Histone Modification Pattern of the T-Cellular Herpesvirus Saimiri Genome in Latency

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Herpesvirus saimiri (HVS) subgroup C strains are able to growth transform human T lymphocytes in vitro. The stably persisting and nonintegrating HVS episome represents an optimal prerequisite for the investigation of the epigenetic state of latent herpesvirus genomes in vitro. Quantitative chromatin immunoprecipitation experiments using seven different histone acetylation- or methylation-specific antibodies revealed repressive marks at four lytic gene promoters and a variable pattern at the weakly transcribed LANA/orf73 promoter. The constitutive stpC/tip promoter regulating the viral oncoproteins and, more interestingly, the noncoding repetitive H-DNA elements flanking the coding region, showed a permissive chromatin structure. This study provides an appropriate model for the analysis of epigenetic herpesvirus genome modifications and their dynamics in T cells.

Gene expression in eukaryotic cells is regulated at multiple distinct levels. “Epigenetic” modifications of deoxyribonucleic acid and the associated histones play a key role in transcriptional regulation and for the inheritance of gene expression patterns. Besides the “traditional” CpG methylation of DNA, the regulation of chromatin accessibility and activity mediated by covalent modifications of core histone tails are increasingly recognized and have led to the proposal of a “histone code” (22, 24, 43, 54, 55).

DNA associates with cellular histones and forms a nucleosomal structure. The N-terminal tails of the core histones H2A, H2B, H3, and H4, which protrude from the nucleosomes, are subject to covalent modifications such as acetylation and methylation by specific cellular factors. These modifications are markers for the transcriptional state of the associated genes. In general, acetylation of lysine residues of histones H3 and H4 is linked to an active state of gene expression. The acetyl marks are recognized by bromodomain-containing adapter proteins that presumably mediate the activating effect. Alternatively, structural changes due to a removal of the positive charge at the lysine side chain and enhanced accessibility for the transcription machinery are discussed for causing increased transcriptional activity (56). Mono-, di-, or trimethylation of lysine residues of histone H3 can be either markers for activation or repression depending on the residue modified (for a review, see reference 35). Here, chromdomain-containing proteins are responsible for downstream effects.

Herpesviruses regularly establish a persistent state termed latency after primary infection of host organisms. Similar to other DNA viruses such as polyomaviruses and papillomaviruses, the persisting circular viral genomes (episomes) of herpesviruses adopt nucleosomal structures upon infection of the host cells (10, 50). This state is characterized by a strict down-regulation of most viral genes and a bidirectional mode of genome replication that is mostly carried out by the cellular replication machinery and that is synchronous to cellular DNA synthesis (47). The viral genome is passed on to progeny cells as an episome that is associated with cellular histones. Specific herpesviral proteins such as Epstein-Barr virus nuclear antigen 1 (EBNA1) or latency-associated nuclear antigen (LANA) from the Kaposi’s sarcoma-associated herpesvirus (KSHV, human herpesvirus 8) are thought to mediate efficient segregation. They attach the viral genomes to host chromosomes, preventing their loss during mitosis (30, 45). In contrast, productive lytic replication within permissive cells, which is usually tightly regulated by a cascade of viral immediate-early, early, and late genes, is effected by a rolling-circle mechanism and viral replication proteins (47). This results in the production of a huge amount of viral genomes in subnuclear structures which are termed replication compartments (29). Here, only a minor portion of the viral genome seems to be associated with histones (27, 38).

Covalent histone modifications as markers for the transcriptional state of genes have also been found in association with herpesvirus genomes during latent infection. In the case of herpes simplex virus type 1, ex vivo studies in dorsal root ganglia revealed histone acetylation at the promoter and the enhancer of the latency-associated transcript (LAT), which is the only transcribed region during latency (31, 32). Correspondingly, the promoters of the nonexpressed lytic genes display modifications typical for heterochromatin (58). In turn, induction of lytic replication goes along with the loss of acetylation marks in the LAT enhancer region (3). Studies on KSHV in semipermissive primary effusion lymphoma-derived B-cell lines showed a high level of histone acetylation in the noncoding terminal repeats of the viral genome, which also serve as the latent origin(s) of replication (52).

The T-lymphotropic Herpesvirus saimiri (HVS) is the prototype of the gamma-2 herpesviruses and related to KSHV. HVS strains form three subgroups (A, B, and C) of different gene content and oncogenicity (36, 37). Subgroup C strains are the
most oncogenic and capable of inducing disease in Old World primates (2, 28), as well as in lagomorphs (36). Interestingly, human T cells are also transformed to antigen-independent growth after in vitro infection with HVS subgroup C strains such as C488 (5). HVS-transformed human T-cell lines harbor multiple latent copies of intact viral genomes and do not secrete viral particles. Integration in primate or human cells is not observed (51, 53, 60).

The approximately 155-kb double-stranded DNA genome of HVS strain C488 harbors at least 77 open reading frames (ORFs). The coding 113-kb L-DNA is flanked by noncoding repetitive DNA segments (H-DNA) (14). In human T cells, only few ORFs are known to be expressed in latency. Consistently detectable is the expression of ORF1 (stpC/tip) (16) encoding a bicistronic transcript which is translated into StpC (for Saimiri transformation-associated protein) and Tip (for tyrosine-kinase interacting protein). Both gene products are oncogenes (26, 39, 59) and necessary for the transformation of T lymphocytes (1, 11). Low-level transcription is detectable for ORF73 (13, 49), which codes for the LANA homolog. Like its counterpart in KSHV or EBNA1 from Epstein-Barr virus, ORF73/LANA tethers the viral episome to metaphase chromosomes and is therefore necessary for the maintenance of the HVS genome during cell division (57). In addition, it also mediates repression of the lytic replication cascade (49).

The cell system chosen for this investigation were primary human cord blood lymphocytes (CBL) infected and transformed with *Herpesvirus saimiri* strain C488 (14). Briefly, CBL were infected with HVS strain C488 wild type and maintained in 45% RPMI 1640 and 45% Panserin (PAN) medium supplemented with 10% fetal calf serum (PAN) and the following additives: 10 U of recombinant human interleukin-2 (Roche Diagnostics)/ml, 1 mM sodium pyruvate (PAN), 50 μM monothioglycerol (Sigma), 20 mM bactoprenone disulfonic acid (Sigma), glutamine, and gentamicin. Noninfected control cells that were cultivated in parallel usually ceased growing after 3 to 6 weeks; the infected CBL were cultivated further on and were considered transformed after 12 weeks of continuous expansion. The presence of HVS DNA in multiple viral copies in the transformed cells was verified by semiquantitative PCR (data not shown).

HVS-transformed CBL were harvested by adding fixative directly to the cell culture medium and subjected to chromatin immunoprecipitation (ChIP) analysis according to a standard protocol (Upstate, Charlottesville, VA). The antibodies used were purchased from Upstate (anti-acetyl H3, anti-acetyl H4, anti-acetyl H3 K9, anti-trimethyl H3 K4, and anti-trimethyl H3 K9) or from Abcam (anti-dimethyl H3 K4 and anti-dimethyl H3 K9). We show two representative datasets out of five experiments (from different lymphocyte donors); the distribution pattern of modifications was consistent in itself and comparable between the data sets. Standard deviations between experiments were not calculated due to a variable efficiency of immunoprecipitation experiments performed at different dates. The cycling conditions were optimized for each primer pair
by running gradient PCRs (Eppendorf Mastercycler gradient), verifying by agarose gel electrophoresis that a specific product is amplified under the respective conditions. The precipitated histone-associated DNA was detected by quantitative SYBR green PCR (Fig. 2).

Histone acetylation. HVS-C488 transformed CBL were harvested and the histone acetylation pattern of the HVS H-DNA and promoter regions scattered over the HVS coding sequence was assayed. Three different antibodies specifically directed against a range of acetylated lysine residues of histones H3 and H4 were used for ChIP, all of them indicative of a permissive chromatin environment. Quantitative SYBR green PCR was performed using primer pairs that bind the noncoding H-DNA, promoter regions of transcribed latent (orf1, spcC/lip oncogenes; orf73, LANA) and presumably repressed lytic genes, including immediate-early (orf50, R-transactivator), early (orf6, major single-stranded DNA-binding protein) and late (orf17, protease; orf25, major capsid protein) gene promoters (14). Cellular promoters of the housekeeping genes alcohol dehydrogenase 5 (ADH5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as euchromatic (EU) controls, and cellular juxtacentromeric and centromeric satellite regions served as controls for heterochromatin (HE). For a no-antibody control, see Fig. 3C.

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H-DNA and at the stpC/tip promoter, whereas the promoter of the latently transcribed orf73 is not acetylated at a higher level than repressed lytic gene promoters. Interestingly, the promoter of the related KSHV orf73/LANA also displays only weak acetylation (52). This is surprising since the orf73 gene product LANA is indispensable for latent persistence of the viral episomes of gamma-2 herpesviruses (6, 7, 19). In HVS, only low levels of the orf73 transcript (13) and no protein could be detected, which argues for low expressional rates. The stable expression of stpC and tip in transformed human T cells (16) is reflected by a significantly higher degree of acetylation of stpC/tip promoter associated histones compared to the promoters of the lytic genes. This has been detected by three different antibodies.

The consistent high signal obtained for H-DNA-associated histone acetylation clearly identifies a permissive chromatin structure within the repeats, although no genes have been found here to date. The copy number of the repetitive elements is unlikely to be the cause since this has been corrected by the equally high number of repeats in the input DNA.

**Histone trimethylation.** Specific lysine (K) residues of the N-terminal histone tails can also be covalently linked to methyl groups. Up to three methyl groups can be deposited at the terminal nitrogen (N6) of lysine side chains by the respective methyltransferases. The correlation between methylation and transcriptional activity of associated genes is not as uniform as with acetylation: trimethylation of histone H3 lysine residue 4 (K4) (A) and trimethylation of histone H3 lysine residue 9 (K9) (B). Cellular promoters of the housekeeping genes ADH5 and GAPDH served as euchromatic (EU) controls, and cellular juxtacentromeric and centromeric satellite regions served as controls for heterochromatin (HE). (C) No-antibody control.

FIG. 3. Histone trimethylation profile of HVS episomes. Latent viral genomes from transformed human T cells were investigated by ChIP and quantitative SYBR green PCR. The results from two independent experiments are shown. (A and B) Trimethylation of histone H3 lysine residue 4 (K4) (A) and trimethylation of histone H3 lysine residue 9 (K9) (B). Cellular promoters of the housekeeping genes ADH5 and GAPDH served as euchromatic (EU) controls, and cellular juxtacentromeric and centromeric satellite regions served as controls for heterochromatin (HE). (C) No-antibody control.
ing episomes which carry one or the other epigenomic type, or even both modified variants may reside within the same cells.

**Histone dimethylation.** The addition of two methyl groups to the respective lysine residues constitutes the precursor of the final trimethylation state. However, both di- and trimethylation of histone H3 K9 provide the basis for HP1 binding, a family of adapter proteins with chromo domain, which mediate subsequent heterochromatinization (12, 33, 41). Furthermore, adapter proteins recognizing H3 K4 di- and trimethylation have been described (17, 21, 23, 44). Thus, similar to trimethylated lysines 4 and 9 of histone H3, the respective dimethylation holds the equivalent information about transcriptional stimulation or silencing.

ChIP analysis of the HVS genome loci in terms of dimethylation of the aforementioned lysine residues reveals a distinctive pattern: it appears that permissive chromatin is located toward the H-DNA, while the central part of the coding sequence displays a rather heterochromatic phenotype (Fig. 4). Elevated levels of histone H3 K4 modifications were found at the latent HVS promoters and the H-DNA (Fig. 4A). Dimethylation of histone H3 K9, in contrast, peaked at lytic gene promoters (Fig. 4B). Taken together, these results are well in accordance with the transcriptional profile during latency. The cellular heterochromatic controls do not show a strict analogy between the clearly repressive trimethyl H3 K9 (Fig. 3B) and the dimethyl H3 K9 (Fig. 4B). This is not unexpected, given that centromeric constitutive heterochromatin holds predominantly the trimethylated state (42).

The HVS terminal repetitive H-DNA was constantly associated with euchromatic marks. HVS H-DNA is made up of two units of G+C-rich repeats with different sizes (1,318 or 1,458 bp), the shorter form being identical to the longer form except for the deletion of 140 bp. Tandem repeats have long been known to initiate heterochromatin formation also by involving the small interfering RNA machinery (20, 34). Evidence for heterochromatinization of herpesviral terminal repeats has been provided by Sakakibara et al. for the KSHV genome (48). These researchers found that KSHV LANA, when bound to the terminal repeats, interacts with HP1 and also with the histone H3 K9 specific methyltransferase SUV39H1. Conversely, throughout the investigations performed here using various antibodies, the HVS H-DNA consistently showed a euchromatic modification pattern. The primers chosen for quantitative PCR of H-DNA lie within a region present in both types of repeats. A better resolution is hardly achievable even by using primers homologous to other regions of the −1.4-kbp H-DNA repeat (which are difficult to find because of the repetitive structure), since sonification yields fragments of about 200 to 1,000 bp. Fragments are subsequently precipitated only by antibodies directed toward activation-specific but not toward repressive histone modifications. Thus, HVS H-DNA repeats harbor predominantly euchromatic marks.

**FIG. 4.** Histone dimethylation profile of HVS episomes. Latent viral genomes from transformed human T cells were investigated by ChIP and quantitative SYBR green PCR. The results from two independent experiments are shown. (A and B) Dimethylation of histone H3 lysine residue 4 (K4) (A) and dimethylation of histone H3 lysine residue 9 (K9) (B). Cellular promoters of the housekeeping genes ADH5 and GAPDH served as euchromatic (EU) controls, and cellular juxtacentromeric and centromeric satellite regions served as controls for heterochromatin (HE). (C) No-antibody control.
euchromat marks. This result is in accordance with data from the closely related KSHV genome. Stedman et al. have demonstrated hyperacetylation in the KSHV terminal repeats, which can also function as the latent origin of replication (52). In analogy, it can be speculated that the latent origin of replication of HVS may be located in these repetitive elements. Alternatively, an accessible chromatin structure may be a prerequisite for the binding of HVS LANA to the terminal repeats to ensure the maintenance of the viral episome (7, 57), similar to the KSHV LANA (4, 15, 52).

The analysis of epigenetic modifications has already proven its relevance for the understanding of viral latency. At the same time, new discoveries expand our knowledge about the regulatory processes that determine transcriptional activity. For example, histone trimethylation is very stable and was considered a permanent mark, since no enzyme for the removal of epigenetic marks underlie dynamic and well-regulated processes. With regard to this, future studies addressing changes of histone modifications of this T-lymphotropic virus upon induction of the lytic phase will be promising.

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