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[title]

HDAC inhibitor increases *Ac*MNPV gene expression in mammalian cells

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[Abstract]

The baculovirus *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) is used as a safer viral vector in mammalian cells with potential applications in gene therapy. However, the mechanism for the insusceptibility of mammalian cells to proliferative infection by entomopathogenic viruses is not well understood. Here we studied the significance of epigenetic modifications such as histone acetylation, histone methylation and HP1 accumulation to *Ac*MNPV gene expression in mammalian BHK cells. Real-time PCR and chromatin immunoprecipitation with sodium butyrate revealed an important relationship between viral gene expression and histone acetylation, having implications for a mechanism of suppression of *Ac*MNPV gene expression in BHK cells.

[Keywords]

baculovirus, *Ac*MNPV, histone deacetylation, nucleosome, epigenetic, gene therapy

[Introduction]

Autographa californica multiple nucleopolyhedrovirus (*Ac*MNPV), a type member of the invertebrate-specific virus family *Baculoviridae* (genus *Nucleopolyhedrovirus*, NPV), is a large rod-shaped virus with a double-stranded closed circular DNA genome (130 kbp) containing

approximately 150 genes [2]. Studies have demonstrated that *Ac*MNPV can enter the nucleus of mammalian cells independently of the cell cycle without multiplication [20, 21] and effectively express foreign genes [13], suggesting that the baculovirus can be used as a low risk viral vector in the field of gene therapy [5]. More recently, we elucidated that *Ac*MNPV expressed viral genes at least partly in mammalian cells through the usual infection pathway, though there was no evidence for the functional expression of viral genes [8]. Studies focusing on improving the expression from baculoviral vectors in mammalian cells have demonstrated that adding histone deacetylase (HDAC) inhibitors, trichostatin A (TSA) and sodium butyrate (NaBt), to the culture medium activated expression of foreign genes [9, 11, 19]. Moreover, HDAC inhibitors-induced gene expression from the viral vectors has been studied with the aim of application in clinical gene therapy [15, 17,]. HDAC changes the conformation of nucleosomes by removing acetyl groups from histones, resulting in an inactivated form of chromatin [4]. In this process, the lysine 9 residue in histone H3 (H3K9) is usually methylated after deacetylation, followed by the binding of heterochromatin protein 1 (HP1) binding and formation of heterochromatin. Considering the function of HDAC inhibitors, these reagents should affect not only foreign genes but also viral and cellular genes, presenting an important issue for the application of baculovirus vectors in the clinical field. Here we performed quantitative RT-PCR (qRT-PCR) and chromatin immunoprecipitation (ChIP) assays in this study to investigate the association of *Ac*MNPV DNA with histone proteins and the state of epigenetic

modification of the *Ac*MNPV genes in mammalian BHK cells.

[Materials and Methods]

Cells and virus

Sf-9 cells and BHK cells were maintained as previously described [8]. Treatment (inoculation) of the BHK cells with *Ac*MNPV was carried out by incubating the cells in DMEM containing *Ac*MNPV (30pfu/cell) for 2 hours at 37°C followed by cultivation in DMEM containing 10% FBS in the presence or absence of NaBt.

Quantitative RT-PCR

Total RNA was isolated from BHK cells 48 hours after the introduction of viruses. The first strand of cDNA for quantitative RT-PCR was synthesized from 1 μg of total RNA with an oligo-dT primer using an RNA PCR kit (AMV), ver.3.0 (Takara). Real-time PCR was performed using a One Step RT-PCR Kit (Perfect Real Time) (Takara) with gene-specific primer sets (Table 1).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using Chromatin immunoprecipitation (ChIP) assay kit (Upstate) according to the manufacturer's instructions. BHK cells were infected

with the viruses and incubated for 48 hours with or without 10mM NaBt. The precipitation was performed with anti-histone H3, anti-acetylated histone H3, anti-H3K9 dimethyl, anti-HP1 (heterochromatin protein) α, anti-HP1β or anti-HP1γ antibody (Upstate). After washing, the DNA/protein complexes were eluted and cross-linking was reversed. Precipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Real-time PCR was performed as described above with primer sets specific for each gene region (Table 1).

[Results]

We carried out a preliminary experiment to examine the effect of NaBt on *Ac*MNPV gene expression in BHK cells by using a NPV DNA microarray as described elsewhere [8]. The result showed that, in the absence of NaBt, the transcripts from about 10 *Ac*MNPV genes were detected with very slight signal intensities, being consistent with the result of the previous DNA microarray analysis [8]. On the other hand, more than 40 *Ac*MNPV genes expression was observed with strong signals in the presence of 10mM NaBt (data not shown). As DNA microarray analyses can have problems with the standardization of data and a narrow dynamic range for quantification [6], we adopted a quantitative RT-PCR method to further investigate/quantify the increase in *Ac*MNPV gene expression by NaBt. In this analysis, we selected 6 targets categorized as immediate-early (*ie-1* and *pe38*), delayed-early (*gp64* and *p35*), late (*vlf-1*), and very-late (*p10*) genes. By considering the

possible effects of *Ac*MNPV infection[8] and NaBt treatment on the host gene expression, we normalized the result with the amounts of template total RNA in this RT-PCR analysis. As a result, we detected the enhanced expression of all the target genes except for *p35* with the increase in NaBt concentration (Fig. 1). A significant increase in early gene expression was observed (more than 30-fold) in the presence of 10mM NaBt. On the other hand, the expression of *p35* in BHK cells was detected with neither the DNA microarray nor qRT-PCR regardless of NaBt concentrations. In addition, the expression from *vlf-1* is less responsible for NaBt treatment than the other genes. Thus, the results demonstrated gene-dependent (in responsiveness) and dose-dependent effects of NaBt on the transcription from *Ac*MNPV DNA in BHK cells.

We subsequently conducted chromatin immunoprecipitation (ChIP) assays to investigate the association of histone proteins with *Ac*MNPV DNA in BHK cells and the effect of NaBt on the modification of histone proteins (Fig. 2). ChIP with an anti-H3 antibody effectively precipitated DNA with *Ac*MNPV gene (*ie-1*, *pe38*, *gp64* or *vlf-1*) promoter sequences and a *pe38* coding sequence in BHK cells, but the amount of ChIP-precipitated viral DNA was not significantly changed by NaBt treatment (Fig. 2A). On the other hand, NaBt treatment markedly increased the amount of ChIP-precipitated *Ac*MNPV DNA when an anti-acetylated histone H3 antibody was used. This increase was significant ($p<0.05$, in t-test) both in the data normalized by the amount of input DNA in lysate (Fig. 2) and by cell numbers used in the ChIP assay (data not shown). This result

indicated that histone acetylation in *Ac*MNPV DNA was increased by NaBt treatment (Fig. 2B). We also observed the increases of the amount of ChIP-precipitated viral DNA with anti-HP1α (Fig. 2D; *ie1* and *gp64* promoter, and *pe38* CDS) and -HP1β (Fig. 2E; *ie1*, *gp64*, and *vlf-1* promoter) antibodies (Fig. 2C-F). However, the increases were not confirmed when normalization by cell numbers (data not shown), suggesting that the H3K9 dimetylation and association of HP1s on *Ac*MNPV DNA were not significantly altered upon NaBt treatment.

[Discussion]

The genomes of many DNA viruses such as herpes simplex virus (HSV), human papillomavirus (HPV), Epstein-Barr virus (EBV), and simian virus 40 (SV40) are assembled into chromatin-like structures in host cells, and the assembly and organization of nucleosomes are thought to be linked to the mechanism of replication and life cycle of the viruses [16]. Furthermore, the acetylation of histones plays a key role in the transcription of mammalian DNA viruses. For example, HSV-1 disrupts HDAC by using a viral protein, ICP0, resulting in successful viral gene expression [18]. HDAC inhibitors prevent the deacetylation of histones composing nucleosomes and accelerate acetylation catalyzed by HAT, resulting in a relaxation of the nucleosomal structure and increased gene expression [4]. *Ac*MNPV (both parental and progeny) DNA also assumed a chromatin-like structure in *Spodoptera frugiperda* cellular nuclei at least early in infection though the importance of

this structure to viral gene expression is not clear [22]. The ChIP analysis demonstrated that *Ac*MNPV DNA associated with histones also in mammalian cells (Fig. 2), indicating that *Ac*MNPV DNA associated with histone proteins after invasion into mammalian cells because viral DNA produced in insect cells was not associated with histones in parental virions [22]. Previous studies using baculovirus vectors demonstrated that HDAC inhibitors upregulated foreign gene expression from viral DNA in mammalian cells [9, 11, 19]. We demonstrated that *Ac*MNPV gene expression was also upregulated in BHK cells by an HDAC inhibitor, NaBt (Fig. 1), though not all the viral genes were equally activated upon NaBt treatment. The expression from *vlf-1* was less responsive to NaBt. Furthermore, the transcripts from *p35* was detected in neither the presence nor absence of NaBt. These observations might suggest that other mechanisms, in addition to the acetylation of histones, also functioned as determinants of *Ac*MNPV gene promoter activity in mammalian cells.

The treatment with NaBt increased the binding of the acetylated histone H3 to viral DNA, at least in the viral gene promoter regions examined (Fig. 2B). We also observed an increase in transcripts with the increase in histone acetylation (Fig. 1), suggesting that the deacetylation of histones binding to *Ac*MNPV DNA suppressed viral transcription in mammalian cells. Generally, it is believed that deacetylation of H3K9 causes the methylation of H3K9, which acts as a marker of HP1-associated repression [4]. However, no significant change in the dimethylation of H3K9 and binding of HP1s in *Ac*MNPV DNA upon NaBt treatment was observed. These results suggested that low level

expression of *Ac*MNPV genes in BHK cells (at least a part) was associated with histone deacetylation on viral DNA but not with H3K9 dimethylation or HP1 binding. Since high level of DNA methylation is also a trigger of epigenetic gene suppression [12],we also analyzed the status of the *Ac*MNPV *ie-1* promoter region in BHK cells but found no significant methylation (approx. 5% of cytosine residues) (data not shown), consisting with previous report [1]. The epigenetic modification involving the gene expression of *Ac*MNPV with circular genome DNA is thus still obscure in detail.

It was reported for DNA viruses such as HSV-1, HCMV, adenovirus, and papovavirus that the viral DNA localized to a specific nuclear architecture, so called promyeolocytic leukaemia nuclear bodies (PML-NBs), in host mammalian cells [7]. PML-NBs have been known to function in transcriptional regulation [3],and Daxx, a component of PML-NBs, associates with HDAC [10]. It was observed that these viruses sometimes disrupted PML-NBs and changed the distribution of PML proteins though the significance of these observations to viral replication is not clear [7]. Interestingly, it was also reported that *Ac*MNPV genomic DNA localized to PML-NBs in infected mammalian cells [14]. These observations imply relationships between the acetylation of histone H3 and the colocalization of *Ac*MNPV with PML-NBs, though the details await further investigation.

In conclusion, *Ac*MNPV genomic DNA was associated with histones in BHK cells where deacetylation probably acted as a suppressing factor for transcription from the viral genomic DNA,

emphasizing the importance of the deep insight into the effect of epigenetic modifications in the viral gene expression in gene therapeutic field.

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[Figure legends]

Figure 1

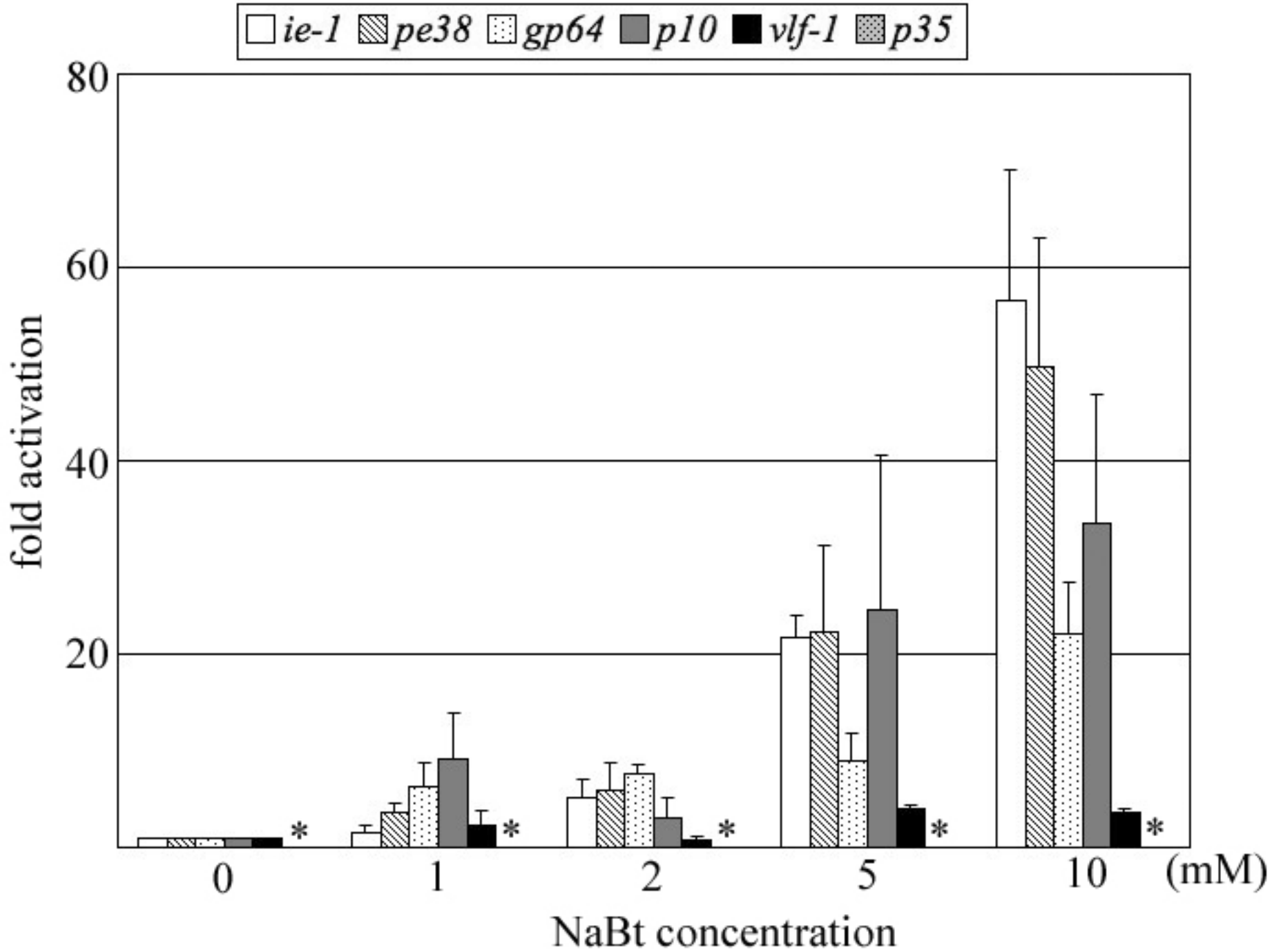
The effect of sodium butyrate on the *Ac*MNPV gene expression in BHK cells was analyzed by real-time PCR at 48 hours post inoculation in the presence of 0 ~ 10mM of NaBt. The individual bars indicate the genes analyzed in this assay (*ie-1*, *pe38*, *gp64*, *p10 vlf-1*, *p35*: from left to right). Quantitative RT-PCR was also adapted for *p35*, but we could not detect any transcripts regardless of the presence of NaBt (indicated as asterisks). The real-time PCR analysis was performed three times and standard deviations are indicated.

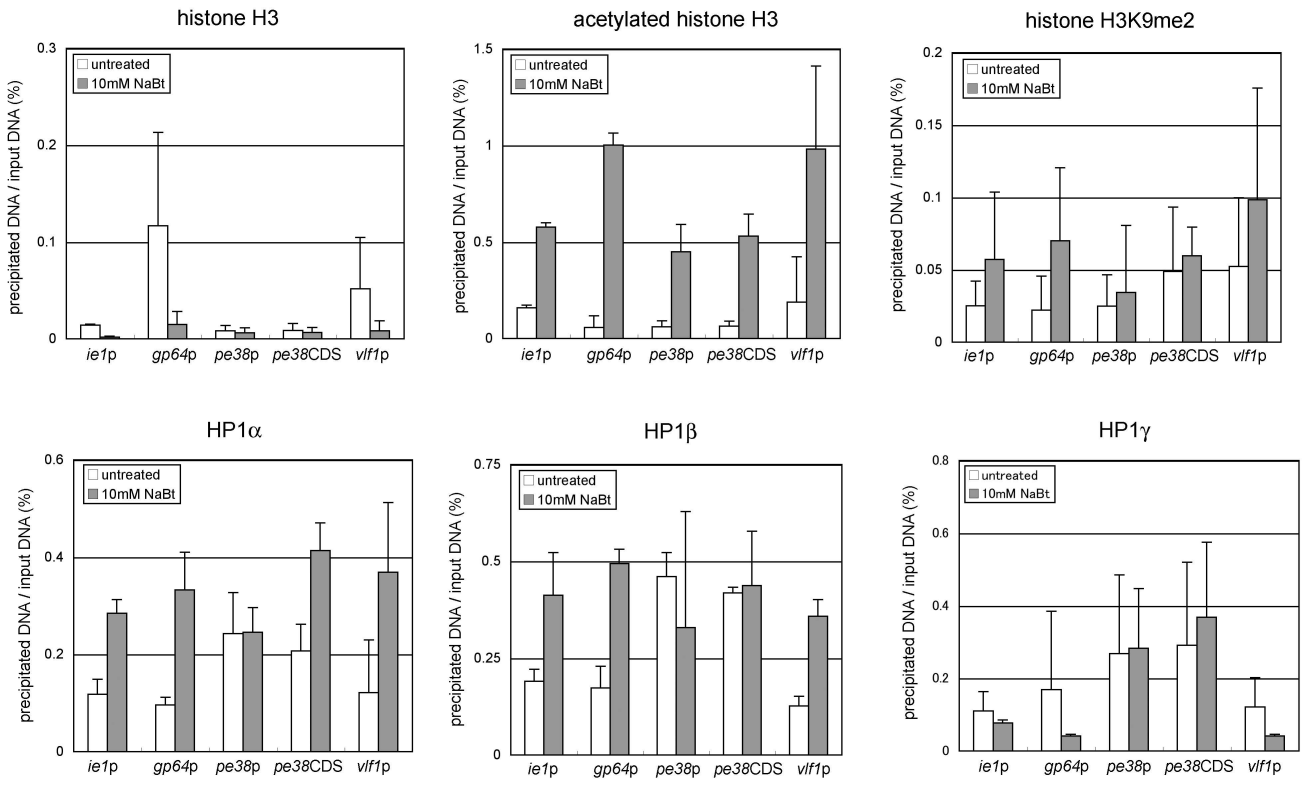
Figure 2

The ChIP-based analysis was carried out with anti-histones or HP1 proteins. The precipitated DNA fragments were quantified by real-time PCR using region-specific primers. A: Histone H3, B: Acetylated histone H3, C: Histone H3K9-dimethyl, D: HP1α, E: HP1β, and F: the binding of HP1γ on the individual panels indicate the antibodies used for each immunoprecipitation. The Y-axis shows the percentage of precipitated DNA to input DNA. The white and black bars indicate the amount of precipitated DNA in the absence or presence of 10mM of NaBt, respectively. The ChIP was performed three times and standard deviations are indicated.

Table 1 The primer oligonucleotide sets used in the quantitative RT-PCR and ChIP analysis

quantitative RT-PCR	
Target genes	Oligonucleotide sequences
ie-1	5′-GCGTCGTACACCAGCGCTTC-3′ and 5′-TGAAGCTGCCGCAGTCTCGC-3′
pe38	5′-AAGGGACACCAACAATCGCC-3′ and 5′-GAGCTGGTTTTGACGCTGCC-3′
gp64	5′-TATGTGCTTTTGGCGGCGGC-3′ and 5′-GCATACGCCTGGTAGTACCC-3′
p35	5′-GAAATCGACGTGTCCCAGAC-3′ and 5′-GTTCTTGCGCGTAACGCTTC-3′
p10	5′-TGACGCAAATTTTAGACGCC-3′ and 5′-GTCAATTGAGCGGGCAAACC-3′
vlf-1	5′-TTCAATCCGCTCCCCGGTTC-3′ and 5′-ACGGTTTGGGGAACATGTAC-3′
ChIP analysis	
Target regions	Oligonucleotide sequences
ie-1 promoter	5′- GTCGGCGGATGTTCTATATC-3′ and 5′- TCACTTGGTTGTTCACGATC-3′
pe38 promoter	5′-GAACGGCAGCTGTATTTATACT-3′ and 5′-TGTTGGTGTCCCTTGGCATTTT-3′
gp64 promoter	5′- AGCGTCCGTGTTCATGATCC-3′ and 5′- GTGTGTTCCTTATTGAAGCC-3′
vlf-1 promoter	5′- CAATAAACGACATGGTGGGC-3′ and 5′- ACAAAAGGAGAATCGGTGTC-3′
pe38 CDS	5′-ATTCCGACTACGTGCGACCAC-3′ and 5′-CTCACCTGATGCTGCAACTCTT-3′


ie-1
pe38
gp64
p10
vlf-1
p35
80
60
40
20
fold activation
0
1
2
5
10
(mM)
NaBt concentration
*


histone H3
acetylated histone H3
histone H3K9me2
HP1α
HP1β
HP1γ
untreated
10mM NaBt
precipitated DNA / input DNA (%)
ie1p
gp64p
pe38p
pe38CDS
vlf1p