(b) TPA/Butyrate

(c) R

Fig. 5. Butyrate-increased expression of transfected EB1<sup>gen4</sup> increases the expression of EB1, R and EB2 from the Raji viral genome. (a) Raji cells were either treated by TPA plus butyrate, or mock-transfected (pAAC, 10 µg/10<sup>6</sup> cells) or transfected with pAAC-EB1<sup>gen4</sup> (pEB1<sup>gen4</sup>, 10 µg/10<sup>6</sup> cells). Western blot (b) and Northern blot (c) analysis of proteins and poly(A)<sup>+</sup> RNAs isolated from Raji cells treated as indicated in the upper part of the panel. The antibodies used for the immunodetection are indicated at the left of (b); the position and the size of the mRNAs detected by DBD-EB1 and LZ-EB1 probes are indicated by arrows on the right-hand side of (c).

Butyrate-increased expression of transfected EB1<sup>gen4</sup> is followed by increased expression of EB1 and R from the viral genome

The transfected EB1<sup>gen4</sup> protein, when expressed in Raji cells, induced only very inefficiently the expression of the EBV early proteins R (Fig. 2b, mAb8C2) and EB2 (Fig. 2b, AbEB2), whereas the expression of the early protein EB1 was not detectable (Fig. 2b, AbLZ<sub>eb</sub>). This was also seen at the level of the transcripts initiated at promoter PZ and PR in the viral genome (Fig. 4b). Since butyrate potentiates the activation of the EBV early gene expression by TPA (Ito et al., 1981), we reasoned that butyrate might also potentiate the effect of transfected EB1<sup>gen4</sup>

As shown in Fig. 5(b), treatment of Raji cells with butyrate was not followed by a detectable expression of R (mAb8C2, lane 2) or EB2 (AbEB2, lane 2). In Raji cells transfected with pAAC-EB1<sup>gen4</sup>, EB1<sup>gen4</sup> (mAbZ125, lane 3) was expressed but only the induction of R from the viral genome was weakly detectable (mAb8C2, lane 3). Butyrate increased the amount of EB1<sup>gen4</sup> protein expressed from plasmid pAAC-EB1<sup>gen4</sup> (mAbZ125, compare lane 3 to lane 4) and efficient expression from the viral genome of both R (mAb8C2, lane 4) and EB2 (AbEB2, lane 4) proteins was also observed, their expression was as efficient as that observed in Raji cells treated with TPA/BA (mAb8C2 and AbEB2, lanes 5). Moreover, in Raji cells transfected with plasmid pAAC-EB1<sup>gen4</sup> and treated with butyrate, the EB1 protein was now expressed from the viral genome (AbLZ<sub>eb</sub>, lane 4), although in very low amounts compared to R and EB2 (mAb8C2 and AbEB2, lanes 4). Thus, it would appear that EB1<sup>gen4</sup> plus butyrate induced, although very inefficiently, the expression of viral mRNAs from which EB1 could be translated. To characterize these mRNAs, we analysed by Northern
blotting the poly(A)$^+$ RNAs transcribed from the BZLF1/BRLF1 region in Raji cells transfected with the expression vector pAAC-EB1$^{gen4}$ and treated or not with butyrate.

As shown in Fig. 5(c), for the poly(A)$^+$ RNAs isolated from Raji cells transfected with plasmid pAAC-EB1$^{gen4}$, the $^{32}$P-labelled DBD-EB1 probe hybridized to a 1.5 kb RNA species corresponding to the EB1$^{gen4}$ mRNA (upper panel, lane 3), but did not detect the 1 kb RNA species found in the poly(A)$^+$ RNAs isolated from Raji cells induced by TPA/BA (upper panel, lane 5). Moreover, although the R protein was detectably induced by transfected EB1$^{gen4}$ (Fig. 5b, mAb8C2, lane 3), no 30 kb RNA could be detected (Fig. 5c, upper panel, lane 3). However, when pAAC-EB1$^{gen4}$ transfected Raji cells were treated with butyrate, more 1-5 kb mRNA was expressed from the plasmid (Fig. 5c, upper panel, lane 4), corresponding to the increased expression of EB1$^{gen4}$ protein (Fig. 5b, mAbZ125, lane 4). The 30 kb mRNA was now detectable and abundant, but no 1 kb mRNA expressed from promoter PZ could be detected (Fig. 5c, upper panel, lane 4), despite a clear but weak expression of EB1 protein from the viral genome (Fig. 5b, AbLZ$^{emb}$, lane 4). To ascertain that the 1-0 kb transcript was not detectably transcribed from the PZ promoter, we used as a probe the $^{32}$P-labelled DNA fragment LZ-EB1 which should not hybridize to the EB1$^{gen4}$ 1.5 kb mRNAs expressed from the transfected plasmid. As shown in Fig. 5(c), lower panel, lane 4, a weak hybridization with the EB1$^{gen4}$ 1.5 kb transcript was observed probably due to the large amount of 1-5 kb mRNA. However and more importantly, the bicistronic mRNA was strongly detected but again no 1 kb mRNA could be seen (Fig. 5c, lower panel, lane 4). In conclusion, the PZ promoter, when compared to the PR promoter, is very poorly, if at all, activated by EB1$^{gen4}$ in Raji cells. Moreover, in conditions where efficient expression of the R protein was detected (Fig. 5b, AbLZ$^{emb}$, lane 4), very little EB1 protein is detected (Fig. 5b, AbLZ$^{emb}$, lane 4) demonstrating that the BZLF1 ORF is poorly translated from the bicistronic mRNAs.

**Promoter PZ transfected in Raji cells is also not activated by EB1**

It is difficult to formally conclude that promoter PZ is not activated by EB1$^{gen4}$ in the viral genome, since a very small amount of EB1 protein was found in Raji cells transfected with pAAC-EB1$^{gen4}$ and treated with butyrate. However, showing that the promoter PZ transfected on a plasmid in Raji cells would also not respond to EB1 or EB1$^{gen4}$, would reinforce the idea that it is also not activated in the viral genome. The PZ promoter carrying the two EB1 binding sites and the AP-1 site was cloned upstream of the CAT gene in plasmid PZ-CAT (Fig. 6a) (Urier et al., 1989). Upon transfection in Raji cells, PZ-CAT had a weak basal activity (Fig. 6b, lanes 1 and 5), which was not increased by treatment of the cells with 3 mm-butyrate (Fig. 6b, lanes 9 and 13). Neither EB1$^{gen4}$ (Fig. 6b, lanes 2 to 4), nor EB1 (Fig. 6b, lanes 6 to 8) increased the PZ-CAT basal activity, although EB1$^{gen4}$ (Fig. 6c, lanes 2 to 4) and EB1 (Fig. 6c, lanes 6 to 8) proteins were detectably expressed. Moreover, PZ-CAT activity was also not influenced by EB1$^{gen4}$ (Fig. 6b, lanes 10 to 12) nor by EB1 (Fig. 6b, lanes 14 to 16) in the presence of 3 mm-butyrate, although butyrate dramatically increased the amount of EB1$^{gen4}$ (Fig. 6c, mAbZ125, lanes 10 to 12) or of EB1 proteins (Fig. 6c, mAbZ125, lanes 14 to 16) expressed from the transfected plasmids. The transfected EB1$^{gen4}$ and EB1 proteins were functionally active since both induced the expression of the R protein from the Raji viral genome (Fig. 6c, mAb8C12, lanes 10 to 12 and 14 to 16).
As a further control for functionality, Raji cells were treated with butyrate and cotransfected with PZ-CAT together with Jun plus Fos, or Jun plus Fos plus EB1\(\text{gen4}\) expressed from appropriate expression vectors. The basal activity of PZ-CAT (Fig. 6b, lane 17) was activated by Jun plus Fos (Fig. 6b, lane 18), but the Jun/Fos activated transcription of PZ-CAT was not further affected by EB1\(\text{gen4}\) (Fig. 6b, lane 19). Thus, the PZ promoter was potentially functional, and overexpression of EB1\(\text{gen4}\) did not prevent its activation by Jun and Fos. In conclusion, the PZ promoter was not activated by EB1 in Raji cells both in the viral genome and when carried on a plasmid.

Discussion

In the current model for the activation of the EBV lytic cycle genes (Fig. 1), one limiting step is the translation of EB1 and the autoactivation of promoter PZ. Once made, EB1 will bind to EB1-responsive elements in the viral genome and activate transcription. If this is true, then transfection of an EB1 expression plasmid in EBV-latently infected with EBV was not sufficient to overcome the downregulation of promoter PZ. These results suggest that the expression of an EB1 protein competent for lytic cycle activation in B cells latently infected with EBV was not sufficient to overcome the downregulation of promoter PZ. This downregulation could be due to a repressor, to a lack of competent cellular factors or both. Interestingly, negatively cis-acting elements have been identified in the PZ promoter (Schwarzmann et al., 1994; Montalvo et al., 1995). Moreover, the cellular repressor YY1 has been shown to bind in vitro to two elements in the PZ promoter and a loss of YY1 binding-activity was observed after TPA treatment (Montalvo et al., 1995). Whether these negatively cis-acting elements are occupied by factors in the viral genome in the presence of EB1 has not yet been evaluated. Moreover, in PZ-CAT, the minimum PZ promoter devoid of all negatively cis-acting elements except for that located next to the AP-1 site, responded to Jun and Fos but did not respond to EB1. These results strongly suggest that the lack of EB1-responsiveness of promoter PZ is not directly linked to the presence of repressors. Viral latency has been proposed to be maintained via antisense hybridization of EBNA1 and EB1 hnRNAs (Prang et al., 1995), via the direct interaction of EB1 with NF-\(\kappa\)B p65 (Gutsch et al., 1994), the retinoic acid receptor (Sista et al., 1993, 1995) or p53 (Zhang et al., 1994) leading to inactivation of the EB1 transactivating function. Our results clearly show that the function of transfected EB1 is not inhibited by any of the factors described above, since transfected EB1 proteins activated many if not all of the EBV early genes in the viral genome. Furthermore, as shown in Fig. 2 (lanes 2 to 4), EB1 and EB1\(\text{gen4}\) were functionally equivalent as they induced both the BRLF1 and BMLF1 genes from which R and EB2 mRNAs were transcribed. However, these activations were very inefficient when compared to the induction of the BRLF1 and BMLF1 genes in Raji cells treated with TPA and butyrate, even though the amounts of EB1 proteins expressed were almost equivalent in extracts prepared from TPA/BAtreated cells or from Raji cells transiently expressing EB1 or EB1\(\text{gen4}\) (see Fig. 2b). It could be that EB1 induced by TPA is a more competent transcription factor than EB1 expressed from a plasmid, due to post-transcriptional modifications induced by TPA (Daibata et al., 1992; Hunter & Karin, 1992; Kolman et al., 1993). Another possibility is that butyrate-induced chromatin alterations (Klehr et al., 1992) in the viral genome render the BRLF1 and the BMLF1 promoters more accessible to EB1. However, although TPA alone did not affect the transcription of the early genes in Raji cells expressing transfected EB1\(\text{gen4}\) (data not shown), butyrate dramatically increased the amount of EB1\(\text{gen4}\) protein expressed from the transfected plasmid and a strong induction from the viral genome of R, EB2 and to a much lower extent EB1, was observed (see Fig. 5b). The increased expression of R, EB2 and EB1 from the viral genome was probably due to the large amount of EB1\(\text{gen4}\) protein translated from butyrate-induced BZLF1 mRNAs initiated at the CMV promoter in plasmid pAAC-EB1\(\text{gen4}\) (see Fig. 5c). It should be noted that the CMV promoter was found to be responsive to butyrate although the herpes simplex virus thymidine kinase (HSV-tk) promoter was not (not shown). The effect of butyrate is therefore likely to be due to a direct induction of promoter CMV in plasmid pAAC-EB1\(\text{gen4}\) rather than an effect on the EBV chromatin. Finally, it is possible that EB1 and EB1\(\text{gen4}\) are not equivalent at activating the PZ promoter. However, this is unlikely. Indeed, the ‘authentic’ P3HR1 EB1 protein can be clearly separated by SDS-PAGE from EB1 expressed from the Raji genome (Manet et al., 1989). When EB1-P3HR1 was expressed in Raji cells, it activated all the EBV early promoters except the PZ promoter (not shown).

Increased amounts of EB1\(\text{gen4}\) in transfected Raji cells treated with butyrate strongly induced the mRNAs carrying both the BZLF1 and the BRLF1 ORFs from which R was efficiently translated but not EB1. However, even in these conditions, the 10 kb mRNA carrying the BZLF1 ORF and initiated at promoter PZ was
undetectable (Fig. 5c), although some EB1 protein was translated from mRNAs initiated at promoters in the viral genome (Fig. 5b). Thus, it remains to be established whether the EB1 protein expressed from the viral genome is due to an undetectable induction of the PZ promoter or to inefficient translation of the EB1 protein from bicistronic mRNAs. Nevertheless, in conditions where efficient expression of the 3-0 kb mRNA was detected (Fig. 5c, lane 4) and efficient translation of R from this mRNA was observed (Fig. 5b, mAb8C2, lane 4), very little EB1 protein was detected (Fig. 5b, AbLZ 


the Z transactivator is mediated in a cell-type-specific manner by two independent signal regions. *Journal of Virology* **63**, 3040–3050.


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