Histone Hyperacetylation Occurs on Promoters of Lytic Cycle Regulatory Genes in Epstein-Barr Virus-Infected Cell Lines which are Refractory to Disruption of Latency by Histone Deacetylase Inhibitors

Jill K. Countryman a)
Lyndle Gradoville b)
George Miller a,b,c)*

Departments of a) Molecular Biophysics and Biochemistry, b) Pediatrics and c) Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06520

*Communicating author
Tel.: 203-785-4758
Fax: 203-785-6961
Email: George.Miller@yale.edu
ABSTRACT

Activation of the Epstein-Barr virus (EBV) lytic cycle is mediated through the combined action of ZEBRA and Rta, the products of the viral BZLF1 and BRLF1 genes. During latency these two genes are tightly repressed. Histone deacetylase inhibitors can activate viral lytic gene expression. Therefore, a widely held hypothesis is that Zp and Rp, the promoters for BZLF1 and BRLF1, are repressed by chromatin and that hyperacetylation of histone tails, by allowing the access of positively acting factors, leads to transcription of BZLF1 and BRLF1. To investigate this hypothesis we used Chromatin Immunoprecipitation (ChIP) to examine the acetylation and phosphorylation state of histones H3 and H4 on Zp and Rp in three cell lines, Raji, B95-8, and HH514-16, which differ in their response to EBV lytic induction by histone deacetylase inhibitors (HDACi). We studied the effects of three HDAC inhibitors, sodium butyrate (NaB), trichostatin A (TSA) and valproic acid (VPA). We also examined the effects of tetradecanoyl phorbol acetate (TPA) and 5-aza 2’-deoxycytidine (AZC), a DNA-methyltransferase inhibitor, on histone modification. In Raji cells, TPA and NaB act synergistically to activate the EB lytic cycle and promote an increase in histone H3 and H4 acetylation and phosphorylation at Zp and Rp. Surprisingly, however, when Raji cells were treated with NaB or TSA, neither of which is sufficient to activate the lytic cycle, an increase of comparable magnitude of hyperacetylated and phosphorylated histone H3 at Zp and Rp was observed. In B95-8, NaB inhibited lytic induction by TPA, yet NaB promoted hyperacetylation of H3 and H4. In HH514-16 cells NaB and TSA strongly activated the EBV lytic cycle and caused hyperacetylation of histone H3 on Zp and Rp. However, when HH514-16 cells were treated with VPA, lytic cycle mRNAs or proteins were not induced, although histone H3 was hyperacetylated as measured by immunoblotting or by ChIP on Zp and Rp. Taken together, our data suggests that open chromatin at EBV BZLF1 and BRLF1 promoters is not sufficient to activate EBV lytic cycle gene expression.
INTRODUCTION

The short chain fatty acid n-butyric acid, or its salt, sodium butyrate (NaB), promotes differentiation and decreases growth of eukaryotic cells. In early experiments, butyrate caused Chinese hamster ovary cells to assume a fibroblastic shape (76), promoted differentiation of Friend erythroleukemia cells leading to production of $\beta$ globin (40) and induced neurite formation in cloned murine neuroblastoma cells (67). In chick embryo fibroblasts and in HeLa cells n-butyric acid arrested cell proliferation (31). A clue to the mechanism of action of butyrate was that histone hyperacetylation was observed in HeLa and Friend leukemia cells within one hour after treatment (62). The pattern of histone hyperacetylation was reversible when n-butyrate was removed.

NaB does not directly promote acetylation of histones in vitro. Instead NaB inhibits the action of histone deacetylases (HDAC) (8). Other HDAC inhibitors, chemically distinct from NaB, were found to produce similar biologic and biochemical alterations. Trichostatin A, (TSA), a fungistatic antibiotic, induced differentiation of Friend erythroleukemia cells and arrested the cell cycle. TSA also manifested profound anti-tumor effects on transplanted mouse mammary tumor cell lines (80). Valproic acid (VPA), 2-propylpentanoic acid, a drug widely used in the treatment of epilepsy, also caused histone hyperacetylation in cultured cells and induced differentiation of carcinoma cells and leukemia blasts (28). These profound effects on differentiation of cancer cells have led to clinical trials in which HDAC inhibitors are being evaluated for anti-neoplastic effects (50).

HDAC inhibitors globally alter cellular gene expression. The expression of 2 to 10% of cellular genes may be affected, either increased or decreased (37, 52). In the hyperacetylated N-terminal tails of histones the positively-charged epsilon amino groups of lysines are modified...
with a negatively-charged acetyl group (34). This change in charge markedly lowers the affinity of the nucleosome for DNA (35). Relief of chromatin repression allows the access of transcription factors which modulate gene expression (41). One prominent target gene whose expression is enhanced in many cell backgrounds is p21\textsuperscript{waf/cip}, an inhibitor of cyclin dependent kinase 2 (CDK2). Increased p21 expression may account for cell cycle arrest induced by HDAC inhibitors. The p21 promoter, and some other butyrate-responsive target promoters, such as that of the KSHV ORF 50 gene, contain butyrate responsive elements which co-localize with Sp1/Sp3 sites (54, 79). One model for how NaB activates gene expression postulates that under basal conditions Sp1/Sp3 recruit HDACs which form a complex with co-repressors. HDAC inhibitors alter the activity of these repressive complexes, and allow access to DNA of transcription factors complexed with histone acetyl transferases (HATs) (71). The mechanism by which HDAC inhibitors are able to repress gene expression is not understood.

The genome of Epstein-Barr virus is usually not integrated into the cell genome; it exists as mini-chromosomes in latently infected cells (56). Experiments with micrococcal nuclease digestion have shown that EBV DNA is nucleosomal in several cultured cell lines (69). EBV DNA which is newly replicated during the lytic cycle and destined for incorporation into virions may not be nucleosomal (19). In Burkitt lymphoma cells EBV DNA is also heavily methylated (21). Reversible methylation and demethylation of EBV DNA in the region of the “C promoter,” one of the major promoters utilized during latency, appears to play an important role in the switch between different latency types (64). Thus, the EBV genome is a good model for understanding how epigenetic changes may affect eukaryotic gene expression.

The EBV lytic cycle can be induced by HDAC inhibitors, such as NaB and TSA, in some cell lines derived from Burkitt’s lymphoma (29, 47, 63). This finding has significant implications. It offers a window of opportunity to understand the role of chromatin structure on
EBV gene expression. Since HDAC inhibitors manipulate chromatin modifications, such experiments might offer clues about the role of chromatin alterations in the switch between EBV latency and the lytic cycle. Activation of the EBV lytic program in Burkitt lymphoma cells, perhaps accompanied by B cell differentiation, following treatment with HDAC inhibitors offers promise of clinical application as “oncolytic” therapy (22, 48, 68, 75).

The lytic cycle of EBV is controlled by two viral proteins, ZEBRA and Rta, encoded in the BZLF1 and BRLF1 genes (13, 14, 61, 82). These proteins function as transcription factors which control viral lytic gene expression. ZEBRA also plays an essential role as an origin binding protein in lytic viral DNA replication (23, 32, 65, 66). Both ZEBRA and Rta have the capacity to alter chromatin. ZEBRA and Rta, like many cellular transcription factors which bind DNA, directly interact with HATs, such as cyclic AMP response element binding protein-associated protein (CBP) (1, 17, 72, 83). The interaction between ZEBRA or Rta and CBP enhances the potential of each of the two viral proteins to activate transcription (16, 72, 83). Conversely, ZEBRA reciprocally enhances the histone acetyl transferase activity of CBP, especially on di-nucleosomes and oligo-nucleosomes (10).

A prevailing view is that EBV latency is maintained, in part, as the result of chromatin’s capacity to repress Zp and Rp, the promoters of the BZF1 and BRLF1 genes. This concept is supported by considerable evidence. Digestion with micrococcal nuclease shows that Zp and Rp are nucleosomal (29). EBV lytic cycle activation induced in Akata Burkitt lymphoma cells by cross-linking the B cell antigen receptor with anti-Ig leads to hyperacetylation of histone H4 associated with Zp (36). EBV lytic cycle activation in the P3J-HR-1 cell line treated with TSA is also accompanied by hyperacetylation of histone H4 associated with Rp (9). Hyperacetylation of histone H3 and H4 are markers for open or non-repressive chromatin.
Zp contains a class of response elements called ZI sites that may mediate repression by chromatin (6, 24, 25, 70). Three of these sites, ZIC, ZIA, and ZID, bind Sp1 and Sp3 that can recruit HDACs and repressive complexes. Additional Sp1 sites have been found upstream in Zp (at −360) by genome footprinting techniques (55). Some ZI sites, ZIA, ZIB, and ZID, also bind MEF2D, which recruits class II HDACs (30, 45, 46, 51). The ability of cyclosporin A to inhibit EBV lytic cycle induction in the Akata Burkitt lymphoma cell line, following cross-linking of the B cell antigen receptor, maps to the MEF2D sites (44). Thus, the MEF2D sites are potentially repressive. Anti-Ig cross-linking in Akata cells activates a signal transduction pathway which can dephosphorylate MEF2D, reduce its association with HDACs and promote its association with HATs. Transfection of a chimeric protein in which the potent transcriptional activation domain of Herpes simplex VP16 has been fused to HDAC 4 or 5 activates the EBV lytic cycle in Akata cells, presumably by changing a repressive MEF2D/HDAC complex into an activating one (7, 30). Rp also contains a potentially repressive Sp1 site near the start of transcription. Autostimulation of Rp by Rta maps to this site (60). Autostimulation of Rp may exchange a repressive complex containing Sp1 and HDAC for an activating complex containing Rta.

Despite evidence supporting the role of chromatin repression of Zp and Rp in maintenance of EBV latency, it has not yet been proved that formation of open chromatin on these promoters is a sufficient or even necessary stimulus for activation of the EBV lytic cycle. A variety of experimental findings complicate the interpretation of the role of nucleosomal organization of EBV DNA on the latent to lytic cycle switch. Not all lymphoid cell lines which harbor EBV can be induced into the lytic cycle by HDAC inhibitors (29). For example, neither B95-8 cells, which are efficiently induced by phorbol esters, or Akata cells, which are efficiently induced by anti-Ig, can be induced into the EBV lytic cycle by TSA (29, 36). The nucleosomal
organization of Zp and Rp is grossly similar in cell lines in which the EBV lytic cycle can or cannot be induced by HDAC inhibitors (29). Even in cell lines which are highly responsive to EBV lytic induction by TSA, such as the HH514-16 subclone of the P3J-HR1-K cell line, only a subpopulation of cells are activated into the EBV lytic cycle (3). In early experiments it was found that fewer than 4% of Raji cells were activated into the viral lytic cycle following treatment with NaB. However, more than 80% of Raji cells treated with butyrate were seen to have undergone plasmacytoid differentiation when examined by electron microscopy (2). Cells which are susceptible to differentiation induced by NaB may not always be activated into the EBV lytic cycle.

This report begins to address the conundrum that some lymphoid cell lines containing EBV are susceptible and others are refractory to lytic cycle induction by HDAC inhibitors. We asked the following questions: 1) Does treatment with NaB and TSA induce histone hyperacetylation at Zp and Rp in cell lines, such as Raji and B95-8, which are refractory to lytic induction by these agents? 2) Does histone hyperacetylation of Zp and Rp occur in a cell line in which the EBV lytic cycle is induced by 5-Aza2’-deoxycytidine, a DNA methyl transferase inhibitor? 3) In a cell line, such as HH514-16, which responds with EBV lytic cycle activation following exposure to NaB and TSA, is histone hyperacetylation seen following treatment with another HDACi, VPA, which is a poor activator of the EBV lytic cycle? These experiments indicate that open chromatin formation on Zp and Rp is not sufficient to activate expression of the BZLF1 and BRLF1 genes, and show that functions in addition to open chromatin are required to activate the EBV lytic cycle.
MATERIALS AND METHODS

Cell Culture: Raji, B95-8 and HH514-16 cells were maintained in RPMI 1640 culture medium with 8% fetal bovine serum at 37°C with 5% CO₂. HH514-16 cells do not contain defective viral genomes as assessed by PCR and Southern blot for the WZhet rearrangement (13, 14). To induce the EBV lytic cycle, 1x10⁶ cells /ml, were treated with 20 ng/ml tetradecanoylphorbol acetate (TPA, Calbiochem, #524400), with 3 mM n-butyrate (NaB, Sigma, #B5887), with 5 µM Trichostatin A (TSA, Wako Chemicals, Richmond, VA, #204-11991), with 10 mM Valproic acid (VPA, Sigma #P4543) or with 5 µM 5-aza 2’-deoxycytidine (Sigma#A3656) for 18 hrs. This duration of stimulus has been previously determined to yield maximal viral lytic reactivation for all three cell lines. All doses of chemicals were determined to be in the optimal range for a maximal response.

Chromosome Immunoprecipitation (ChIP): Untreated cells or cells treated for 18 hrs with lytic cycle inducing agents were cross-linked with 1% formalin for 10 min at 37°C in culture medium. 1x 10⁶ cells were washed twice in cold phosphate-buffered saline (PBS) containing a mixture of protease inhibitors (PI) (1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride). The cell pellet was lysed in Tris/SDS/EDTA (50 mM Tris, pH 8.1, 1% SDS, 10 mM EDTA with PI) for 10 min at 4°C. The cell lysate was sonicated for three cycles of 20 sec per cycle at power setting 7 using a Branson sonifier equipped with a microtip. The sonicated cell lysate was centrifuged for 20 min in a microfuge at 4°C. The supernatant was diluted 10-fold with immunoprecipitation (IP) buffer (IP buffer: 0.01%SDS, 1.1% Triton-X, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 16.7 mM NaCl and PI); 1% was removed for input control. The remainder of the supernatant was pre-cleared with proteinA or proteinG-agarose beads containing 20 µg sonicated salmon sperm DNA and 1 mg/ml BSA (Upstate) by rotating the
sample for 30 min at 4°C. The mixture was spun at low speed. Antibody was added to the
supernatant which was incubated overnight at 4°C with rotation. ProteinA or proteinG-agarose
beads were added for 1 hr at 4°C with rotation. The immune complex was collected by brief
centrifugation, and washed sequentially with 150 mM NaCl buffer (0.1% SDS, 1% Triton-X, 2
mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl), with the same buffer containing 500 mM
NaCl, with LiCl buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris,
pH 8.1) and two washes with Tris/EDTA (TE)(pH 8.0). The immune complexes were eluted
from the beads in 1% SDS and 100 mM NaHCO₃; cross-links were removed by heating the
supernatant at 65°C for 4 hrs. To isolate the DNA, the sample was treated with proteinase K for
60 min at 45°C, extracted with phenol/chloroform and precipitated with alcohol. The DNA was
resuspended in 50 µl TE and used for PCR.

**PCR:** Conventional and quantitative PCR were performed in similar conditions. Reactions
contained 5 µl template, 20 mM each nucleotide, 50 ng of each primer, and 1.25 units of
Platinum Taq polymerase (Invitrogen) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 2.5 mM
MgCl₂. Conventional PCR reactions were electrophoresed in 1.2% agarose gels and visualized
by staining with ethidium bromide. Real-time PCR reactions contained SYBR green® (0.25X,
Molecular Probes) and 4 mM MgCl₂. Quantitative real-time PCR was measured with a Smart
Cycler (Cepheid); DNA concentration was determined using a standard curve generated from
purified plasmid DNA containing each promoter tested. Primers for conventional PCR were as
follows: Rp (5’CCCTGGAGGATTGTCTACCA3’; 5’GCTGACATGGATTACTGGTC3’), Zp
(5’GACACTGTTATTCcccAG3’; 5’CCTGTCTAACATCTCCCC3’), EAp
(5’GCGGTGGAGGTAGAGACTGC3’; 5’CCAGAGCAGGCGAGGCAGGCAGG3’). Primers for
quantitative PCR (Q-PCR) were as follows: Rp (5’TTAGTTAATGGCCcccAGccAGA3’;
5’TTAAAAAAGGCGGCTGAC3’), Zp (5’TTACCCTGTCTAACATCTCCCCCTTTAAA3’;
Western Immunoblotting and Antibodies: Cells were washed in cold PBS and resuspended in sodium dodecyl sulfate (SDS) sample buffer at 5x10^7 cells/ml. The sample was heated at 100°C for 5 min. 20 µl of cell extract was loaded onto SDS, 8% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose that was blocked with 5% nonfat dry milk overnight at 4°C. Blots were incubated for 1 hr at 25°C with antiserum diluted in 5% nonfat dry milk, washed twice with 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% Tween 20, incubated with 125I-protein A for 1 hr at 25°C and washed again prior to exposure to Kodak XAR film with intensifying screens. Antibodies to acetylated histone H4 (K5, K8, K12, K16) (cat# 06-598), acetylated histone H3 (K9, K14) (cat# 06-599), unmodified histone H3 (cat# 05-499), dimethyl histone H3(K4)(cat# 07-030) and phosphorylated S10 of histone H3 (cat# 05-598) were obtained from Upstate Biochemicals. Rabbit polyclonal antibodies to ZEBRA and Rta have previously been described (59, 73). EA-D was detected with mouse monoclonal antibody R3.1, a gift from G. Pearson, and a rabbit anti-mouse Ig bridge (57). Protein loading was controlled with an antibody to β-actin (cat# A5316, Sigma).

Northern Blotting: Total cellular RNA was prepared from HH514-16 cells at intervals up to 12 hrs after application of an inducing stimulus. The procedures for preparation of total cellular RNA, electrophoresis, transfer to nylon membranes, and preparation of the radioactive probes have been described recently (78).
RESULTS

Sodium butyrate activates the EBV lytic cycle only in some cell backgrounds, but invariably induces global histone hyperacetylation. In initial experiments (Fig. 1) we examined the capacity of sodium butyrate, (NaB), a well studied histone deacetylase inhibitor (HDACi), to activate the EBV lytic cycle in three well characterized lymphoid cell lines, Raji and HH514-16 (Cl16) from Burkitt lymphoma and B95-8 marmoset lymphoblastoid cells. The EBV in Raji and Cl16 cells is tightly latent, whereas B95-8 cells spontaneously express EBV lytic cycle products and release virions (49). All three cell lines can be efficiently activated into the EBV lytic cycle; however, each cell line responds differently to inducing stimuli (29).

Expression of the EBV BMRF1 (EA-D) early gene protein, the DNA polymerase processivity factor, was used as a marker for lytic cycle induction. Figure 1A shows that NaB failed to induce EA-D expression in Raji or B95-8 cells. Nonetheless, whenever Raji or B95-8 cells were treated with NaB there was a marked increase in the amount of hyperacetylated histone H4 evident on an immunoblot. TPA induced the lytic cycle in both cell backgrounds but did not increase the abundance of acetylated histone H4 (Fig. 1A; lanes 3 and 8). NaB augmented EA-D expression in response to TPA treatment of Raji cells (lane 2), but dampened the EA-D response to TPA in B95-8 cells (lane 6). Nonetheless, when NaB and TPA were present together the level of hyperacetylation of histone H4 was similar to that observed with NaB alone, whether NaB was synergistic with TPA in activating the EBV lytic cycle, as in Raji cells, or inhibitory of lytic cycle activation, as in B95-8 cells.

NaB was a potent activator of lytic cycle gene expression in Cl16 cells (Fig. 1B). NaB treatment was accompanied by an increase in the amount of hyperacetylated histone H4. TPA by itself did not activate the lytic cycle in Cl16 cells, did not synergize with NaB to activate EBV
lytic proteins and did not alter the overall level of histone H4 acetylation either alone or in combination with NaB.

These experiments showed that NaB treatment induced hyperacetylation of histone tails in all three cell backgrounds, but only activated lytic cycle gene expression in one of the three cell lines, namely HH514-16. Therefore, global hyperacetylation of histone H4 did not correlate with activation of the EBV lytic cycle. Conversely, activation of the lytic cycle by TPA was not accompanied by global histone H4 hyperacetylation that was detectable on an immunoblot.

Butyrate treatment induces histone hyperacetylation on promoters of EBV lytic cycle genes in cell backgrounds refractory to lytic cycle induction. The findings of Fig. 1, indicating that NaB treatment alone, or in combination with TPA, resulted in global hyperacetylation of cellular histone H4, left unanswered the question about the state of histone hyperacetylation on the promoters of specific EBV lytic genes. Chromatin immunoprecipitation (ChIP) with antibodies to acetylated histones H3 and H4 was used to study this question (Fig. 2). Figure 2B examines the state of histone hyperacetylation on Rp, the promoter of the BRLF1 gene, in the three cell lines studied in Fig. 1. Treatment of all three cell lines with either NaB (B) or a combination of NaB and TPA (T/B) resulted in increased amounts of Rp being immunoprecipitated by antibodies to acetylated histone H3 and H4. The capacity to immunoprecipitate Rp following treatment with NaB was not altered by phosphonoacetic acid, an inhibitor of viral DNA replication. In these experiments treatment of the cells with TPA did not increase the amount of Rp which could be immunoprecipitated by antibodies to acetylated H3 or H4.

We examined the acetylation state of histones associated with three early lytic promoters, Zp, Rp and EAp, in Raji cells (Fig. 2C). NaB treatment alone, which does not induce the EBV lytic cycle in Raji cells, nonetheless caused hyperacetylation of histones H3 and H4 on all three promoters. TPA alone did not increase the ability to immunoprecipitate lytic cycle promoters
with antibodies to acetylated histone H3 or H4. The data of Fig. 2 show that histone H3 and H4
hyperacetylation of three EBV early lytic cycle promoters induced by NaB does not correlate
with EBV lytic cycle activation.

Quantitative comparison of the effects of NaB and TPA alone and in combination on
histone acetylation of EBV early lytic cycle gene promoters in Raji cells. EBV in Raji cells
is not activated into the lytic cycle by NaB, but NaB synergizes with TPA to activate EBV lytic
gene expression (Fig. 1). This finding raised the possibility that the synergistic combination of
TPA and NaB might result in more extensive histone hyperacetylation on the early lytic cycle
promoters than that induced by NaB alone. ChIP experiments using standard PCR (Fig. 2) were
not amenable to quantitation. Therefore, quantitative real time PCR was used to measure the
extent of chromatin modification on Zp, Rp and EAp following treatment of Raji cells with NaB
and TPA, singly and in combination (Fig. 3). NaB treatment markedly enhanced (25- to 60-fold)
the capacity of antibodies to acetylated H4 to immunoprecipitate all three early promoters. NaB
also dramatically increased the amount of Rp precipitated with antibodies to acetylated H3 (50-
fold relative to untreated cells), although in this experiment these antibodies were less effective
at immunoprecipitating Zp or EAp. As compared with NaB alone, the combination of NaB and
TPA produced no significant enhancement of the capacity to immunoprecipitate any of the three
promoters with antibodies to acetylated H3 or acetylated H4.

Quantitative PCR showed that treatment of Raji cells with TPA alone led to a small but
reproducible increased capacity to ChIP all three promoters with antibody to acetylated H4 (Fig.
3). Treatment with TPA also slightly increased capacity to precipitate Rp and EAp with
antibodies to acetylated H3. This effect was always observed at a low level, less than 5-fold
above the background, and was not detectable using conventional PCR (see Fig. 2). These
experiments showed that NaB and TPA, which were synergistic in activating the EBV lytic cycle in Raji cells, did not synergize to increase histone hyperacetylation.

**Comparing effects of Trichostatin A (TSA) and NaB on histone H3 modifications in Raji cells.** TSA, another HDAC inhibitor, was more potent than NaB in inducing the EBV lytic cycle in HH514-16, a responsive cell line (Fig. 6A and B). Yet TSA did not activate the EBV lytic cycle in Raji cells (Fig. 8). In separate experiments we compared the ability of TSA and NaB to cause hyperacetylation of histone H3 on Zp, Rp and EAp (Fig. 4). Both HDAC inhibitors caused comparable (12- to 35-fold) histone H3 acetylation on all three promoters. Both TSA and NaB treatment of Raji cells led to another histone modification characteristic of open chromatin, namely phosphorylation of S10 of histone H3 (phosH3, Fig. 4A, B, C). Using antibodies to phosphorylated S10, 5- to 15-fold more lytic cycle promoter DNA was immunoprecipitated from NaB and TSA treated cells than from untreated cells. TPA treatment of Raji cells led to small increases in H3 acetylation and H3 S10 phosphorylation which were 2 to 3-fold above that in untreated cells. These experiments showed that NaB and TSA induced equivalent hyperacetylation and phosphorylation of histones H3 on EBV lytic cycle promoters in the Raji cell line. Yet, neither HDAC inhibitor was sufficient to activate the EBV lytic cycle in this cell background.

**EBV lytic cycle activation by 5-aza 2’- deoxycytidine (AZC) does not increase histone H3 hyperacetylation.** The findings of experiments, illustrated in Fig. 3 and 4, showing that TPA treatment for 18 hrs led to a low level of H3 hyperacetylation on the three EBV lytic cycle promoters in Raji cells, raised the question whether H3 hyperacetylation invariably accompanied EBV lytic cycle activation. In HH514-16 (Cl16) cells EBV can be rapidly reactivated into the lytic cycle by HDAC inhibitors and by AZC, a DNA methyl transferase inhibitor (Fig. 5). EBV BRLF1 and BZLF1 mRNAs were detected by Northern blotting in Cl16 cells within 4 hrs after
treatment with AZC and by 6 hrs after treatment with NaB (Fig. 5A). AZC induced EBV lytic mRNAs more rapidly than did NaB, reaching a near maximum effect at 6 hrs. At 4 hrs and 8 hrs after application of the two inducing stimuli there was a marked increase in global acetylation of histone H4 above background in cells treated with NaB, but AZC did not alter acetylation of histone H4 above background levels (Fig. 5B).

ChIP and quantitative PCR were used to compare histone H3 acetylation on Zp and Rp at early times, 4 hrs and 6 hrs after treatment with NaB and AZC, when BZLF1 and BRLF1 mRNAs were first detectable (Fig. 5C and 5D). At these times NaB treatment induced 4 to 6-fold increases in acetylated histone H3 on Zp, and 2 to 4-fold increases in acetylated H3 on Rp. However, treatment with AZC induced less than 2-fold histone H3 acetylation on Zp and less than background levels of H3 acetylation on Rp. Thus, at times when expression of mRNAs controlled by Zp and Rp was markedly induced by AZC treatment, histone H3 bound to these promoters was not appreciably acetylated.

Valproic Acid induces histone H3 hyperacetylation and H3(K4) dimethylation but does not activate the EBV lytic cycle in HH514-16 cells. A recent report indicated that another HDAC inhibitor, Valproic Acid (VPA), can synergize with chemotherapeutic agents, such as doxorubicin, cis-platinum and 5-Fluorouracil, to induce the EBV lytic cycle (22). We found that at times when EBV in HH514-16 cells was susceptible to lytic cycle activation by other HDAC inhibitors, such as NaB and TSA, VPA did not activate BZLF1 or BRLF1 mRNA expression (Fig. 6A). Moreover, VPA did not induce expression of ZEBRA or EA-D proteins even though VPA caused global acetylation of cellular histone H3 at levels comparable to NaB and TSA (4- to 5-fold above background) (Fig. 6B).

Valproic acid, as well as the other HDACi tested, was able to hyperacetylate histone H3, but it was important to determine whether it also caused histone H3(K4) to become
dimethylated, another marker for open chromatin. HH514-16 cells were treated with AZC, TSA, NaB and VPA for 18 hours. As shown in Figure 7A, VPA failed to activate the EBV lytic cycle, as no ZEBRA or EA-D was expressed. The level of unmodified histone H3 remained the same, regardless of the treatment. When these same extracts were examined for the presence of dimethyl histone H3(K4), a two-fold increase in dimethyl H3(K4) was observed in cells treated with each of the three HDACi (Fig. 7B, lanes 3-5). Therefore, all of the HDACi tested were competent to open chromatin, whether monitored by acetylation, phosphorylation or dimethylation of histone H3, although only NaB and TSA activated the viral lytic cycle.

Chromatin immunoprecipitation experiments (Fig. 8) showed that VPA caused Zp, Rp and EAa to become associated with hyperacetylated histone H3, about 4 to 5-fold above the background level in Cl16 cells. However, treatment with the two HDAC inhibitors which activated the EBV lytic cycle in Cl16 cells induced a higher level of histone H3 acetylation (7-16-fold above background) than did VPA. The relative amount of acetylated H3 associated with the lytic cycle promoters was always 2 to 3-fold higher following treatment with VPA, which failed to induce the EBV lytic cycle, than following treatment with AZC, which was competent to do so. AZC never induced H3 hyperacetylation more than 2-fold above background. These experiments emphasized that even in a cell line in which some HDAC inhibitors mediate EBV lytic reactivation, histone hyperacetylation per se on EBV lytic cycle promoters is not sufficient to mediate a lytic cycle switch.

Valproic acid can act in synergy with TPA to activate the EBV lytic cycle in Raji cells. VPA induced an approximately 3-fold increase in global H3 hyperacetylation in the B95-8 and Raji cell lines but, by itself, did not activate the EBV lytic cycle in either of these two cell lines (Fig. 6C and Fig. 9). However, VPA acted in synergy with TPA to increase lytic gene expression in Raji cells (Fig. 9). The synergistic effect of VPA with TPA was similar to that of
NaB and TSA on expression of ZEBRA, although VPA was slightly less active than the other HDAC inhibitors at induction of EA-D. VPA strongly synergized with suboptimal doses of TPA which, by themselves, were unable to activate lytic gene expression in Raji cells (data not shown). These experiments indicated that VPA can exert a positive effect on EBV lytic cycle activation in some cell backgrounds, provided that an additional stimulus is present.
DISCUSSION

Histone hyperacetylation is not a sufficient stimulus for EBV lytic cycle activation. Before the experiments described in this manuscript were performed it was known that the HDAC inhibitors, NaB and TSA, did not activate EBV lytic cycle gene expression in some cell lines, such as B95-8 and Akata (29, 36). Since Zp and Rp were similarly nucleosomal in cell lines in which the EBV lytic cycle was or was not activated by HDAC inhibitors, the failure to induce the EBV lytic cycle was not likely to be related to the presence or absence of chromatin repression of EBV DNA (29). Even in cell lines which were highly susceptible to lytic cycle activation by NaB and TSA there was always a subpopulation of cells which were not induced into the lytic cycle (3). Two sets of questions were left unanswered which were addressed in this report. First, in cell lines in which the EBV lytic cycle could not be induced by HDAC inhibitors were these agents causing global histone hyperacetylation and, more importantly, were they specifically causing hyperacetylation on the promoters of BZLF1 and BRLF1, the genes whose products initiate the lytic cascade? Second, do all stimuli which induce the EBV lytic cycle promote histone hyperacetylation on Zp and Rp?

Here we present extensive evidence that lack of an EBV lytic response is not due to failure of the HDAC inhibitors to open chromatin. Global histone hyperacetylation is observed after treatment with HDAC inhibitors in all cell lines, those which are and those which are not activated into the EBV lytic cycle (Fig. 1 and Fig. 6). Hyperacetylation of H3 and H4 tails, which is a well accepted marker for open chromatin, is detected on Zp and Rp, the promoters of the lytic cycle activator genes in two cell lines, Raji and B95-8, which are not induced into the EBV lytic cycle by NaB or TSA (Fig. 2-4). Even in HH514-16, a cell line in which EBV lytic cycle gene expression is activated by NaB and TSA, another HDAC inhibitor, VPA, opens
chromatin but fails to induce the lytic cycle (Fig. 6-8). In Raji cells, the capacity of VPA to open chromatin must be complemented by treatment with TPA in order to activate EBV lytic genes (Fig. 9). Taken together this new data indicates that hyperacetylation of H3 and H4 on Zp and Rp is not sufficient for lytic cycle activation. Additional events are required.

**Does lytic cycle activation invariably involve histone hyperacetylation?** Our findings with HDACi show that histone hyperacetylation occurs in the absence of induction of the EBV lytic cycle, but the experiments also provide some insight on the more difficult question whether lytic cycle inducing agents, such as TPA or AZC, which are not known to alter chromatin directly, nonetheless promote histone hyperacetylation during lytic cycle induction. We could detect little or no hyperacetylation of histone H3 or H4 on Zp or Rp in TPA-treated Raji or B95-8 cells, which were induced into the lytic cycle (Fig. 2-4). After treatment of HH514-16 cells with AZC we could not detect hyperacetylated histone H3 on Zp or Rp, using real-time PCR, at times when mRNAs from these promoters were abundant (Fig. 5 and Fig. 8). These results are consistent with the conclusion that TPA and AZC do not modify chromatin. However, this conclusion must be tempered with the following qualifications which might account for these results: 1) too few B95-8 or Raji cells were induced into the lytic cycle by TPA; therefore, detection of hyperacetylated histones in lytically induced cells could not be observed over the background of hypoacetylated histones in uninduced cells. 2) Each EBV-infected cell contains multiple copies of the EBV genome. Not all genomes within a responsive cell may become hyperacetylated. Thus, it may be very difficult to detect the rare responsive genome against a large background of unresponsive genomes. 3) We may have looked for hyperacetylation, as a result of AZC or TPA treatment, at the wrong time. In contrast to the action of the HDACi, which maintain the hyperacetylated state by preventing deacetylation, the hyperacetylation events following AZC and TPA may be transitory.
What upstream events are required for EBV lytic cycle activation? At least four different types of events may participate in the process of activation of the EBV lytic cascade. These include signal transduction, synthesis of new proteins, epigenetic alterations and inactivation of repressors bound to Zp and Rp. Experimental evidence supports the requirement for each of these events, though all the events have yet to be analyzed simultaneously in one experimental system.

In Akata cells, EBV lytic cycle activation, triggered by cross-linking of the B cell antigen receptor, can be blocked by Cyclosporin A and FK506 which inhibit the Ca++/calmodulin dependent protein kinase pathway (27). In B95-8 cells EBV lytic cycle activation mediated by treatment with TPA is blocked by bisindoylmaleimide, a specific inhibitor of protein kinase C (29). These results argue for the importance of kinase-mediated signal transduction as a component of the upstream events needed for activation of the BZLF1 and BRLF1 genes. New protein synthesis is also needed for activation of BZLF1 and BRLF1 expression since cycloheximide inhibits EBV lytic cycle activation induced by three stimuli, the HDAC inhibitors, TPA and AZC (78). The identity of these newly synthesized proteins is not known. Among the candidates for a protein that might be required for EBV lytic cycle activation is XBP-1, a B cell differentiation factor which, in conjunction with constitutively active protein kinase D, is competent to activate the EBV lytic cycle in some cell backgrounds (4).

The requirement for epigenetic modification, such as demethylation of CpG on the DNA of Zp and Rp, is suggested by the ability of a DNA methyl transferase inhibitor, such as 5Aza2’deoxycytidine (AZC), to activate the EBV lytic cycle in some cell backgrounds (Figs. 5-8). However, the mechanism by which AZC activates the EBV lytic cycle remains to be elucidated. AZC activates BZLF1 and BRLF1 gene expression very rapidly, within 4 hours (Fig.5). This rapid mode of action is not likely to be explained by demethylation of DNA...
occurring as the result of AZC being incorporated into newly replicated DNA (11). Acetylation, methylation, and phosphorylation of histone tails and nucleosome remodeling are also likely to be components of the EBV lytic activation pathway, although, as this study emphasizes, their exact role or even their requirement is far from clear. However, any conclusions about the role of epigenetic changes must be tempered by the reservation that it is not yet possible experimentally to analyze such changes on individual EBV genomes. If only one or a few EBV genomes in a cell is so affected, this event would not be detected by assays of cell populations.

At least four candidate repressors are known to bind Zp. Sp1/Sp3 and MEF2D can bind complexes containing HDACs (18, 30, 43, 44). ZEB1 binds near the start of transcription of BZLF1 (39). YY1 has been shown to exert negative control on Zp (53). These repressive activities must be relieved before RNA polymerase can bind to the promoter and initiate transcription. How do lytic cycle activating agents relieve this repression? More experiments are necessary to explore the hypothesis that inducing stimuli alter the status of these repressor proteins at Zp.

What is different between cell lines which are responsive and those which are refractory to EBV lytic cycle activation by HDAC inhibitors? In cells which respond to HDAC inhibitors with BZLF1 and BRLF1 expression it may be assumed that the required signaling pathways are intact, the newly synthesized proteins which are essential can be made, are properly modified and functional, and the repressors rendered inactive. HDAC inhibitors are known to induce and to inhibit the expression of a large number of cellular genes (37, 52). Activation and inhibition of cellular gene expression seems to occur to an equal extent. In cells which respond with EBV lytic gene activation it is likely that there must be an optimal mixture of activated and repressed cellular genes.
Among the genes whose expression is induced by HDAC inhibitors there are likely to be those which potentiate lytic activation and those which are repressive in nature. In cells which fail to be activated into the lytic cycle there may be an unfavorable ratio of repressive to potentiating products. Moreover, the non-responding cells might express the required proteins, but they may not be appropriately modified to facilitate EBV lytic gene expression. Lack of modification might also explain failure to remove a repressive complex. For example, a signal transduction cascade is thought to modify MEF2D thereby decreasing its association with HDACs and promoting the exchange of a histone acetyl transferase (7, 45, 81). In refractory cells this signal to remove a repressor may not occur.

Our experiments make it unlikely that in the refractory population cells Zp and Rp have not undergone the required histone modifications which allow active transcription factors to bind to these promoters. We have shown that H3 and H4 acetylation and H3S10 phosphorylation occurs in non-responding cells, but we have not yet explored the requirement for histone methylation on the promoters or the open reading frames of the BZLF1 and BRLF1 genes (74). HDAC inhibitors may promote the acetylation of non-histone proteins (26). In cells which do not respond with lytic activation these acetylated target proteins may not be present or the modification may not have occurred.

An equally intriguing series of questions focuses on the lack of a lytic cycle response to VPA (Fig. 6-9). TSA is a broad spectrum inhibitor which blocks the activity of class I and class II HDACs (5, 77). NaB and VPA are thought to have a more limited scope of action, inhibiting class I and IIa HDACs (5). Therefore, the lack of a lytic cycle response to VPA may be explained by its inability to target the specific HDAC involved in repressing Zp and Rp. VPA may induce or inhibit expression of a different subset of cellular genes than that affected by NaB and TSA. VPA may possess properties in addition to those affecting modification of histones.
The capacity of VPA to alter cytoplasmic membrane ion channels may influence its capacity to activate the EBV lytic cycle (20). Nonetheless VPA can synergize with other agents to activate lytic cycle genes in some cell backgrounds (22) (Fig. 9).

On the basis of the original description of the capacity of NaB to affect cell differentiation and growth, HDAC inhibitors are being widely considered for clinical applications. HDAC inhibitors were originally used in patients with sickle cell anemia to promote production of fetal hemoglobin (58). VPA has been employed to activate HIV-1 from its latent reservoir, allowing the virus to become susceptible to anti-retroviral drugs (15, 42). HDAC inhibitors are being evaluated as therapy for leukemias and multiple myeloma, based on their potential to induce terminal differentiation and apoptosis (12, 33, 38). HDAC inhibitors, in conjunction with chemotherapeutic agents, have been proposed to serve as oncolytic therapy for EBV-associated tumors since they can activate the EBV lytic cycle (22). In all these clinical settings it becomes important to understand the mechanism by which HDAC inhibitors activate cellular and viral gene expression. Our experiments indicate that the mechanisms of action of HDAC inhibitors are considerably more complex than induction of histone hyperacetylation on the promoters of responsive genes.

Acknowledgement: Supported by grants from the National Cancer Institute, CA16038 and CA12055. We are grateful to Karen Lavery and Susan Prisley for preparation of the manuscript and to Derek Daigle and Ayman El-Guindy for helpful discussions and critical reading of the manuscript.
REFERENCES


targeting are required for Zta-directed nucleosome acetylation and transcription activation. Mol Cell Biol 23:2633-44.


**FIGURE LEGENDS**

Figure 1. *Sodium butyrate induces the EBV lytic cycle only in some cell backgrounds, but invariably causes global histone hyperacetylation.* A) Raji or B95-8 cells, at a concentration of $1 \times 10^6$ cells/ml, were untreated (O) or treated with tetradecanoylphorbol acetate (T), sodium butyrate (B) or a combination of the two agents (T/B) for 18 hrs. Extracts of $1 \times 10^6$ cells were loaded into each lane of an SDS-polyacrylamide gel. An immunoblot was probed sequentially for EA-D using monoclonal antibody R3.1, with rabbit anti-acetylated histone H4 (K5, K8, K12 and K16) (acH4), with human antibody SJ that detects the p21 (BFRF3) component of small viral capsid antigen (VCA) and with rabbit polyclonal antibodies that detect ZEBRA and Rta. B) HH514-16 cells were treated with inducing chemicals as described in A). The immunoblot was probed sequentially with R3.1, with rabbit anti-acetylated histone H4 and with rabbit polyclonal antibodies that detect ZEBRA and Rta.

Figure 2. *Sodium butyrate induces histone hyperacetylation on the promoters of EBV lytic cycle activator genes in cell lines which do not respond with lytic cycle activation.* A) Schematic diagrams of EBV early lytic promoters analyzed by chromatin immunoprecipitation. Numbers under each promoter indicate nucleotides relative to the start of transcription. The location of primers used for standard PCR (solid arrows) and quantitative PCR (dashed arrows) is illustrated, and the size of expected PCR fragments is indicated. The ZEBRA response elements (ZRE) in each promoter are illustrated: B) Sodium butyrate induces hyperacetylation of the promoter of the EBV BRLF1 gene in three cell EBV-containing cell lines. B95-8, Raji and HH514-16 cells were untreated (O) or treated with chemical inducing agents for 18 hrs. B95-8
and HH514-16 cells were also treated with phosphonoacetic acid (PAA) or butyrate plus PAA to inhibit viral DNA replication. Chromatin immunoprecipitation (ChIP) was carried out using rabbit antibodies to acetylated histone H4 (anti-acH4) or acetylated histone H3 (anti-acH3) (K9 and K14) or non-immune rabbit serum (preI). Input represents 1% of total cellular DNA before immunoprecipitation. The DNA was amplified by PCR using primers from Rp, the promoter for BRLF1 (see Panel A). Shown are negative images of ethidium bromide stained agarose gels containing the PCR products. C) Sodium butyrate induces hyperacetylation of histones H3 and H4 on promoters of three early lytic cycle genes in Raji cells. Shown are ChIP experiments in which antibodies to acetylated histone H4 (top) and to acetylated histone H3 (bottom) were used to precipitate DNA from Raji cells that were untreated or treated with chemical inducing agents for 18 hrs. Immunoprecipitated DNA was analyzed by PCR using primers from the promoters of the BZLF1 (Zp), BRLF1 (Rp) or BMRF1 (EAp) genes.

Figure 3. Quantitation of histone hyperacetylation of the promoters of BZLF1, BRLF1 and BMRF1 genes in Raji cells treated with sodium butyrate or TPA. Raji cells were untreated or treated with inducing chemicals for 18 hrs. Shown are results of quantitative (q) PCR for the amount of EBV DNA precipitated by antibodies to acetylated histone H4 (acH4) or acetylated histone H3 (acH3). DNA was amplified with primers for Zp (A), Rp (B), or EAp (C) (see Fig. 2A). The error bars show standard error of the mean.

Figure 4. Sodium butyrate and Trichostatin A induce hyperacetylation and phosphorylation of histone H3 in Raji cells. Shown are results of qPCR of ChIP from untreated Raji cells or those treated with TPA, NaB or Trichostatin A (TSA). DNA was immunoprecipitated with specific antibodies to acetylated histone H3 (K9 and K14) and
phosphorylated histone H3 S10 (phosH3) or with pre-immune rabbit serum. DNA was amplified with primers for Zp (A), Rp (B), or EAp (C).

Figure 5. **Comparison of induction of the EBV lytic cycle and histone hyperacetylation at early times after treatment of HH514-16 cells with sodium butyrate or Azacytidine.** A) Kinetics of BRLF1 and BZLF1 mRNA expression in HH514-16 cells at 2 hour intervals following treatment with sodium butyrate (B) or Azacytidine (A). Total RNA was prepared and examined for mRNA by Northern blotting with a probe, Z(301), that detects the 3.0Kb BRLF1 and 1.0Kb BZLF1 mRNAs. RNase P served as a loading control. B) Kinetics of expression of EBV lytic cycle proteins and acetylated histone H4 in HH514-16 cells following treatment with sodium butyrate (B) or Azacytidine (A). Extracts from untreated or chemically treated cells prepared at 4 hr intervals were examined for expression of ZEBRA, Rta and EA-D and acetylated histone H4 by immunoblotting. β-Actin served as a loading control. C and D) Kinetics of histone H3 acetylation on Rp and Zp following treatment with sodium butyrate (NaB) or Azacytidine (AZC). Chromatin immunoprecipitation with antibody to acetylated histone H3 was performed at time zero and 4 hrs and 6 hrs after chemical treatment. The immunoprecipitated DNA was analyzed for Zp (C) and Rp (D) by qPCR. Results are expressed as fold stimulation of untreated cells at time zero.

Figure 6. **Valproic acid does not induce the EBV lytic cycle but does induce histone hyperacetylation.** A) Valproic acid fails to induce BZLF1 or BRLF1 mRNA. HH514-16 cells were treated with NaB, AZC, TSA or VPA for 12 hrs. Total RNA was isolated and examined for mRNA by Northern blotting with the Z301 probe that detects the 3.0Kb BRLF1 and 1.0Kb BZLF1 mRNAs. RNase P served as a loading control. B) HH514-16 cells, at 1x 10^6 cells/ml,
were treated with sodium butyrate (B), AZC, TSA or Valproic Acid (VPA) at 1mM, 10mM and 50mM for 12 hrs. Extracts of $1 \times 10^6$ cells were loaded into each lane of an SDS-polyacrylamide gel. The immunoblot was probed sequentially for EA-D with monoclonal antibody R3.1, with rabbit polyclonal antibody to ZEBRA, with rabbit anti-acetylated histone H3, and with monoclonal antibody to β-Actin. C) B95-8 cells were treated for 12 hrs with TPA or VPA; Raji cells were treated for 12 hrs with TPA plus NaB or VPA alone. The immunoblot was probed with antibodies to EA-D, Rta, ZEBRA, acetylated-H3 (K9 and K14) and β-actin. In panels B and C the signals from acH3 and β-actin on the immunoblots were scanned by densitometry. Fold stim: = Fold stimulation of acH3 relative to unstimulated cells and corrected for β actin.

Figure 7. **Tricostatin A, Sodium butyrate and Valproic acid induce dimethylation of histone H3(K4) in HH514-16 cells.** HH514-16 cells were treated with AZC, TSA, NaB and VPA for 18 hours. Extracts from $1 \times 10^6$ cells were loaded onto an SDS-polyacrylamide gel. Immunoblots were probed with A) antibody to unmodified histone H3 or B) antibody to dimethyl histone H3(K4).

Figure 8. **Valproic acid causes hyperacetylation at Zp, Rp and EAp in HH514-16 cells.** Shown are results of qPCR for the amount of EBV lytic cycle promoter DNA precipitated from HH514-16 cells treated with NaB, AZC, TSA or VPA for 12 hrs. Chromatin was immunoprecipitated with antibodies to acetylated histone H3 (anti-acH3). DNA was amplified with primers for Zp (A), Rp (B), or EAp (C) (see Figure 2A).

Figure 9. **Synergistic activation of the EBV lytic cycle in Raji cells by TPA and the HDAC inhibitors, NaB, TSA and VPA.** Raji cells were treated with TPA plus NaB, TSA or VPA or
TPA alone for 24 hrs. Extracts from 1 x10^6 cells were loaded onto each lane of an SDS-polyacrylamide gel and the immunoblot was sequentially probed for EA-D using monoclonal antibody R3.1, for ZEBRA using a rabbit polyclonal antibody, and for β-actin using a monoclonal antibody.
Figure 1
Figure 2
Figure 3

RAJI
Figure 4

RAJI
Figure 5
Figure 7
Figure 8

A

B

C

HH514-16

Zp

Fold Stimulation

TSA

VPA

Treatment

B

AZC

anti-acH3

pref

Rp

Fold Stimulation

TSA

VPA

Treatment

B

AZC

anti-acH3

pref

EAp

Fold Stimulation

TSA

VPA

Treatment

B

AZC

anti-acH3

pref
Figure 9

RAJI