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Evidence for Association of Non-acetylated Histones with Newly Replicated Epstein-Barr Virus DNA

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EVIDENCE FOR ASSOCIATION OF NON-ACETYLATED HISTONES WITH NEWLY REPLICATED EPSTEIN-BARR VIRUS DNA.

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ABSTRACT

Epstein-Barr Virus (EBV) has two states of infection, latent and lytic. During the latent state the viral genome remains stable in cells as episomes and replicates with cellular DNA. During the lytic cycle the viral DNA becomes amplified and packaged in newly formed virions. An unsolved problem is whether newly replicated EBV DNA produced upon lytic cycle activation is associated with histones, and if so, whether these histones are acetylated. This question has biological significance as knowing the chromatin structure of genes is important in determining their function and expression profile. Our hypothesis is that newly synthesized EBV lytic DNA is associated with histones and the histone tails are selectively acetylated. To investigate our hypothesis we performed chromatin immunoprecipitation (ChIP) in HH514-16 cells, a Burkitt's Lymphoma cell line, during latent and lytic replication. We used quantitative PCR (qPCR) to detect the relative concentration of DNA among the different samples. We tested three different variables: type of inducing agent, duration of treatment, and different regulatory regions in the genome of Epstein-Barr Virus. We found that in cells induced into the lytic cycle with Trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), association of newly replicated EBV DNA with acetylated histone 3 (H3) increased ~ 6-10 fold. This increase in association was greatest 72 hrs after treatment. Furthermore, activation of lytic viral replication in HH514-16 cells using a different inducing agent, Azacytidine (AZC), which is known to function as a DNA methyltransferase inhibitor, increased binding of H3 with viral DNA ~8 fold. However, unlike TSA, AZC increased the acetylation state of histones bound to newly synthesized viral DNA only ~ 2 fold. Changing the regulatory region of the EBV genome analyzed in qPCR did not affect our results. Our results suggest that newly replicated viral DNA is associated with histones, a fraction of which are acetylated. The degree of acetylation likely depends on the agent used to induce the lytic cycle. H3 is highly acetylated when an HDACi is used and less acetylated when AZC is used. Our study provides new insight on the epigenetic profile of newly replicated viral DNA during the lytic cycle. It remains to be determined whether histones are packaged together with viral genomes into virions and whether the chromatin state of virion DNA affects gene expression after the virus enters uninfected cells.

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Characteristics of the Virus

Epstein-Barr Virus (EBV), a member of the herpesvirus family, primarily infects B-lymphocytes but sometimes infects epithelial cells, Natural Killer cells, and T-cells ((1-3), as reviewed in (4, 5)). Infection with EBV is common. According to the CDC, in the United States approximately 95% of adults between ages 35 and 40 are infected. Worldwide, 90% of adults are infected (6).

Most people become infected during childhood when the infection is asymptomatic. An early age of infection is especially true in the developing world, as rates of infection in children of developed countries are lower than in developing countries. Conversely, the rates of infection in adolescence or early adulthood are higher in developed countries than in developing countries. When an infection occurs during adolescence or early adulthood the disease often manifests as infectious mononucleosis (IM) (7) .

Once someone becomes infected with EBV the viral DNA will remain indefinitely as an episome[1] in a latent[2] stage in about 10% of the exposed cells (8, 9). This episome can be found in the nucleus of B-cells (10). The lytic[3] cycle can become

[1] An episome is a double-stranded, circular, extra-chromosomal DNA molecule.
[2] Latent cycle, also referred to as lysogenic cycle, is where the virus remains dormant. Gene expression of most of the viral reading frames is turned off except for a few genes that are sufficient to cause transformation and immortalization of infected cells. Very little viral replication takes place in this cycle.
[3] The lytic cycle is when active viral replication takes place and viral particles (virions) are formed. Most of the genes are expressed during this stage.

activated at any point in time in these cells. If it does become activated EBV DNA is amplified 100-1000 times and repackaged, as a 184 kb double-stranded linear genome, into virions[4] (11, 12). The DNA is surrounded by a capsid, tegument[5] and a glycoprotein spiked envelope (10, 13, 14). Then the virion is released. In epithelial cells in the mouth the virus remains in the lytic stage; it is shed in the saliva and can be passed on orally (15, 16).

Latent Infection

The EBV genome is linear in virus particles but when it infects B-cells it remains in the cell as a multicopy episome. During latency the virus expresses a small number of viral genes. One such class of genes is the six EBV nuclear antigen (EBNA) genes, the expression of which are driven by either the *Bam*H1[6] W promoter (Wp) during initial infection, the *Bam*H1 C promoter (Cp) during the most transcriptionally active latent state (type III latency), and the *Bam*H1 Q promoter (Qp) during a less transcriptionally active latent state (type I latency) (17-22). The gene products (except for EBNA-1) are targets of cytotoxic T-cells ((23), as reviewed in (24)). EBNA-1 protein also recognizes the EBV latent origin of replication (oriP) (25). The binding of EBNA-1 to oriP is necessary for replication to take place. Replication in latently infected lymphocytes will only occur once per cell cycle (26, 27). EBNA-1 is also important for episome maintenance as it tethers EBV DNA to cellular chromosomes (28-30). EBNA-2, the first

[4] A virion is a complete virus particle that can exist outside of the cell.
[5] Tegument refers to the proteins that fill the space in between the envelope and nucleocapsid of a virus.
[6] *Bam*H1 is a restriction endonuclease that recognizes the 6bp sequence of DNA 5'-GGATCC-3', and cleaves after the 5'-G. It was used to digest the EBV genome, and the genes were named accordingly (see footnote on naming EBV genes).

protein that is expressed when EBV infects B-cells, is a transactivator that regulates the expression of genes encoding latent membrane protein 1 (LMP1), LMP2A, and also many cellular genes (4, 31). LMP1 is an inhibitor of apoptosis and functions by upregulating expression of the BCL2 and A20 genes (which encode anti-apoptotic proteins) (32, 33). In fact, LMP1 has been shown to be an oncogenic protein – expression of the gene encoding LMP1 in the B-cells of transgenic mice results in the mice developing lymphomas (34). LMP1 also mimics tumor necrosis factor (TNF) receptors, which are constitutively expressed (35). LMP2A inhibits B-cell receptor (BCR) function by binding to and sequestering tyrosine kinases[7] (36). This binding prevents EBV in infected B-cells from being activated by antigens into the lytic cycle. Antigenic binding also mildly stimulates the tyrosine kinases. This stimulation is important for the survival of B-cells (37). EBV also expresses non-translated RNAs: EBERs (EBV-encoded RNAs) and BARTs (BamH1-A rightward transcripts) (38-41). EBERs are thought to induce the secretion of interleukin-10, which may be responsible for stimulating the growth of EBV-infected B-cells as well as suppressing cytotoxic T-cells (42). However, the exact function of EBERs in the viral life cycle is not clear.

There are four different patterns of EBV latency that are known (as reviewed in (4, 5)). In type I latency, found in Burkitt Lymphoma cells, the viral genes expressed include two EBER genes, the BARTs and EBNA-1 (43, 44). In type 2 latency, seen in Hodgkin's disease, LMPs 1, 2A, and 2B are expressed in addition to the genes expressed in type I latency (45). In latency type III, seen in immunocompromised patients with lymphoproliferative diseases as well as lymphoblastoid cell lines transformed with EBV,

[7] Tyrosine kinases are cell membrane enzymes involved in signal transduction. They transduce signals by phosphorylating tyrosine residues in proteins.

the two EBER genes, the three LMP genes, and all six EBNA genes are expressed (46). Latency type 0 is found in memory B-cells and has no detectable gene expression (47).

Genes Critical to Lytic Activation of Epstein-Barr Virus

ZEBRA, a protein encoded by the viral gene BZLF1[8], is a major transcription factor critical to lytic replication of EBV (48, 49). Expression of ZEBRA is sufficient to activate the lytic cycle in latently infected cells (48, 50). ZEBRA was originally thought to be an immediate early protein, but an experiment with the inhibitor of protein synthesis, cyclohexamide, proved otherwise (51). Blocking protein synthesis prevented ZEBRA mRNA from being produced. This discovery suggests there is activation of a gene upstream of BZLF1 responsible for initiation of transcription of BZLF1. Nevertheless, ZEBRA is a very early protein that activates expression of early viral lytic genes. ZEBRA also binds to its own promoter, Zp, and autoactivates transcription of itself, enhancing the switch from latent to lytic cycle (52). One of the early proteins ZEBRA activates is R transactivator (Rta), encoded by the BRLF1 gene (53). Na, another early viral protein encoded by the open reading frame BRRF1, works with Rta to activate ZEBRA (54, 55). ZEBRA and Rta work synergistically to activate expression of downstream genes such as BMRF1, which encodes the viral DNA polymerase processivity factor, also known as Early Antigen Diffuse (EA-D) (56). Both ZEBRA and Rta bind to the BMRF1 promoter. ZEBRA also binds to the lytic origin of replication,

[8] Naming EBV genes: the EBV genome was digested with *Bam*H1, thus the first letter for EBV genes is B. The fragments were labeled A-Z, according to decreasing size, so the second letter corresponds to the size of the fragment. The third letter corresponds to left-ward (L) or right-ward (R) transcription. The fourth letter stands for frame and the following number is which specific frame in the *Bam* fragment is being referred to.

oriLyt, where it acts as a replication protein (57). Expression of late genes, such as viral capsid antigens, occurs after DNA replication is initiated (58-60). Late gene expression is generally dependent on viral replication, as blocking replication results in the absence of late gene expression (61). However, Rta was found to be capable of activating some late genes, such as BRLF2 and gp350, independent of ZEBRA and in the absence of DNA replication (62). Rta was shown to actually activate these genes to a greater degree when ZEBRA was absent than when ZEBRA was present. Thus, ZEBRA is thought to play a repressive role in Rta's ability to activate some late genes at early times.

The Structure of ZEBRA Protein

ZEBRA has 4 domains: a transcriptional activation domain (aa 1-166), a regulatory domain (aa 167-177), a basic domain (aa 178-194) and a dimerization domain (aa 195-225) (63-65). Two of the main functions that ZEBRA performs during the lytic phase of the EBV life cycle are activation of the expression of Rta and other early lytic genes and promotion of viral replication. Both functions are dependent on the capacity of ZEBRA to recognize specific DNA sequences, known as ZEBRA response elements (ZREs), through its basic domain (66). Studying 48 single point mutations, all installed in the DNA binding domain of ZEBRA, resulted in four different phenotypes. The first phenotype was identical to the phenotype of wild type (wt). The second phenotype was inability to activate expression of Rta. These mutants also bound weakly to DNA. The third phenotype was inability to syngergize with Rta to activate expression of EA-D. The last phenotype was inability to activate expression of late viral genes. There are a few hypotheses as to why these mutants were able to activate early but not late genes. One

hypothesis is that perhaps the mutants were unable to activate a specific early gene that is responsible for activating late genes. Another hypothesis is since the mutants seem to be more soluble in 0.3M NaCl than wt, they may be defective in nuclear compartment localization (described below). Lastly, the mutants could be defective at binding oriLyt.

Two amino acids in ZEBRA, S167 and S173, have been found to be phosphorylated *in vivo*, most likely by casein kinase 2 (CK2) which phosphorylates those amino acids *in vitro* (65, 67). These amino acids are critical to ZEBRA's ability to repress Rta from activating some of the late viral genes at early times. If the S167 and S173 amino acids are not phosphorylated ZEBRA cannot repress Rta. Additionally, when S173 was mutated to alanine so that it could not be phosphorylated ZEBRA was unable to bind to the origin of replication and initiate replication (64). The ZEBRA S173A mutant was, however, able to activate Rta and EA-D. This discovery indicates that phosphorylation is important for DNA binding and also suggests that stronger DNA binding is needed for activation of replication than for activation of transcription. When S173 was mutated to aspartic acid, which resulted in a phosphomimetic mutant (the mutant mimics the activity of phosphorylated S173), ZEBRA was still able to activate DNA replication. It did so, in fact, with greater efficiency.

Lytic Replication Occurs in Nuclear Compartments

When fluorescent in situ hybridization (FISH)[9] was used to probe viral DNA (specifically the BAMH1W gene) in EBV-infected cells, the viral DNA was found in small intranuclear dots (68). When these cells were induced into the lytic cycle the viral

[9] FISH is a technique where fluorescent DNA probes are used to find the location of certain DNA sequences on chromosomes, or in this case episomes.

DNA was found either in course granules or larger globules. The thought was that these larger globules were actually compartments in the nucleus where replication of the virus was taking place. Compartmentalization in the nucleus appears to be characteristic to lytic replication of all of the herpesviruses, including HSV and CMV. Upon further investigation ZEBRA, which is diffusely distributed throughout the nucleus immediately after activation, was also found to localize to these compartments about 46 hours after the lytic cycle was activated (69). In addition, the protein EA-D colocalized with ZEBRA around this time period to form mature compartments. If viral replication was blocked by the inhibitor of viral DNA polymerase, phosphonoacetic acid (PAA) (70), however, ZEBRA and EA-D did not colocalize after 46 hours.

The point mutations in ZEBRA's basic domain that were described earlier also affected the ability of ZEBRA to localize to these replication compartments (71). S186A, a mutation that led to ZEBRA's inability to activate Rta, resulted in ZEBRA being diffusely distributed through the nucleus and not localized to replication compartments. The mutation R179A, which resulted in ZEBRA being unable to activate EA-D, was found in many discrete punctate foci. Cells transfected with these two mutants, S186A and R179A, did not express EA-D. The mutation unable to activate late genes, Y180E, also caused ZEBRA to be found in punctate foci. In this cell EA-D was present but diffusely located instead of localized to replication compartments. The reason the latter two mutations led to ZEBRA being found in a speckled appearance is not clear, but the punctate foci could represent an intermediate stage between lytic activation and viral DNA replication. Thus, the speckles wouldn't be found when the S186A mutant was used as this mutant is not able to activate the lytic cycle.

Induction of the Lytic Cycle

The original thought was that cells infected with EBV could be induced into the lytic cycle mainly through the Protein Kinase C (PKC) pathway[10] ((72), as reviewed in (68)), as phorbol esters activate the PKC pathway and also activate the lytic cycle of EBV (73). However, subsequent evidence suggests that there is more than one pathway to lytic activation. This evidence comes from the observation that there are many ways to activate the lytic cycle of EBV-infected cells. Also, different cell lines are activated in different ways. For example, HH514-16 cells (a Burkitt's Lymphoma cell line) can be activated by HDAC inhibitors such as TSA and sodium butyrate as well as the demethylating agent Azacytidine (AZC), but cannot be activated by phorbol esters (68). In B95-8 cells (a lymphoblastoid cell line), however, the opposite is true – phorbol esters activate the lytic cycle but HDAC inhibitors do not (74). In the lymphoid Raji cells, where HDAC inhibitors don't activate the lytic cycle but phorbol esters do, HDAC inhibitors actually enhance the effect of phorbol esters. In Akata cells, which are derived from a Japanese patient with Burkitt's, the main activator is anti-IgG (75). The other agents do not activate the lytic cycle in this cell line.

Why different cell lines respond differently to inducing agents is not clear. For those cells that are not respondent to phorbol esters, the lack of response does not have to do with failure to activate PKC because PKC was induced by the phorbol ester in all cell lines regardless of whether or not the lytic cycle was induced (68). Also, the differences in response to HDAC inhibitors do not have to do with chromatin configuration of the DNA, as the nucleosomal structure of EBV in the responsive HH514-16 and the

[10] The PKC pathway is a signal transduction pathway.

refractory B95-8 cells were similar to each other when digested with micrococcal nuclease. This mystery of why different cell lines respond differently to inducing agents is still under investigation.

As a note, the inducing agents aren't sufficient to induce the lytic cycle in all EBV infected cells (76). Only 20-30% of cells enter the lytic cycle when treated *in vitro*. The refractory cells aren't permanently so – if they are recultured for several weeks they can also be induced into the lytic cycle. Why some cells are able to be induced and some are not is also something that is still under investigation.

Negative Regulation of the Lytic Cycle

While there exists several agents that can induce the lytic cycle, there also exist agents that can inhibit the cycle from being activated. Retinoic acid (RA) is one such agent that has been shown to negatively regulate lytic induction of the virus by the phorbol ester TPA (77). RA prevents induction by blocking ZEBRA's ability to activate downstream promoters (78). The receptors for RA (RAR) appear to be involved, as expression of RAR plasmids blocks ZEBRA from transactivating the downstream gene BMRF1.

Endogenous proteins also serve to regulate lytic activation. The p65 subunit of NF-κB[11] blocks ZEBRA's function in EBV-infected cells (79). In fact, the two proteins have been shown to physically interact. The thought is that perhaps NF-κB's inhibition of lytic activation is a mechanism to prevent the latent cycle from being disrupted. Further evidence supporting this theory comes from the fact that when the negative inhibitor of

[11] NF-κB stands for nuclear factor kappa-light-chain-enhancer of activated B cells, and has become widely accepted to be a major transcription factor in most animal cells.

NF-κB, IκB, was overexpressed in Raji cells ZEBRA's ability to transactivate the promotor of early gene BHRLF1 increased 4 fold. Another endogenous protein, the tumor suppressor p53, was also shown to interact with ZEBRA (80). P53's overexpression blocked ZEBRA from activating the lytic cycle.

Epigenetics

Epigenetics is the study of the ability of cells to inherit phenotypic variations that do not manifest from changes in the DNA sequence (as reviewed in (81)). Epigenetics plays a major role in transcriptional regulation of genes. The way the phenotypic variations are thought to arise is from changes in the DNA methylation pattern or alterations of the chromatin structure associated with genes. These alterations are often in the form of post-translational modifications of the N-terminal tails of histones. Histones are the core proteins that DNA is wrapped around to form nucleosomes. The four core histone proteins are H2A, H2B, H3, and H4. One nucleosome is an octamer that contains 2 of each core histone protein along with 147 bp of DNA. Modifications of the histone tails, typically those found on H3 and H4, are thought to influence transcriptional activity. These post-translational modifications include acetylation[12], methylation[13], phosphorylation[14], ubiquitination[15], and sumoylation[16] (as reviewed in (81, 82)). The modification state of chromatin can then be passed on to daughter cells during

[12] Acetylation is the addition of a CH_3CO group.
[13] Methylation is the addition of a CH_3 group.
[14] Phosphorylation is the addition of a PO_4 group.
[15] Ubiquitination is the addition of ubiquitin, a small protein. More than one monomer of ubiquitin can be added. Often these are used to tag proteins for degradation.
[16] Sumoylation is the addition of a SUMO protein, which is similar to ubiquitin but does not typically tag proteins for degradation.

mitosis. The mechanism of conserving histone changes among progeny is not yet completely elucidated, but a semi-conservative model has evidence supporting it. In this model the H3 and H4 histones split into 2 identical dimers, each of which go to the daughter strand. These dimers can then be used as a template for the new H3 and H4 in the nucleosome. The evidence supporting this model includes the finding that the form of H3/H4 that was shown to be deposited was a dimer and not a tetramer (83).

Chromatin Structure of Herpesviruses

The chromatin structure of another herpesvirus, Herpes Simplex Virus 1 (HSV1), has been largely determined. During the latent cycle the HSV1 DNA is packaged into chromatin (84). The HSV1 DNA is also associated with histones early in the lytic cycle, but the histones dissociate from viral DNA during the process of packaging viral DNA into capsids (85, 86). Additionally, during the lytic cycle the N-terminal tails of H3 associated with viral DNA are acetylated such that transcription is facilitated.

While there is not a lot of information on chromatin structure of EBV during the lytic cycle, there is some literature on EBV's chromatin structure during the latent cycle. For the most part EBV DNA is wrapped around histone octamers during latent infection (87). However, the chromatin structure was found to be different around the latent origin of replication (OriP) and the gene encoding EBER1 (88). These areas of DNA are easily digested by micrococcal nuclease while the remaining DNA is not. This finding suggests that these areas of DNA are not protected in nucleosomes. That the region that encodes the EBER gene is not chromatinized might explain why the EBER1 gene is transcribed in latent infection. For the case of OriP, this area of DNA appears to be attached to cellular

chromosomes (88, 89). The ZEBRA promoter (Zp) and Rta promotor (Rp), on the other hand, were both found to be in nucleosomes when digested with micrococcal nuclease (68). This finding makes sense, as the corresponding genes are transcriptionally silent during the latent cycle.

Acetylation

Acetylation of the N-terminal tails of histones is often a form of regulation of gene expression (Figure 1). Acetylation can also occur on proteins other than histones, such as transcription factors like p53 (90). Like the majority of post-translational modifications, acetylation is reversible. The enzyme responsible for acetylation is histone acetyl transferase (HAT), with the main mammalian members being p300, CBP, and pCAF (as reviewed in (82)). HATs were first discovered to target the lysine residues in the N-terminal tails of histones (especially histone 3). HATs are typically found in multi-protein complexes with other transcription factors. Since acetylation neutralizes positively charged lysine residues it makes the histones less attracted to negatively charged DNA. Thus, acetylated chromatin is more loosely packed and more accessible to transcription factors (as reviewed in (82, 91)). Conversely, enzymes that deacetylate histones, HDACS, are thought to be associated with decreased transcription as they lead to DNA being more tightly packed around the chromatin. There are eleven different isoforms of HDACs that are numbered from 1 to 11. The role of these HDACs is not only to remove acetyl groups from histone tails to induce transcriptional repression, but also to allow other post-translational modifications to occur at the N-termini of these deacetylated histones (82).

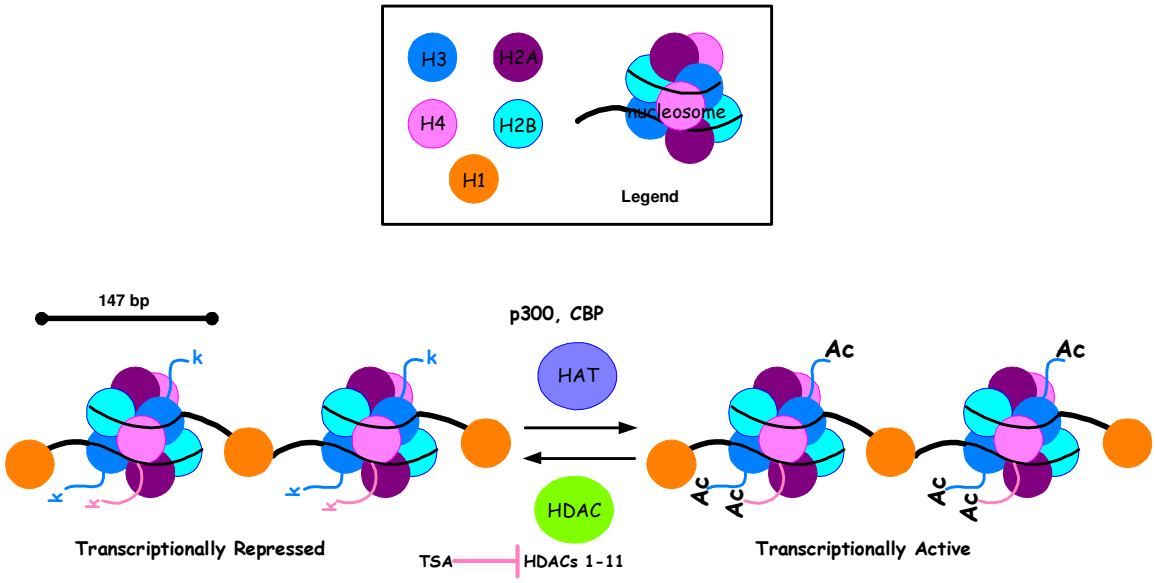


Figure 1: Histone acetylation as a form of transcriptional regulation. DNA, when arranged in chromatin, is wrapped around an octamer of histone proteins with H3 and H4 both having N-terminal tails that extend out. Acetylation of the lysines on these tails can neutralize positive charge and thus loosen the association of DNA with the histones. This loosening allows greater access of transcription factors to the DNA, often causing activation of transcription. The enzymes responsible for acetylation are Histone Acetyl Transferases (HATs), and the enzymes responsible for the counter action, deacetylation, are Histone Deacetylases (HDACs). Generally, these enzymes are recruited to chromatin via transcription factors.

These post-translational modifications of histones add another level of regulation during gene expression. Different modifications of histone tails will have a specific effect on the activity of a promoter, a process known as the "histone code" (as reviewed in (92)).

Acetylation May be Important for Lytic Activation of EBV

Some experiments have been performed which demonstrate that acetylation of histones associated with Zp is necessary for the switch from latent to lytic cycle to occur (93). Researchers stably transfected episomes containing Zp into Akata cells and found

that the promoter was chromatinized during the latent cycle (87). They also found an increase of Zp associated with acetylated histones (specifically H4) upon induction of the lytic cycle. Acetylation, however, is not sufficient for activation of the lytic cycle (74). A study was done looking at the effect of the HDAC inhibitors TSA and sodium butyrate on the acetylation state of the histones associated with Zp and the Rp in cell lines that are both responsive (HH514-16) and refractory (Raji, B95-8) to lytic activation by these agents. Hyperacetylation of H3 and H4 on these histones occurred in both types of cell lines, but lytic activation did not occur in the refractory ones. In addition, valproic acid (VPA), an HDAC inhibitor (HDACi) that does not induce the lytic cycle in HH514-16 cells, also leads to hyperacetylation of H3. Both of these experiments demonstrate that hyperacetylation of either Zp or Rp is not sufficient to activate the lytic cycle.

Another study found that transfecting CBP and p300 into cells in the presence of ZEBRA can enhance induction of the lytic cycle by ZEBRA (94). Also, ZEBRA was discovered to be associated with CBP in cells that were lytically but not latently infected with EBV. Further experiments were able to show that ZEBRA specifically activated the HAT function of CBP (95). *In vitro* acetylation of small oligonulcotides by CBP only occurred when the ZEBRA protein was added to the reaction mixture, and was dependent on the presence of a functional HAT domain. If the HAT domain of CBP was deleted, acetylation of small oligonucleotides did not occur.

In addition to the HAT domain CBP also has two cysteine-histidine (C/H) -rich domains, referred to as C/H1 and C/H3, and a bromodomain that binds acetyl-lysines ((96), as reviewed in (97, 98)). The transcriptional activation domain of ZEBRA interacts with CBP at C/H1 and C/H3 (94). The presence of the C/H3 and bromodomain

of CBP are both required for ZEBRA to be able to activate transcription of Rta (99). These two domains are also required for ZEBRA to stimulate HAT activity of CBP. This finding was demonstrated using an approach similar to the one above, where acetylation of small olignoucleotides was assessed in the presence of ZEBRA protein with intact CBP or CBP lacking either the C/H3 or bromodomain. ZEBRA was not able to stimulate HAT activity of the mutant forms of CBP protein.

This same study also looked at which particular lysines CBP acetylates on the N-terminal tails of the H3 and H4 histones associated with viral DNA. ZEBRA was found to stimulate acetylation of all of the lysines. This discovery was proven using western blot with various antibodies containing different patterns of acetylation on H3 and H4. Edman degradation[17] was also used on H3 (Edman degradation could not be done on H4 as in HeLa cells the N-termini of H4 are blocked) with ^{3}H-acetylated histones to show that ZEBRA stimulates the acetylation of all lysines.

The necessity of acetylation in the induction of the lytic cycle is controversial, as there exists some data that shows the lytic cycle can be activated in the absence of histone acetylation (74). This evidence comes from using AZC to induce the lytic cycle. When AZC was used, the acetylation state of Zp and Rp did not significantly increase despite lytic activation. Thus, acetylation is not sufficient and may not even be necessary for activation of the lytic cycle.

[17] Edman degradation is an experimental technique used to determine the sequence of amino acids in a peptide.

Methylation of EBV DNA

Methylation of the cytosine bases in DNA also plays a role in controlling gene expression for EBV, as it does for many other cell systems. In DNA there exists CpG islands[18], 70% of which are methylated in mammalian cells (as reviewed in (100, 101)). The level of methylation is typically inversely related to the transcriptional activity of the corresponding genes. Methylation prevents transcription a few different ways, including preventing transcription factors from binding to DNA as well as recruiting HDAC inhibitors to deacetylate histones (as reviewed in (102)).

DNA Methylation is Important for EBV

In latently infected cells the DNA of EBV is heavily methylated (103, 104). How ZEBRA can activate gene transcription when the DNA is in this state then becomes a question. It turns out that ZEBRA is quite unique in that it preferentially binds to Rp when it is methylated (105). Rp contains three Z response elements (ZREs), two of which contains a CpG motif. This CpG island was examined in five different cell lines and was found to be methylated in every one of them. When this sequence of DNA was used as a probe for ZEBRA the methylated form was 10 times more efficient at binding ZEBRA than the unmethylated form. Also, using a construct of Rp linked to a

[18] CpG refers to a sequence of DNA where there is a cytosine nucleotide linked linearly with a guanosine nucleotide. CpG islands are regions in the DNA where several of these pairs occur.

chloremphenicol acetyl transferase (CAT) gene[19], ZEBRA was shown to activate methylated Rp at a much higher rate than unmethylated Rp. This phenomenon is unique to ZEBRA – it has not been described in any other system to date. Recently ZEBRA was also found to preferentially bind to the methylated promoter of another early viral gene, Na (106). Na also contains methylated ZRE's that are activated by ZEBRA at a higher rate than when the ZREs are unmethylated. The serine 186 amino acid of ZEBRA is required for binding to both methylated promoters (106, 107).

The latent C promoter of EBV, on the other hand, does not behave like Rp. As mentioned earlier, the EBNA proteins driven by EBV latent C promoter are targets of CD8+ cytotoxic T-cells. In certain cancers associated with EBV, such as Burkitt's lymphoma and Hodgkin's disease (which will be discussed later), this promoter is not transcriptionally active and thus the cells are able to evade T-cell destruction (reviewed in (24)). The reason the promoter is inactive is that a CpG island upstream of the promoter is methylated in these cancers (103). Evidence that supports the idea that methylation is responsible for EBNA gene repression comes from the experiment that treated these cells with AZC (100). When this DNA methyltransferase inhibitor was added to a Burkitt's lymphoma cell line the latent C promoter was activated and EBNA-2 was transcribed.

Medical Relevance

While EBV is known to cause infectious mononucleosis (IM) during primary infection in adolescents, reactivation of a latent infection also has a role in several

[19] This gene is a reporter gene used in assays to assess function of the promoter being studied. The protein produced from this gene renders cells resistant to the antibiotic chloramphenicol.

cancers. If the lytic cycle is activated in latently infected cells EBV can predispose to lymphomas such as Hodgkin's disease and Burkitt's lymphoma (76). It can also lead to lymphomas in immunocompromised patients and natural killer (NK) and T-cell lymphomas in patients that are chronically infected with EBV (108, 109). Evidence that it is the activation of the lytic cycle that favors malignancies comes from the fact that elevated antibody titers against lytic antigens precede such malignancies (110, 111). Also, despite the fact that latent infection of cells with EBV is critical for malignancy, EBV strains not capable of lytic viral replication do not lead to lymphoma mice with Severe Combined Immunodeficiency (SCID) (112).

Infectious Mononucleosis

According to the CDC approximately 50% of adolescents or young adults who have a primary infection with EBV experience IM. Why children don't experience IM is not clear, but it could have to do with the fact that adolescents may be more likely than children to receive the virus in higher doses through oral routes (hence the term "kissing disease"). The different responses could also have to do with a different immune response, as IM is an immunological disease. Pathologically, the site for initial infection is thought to be the B-cells or epithelial cells located in the oropharynx (113). There the tonsils become invaded with lymphoblastoid cells that contain EBV in the three different latency patterns ((114, 115), as reviewed in (116)). These infected cells also circulate among the B-cells (117). The virus in these cells can also become lytically activated. A CD8+ cytotoxic T-cell response is triggered by these latently or lytically infected cells, producing the atypical lymphocytes characteristic of IM (118, 119). Of course those cells

lytically infected trigger a greater response due to the higher number of antigens produced. This T-cell response leads to a rapid decrease in viral shedding and in the number of infected B-cells, resulting in convalescence (120). The T-cell numbers are then thought to be reduced by apoptosis. Subsequently, the number of genes expressed in the cells that are indefinitely infected with EBV in the latent cycle is somehow downregulated so T-cells do not also clear these cells.

Clinical symptoms can vary so patients could simply have a low-grade, transient fever or they could endure lymphadenopathy, malaise, and pharyngitis for weeks. Unfortunately no medical treatments have been shown to effect the course of the disease, including acyclovir (the nucleoside analog that blocks viral replication in vitro) (121). Acyclovir also had no effect on the number of EBV infected B-cells in patients during treatment (122). Acyclovir did, however, stop the virus from shedding (although viral shedding went back up after treatment was stopped). The fact that an inhibitor of replication could not reduce clinical symptoms indicates that perhaps the symptoms are caused by the proinflammatory cytokines like IL-1, IFN-γ, and TNF-α that are released by the T-cells, and not by viral replication (123). However, if prednisolone is added to acyclovir there is again no effect on the duration of the clinical course (although there is a slight improvement during the first few days) or number of latently infected B-cells (124). The failure of steroids to have an effect could be due to the fact that IFN-γ is able to overcome glucocorticoid's effect on decreasing the amount of TNF-α produced by macrophages (125). IFN-γ is present at elevated levels during infection, which would explain why glucocorticoids don't have a profound affect in IM (126).

Burkitt's Lymphoma

Burkitt's Lymphoma was originally described by Denis Burkitt, who found a high incidence of the disease in equatorial Africa (127). Most of those affected were under 15 years of age (128). Endemic Burkitt's lymphoma typically presents as extranodal tumors in the jaw, orbit, central nervous system, or as an abdominal mass. The histology of the tumor is similar to that of germinal B-cell centers, with macrophages spread throughout, giving the classic "starry sky" appearance (129). The classic molecular mutation is a reciprocal translocation between chromosome 8 (at the site of the *c-myc* proto-oncogene) and chromosome 14 (which is where the *Ig* heavy chain gene resides) (130). There are cases of sporadic Burkitt's lymphoma, which typically manifests as an abdominal mass or in a leukemic form, with the same translocation (as reviewed in (131)). Most of these sporadic cases occur in the United States. The sporadic form has a lower association with Epstein-Barr Virus than the endemic form (15-25% of tumors are EBV genome positive in the sporadic form verses 100% in the endemic form) ((10), as reviewed in ((131)).

The mutation that leads to Burkitt's lymphoma is thought to arise from the stimulation of somatic hypermutation in germinal center B cells by Epstein-Barr Virus (132). There is also some evidence that the virus helps the tumor cells continue to grow (133). Akata cells that had lost the EBV DNA also lost the malignant properties of Burkitt's Lymphoma, such as the ability to grow in low serum and the ability to grow tumors in nude mice. Also, dominant negative forms of EBNA-1 protein impair the growth of Burkitt's Lymphoma cells (134). Thus, EBV seems to play a significant role in Burkitt's Lymphoma.

Hodgkin's Lymphoma

EBV is also associated with Hodgkin's lymphoma (135). The tumors in Hodgkin's disease are made up of a mixture of mononuclear lymphocytes, multinuclear, malignant Reed-Sternberg cells derived from B-cells, and non-malignant cells (which make up greater than 98% of the tumor) (as reviewed in (136)). In the United States and European countries Hodgkin's has a low incidence in childhood and a higher incidence in young adults (137). Childhood Hodgkin's is more common in countries that are less developed. This difference suggests that perhaps in more developed countries the age of incidence is higher due to delay of exposure to EBV. Evidence to support this theory comes from studies that showed that children with factors that lead to delayed exposure to infectious agents (early birth order, small family, high maternal education) are at greater risk of getting Hodgkin's as a young adult (137, 138).

A study was done looking at titers of IgG antibodies against the viral capsid antigen of EBV as well as antibodies against early antigens (111). Titers were found to be higher than normal in patients with Hodgkin's disease. In blood that had been drawn at an average of 50.5 months before diagnosis (compared to controls from the same population) the relative risk of Hodgkin's disease associated with higher antibody titers was 2.6 (90% confidence interval was 1.1 to 6.1) for IgG and 3.7 (90% confidence interval was 1.4 to 9.3) for IgA. The relative risk for Epstein-Barr nuclear antigen was 4.0 (90% confidence interval was 1.4 to 11.4). Additionally, there have been a number of studies that show that patients with a history of infectious mononucleosis are ~ 3 times as likely to get Hodgkin's (139, 140). Further evidence indicating that EBV is associated with Hodgkin's is that 19% of biopsies from patients with Hodgkin's had EBV DNA

(detected by slot blot hybridization[20]) (141). In situ hybridization also showed great intensity of EBV DNA in Reed-Sternberg cells. All of this evidence seems to indicate EBV is strongly associated with Hodgkin's lymphoma.

Malignancies in Immunocompromised Patients

Patients who are immunocompromised are also subject to EBV induced B-cell lymphomas. Included in this category are patients with congenital immunodeficiencies, patients who have received organ transplants, and patients who have AIDS ((142), as reviewed in (143, 144)). Congenital immunodeficiencies that result in patients being susceptible to these B-cell lymphoroliferative diseases include XLP (145), SCID (146), Wiskott-Aldrich syndrome (147), and common variable immunodeficiency (148), as these patients are unable to mount a proper cell-mediated immune response over the virus ((149), as reviewed in (144)).

Transplant patients, because of their persistent immunocompromised state, have been found by numerous studies to be at a higher risk to develop lymphomas (as reviewed in (116, 143)). The lymphoma in question is typically post-transplant lymphoproliferative disease (PTLD), and has been found to be associated with EBV (150, 151). The estimate is that about 6% of patients get cancers post-transplant (152). 23% of these cancers are PTLD, resulting in an overall incidence of 1.4%. Who gets PTLD depends on a number of things, including type of transplant and whether or not the patient is EBV seropositive at the time of transplant. The higher the immunosupression

[20] Slot blot hybridization, also known as dot blot, is a technique similar to Western or Southern blots. However, instead of separating the molecules by chromatography, the mixture that contains the molecule in question is applied to a membrane as a dot. Then DNA or antibody probes are used to detect the molecule in question.

needed the greater the risk of PTLD (153, 154). Thus, patients who have heart/lung transplants have a 5-9% risk while patients who have renal or liver transplants have a 1-2% risk ((155), as reviewed in (143)). Patients receiving bone marrow transplants also have a ~ 1% risk. This risk can increase to 12-24%, however, if the donor T-cells are depleted (156). On the other hand, if both donor T- and B-cells are depleted the risk of developing PTLD is greatly reduced. This risk reduction occurs because the EBV-infected donor B-cells are the cells that PTLD is derived from in these patients (the hosts immune system is usually irradiated) (157). Host irradiation does not occur in solid organ transplants, however, and it is thought that the source of PTLD in these patients is from the host (158).

A patient's EBV status can influence their risk of PTLD. Those who are EBV negative have a 20-fold higher incidence of PTLD than those who are positive, indicating that a primary infection is more dangerous than reactivation of a latent infection (159). This phenomenon was proven using DNA hybridization for the EBNA gene in tumor cells of patients post-transplant. This discovery indicates why pediatric patients are at higher risk than adults for PTLD (160).

The types of PTLD vary greatly both pathologically and clinically (as reviewed in (143)). Different pathological lesions include plasma cell hyperplasia with reactive elements similar to acute IM, polymorphic lesions that look like B-cell lymphoma, and monomorphic lesions that resemble immunoblastic lymphoma. Clinically, PTLD can present in lymph nodes or extranodally. It can also present as one or multiple masses. These are just some of the clinical variations of PTLD.

The first line treatment of PTLD is typically to reduce the amount of immunosupression drugs so a patient's T-cell response against EBV can take over (161). This form of treatment has been found in some studies to control PTLD in 31% of cases (162). A risk of immunosupression reduction is allograft rejection so reduction has to be done carefully and the patient has to be closely monitored. Acyclovir and Gancyclovir have also been used prophylactically in some patients as there has been evidence that this is somewhat effective (although not enough evidence to make it standard practice) (163). Radiation (when the CNS is involved) and chemotherapy also have some role in treatment of PTLD (164, 165). The overall outcome of patients with PTLD depends on type, as the disease varies greatly both clinically and pathologically.

Patients who have HIV are at an even higher risk of developing B-cell lymphomas than post-transplant patients (142). They are 60 times more likely to develop non-Hodgkin's lymphomas than the general population. Half of these lymphomas are associated with EBV, and different types of lymphomas have different strengths of association (as reviewed in (166)). These lymphomas are thought to arise from a deficient T-cell response to the virus (146).

Manipulation of EBV to Aid in Chemotherapy

Knowledge of how EBV functions in cells can provide ways to target tumor cells in EBV-associated malignancies. There have been a few studies looking at treatments that take what is known about the virus into consideration. For example, certain chemotherapy drugs have been shown to induce the lytic cycle, including 5-flourouricil (5-FU) in epithelial cells (167) and methotrexate in both epithelial and B-cells (167, 168).

These drugs seem to require the P38 stress MAPK, P13 kinase, and PKC signaling pathways. Since gancyclovir is more capable of killing EBV-positive cells in the lytic form (acyclovir becomes phosphorylated in cells that are lytically infected to become active) (169), these chemotherapeutic agents actually enhance gancyclovir's ability to kill EBV-positive tumor cells (167). Thus, the combination of gancyclovir and chemotherapy agents could be used to specifically target EBV-positive cells; the chemotherapy agent would activate the lytic cycle and then gancyclovir would kill the cell.

Valproic Acid (VPA), an anti-seizure drug that has been shown to weakly induce the lytic cycle in EBV-positive tumor cells, has been found to work synergistically with other chemotherapy agents to induce the lytic cycle (170). This same synergistic effect is not shown when AZC is used with chemotherapy drugs. Also, VPA greatly enhances the ability of agents such as 5-FU and cisplatin along with gancyclovir to kill EBV-positive tumor cells (both epithelial and lymphoblastoid). These experiments show that there is some unique property of VPA that could make it a very useful drug in cancers associated with EBV. As a note, VPA has been shown to block induction of the lytic cycle by TSA and AZC, so whether or not VPA can be used as a chemotherapy drug is controversial (Derek Daigle, unpublished data).

STATEMENT OF PURPOSE

Hypothesis: During EBV lytic infection, the virus replicates its genome several hundred fold. It is not known whether the newly synthesized EBV genomes interact with histones to form nucleosomal DNA and whether the tails of these histones are modified. My

hypothesis is that newly synthesized EBV lytic DNA is associated with histones and the histone tails are selectively acetylated.

Experimental Design: To test our hypothesis we will employ the technique chromatin immunoprecipitation (ChIP) performed on a Burkitt's Lymphoma cell line. In our experiments we will test three different variables: type of inducing agent, duration of treatment, and different regulatory regions in the genome of Epstein-Barr Virus.

METHODS

In this thesis, the cell line used was provided by Lee Heston. The reagents used to induce the lytic cycle were provided by Lyn Gradoville. I performed the cell treatments, ChIP and quantitative real time PCR (qPCR) experiments. Dr. Ayman El-Guindy provided the primer pairs as well as the samples used for the standard curve.

Cell Culture and Activation of EBV Lytic Cycle

The cell line used was HH514-16, an EBV-positive cell line that was cloned from the human Burkitt's lymphoma cell line P3HR1 (171). This cell line was cloned by Lee Heston of the Miller Lab. I maintained the cells in RPMI 1640 media containing 8% fetal calf serum, 50 units/mL antibiotics (penicillin and streptomycin), and 1 g/ml fungizone.

I treated HH514-16 cells during the logarithmic growth phase, during the initial 48 hours after subculture. At this phase the cell count ranged between 0.8-1.1 10^6

cells/ml. I treated cells with 5 M TSA or 5 M AZC. 500 M PAA was added to some samples as a control. These reagents were prepared by Lyn Gradoville. I harvested cells at several time points between 12 and 72 hours after treatment.

Chromatin Immunoprecipitation

I cross-linked ~1x10^7 cells with 1% formaldehyde for 10 minutes at 37 C in the growth medium to covalently link DNA-protein complexes. I then washed the cells twice with phosphate-buffered saline containing the protease inhibitors Pepstatin A and PMSF at concentrations of 1 g/mL and 500 M, respectively (Roche). After re-suspension in SDS lysis buffer (containing 1% SDS, 50mM Tris-HCL pH 8.1, and 10 mM EDTA), made by me or purchased from Millipore for my last set of experiments (in which AZC was the inducing agent), I sonicated the cells for 10 s x 4, using a Sonifier 450 apparatus (Branson). The supernatant was obtained by centrifuging the cell lysates (14,000 rpm) for 10 minutes at 4ºC. I transferred the supernatant to a new tube and diluted the samples 10x in ChIP dilution buffer (also made by me or purchased from Millipore for my last set of experiments) containing 0.01% SDS, 16.7 mM Tris-HCl pH 8.1, 1.1% Triton X-100, 167 mM NaCL, 1.2 mM EDTA, and the protease inhibitors listed above. 100 L was taken from each sample as input. The samples were then incubated with 80 L of Protein A Agarose – 50% slurry containing Salmon Sperm DNA at 4 C for half an hour. The samples were centrifuged for 1 minute at 1000rpm, and the supernatant was transferred to a new tube. Subsequently I added 10 g IgG along with 60 L of agarose beads to the samples to clear nonspecific antibody interactions. This incubation was for two hours, at 4 C. The samples were then centrifuged for 1 minute at 1000rpm and the

supernatant was transferred to a new tube containing 10 g of a polyclonal rabbit antibody against H3 or AcH3 (purchased from Millipore). The protein-DNA complexes were incubated with the antibody overnight at 4 C. The complexes were collected using the agarose beads. I then washed the beads five times with different solutions made by me (or purchased from Millipore for my last set of experiments): 1x with a low concentration salt solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), 1x with a high concentration salt solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), 1x with a lithium chloride solution (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris pH 8.1), and 2x with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was eluted off the beads with a solution of 100 mM sodium bicarbonate and 1% SDS. I then reversed the crosslinks by adding 20 L of 5M sodium chloride and heating at 65ºC for four hours. The protein was digested at 45ºC for 2 hours with 20 g proteinase K, 10 L of 0.5 M EDTA, and 20 L of 1 M Tris-HCl pH 6.5. The DNA was then precipitated at 20ºC overnight with 10% sodium acetate, 2.5x volume 95% ethanol, and 20 g glycogen. The samples were then spun down for 30 minutes at 4ºC (13,000 RPM). I washed them with 70% ethanol, spun for another 5 minutes at 4ºC (13,000 RPM), and then the ethanol was aspirated. After the samples dried I added 15 l of TE and left the samples to dissolve at room temperature for ½ hr.

Quantitative Real Time PCR

I next used Real-time PCR (qPCR) to amplify and quantitate the immunoprecipitated DNA, using the Biorad iCycler. Standard curves prepared from

different concentrations of plasmids containing oriLyt or Zp (obtained from Dr. Ayman El-Guindy) were used to calculate relative concentrations of immunoprecipitated DNA. We used concentrations of 10, 1000, and 100,000 fg/ L. The sequences for oriLyt and Zp, which were also obtained from Dr. Ayman El-Guindy, are listed below (Table 1). Also, the relative concentration of immunoprecipitated DNA by the antibodies was divided by the amount of OriLyt or Zp present in the inputs to control for the amount of viral DNA present in each sample.

Table 1: Primer pairs

	Forward	**Reverse**
Zp	TTGACACCAGCTTATTTTAGACACTTCT	TTACCTGTCTAACATCTCCCCTTTAAA
oriLyt	TCCTCTTTTTGGGGTCTCTG	CCCTCCTCCTCTCGTTATCC

RESULTS

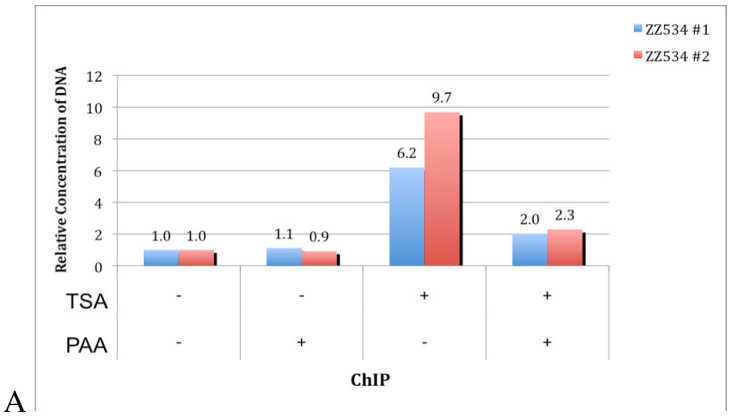
TSA Increases Acetylation of Histones Associated with EBV DNA

The first experiment was to see the effect of TSA, a known lytic cycle inducing agent, on the association of viral DNA with acetylated histones. HH514-16 cells were either left untreated, treated with PAA[21], or had both TSA and PAA added. Cells were treated for 24 hours and then we performed chromatin immunoprecipitation (ChIP) using an anti-acetyl H3 (AcH3) antibody. We used qPCR with primers specific for the upstream region of oriLyt (origin of lytic replication). We found that when the levels of DNA were normalized to the DNA in uninduced cells the addition of PAA by itself had

[21] PAA stands for phosphonoacetic acid. It inhibits viral DNA polymerase.

no effect. Treatment with TSA however, seemed to greatly increase the amount of viral DNA associated with acetylated histones (Figure 2A). This increase was ~ 6-10 fold. When PAA was added to TSA-treated cells the amount of DNA associated with acetylated histones was significantly reduced by ~3-4 fold. Analysis of DNA nonspecifically precipitated by normal IgG revealed no significant amounts of oriLyt DNA (data not shown). The input (the DNA in the cell before the antibody was added) followed a similar profile as ChIP, indicating that TSA induced while PAA blocked lytic viral DNA replication (Figure 2B). However, when the amount of oriLyt precipitated with the anti-AcH3 antibody was corrected for the level of DNA present in the corresponding input sample we found that TSA-treated samples had a smaller increase in the amount of oriLyt pulled down over uninduced samples (~2-3 fold increase) (Figure 2C). Also, this time, adding PAA to TSA did not seem to decrease the amount of DNA that was precipitated.

Using the same ChIP samples I investigated the association of H3 with Zp (ZEBRA promoter). The results were similar to the previous experiment: TSA increased the amount of viral DNA associated with acetylated histones by ~10 fold (Figure 3A), the input followed a similar profile as ChIP (Figure 3B), and when the amount of Zp precipitated with the anti-AcH3 antibody was corrected for the level of DNA in the corresponding input samples TSA-treated cells had a smaller increase in the amount of Zp pulled down over uninduced (~5.5 fold) (Figure 3C).



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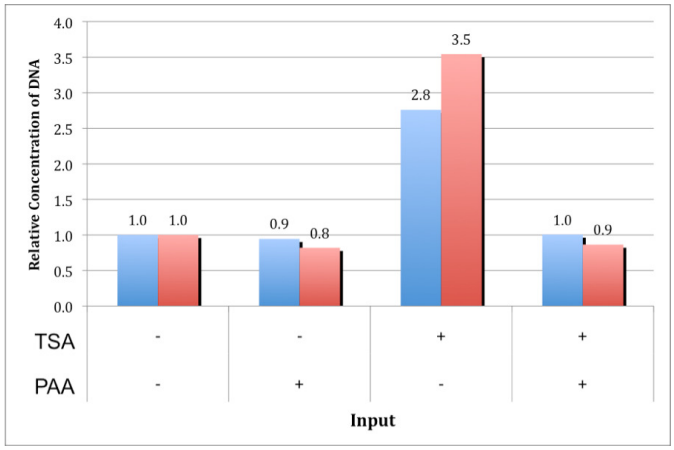

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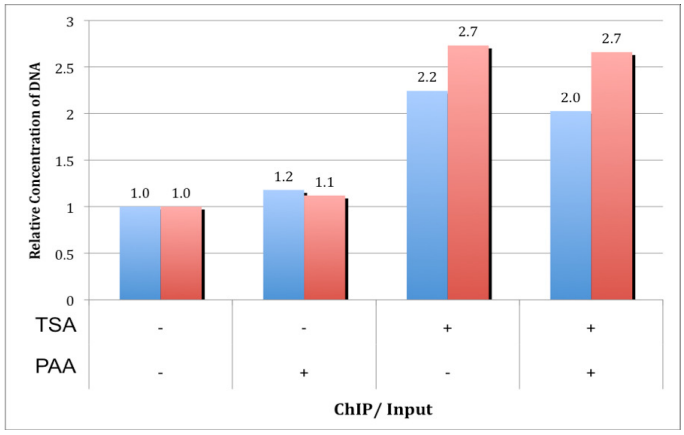
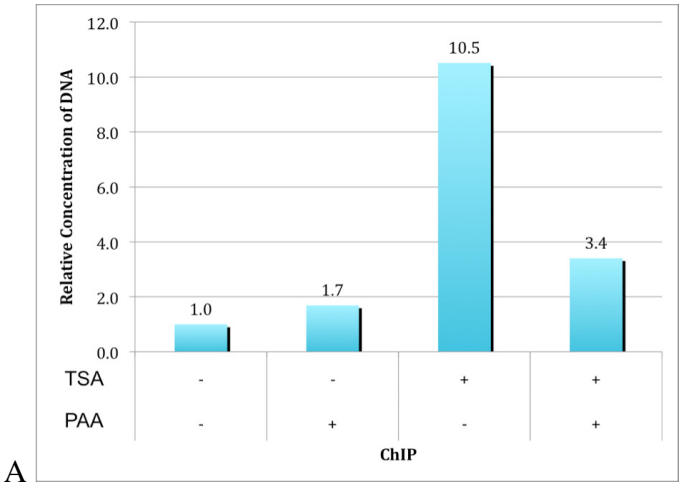


C

Figure 2: Induction of the lytic cycle with an HDAC inhibitor increases acetylation of histones associated with viral DNA. HH514-16 cells were either untreated, treated with PAA, treated with TSA, or treated with TSA and PAA. At 24 hours they were harvested and ChIP was performed using anti-AcH3 antibodies. Primers against the origin of replication, OriLyt, were used in qPCRs. ZZ534 #1 and #2 are technical replicates of the same ChIP experiment. **A.** Relative concentration of DNA associated with acetylated histones, normalized to uninduced. **B.** Relative concentration of total DNA, normalized to uninduced. **C.** Relative concentration of DNA associated with acetylated histones corrected for input DNA, normalized to uninduced.



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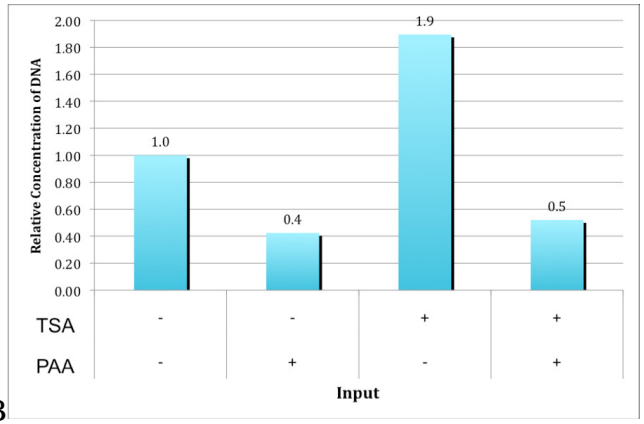

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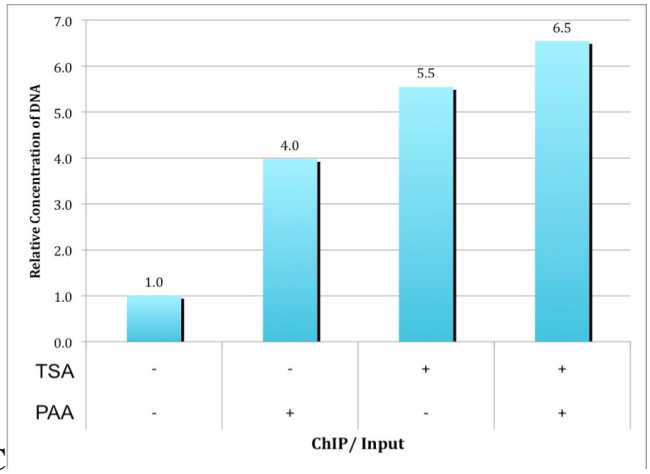


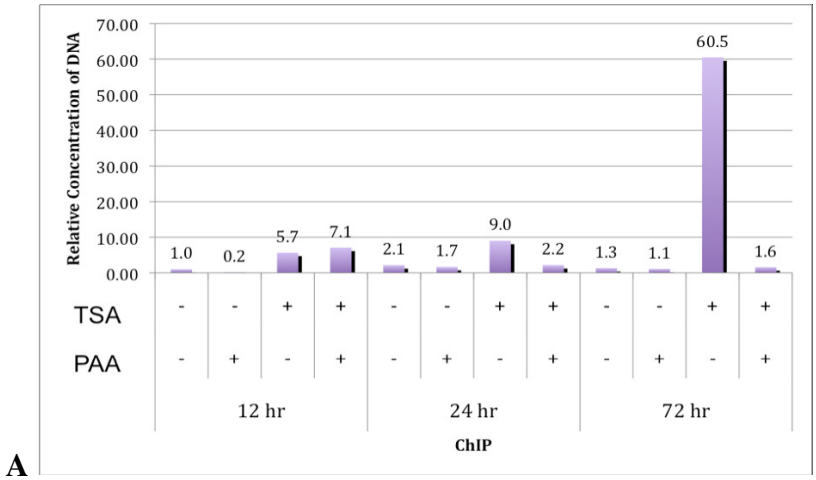
C

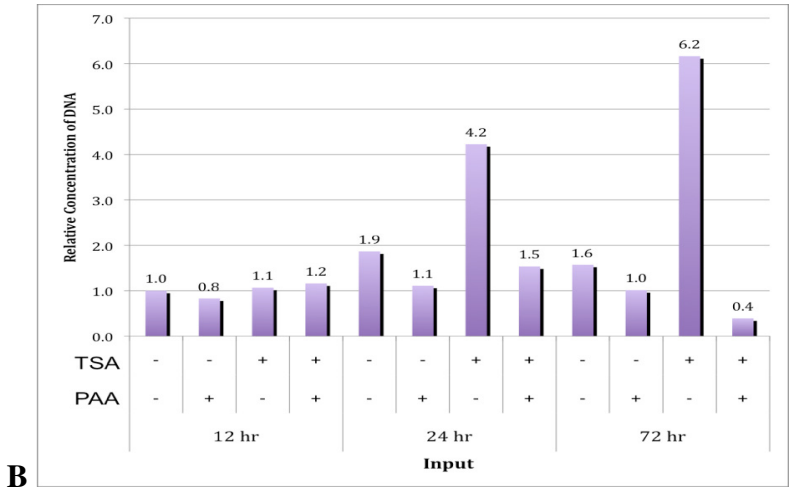
Figure 3: Induction of the lytic cycle with an HDAC inhibitor increases acetylation of histones with viral DNA. HH514-16 cells were either untreated, treated with PAA, treated with TSA, or treated with TSA and PAA. At 24 hours they were harvested and ChIP was performed using anti-AcH3 antibodies. Primers against the promoter for ZEBRA, Zp, were used in qPCRs. **A.** Relative concentration of DNA associated with acetylated histones, normalized to uninduced. **B.** Relative concentration of total DNA, normalized to uninduced. **C.** Relative concentration of DNA associated with acetylated histones corrected for input DNA, normalized to uninduced.

Association of Viral DNA with Acetylated Histones Increases as Replication Does

To examine the effect of viral replication on association of viral DNA with acetylated histones we carried out chromatin immunoprecipitation at three time points: 12 hours, 24 hours, and 72 hours. We chose these time points because we knew from previous experiments performed with the same cell line that replication could not be detected after treating the cells with TSA for 12 hours.

Like the previous experiment, HH514-16 cells were either left untreated, treated with PAA, induced with TSA, or treated with both TSA and PAA. We used Zp primers in the qPCR. We found that at 12 and 24 hrs there was a small increase in the amount of DNA associated with acetylated histones in cells induced with TSA (~6 fold for 12 hours and ~ 9 fold for 24 hours), but at 72 hrs there was a substantial increase of ~60 fold (Figure 4A). Adding PAA slightly increased the association at 12 hours but decreased the association at 24 and 72 hours. As we expected, by analyzing the input we found that no viral replication was detected during the first 12 hours of the EBV viral lytic cycle (Figure 4B). Viral DNA replication was barely detected after 24 hours. Among these three time points viral replication was highest at 72 hours. Analysis of DNA nonspecifically precipitated by normal IgG revealed no significant amounts of Zp DNA (data not shown). When the amount of Zp precipitated with the anti-AcH3 antibody was corrected for the level of DNA present in the corresponding input sample the results for 12 and 24 hours were similar to those in the previous experiment, in that there was no difference between association of acetylated histones with newly replicated viral DNA in TSA-treated cells and non-replicated DNA in the TSA and PAA treated





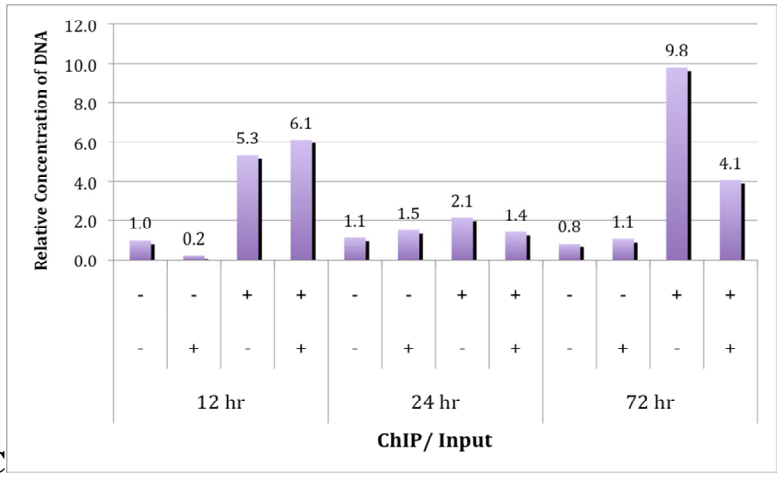


Figure 4: As Lytic Replication Increases, So Does the Amount of Viral DNA Associated with Acetylated Histones. HH514-16 cells were either untreated, treated with PAA, treated with TSA, or treated with TSA and PAA. They were harvested either at 12 hours, 24 hrs, or 72 hrs, and ChIP was performed using anti-AcH3 antibodies. Primers against the ZEBRA promoter (Zp), were used in qPCR. A. Relative concentration of DNA associated with acetylated histones, normalized to uninduced. B. Relative concentration of total DNA, normalized to uninduced. C. Relative concentration of DNA associated with acetylated histones corrected for input DNA, normalized to uninduced.

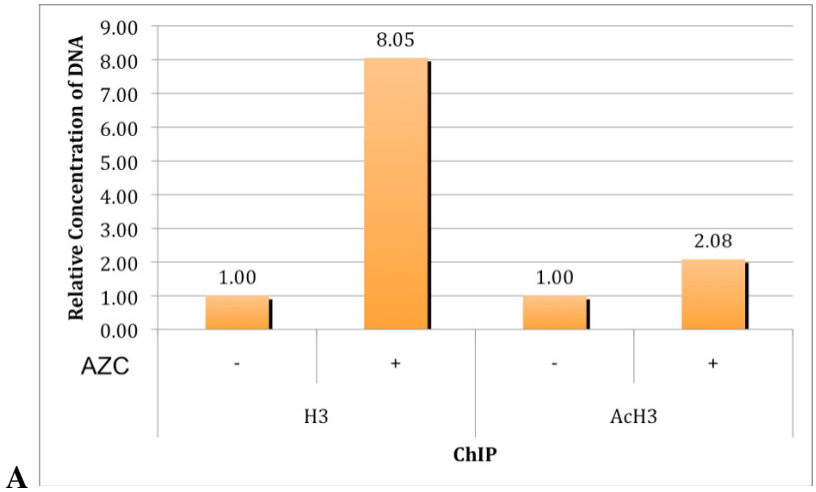
cells (Figure 4C). At 72 hours, however, the results were slightly different. At this time point adding TSA to PAA did lead to a decrease the amount of DNA associated with acetylated histones, about ~2 fold.

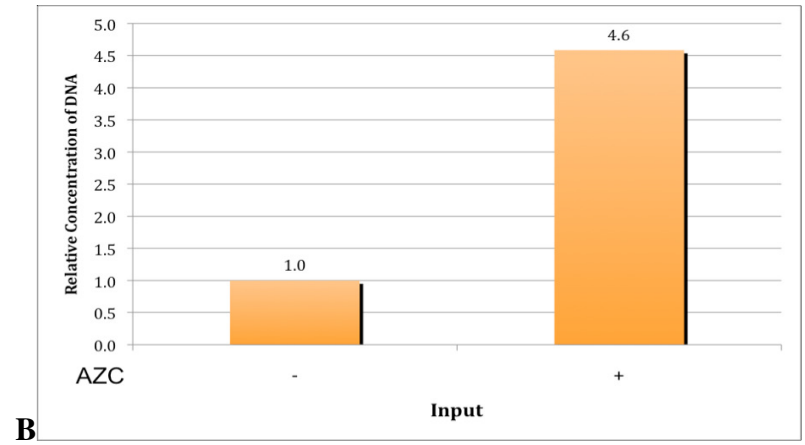
Newly Replicated DNA is Associated with non-Acetylated Histones

One caveat of the previous experiments is that an HDACi was being used to induce the lytic cycle. The worry is that the HDACi could have increased the acetylation state of the histones independently. For that reason we decided to use the DNA methyltransferase inhibitor AZC to induce the lytic cycle, as we know from previous experiments that AZC does not induce acetylation of H3 (74). HH514-16 cells were either untreated or treated with AZC. Cells were harvested after 48 hrs to allow sufficient time for lytic DNA replication to occur. This time both anti-H3 and anti-AcH3 antibodies were used in the ChIP experiment. Zp primers were used in the qPCR. We found that when AZC was added the amount of DNA associated with H3 increased ~ 8 fold while the amount of DNA associated with AcH3 only increased ~ 2 fold (Figure 5A). Thus, the ratio of DNA pulled down for non-acetylated histones to acetylated histones was roughly 4 fold when the lytic cycle was induced.

The input data verified that the induction of viral replication was successful by showing that the overall amount of DNA had also increased, about ~5 fold, with the addition of AZC (Figure 5B). Analysis of DNA nonspecifically precipitated by normal IgG revealed no significant amounts of Zp DNA (data not shown). When the amount of Zp precipitated with the antibody was corrected for the level of DNA present in the corresponding input sample we found that with the H3 antibody there was only a slight

increase in association of viral DNA with histones when AZC was added. With the AcH3 antibody on the other hand, there was a decrease in association (Figure 5C). The findings from this set of experiments were reproducible in three biological replicates (data not shown). We also found that using oriLyt primers did not change our results (Figure 6): newly replicated viral DNA has a greater association with non-acetylated histones than acetylated histones (Figure 6A).





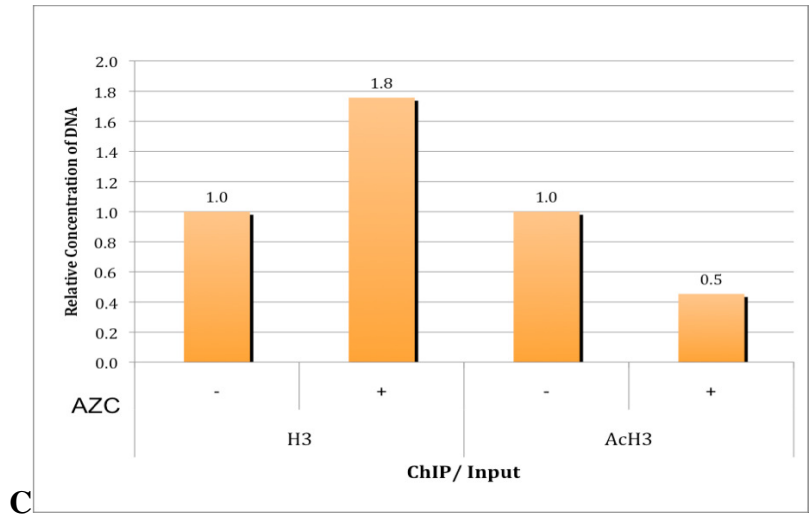


Figure 5: Newly replicated DNA appears to be associated with histones that are not acetylated. HH514-16 cells were either untreated or treated with AZC. They were harvested either at 48 hours, and ChIP was performed using anti-H3 and anti-acetyl-H3 antibodies. Primers against the ZEBRA promoter (Zp), were used in qPCR. A. Relative concentration of DNA associated with acetylated histones, normalized to uninduced. B. Relative concentration of total DNA, normalized to uninduced. C. Relative concentration of DNA associated with acetylated histones corrected for input DNA, normalized to uninduced.

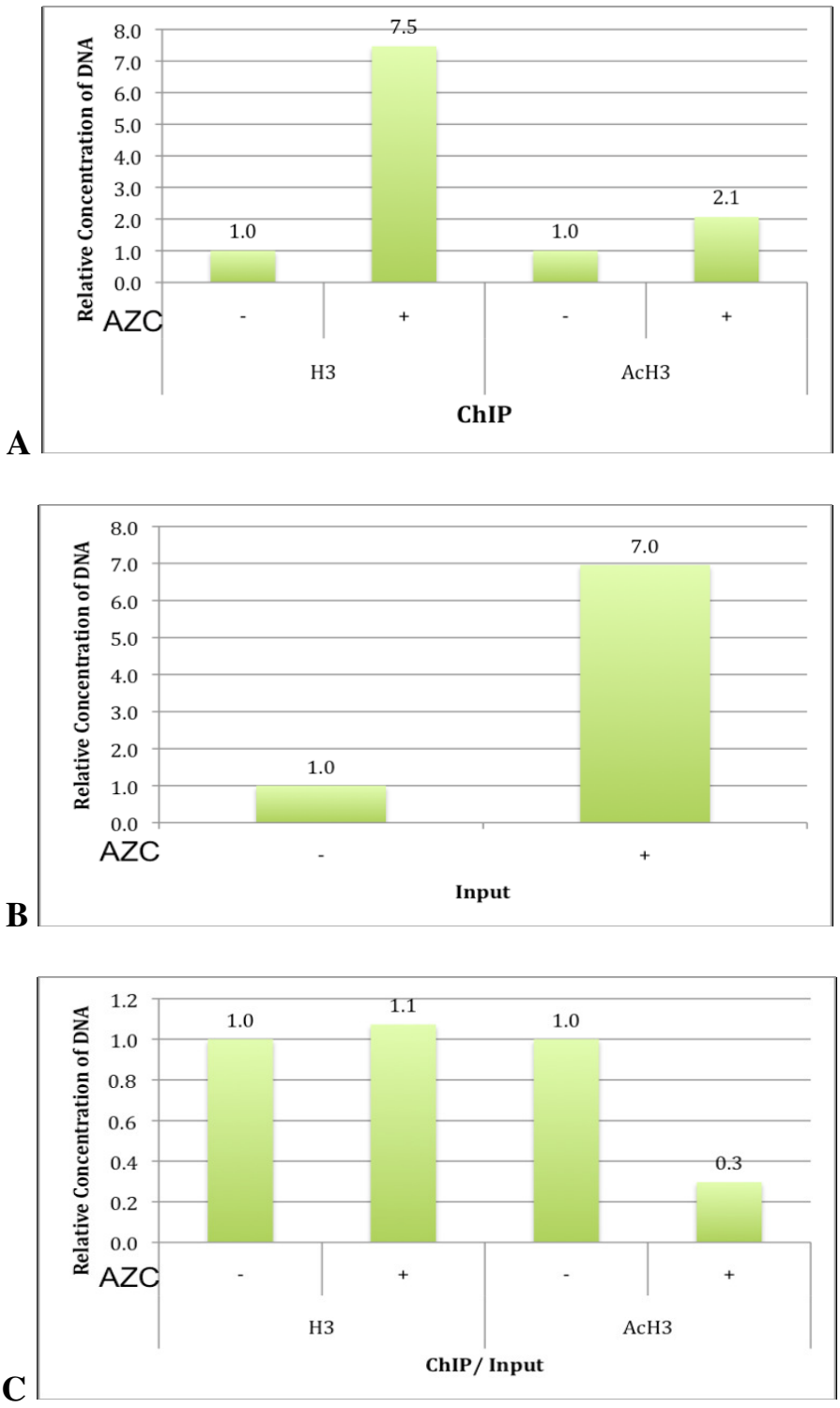


Figure 6: Newly replicated DNA appears to be associated with histones that are not acetylated. HH514-16 cells were either untreated or treated with AZC. They were harvested either at 48 hours, and ChIP was performed using anti-H3 and anti-acetyl-H3 antibodies. Primers against oriLyt were used in qPCR. A. Relative concentration of DNA associated with acetylated histones, normalized to uninduced. B. Relative concentration of total DNA, normalized to uninduced. C. Relative concentration of DNA associated with acetylated histones corrected for input DNA, normalized to uninduced.

DISCUSSION

While there was some information about the chromatin structure of EBV in latently infected cells, not much was known about what happens to the newly replicated viral DNA when the lytic cycle is induced. Here we aimed to find out whether or not the newly replicated viral DNA is associated with histones, and if so whether or not those histones are acetylated. In our experimental plan we studied three different variables: the inducing agent, the length of induction, and association of histones with two DNA regulatory regions (Zp and oriLyt) present in the EBV genome.

We first discovered that when TSA was used to induce the lytic cycle the newly replicated viral DNA in these cells was associated with AcH3 at a ~6-10 fold increase over untreated cells (Figure 2A). When the amount of oriLyt precipitated was corrected for the amount of DNA in the corresponding input sample we found that newly replicated viral DNA was associated with acetylated histones at a level proportional to the association of acetylated histones with non-replicated DNA from cells that were treated with TSA and PAA (Figure 2C). When we used different primer pairs on the same ChIP samples, we found no affect on the association of newly replicated viral DNA with AcH3, as similar results were obtained with primers for both Zp and oriLyt (Figure 3).

We then decided to see how changing the time of induction affected our results. We saw that at a time point where no viral DNA replication would be taking place (12 hours) there was only a ~ 6 fold increase in association with acetylated histones, but at 24 and 72 hours there was a ~9 fold and ~60 fold increase, respectively (Figure 4A). At 12 and 24 hours TSA induced the association of viral DNA with acetylated histones independent of replication (Figure 4C). At 72 hours, however, the amount of newly

replicated viral DNA associated with acetylated histones was greater than the amount of non-replicated viral DNA in the TSA plus PAA treated sample.

We then decided to change the type of inducing agent used. Instead of using an agent that increases acetylation we used the DNA methyltransferase inhibitor AZC. We found that when AZC was added to cells the association of newly replicated viral DNA with non-acetylated histones was increased ~8 fold (Figure 5A). The association of newly replicated viral DNA with acetylated histones increased ~ 2 fold. The association of newly replicated viral DNA with histones was slightly greater than the association of latent viral DNA with histones (Figure 5C). The association of newly replicated viral DNA with acetylated histones, on the other hand, was slightly lower than the association of latent viral DNA with histones.

Different Inducing Agents Impact Association of Viral DNA with Acetylated Histones

Figure 2A showed that when TSA was used as an inducing agent newly replicated viral DNA was associated with acetylated histones. When AZC was used, on the other hand, newly replicated viral DNA appeared to be preferentially associated with non-acetylated histones over acetylated histones (Figure 5A). Thus, our previous results that indicated that the induction of the lytic cycle significantly increases association of newly replicated viral with acetylated histones were most likely a result of using an HDACi to induce the lytic cycle. When cells were treated with AZC the newly replicated viral DNA was associated with non-acetylated histones ~4 fold higher than with acetylated histones. Thus, the HDACi most likely resulted in acetylation of the histones associated

with the viral DNA. We could compare the amount of viral DNA immunoprecipitated with histones to the amount of viral DNA immunoprecipitated with acetylated histones in TSA treated cells to verify our results.

At Early Times, Inducing Agents Increase Level of Association of Viral DNA with Histones Independent of Replication

When the amount of DNA precipitated with antibodies to acetylated histones was corrected for input in the corresponding samples, we found that the association of acetylated histones with viral DNA was very similar for newly replicated viral DNA from TSA treated cells and non-replicated DNA from TSA and PAA treated cells at 12 and 24 hours (Figures 2C, 3C, 4C). Thus, at these times association with acetylated histones occurs independent of viral replication. In all of these experiments the association of histones with viral DNA from cells induced into the lytic cycle was greater than the association of histones with latent DNA from untreated cells, indicating that there is something about the induction itself that changes the association of viral DNA with histones.

At 72 hours, however, when more viral replication had taken place, more newly replicated viral DNA from TSA-treated cells associated with acetylated histones than non-replicated viral DNA from TSA and PAA-treated cells. This finding indicates that as more viral DNA is being replicated the newly replicated viral DNA preferentially associates with acetylated histones.

Association of Viral DNA with Histones Did Not Change Whether Zp or Orilyt Regions were Analyzed

Both the Zp and oriLyt region of EBV have been previously found to be associated with histones in the latent stage (87, 88). We found that these two regions are also associated with histones upon lytic activation. We tested the association of acetylated histones with both Zp and oriLyt in two of our experiments – where we treated cells with TSA for 24 hours (Figure 3) and where we treated cells with AZC for 48 hours (Figure 6). We obtained similar results with primers for both oriLyt and Zp. Thus, changing the region of DNA analyzed for association with acetylated or non-acetylated histones did not affect our results.

Newly Replicated DNA is Packaged into Chromatin that is Partially Acetylated

Our experiment with AZC as an inducing agent demonstrates that newly synthesized viral DNA is associated with histones (Figure 5A). While the strength of association of EBV DNA with acetylated histones does depend on the inducing agent used, the fact that newly replicated viral DNA in AZC-treated cells does to some degree associate with acetylated histones indicates that the chromatin is at least partially acetylated.

Limitations

Chromatin immunoprecipitation is probably the most widely used approach to investigate association of proteins with DNA *in vivo*, however there are several

limitations to chromatin immunoprecipitation. All of the limitations come from the inability to know how efficient each step is. It is impossible to know how efficient cross-linking the DNA to protein, sonication, reversing the crosslinks, and DNA precipitation is. Thus, these steps can vary from ChIP to ChIP. There seems to be no solution to these limitations; they just have to be taken into consideration when interpreting the results of the experiments. However, ChIP is still a "state of the art" experiment, and the only technique for knowing the chromatinization of DNA *in vivo*.

In most of my experiments my results were reproducible among different biological replicates or even when two different regions of the EBV genome were compared for their extent of association with histones. However, that does not exclude the presence of limited fluctuations among different experiments that do not affect the general outcome of the results. For example, in Figure 2B the amount of DNA present in the input sample obtained from PAA-treated cells was 1.1-1.25 fold lower than the DNA present in the uninduced cells. In Figure 3B, however, where the same ChIP samples were analyzed using a different set of primers, the amount of DNA present in PAA-treated cells was lower than that present in uninduced cells by 2.5 fold. To further understand whether these fluctuations are part of the limitations of ChIP or have some biological significance one has to carry out several ChIP experiments to confirm the results as well as to analyze other EBV DNA regulatory regions.

Future Directions

The next step of the project would be to look at the effect of TSA as an inducing agent on the amount of viral DNA pulled down with an H3 antibody versus an acetylated

H3 antibody. If we found that TSA increased association of viral DNA with H3 the result would be consistent with our findings, specifically the finding that lytic induction by AZC increased association of viral DNA with H3.

Another future step of the project would be to determine whether viral DNA that is packaged in virions is chromatinized. We could perform a western blot using an anti-H3 antibody on isolated virions. If the histones were found to be associated with the DNA in virions it would mean that EBV episomes don't lose their chromatin by the time they enter the capsid. If virions contain viral DNA bound to histones, the virions isolated from cells treated with TSA could possibly have a higher level of acetylated histones. To investigate this possibility we could see what happens when virions are created by inducing cells with TSA vs. AZC. If we find that the virions from the TSA-treated cells have a higher level of association with acetylated histones than the virions from AZC-treated cells, we could then investigate the effect of this increase in histone acetylation on the establishment of latency.

Another experiment would be to see whether or not the histones that are associated with newly replicated DNA are in the replication compartments previously identified (68, 71). This information would tell us whether or not DNA is chromatinized as its being replicated, or if chromatinization takes place later on.

The Importance of Epigenetics in EBV

As outlined earlier in the literature review, epigenetics appears to play a significant role in the life of Epstein-Barr virus. Acetylation of the histones associated with EBV DNA and methylation of the EBV viral DNA have been shown to be critical in

activation of the lytic cycle. Typically, acetylation of histones marks transcriptionally active DNA regions, while methylation of DNA occurs at regions that are transcriptionally repressed. In, EBV, however DNA methylation plays a different role; ZEBRA, a transcription factor capable of inducing the full EBV lytic cycle when expressed in latently infected cells, can only activate the methylated form of the Rta promoter. This anomaly is one example of how epigenetics is actually quite complex. In addition to the modifications that can occur on DNA there are also numerous post-translational modifications that can take place on histones, which can lead to the binding of various transcription factors and inhibitors. These modifications can also result in blocking transcription factors and other proteins from binding. This complexity is the reason the term "histone code" was created (as reviewed in (92)). The histone code is the specific pattern of post-translational modifications on histones that determines the activity of genes associated with the chromatin. The particular histone code for EBV has been further elucidated in this study, but it is by no means complete. We now know that chromatin associated with newly replicated viral DNA is not acetylated, but we don't know if it is methylated, phosphorylated, ubiquitinated or sumoylated. Investigating these other post-translational modifications would be the next step in cracking the histone code of EBV.

Implications

The information elucidated from these and other experiments could perhaps be useful in developing new therapies for EBV-related cancers as well as novel ways to stop the virus from spreading so efficiently. We know that the only way the virus can spread

between cells and humans is if it enters the lytic cycle, as the lytic cycle is when newly packaged virions are created. Knowing that newly replicated DNA associates with histones is another piece of the puzzle of what happens when the lytic cycle becomes activated. The more we know about the lytic cycle the more targets we can find to stop its activation. While our data may not directly provide any new targets it does help us understand what is happening with the newly replicated DNA. Hopefully this information will eventually lead us to develop a new approach to stop the lytic cycle from being activated and thus limit viral propagation.

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