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# Demethylation of the Epstein-Barr virus origin of lytic replication and of the immediate early gene BZLF1 is DNA replication independent

Brief Report

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**Summary.** Epstein-Barr virus (EBV) episomal DNA is extensively methylated in Burkitt lymphoma derived cell lines. In this study we examined whether lytic viral cycle reactivation is dependent on demethylation of critical viral genes. Viral replication was induced in the Burkitt's lymphoma cell line Daudi by the combination of 12-O-tetradecanoylphorbol-13-acetate (TPA) and sodium-butyrate. Two regions necessary for EBV replication, the BZLF1 immediate early region and the origin of lytic cycle replication (ori Lyt) were demethylated during the early phase of the lytic virus cycle. Demethylation was observed while production of new (unmethylated) viral DNA was blocked by phosphonoformic acid (PFA). This suggests that demethylation, which may be instrumental for the onset of the lytic cycle, is an active process independent of viral DNA replication.

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EBV causes infectious mononucleosis (IM) [14] and it is also associated with several human malignancies such as Burkitt's lymphoma (BL) [9], nasopharyngeal carcinoma (NPC) [40], gastric adenocarcinomas [19] and T-cell lymphomas [1]. The latent episomal, nuclear, EB-viral genome is in many ways comparable to cellular genes and is thought to be regulated by similar mechanisms including methylation.

Mammalian genomes are methylated, preferentially in CpG dinucleotides. Approximately 70% of all CpG's are methylated. This methylation is involved in regulation of gene expression [6, 7], and is essential in development, as knock-out mice for the DNA maintenance methylase shows a lethal phenotype at the 8-cells

stage embryo [24]. Both in vivo and in vitro it has been demonstrated that one important function of methylation is modulation of promoter functions.

Unmethylated promoters are easier activated by transcription factors, and methylation of promoters results in lower transcription. In part, this is mediated by methylcytosine binding proteins (MCBP's), which non-specifically recognize and bind to one or several CpGs. Several such proteins are known, including methyl-CpG binding protein 1 = MeCP1 and MeCp2 [25, 30, 31]. In contrast to promoter methylation, CpG methylation in open reading frames is not known to influence the transcription process, and the role, if any, of such methylation is unknown. Methylation has also been proposed to have a role in general organization of the eukaryotic genome into active and inactive regions with respect to gene transcription. Methylation may facilitate loss of expression of genes which are no longer required, and potentially dangerous to the organism [3, 4, 38].

DNA of some mammalian viruses is methylated, either in the nucleocapsid, e.g. Human Papilloma virus [5] or during intracellular persistence [8, 16]. Epstein-Barr virus (EBV) DNA, while it is in a semi-latent state in B cells or epithelial cells is methylated [10, 33]. Previously we have shown that the intracellular EBV DNA is extensively methylated during latent infection of tumor derived B cell lines and in tumor tissue from BL and NPC, with the exception of active promoter regions and the origin of latent DNA replication (oriP) [10, 11]. The role of this methylation has not been clearly identified, but a strict correlation between the unmethylated LMP 1 promoter (LRS) and expression of LMP 1 has been shown [18].

Although methylation has to date only been studied in tumors and tumor derived cell lines, it may reflect a physiologic state of latency in healthy EBV-carriers. Several studies indirectly suggest a positive correlation between the level of demethylation in such cell lines and entry into the lytic cycle [12, 26, 28, 34, 39], although the opposite has also been suggested [35]. In particular our own studies showed that 5-azacytidine (5-AzaC), a well-established methylation inhibitor, can induce the lytic cycle in some BL derived cell lines [28]. It has also been shown that both the cellular and viral DNA of the BL derived cell line, P3HRI, was hypomethylated upon treatment with phorbol ester and sodium butyrate, by measuring overall CpG status with thin-layer chromatography [39]. Although viral replication was not inhibited, they concluded that demethylation took place early and, therefore, was likely to be due to an active process rather than due to blockage of maintenance methylation.

The earliest known marker of re-activation of the lytic viral cycle from latency is the transcriptional activation of the BZLF1 gene. In the latently infected cells, no virus particles are produced. During the lytic infection, the viral DNA is amplified via one or both of the origins of lytic replication (ori LytR and ori LytL) [13]. Many EBV strains carry two copies of ori Lyt, although only one copy is required. In this study we show that induction of the lytic cycle correlates to demethylation of ori Lyts and the BZLF1-gene and that this demethylation does not require replication of viral DNA.

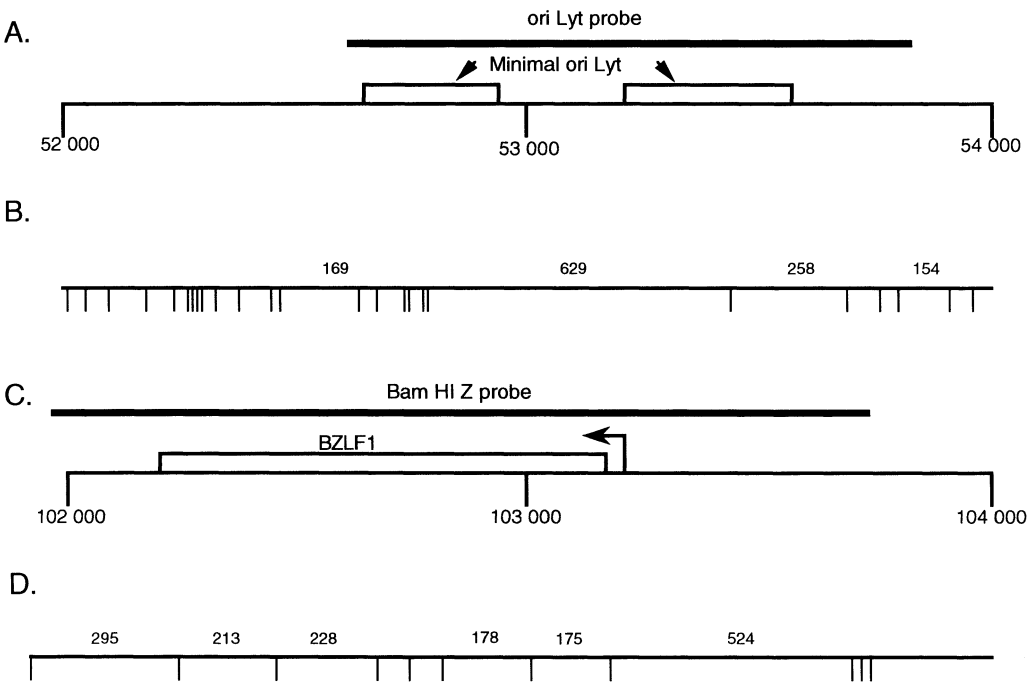


**Fig. 1.** Schematic representation of the B95-8 genome. **A** The bold horizontal bar represents the probe used for analyzing the ori LytL region. The locations of the minimal ori Lyt in the B95-8 genome are indicated. **B** Expected restriction sites when the DNA is digested with the restriction enzyme MspI, as derived from the prototype EBV B95-8 sequence. Numbers indicate expected fragment sizes for fragments over 100 base pairs. **C** The bold horizontal bar represents the BamHI Z probe used for analyzing the BZLF1 gene and promoter. The location of the BZLF1 promoter and the coordinates in the B95-8 genome are indicated. **D** Expected restriction fragment sizes when the DNA is digested with the restriction enzyme MspI, as derived from the prototype EBV B95-8 sequence

In this study we induced the lytic virus cycle in the EBV positive BL cell line Daudi [21] by treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA; 20 ng/ml, Sigma Chemical Co., St. Louis, MO) and Na-butyrate (3 mM). B95-8 and K562 served as positive and negative controls, respectively [32] [27].

In order to block viral replication after the induction of the lytic virus cycle 15 μg/ml Foscarnet (PFA) was added to the cultures 12 h after induction. As a control, DNA was demethylated by treating cells with 4 mM of 5-AzaC (Sigma). Cells were harvested at three different time points after induction (between 27–51 h). To monitor the level of induction, cells were analyzed for early antigen (EA, Fig. 2A) and viral capsid antigen (VCA, Fig. 2B), by direct immunofluorescence, using fluorescein isothiocyanate- or tetramethyl-rhodamine isothiocyanate-conjugated sera from BL (EA) or NPC (VCA) patients, according to the established protocols [15, 22].

The methylation pattern was analyzed by digesting the DNA with the restriction enzyme MspI, and its methylation sensitive isoschizomer HpaII. These two enzymes recognize the sequence CCGG; HpaII does not cut when the second base

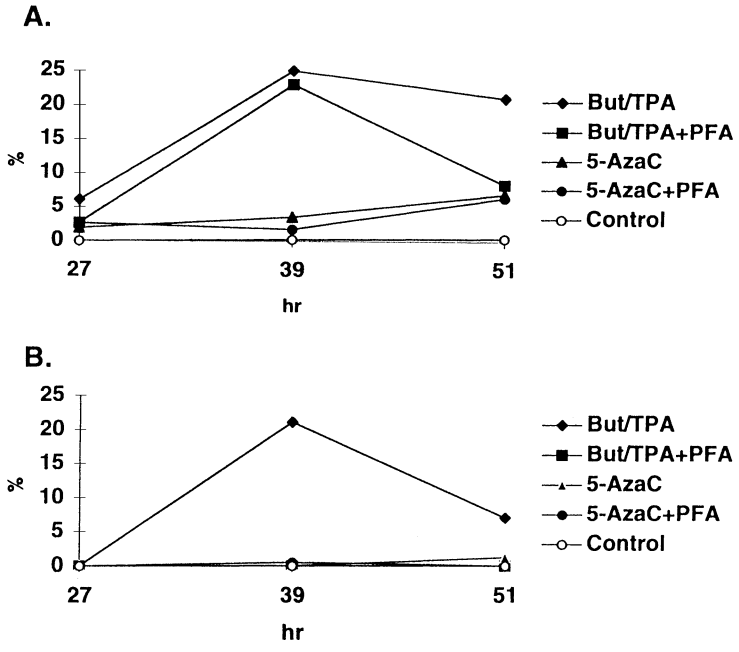


**Fig. 2.** Immunofluorescence detection of EA (**A**) and VCA (**B**) over time in Daudi cells after treatment with drugs in 4 different combinations: sodium butyrate and TPA; sodium butyrate, TPA and PFA; 5-AzaC alone; and 5-AzaC and PFA. Level of protein expression is indicated as % positive cells of the total number of cells

C is methylated. To be able to analyze DNA fragments in the range 100 bp–650 bp the DNA was separated on an 10% polyacrylamide gel in TBE buffer followed by Southern blotting as described previously [11]. Figure 1 shows the probes used in this study.

Prior to induction, no EA or VCA production was seen in Daudi cells, as determined by immunofluorescence (data not shown). Twenty-seven hours after treatment with the different inducers, few of the cells contained EA protein, while no VCA was detected (Fig. 2A and B).

Thirty-nine hours post-treatment, 23% and 25% of the cells treated with butyrate and TPA, with and without PFA respectively, stained positive for EA, indicating that the addition of PFA does not affect the EA production. Of cells treated with 5-AzaC and PFA, 5% stained positive for EA. Treatment with PFA blocked VCA protein expression completely at all time points, i.e. no viral capsid protein was made.

We have shown previously that methylation of the EBV genomes differs depending on form of latency. The only region that was found to be unmethylated in all cell lines and tumors tested was the ori P. Origins of lytic replication (ori Lyt) were found to be unmethylated in lymphoblastoid cell lines, but methylated in BL type I. The ori LytL and the BZLF1 regions are highly methylated in the BL cell line Daudi. The effect of treatment with the inducers and PFA on the methylation pattern showed that both genes became gradually unmethylated afters

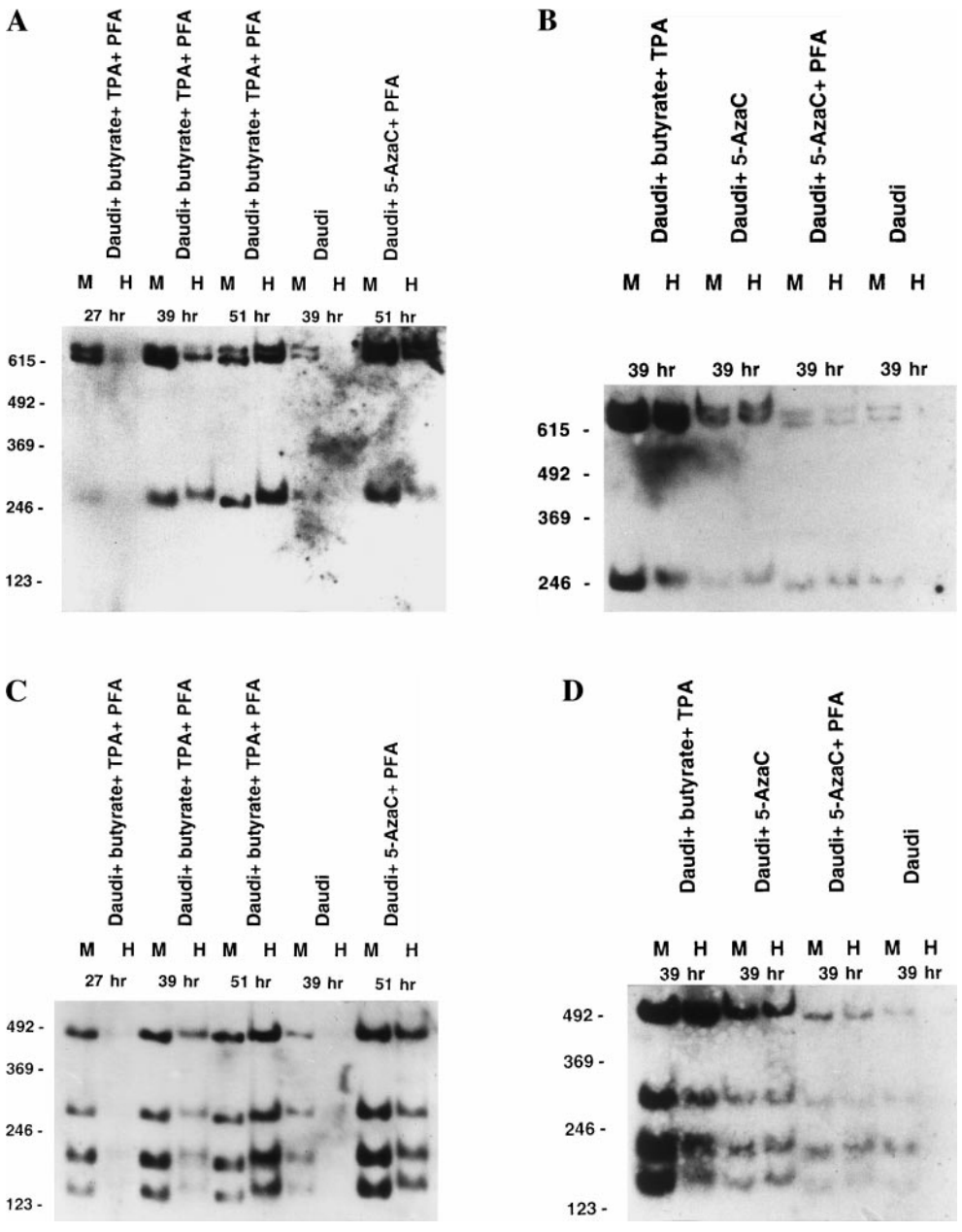


**Fig. 3.** The effect of different inducing agents on the EBV DNA methylation state over time. The ori Lyt region and the BZLF1 gene in Daudi cells at different time points after treatment with drug combinations: sodium butyrate and TPA; sodium butyrate, TPA and PFA; 5-AzaC alone; 5-AzaC and PFA. The DNA was digested with MspI (*M*) and HpaII (*H*) respectively, separated on a 10% polyacrylamide gel, and transfer to filters for Southern blot analysis. Positions of the molecular weight markers are indicated to the left of the blots. **A**, **B** Filter hybridized with an ori LytL probe (coordinates 52 620–53 817). **C**, **D** Blots A and B stripped and reprobed with the BamHI Z fragment (coordinates 10 1947–10 3751 in the B95-8 genome

induction. This demethylation occurs even in the presence of PFA (Fig. 3A–D). Whiles B95-8 has a deletion including ori LytR, the EBV genome in the Daudi cell line has two ori Lyts (ori LytL and ori LytR) which are partially homologous in sequence. Thus, one of the fragments produced from the Daudi cells in Fig. 3 (>600 bp) is most likely derived from ori LytR. This fragment also appears to be methylated before treatment, and becomes demethylated upon induction. Demethylation is seen by 27 h, at which time only the early phase of the viral cycle has been initiated, suggesting that demethylation is an early event occurring at or before the time of the BZLF1 gene transcription.

It has been clearly demonstrated that once the methylation pattern has been established in a DNA region, whether viral or cellular, it is maintained, following the production of newly synthesized DNA during the S-phase, by a maintenance-methylation system [17, 37]. However, there is little known about how the specific methylation pattern is established and modified. It is likely that this is mediated by specific methylases and demethylases, the specificity of which maybe modified by other chromatin associated proteins [2, 20]. In the case of EBV, DNA within the virion is unmethylated [23, 26]. During reactivation to lytic replication, using the methylated, latent, episomes as template, it is likely that either active demethylation of the EBV episome must take place, or the function of the maintenance-methylase is blocked, such that newly synthesized EBV DNA will not be methylated. We have found evidence that two regions instrumental for EBV replication, the BZLF1 immediate early region and the lytic origin of replication (ori Lyt) are, in fact, being demethylated during the early phases of lytic virus replication. Demethylation took place under circumstances where the production of new (unmethylated) linear viral DNA was completely blocked. Demethylation is, therefore, an active process independent of viral replication. On the other hand demethylation is not sufficient for lytic cycle induction as only a proportion of the cells entered lytic cycle.

It remains to be determined whether this demethylation is mediated by one of the immediate early viral gene products itself, or by a cellular protein induced by agents such as TPA and/or sodium butyrate. In either case demethylation maybe an instrumental first step to achieve progression into the lytic cycle. Demethylation in the BL-derived cell line P3HRI have been observed, but not in Raji or Jijoye cells, upon induction by a similar protocol [39]. In that study, however, DNA synthesis was reduced, but not blocked by the treatment with PFA (3.5% of cells expressed VCA protein) suggesting that the demethylation of the viral DNA in their study, was mainly due to viral DNA replication. They also showed that the cellular DNA became demethylated in their study. Thus, they favored the hypothesis that a cellular protein, which is induced by the treatment, executes DNA demethylation. Nonkwelo and Long have shown that in vitro methylation of constructs from the divergent promoter controlling the expression of BZLF1 and BRLF1, resulted in reduced transcription [34]. They also showed that methylation of ori Lyt reduced DNA replication. These data together with our own suggest that the demethylation that we observe early during the lytic cycle, may contribute to induction of the lytic cycle.

Treatment of patients carrying EBV positive tumors with demethylating agents has been suggested, in order to induce immunogenic targets for CTLs. Another interesting and beneficial effect of such treatment may be the desctruction of tumor cells due to the induction of the lytic cycle, and boostering of the immune response to the virus, as suggested by this and earlier studies showing that demethylation correlate to induction of the lytic cycle [29].

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