Genome-Wide Histone Acetylation Profiling of Herpesvirus saimiri in Human T Cells upon Induction with a Histone Deacetylase Inhibitor*

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Herpesviruses establish latency in suitable host cells after primary infection and persist in their host organisms for life. Most of the viral genes are silenced during latency, also enabling the virus to escape from an immune response. This study addresses the control of viral gene silencing by epigenetic mechanisms, using Herpesvirus saimiri (HVS) as a model system. Strain C488 of this gamma-2-herpesvirus can transform human T cells to stable growth in vitro, and it persists in the nuclei of those latently infected T cells as a nonintegrating, circular, and histone-associated episome. The whole viral genome was probed for histone acetylation at high resolution by chromatin immunoprecipitation-on-chip (ChIP-on-chip) with a custom tiling microarray. Corresponding to their inactive status in human T cells, the lytic promoters consistently revealed a heterochromatic phenotype. In contrast, the left terminal region of the genome, which encodes the stably expressed oncogenes stpC and tip as well as the herpesvirus U RNAs, was associated with euchromatic histone acetylation marks representing “open” chromatin. Although HVS latency in human T lymphocytes is considered a stable and irreversible state, incubation with the histone deacetylase inhibitor trichostatin A resulted in changes reminiscent of the induction of early lytic replication. However, infectious viral particles were not produced, as the majority of cells went into apoptosis. These data show that epigenetic mechanisms are involved in both rhabdoviral latency and transition into lytic replication.

Herpesviral infection of a suitable host results in lifelong persistence of the virus within the nuclei of a defined subset of host cells. The viral genomes typically persist in many copies as extrachromosomal nonintegrating episomes and acquire cellular histones to form regular nucleosome-like structures (9, 29, 39). They are duplicated in a bidirectional manner synchronously with cellular DNA.

The virus genomes adopt a state of latency in which the expression of most viral genes is shut down. This enables the herpesviruses to escape from host immune surveillance. Occasionally, the viral genome is reactivated and the lytic replication program, which is characterized by a gene expression cascade of viral immediate-early, delayed-early, and late genes and the production of large amounts of viral DNA by rolling-circle replication, is initiated (23). Finally, viral particles are assembled and released by lysis of the permissive cell. In the process of lytic replication, the viral DNA loses its regular array of nucleosomes, and no histones can be detected in virions (22, 27).

For many herpesviruses, including some of the gammaherpesvirus subfamily, such as Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV), reactivation from latency can be accomplished in vitro. In fact, the addition of small-molecule inducers is routinely used with both viruses to obtain viral particles, since the available standard cell culture systems support lytic replication rather inefficiently (37, 45). Phorbol esters like 12-O-tetradecanoylphorbol-13-acetate (TPA; also called PMA, for phorbol-12-myristate-13-acetate) activate the protein kinase C (PKC) pathway by mimicking diacylglycerol. This finally leads to the activation of cellular transcription factors like AP-1. Other chemicals, like sodium butyrate, trichostatin A (TSA), and sodium valproate, are able to block histone deacetylases (HDACs) and hence shift the equilibrium of histone modifications established on the viral chromatin toward the active acetylated state (35, 45). Even though both PKC activation and HDAC inhibition induce enhanced expression of viral immediate-early genes, such as EBV BRLF1 (R transactivator) and the KSHV homolog orf50, they are independent processes. Which chemical substance is able to successfully trigger or enhance the productive cycle depends on the virus-containing-cell type. For instance, different EBV-positive lymphoblastoid marmoset cell lines have been shown to be inducible by either pathway (18).

Herpesvirus saimiri (HVS), the prototypic gamma-2-herpesvirus, is closely related to KSHV. The double-stranded DNA genome has a variable length of 130 to 160 kb and consists of an AT-rich coding region harboring at least 77 open reading frames (ORFs). The coding region is flanked by a variable number of GC-rich, noncoding repetitive units termed H DNA (13). HVS was isolated from squirrel monkeys (Saimiri sciureus) and presumably persists in the T lymphocytes of its natural host (34). While no symptoms in squirrel monkeys have been described, other susceptible New World monkey species, like common marmosets (Callithrix jacchus) and cottontop tamarins (Saguinus oedipus), develop rapidly growing T cell malignancies after experimental infection (15). HVS strains

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are classified into three subgroups (A, B, and C) according to sequence divergence, especially at the left end of the genome, and transforming potential (33). Subgroup C strains, like C488, are the most oncogenic and are also able to transform human T cells to antigen-independent growth in vitro (3). This cell system supports tightly controlled latency of the virus, with only a few viral genes being abundantly expressed. All of them are located at the left end of the coding region. Specifically, they are the two oncogenes stpC (raimiri transformation-associated protein C) and tip (tyrosine kinase interacting protein), which are carried on a bicistronic transcript, and four of five genes coding for U RNAs (12, 14, 24). The latency-associated nuclear antigen (LANA), which is encoded by orf73, is expressed at such low levels that detection is possible only by reverse transcriptase PCR (RT-PCR) (38). HVS LANA, like its counterpart in KSHV and EBNA-1 in EBV, facilitates correct segregation of the viral copies during cell division by tethering the viral episomes to metaphase chromosomes (4, 7, 19). Furthermore, LANA proteins of both HVS and KSHV also have a function in the suppression of lytic cycle replication (28, 38). It is thought that HVS cannot be reactivated from latency in human T cells. However, available data account only for treatment with TPA and not with HDAC inhibitors (14, 25).

Productive lytic replication of HVS is supported by only one of the monkey kidney (OMK) cells. In homology to that of KSHV, it is initiated by the expression of the immediate-early gene carried on orf50. The R transactivator protein then transactivates other viral replication genes, like the delayed-early gene orf6, which displays homology to a single-stranded DNA (ssDNA) binding protein (43). Other viral genes with immediate-early kinetics are orf14, a viral superantigen, and orf57, which promotes the nuclear export of unspliced viral transcripts (25, 48).

It is not understood how the incoming herpesvirus genomes acquire a nucleosomal structure nor how the repression of viral latent lytic genes in latency is regulated. In recent years, it has become clear that epigenetic processes are involved in the course of viral-gene silencing. In a previous study, we investigated the histone modification status of the latent HVS genome in transformed human T cells. Selected promoter regions of all kinetic classes of replication, including immediate-early, delayed-early, late, and latent promoters, were assessed by chromatin immunoprecipitation (ChIP) experiments with seven different antibodies. The modifications could be correlated with the gene expression pattern observed in T cells. Histone acetylation, which is commonly found in context with actively transcribed genes, had previously been investigated with three different antibodies, and investigation revealed that only the H DNA and the latently transcribed stpC/tip promoter were wrapped up with acetylated histones, while the lytic gene promoters, as well as the orf73/lana promoter, remained free of histone acetylation (1). In this study, we sought to generate a detailed view of the histone acetylation status within the complete HVS genome in latency by performing an investigation of a whole herpesviral genome by a ChIP-on-chip experiment. Further, we investigated the consequences of treatment with the HDAC inhibitor TSA on the histone acetylation profile and on the expression of selected viral genes.

MATERIALS AND METHODS

Cell culture and virus. Primary human cord blood lymphocytes (CBL) from different donors were infected and transformed with Herpesvirus saimiri strain C488 (13). Briefly, CBL were infected with the wild-type HVS strain C488 after it was maintained in 45% RPMI 1640 medium and 45% pansermin medium (PAN Biotech) supplemented with 10% fetal calf serum (PAN Biotech) and the following additives: 10 U/ml of recombinant human interleukin-2 (IL-2; Roche Diagnostics), 1 mM sodium pyruvate (PAN Biotech), 50 μM monothioglycerol (Sigma), 20 mM bactoprophoros disulfonic acid (Sigma), 350 μM/L of glutamine, and 100 μg/ml of 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.

ChIP analysis and quantitative real-time PCR. In order to analyze which histone modifications locate to specific regions of the HVS genome, we performed chromatin immunoprecipitation. To this end, DNA and proteins were temporarily cross-linked and sheared to make shorter fragments of 200 to 1,000 bp. Histone modification-specific antibodies were used to isolate the fragments with the histone modification. Quantification was done in comparison to total input DNA using quantitative PCR of the genomic region of interest. T cells were treated with the customary concentration of 0.08 μM phorbol ester TPA (50 ng/ml; Sigma) or the HDAC inhibitor sodium butyrate at 3 mM (330 μg/ml; Sigma) or TSA at 3 μM (1 μg/ml; Cell Signalling). Treated or untreated transformed lymphocytes were fixed for chromatin immunoprecipitation, incubated with SYBR green PCR. The experiments were performed as described earlier (1) with histone acetylation-specific sera from Upstate (rabbit anti-acetyl H3; catalog number 06-599). Additional primer pairs used were orf75, 5′-TATCATGACAC TGTTTGGAGGCAC-3′ and 5′-TTTGGCCACCTTGGTAACCTTCC-3′, and for a nonpromoter region in the major capsid protein gene, orf25i, 5′-TTCTGCTGATTAGTOCTTACCG C-3′. Total DNA recovery by ChIP varied between 2.6% and 12.1% (untreated) and 5.8% (2-h TSA treatment), 3.2% and 9.6% (4-h TSA treatment), and 1.1% and 3.0% (6-h TSA treatment) of input DNA.

Caspase assay. After T cells were treated with 1 μg/ml of TSA, caspase 3 and 7 (Caspase 3/7) activities were determined with the Caspase-Glo 3/7 assay (Promega) according to the manufacturer’s protocol. Triple values were produced by taking 20,000 cells per sample. Different HVS-transformed CBL lines (numbers 1587 and 1930) in total volumes of 50 μl of Jurkat T cells induced for apoptosis were mixed with 20,000 or 200,000 FasL (clone CH11; ImmunoNet) served as positive controls (see the supplemental material). Luminescence was measured in a Victor 1420 multilabel counter (Wallac).

Annexin V-propidium iodide FACS analysis. For each staining experiment, 500,000 HVS-transformed human CBL derived from three different donors (numbers 1587, 1810, and 1930) were incubated with 1 μg/ml of TSA or remained untreated. Cells were washed with phosphate-buffered saline (PBS), resuspended in 200 μl of Annexin V binding buffer, stained with 1 μl of annexin V-fluorescein isothiocyanate (APC; ApoScreen annexin V-APC; Southern Biotech), and incubated for 10 min. Prior to fluorescence-activated cell sorter (FACS) analysis, 200 ng of propidium iodide (PI) was added to the cells. Jurkat T cells were incubated with mouse anti-C95 Fas IgM (clone CH11; Immunonet) and subjected to annexin V-PI staining: FACS analysis served as a positive control. The analysis was performed on an LSR II flow cytometer (Becton Dickinson) and with BD FASDiva software: further evaluation was done with FCS Express version 3 software (De Novo Software).

Microarray design. Two custom genome-tiling microarrays with the capacity for 15,000 oligonucleotide probes each were purchased from Agilent (Santa Clara, CA). The microarray was equipped with both the HVS coding region, which was covered by 60-mer oligonucleotides with a spacing of 20 bp, and the GC-rich H DNA, which was covered by 45-mer oligonucleotides, to account for the differences in hybridization temperature. The probes were elongated to 60 bp with an Agilent linker-DNA sequence. Probes designed by Agilent covering cellular 5′ coding regions as well as promoter regions of housekeeping genes represented the controls for histone hyperacetylation-associated DNA, while a number of single-cell satellite DNA regions served as histone hypoacetyltransferase controls (see the supplemental material). The probes were synthesized using the phosphoramidite method and printed onto the microarray with Agilent SurePrint technology in a randomized manner.

DNA amplification and microarray hybridization for ChIP-on-chip experiments. DNA amplification of ChIP material from HVS-transformed T cells (21) for the microarray hybridizations was performed by using a protocol adapted from NimbleGen Systems. Whole ChIP samples and 20 ng of the input samples
were used for ligation-mediated PCR (LM-PCR). The ChiP DNA was blunted by using deoxyribonuclease triphosphates (dTTPs; Fermentas) and T4 DNA polymerase (Fermentas). Then the DNA was phosphorylated by the addition of rATP and polyribonucleotide kinase (Fermentas) and ligated with T4 DNA ligase (Fermentas) to partially double-stranded linkers made from high-pressure liquid chromatography (HPLC)-purified oligonucleotides using primers oJW102, 5'-GGCGTGAACCCCGGGAGATCTGAACTC-3', and oJW103, 5'-GGAATTCTAGATC-3'. DNA was purified by phenol extraction and ethanol precipitation and dissolved in water. LM-PCR with oJW102 was performed by using the Phusion Hot Start high-fidelity DNA polymerase system (Finnzymes). An aliquot of the final DNA was separated on an agarose gel for verification of the DNA fragment sizes and for verification with SYBR green PCR (data not shown). The experimental acetyl-H3-associated DNA samples were labeled with Cy5 dye, and the total input amplicons were labeled with Cy3 dye by Imagenes GmbH (Berlin) and then cohybridized to Agilent 15k oligonucleotide tiling arrays. The acetyl-H3 ChiP signal was compared with the control input signal, and the data were extracted according to standard operating procedures and visualized with SignalMap software (version 1.9; NimbleGen/Roche).

Quantitative real-time RT-PCR. Three different human HVS-transformed CBL lines, untreated or treated with TSA (1 μg/ml) for different periods of time, were used for whole-RNA extraction using Trizol reagent (Invitrogen). cDNA synthesis was performed using 2 μg of RNA template and the ThermoScript RT-PCR system (Invitrogen). The cDNA was quantified in duplicate values with 25-μl reaction mixtures of the Platinum SYBR green PCR system (Invitrogen). Two-step PCR amplifications of 40 cycles of denaturing (95°C, 15 s) and annealing/synthesis (60°C, 40 s) were carried out with an Applied Biosystems 7500 sequence detection system. Primer sequences were as follows: RT-HPRT, 5'-ATGGACACACACAGTCCAATG-3' and 5'-TGGCGTCGTGATTAGT-3'; RT-orf25, 5'-TAATTTGTTCTTGGATTCCTCTG-3' and 5'-AGAACGGCAAAGG-3'; RT-orf50, 5'-ATGACACACAAGCCTGTTAAG-3' and 5'-AGACATCTTGGCTGGCCTCAA-3'; RT-orf6, 5'-TGTTGGCCCTGGACTTGATAAC-3' and 5'-GCCCTAGATCATCACGAATGCG-3'; RT-orf14, 5'-CAATGCTAG-3' and 5'-ATGGGCTTAGTGATGCTGAGG-3'; RT-orf50, 5'-ATGACACACACAGTCCAATG-3' and 5'-AGAACGGCAAAGG-3'; RT-orf1, 5'-ACAACTTTTGCGCTCAAGATCATCAGCAATGCC-3' and 5'-ACAGTCTTCTGGGTGGCAGTGATG-3'; RT-orf10, 5'-TTGTTGCCCTGGACTTGATAAC-3'; RT-GAPDH, 5'-ACAGTACTTGCCTGGCCTCAA-3'; RT-orf25, 5'-AATATGCCAAAGG-3' and 5'-AGTTGCTTCTAAGATGCTGAGG-3'; RT-orf50, 5'-ATGACACACACAGTCCAATG-3' and 5'-CAAGTAGAACAAGCCTGAATG-3'; RT-orf57, 5'-GGTGAAAACGTGAAATTG-3' and 5'-CTGTGCGCTCCTCCTCCCTT-3'; and RT-orf73, 5'-CCGGGTATTCGCTAGACTCAGTCTC-3' and 5'-TGCACCTGAGAAGTTCGTGACCCGGGAGATCTGAATTC-3' and 5'-GAATTCAGATATTAAGCTTTGAGATCTGAATTC-3'.

RESULTS
HDAC inhibitors cause an altered acetylation pattern in latent HVS genomes. In an initial set of experiments, we tested the effects of TPA and the HDAC inhibitors sodium butyrate and TSA on histone acetylation at selected loci of the HVS genome in transformed human T cells. The loci were identical to those addressed in a previous study on HVS histone modification status (1). Here, the histone acetylation status was again confirmed with histone H3 (K9 and K14)-specific antisera. The cellular euchromatin controls (housekeeping genes gapdh and adh5) and heterochromatin controls (chromosome 1, satellite 2, and chromosome 4, satellite α) showed expected and constant signals in all experimental settings. In accordance with the known inability of TPA to influence histone deacetylases, a 4-h incubation of the T cells with TPA had no effect on acetylation status (Fig. 1A). In contrast, both HDAC inhibitors led to increased histone acetylation at the orf73 promoter and a minor increase in the orf50 and orf6 promoter regions, indicating histone acetylation activity at these sites, where it is normally balanced by HDACs. In order to shed light on the process of acetylation, we

FIG. 1. (A) Histone 3 lysine 9 (H3 K9) acetylation of HVS episomes changes after treatment with HDAC inhibitors but not after treatment with the phorbol ester TPA. Viral loci and cellular control loci from HVS-transformed human CBL were analyzed by ChiP with acetyl histone 3-specific antisera followed by quantitative SYBR green PCR. Untreated cells were compared to cells treated for 4 h with TPA (50 ng/ml, 0.08 μM) or the HDAC inhibitor TSA (1 μg/ml, 3 μM) or sodium butyrate (330 μg/ml, 3 mM). The promoters of the euchromatic housekeeping genes gapdh and adh5 (adeny) and heterochromatin (chromosome 1, satellite 2, and chromosome 4, satellite α) served as cellular controls (as in reference 1). (B) Time course of histone acetylation and dimethylation at H3 K9 on HVS episomes in T cells treated with 1 μg/ml of the HDAC inhibitor TSA. HVS-transformed human CBL were compared by ChiP with acetyl H3-specific antisera followed by SYBR green PCR. Untreated cells (bottom panels) and cells treated with 1 μg/ml of TSA for 4, 8, 12, and 16 h are shown. Controls were the same as defined for panel A. Mean values from three independent ChiP experiments are shown in panels A and B; each experiment data set (triplicate values) was normalized to the mean value for the experiment, and then means and standard deviations (SD) were calculated.
were in accordance with the inactive state of most viral genes in latency, as the viral genome was in a mainly underacetylated state. The left terminal region containing the orf1 (stpC/tip) promoter and the viral U RNAs was the only region with considerable histone acetylation. Since the stpC and tip gene products are both necessary for the transformation of T lymphocytes, the permissive chromatin structure is not surprising and has been reported previously (1). Interestingly, acetylation was concentrated at the orf1 promoter and was absent from the coding sequence of tip. The most abundant viral transcripts in latency are the U RNAs, which are transcribed by DNA polymerase III and reach copy numbers of up to $2 \times 10^5$ copies per cell (26) even though they are not necessary for transformation (12). This high level of expression is reflected by histone acetylation in the whole U RNA region. This area also contains a gene with strong homology to the family of dihydrofolate reductases (orf2); however, its transcription seems unaffected by histone acetylation, since expression of this gene cannot be detected (14). The HSV coding sequence contains three highly repetitive sequences situated within the coding regions of orf1 (stpC), orf48, and orf73. Precisely at those repetitive regions, a signal on the microarray was apparent, arguing for interference of repetitive sequences with the probe amplification and hybridization required in the ChIP-on-chip procedure, and thus presumably representing a sequence-related artifact. The non-coding H DNA, which also consists of repetitive DNA, also showed an inconclusive signal distribution in the array experiments. However, previous conventional ChIP analysis has already shown the euchromatic character of the repeats (1), which are similar to those of KSHV H DNA (41). Although orf73/lana is ascribed to latently expressed genes due to its

performed a time course experiment, comprising incubation with TSA for up to 16 h (Fig. 1B). Two more sites on the HVS genome were included in this study, the promoter region of orf75, situated immediately adjacent to the H DNA, and orf50i, located in the coding region of the orf25 lytic gene, which is more distant from the promoter. As early as 4 h after the addition of TSA, the right terminal end of the viral genome, including the orf75 and orf73 regions, showed a strong increase in acetylation. The immediate-early promoter of orf50 and the delayed-early promoter of orf6 displayed only weak signals at 4 h after the addition of TSA. These data illustrate that histone acetylation occurs sequentially on different sites of the viral genome.

**Genome-wide analysis after TSA treatment revealed changes in viral histone acetylation reminiscent of reactivation patterns.** Next, we sought to obtain a complete view of the acetylation status of the HVS genome during latency. We therefore designed a microarray covering the HVS coding sequence at a high resolution (probe spacing of 20 bp) and performed a ChIP-on-chip analysis using an antiseraum specific for histone H3 acetylated at lysine residues 9 and 14. Euchromatic, and thereby known, hyperacetylated controls were represented by the promoter and 5′ coding regions of 27 cellular housekeeping genes. Eight cellular heterochromatic DNA satellites of different chromosomes served as controls for hypoacetylation. As expected, the eu- and heterochromatic controls were enriched or depleted, respectively, during histone H3 acetylation (see Fig. S1 in the supplemental material). The results for the genome-wide histone acetylation profile (Fig. 2) were in accordance with the inactive state of most viral genes

![ChIP-on-chip analysis of the Herpesvirus saimiri strain C488 genome using custom oligonucleotide arrays (Agilent).](http://jvi.asm.org/)
weak transcriptional rate in human T cells (11) and its limited homology to KSHV lana, it is not accompanied by histone acetylation in its promoter, which confirms our previous analyses. In summary, these microarray data show that the latency-related transcriptional nonpermissiveness of large parts of the coding viral genome is reflected at the chromatin level.

Blocking the activity of HDACs by TSA leads to a fundamental change in the histone acetylation pattern (Fig. 2). TSA-treated HVS-transformed T lymphocytes have a considerably greater number of additional sites that acquire acetyl groups than untreated HVS-transformed T lymphocytes. Notably, both the left and the right termini of the viral coding sequence become increasingly acetylated. The signals in the central part of the genome allow some interesting conclusions. In accordance with the ability of HDAC inhibitors to induce the productive replication cycle in other gammaherpesviruses, incubation with TSA for 4 h was sufficient to obtain the acetylation of promoter regions in all three HVS immediate-early genes (orf14, orf50B, and orf57) (Fig. 2, red arrows). However, the translated region of orf14 remained completely unaffected, while promoter acetylation of orf50B and orf57 also extended into the 5′ coding region of the genes.

The function of many HVS-encoded proteins is unknown and is mostly deduced from homologies to other gammaherpesviral or cellular proteins. Here, we attempted to categorize genes encoding proteins with known or putative enzymatic or regulatory activities (viral replication, regulation of metabolism, and immune modulation) as delayed-early genes and genes encoding known or putative virion proteins ( tegument and capsid components and glycoproteins) as late genes (13). Bearing this classification in mind, we observed that the microarray data show that several promoter regions of delayed-early genes, like orf6 (ssDNA binding protein), orf15 (vCD59), and orf41 (part of the helicase-primase complex), are subject to histone acetylation, while some are more extensively acetylated throughout the coding region, e.g., orf9 (DNA polymerase), orf36 (phosphotransferase), orf37 (alkaline exonuclease), and orf56 (primase). In contrast, other delayed-early genes, like orf21 (thymidine kinase), orf59 (processivity factor of DNA polymerase complex), orf60 (ribonucleotide reductase, small subunit), orf61 (ribonucleotide reductase, large subunit), and orf70 (thymidylate synthase), are located within the two large genomic areas that were refractory to histone acetylation after 4 h of TSA treatment. The majority of known or putative viral late genes are also located in these regions. Remarkably, other late genes, like orf39 (glycoprotein M), orf47 (glycoprotein L), orf51 (putative virus-specific glycoprotein), and orf53 (glycoprotein N), which are surrounded by acetylated chromatin, retain their underacetylated status.

The genome-wide histone acetylation data of latently HVS-infected human T cells reflect the transcriptionally silent state of all but the left terminal viral oncogenes. The inhibition of HDACs by TSA induces an acetylation pattern that is reminiscent of early lytic replication: active immediate-early genes, partly active delayed-early genes, and inactive late genes.

A 4-h TSA treatment of HVS-transformed T cells was not accompanied by apoptosis. The viability of the TSA-treated T lymphocytes was verified, including by a caspase-3/7 assay to detect extrinsically as well as intrinsically triggered apoptosis. The functionality of the assay was proven in Jurkat T cells activated by cross-linking with the anti-Fas (CD95) receptor IgM-monomoclonal CH11 (Fig. S2). HVS-transformed human T cells exhibit elevated basal caspase activity per se (Stefan Rich-ter, personal communication), but in our experiment with HVS-transformed T cells derived from different donors, no increase in caspase activity 4 h after TSA treatment was observed (Fig. 3A). After 24 h, however, an increase in caspase-3/7 activity was measured.

The TSA-treated T lymphocytes were then tested for externalization of phosphatidyl-serine via annexin V-propidium iodide FACS analysis to further substantiate the data (Fig. 3B). The reliability of the FACS staining was confirmed by parallel analysis of Fas receptor-activated Jurkat T cells (see Fig. S3 in the supplemental material). After the cells were incubated for 4 h TSA, their phenotype was indistinguishable from that of untreated cells. After 24 h, the ratios of both apoptotic and necrotic cells increased in two of the three cell lines. These data demonstrate that transformed T cells remain viable after 4 h of incubation with TSA. However, apoptosis can be induced to various extents—but not completely—after a prolonged TSA incubation of 24 h. This finding led to the question...
of whether productive replication of HVS can be induced by HDAC inhibitors.

**HDAC inhibitor treatment of HVS-transformed human T cells does not result in the production of viral particles.** The supernatants of three different TSA-treated HVS-transformed T cell lines were analyzed for newly released virions in order to test whether the HDAC inhibitors TSA and butyrate, but not the protein kinase C agonist TPA, were able to induce a complete lytic replication cycle (14, 25). Assuming that one replication cycle takes 2 to 4 days, we replaced the cell culture medium with fresh medium after 48 h of HDAC inhibitor treatment. After a further 48 h, these supernatants were transferred from the T cell cultures to permissive OMK cells. In no case could the formation of typical cytopathogenic effects (CPE) be observed in the OMK cultures, indicating that no virions or insufficient numbers of virions had been released by the T lymphocytes. In parallel, a dilution series of HVS virions in which a total of only 10 virus particles per well were still able to induce CPE served as a positive control. Therefore, our studies suggest that HDAC inhibitors such as sodium butyrate and TSA cannot induce the production of HVS virions in human T cells, despite activation of immediate-early and early gene transcription.

**TSA treatment of HVS-transformed T cells alters the transcriptional activity of viral genes.** Next, we tested whether TSA-induced histone acetylation is accompanied by a changed transcriptional pattern of the affected gene loci. The bicistronic orf1 transcript; the transcripts of the immediate-early genes orf14, orf50, and orf57; the orf73 transcript, which is categorized as a latent gene but is not detectable at the protein level; the transcript of the late gene orf25, which encodes the major capsid protein; and the delayed-early gene orf6, which is known to be transactivated by the orf50 gene product, were chosen for analysis. Three independent quantitative RT-PCR experiments were performed with HVS-transformed T cells, both untreated cells and cells incubated with TSA for 8 h and 16 h (Fig. 4). The three cell lines tested were derived from two donors, and line 1587D1 grew more slowly than the other two. The amount of virus-specific mRNA was quantified relative to those of cellular GAPDH (data not shown) and HPRT mRNAs. The expression levels of both cellular mRNAs corresponded with and were unchanged relative to total input RNA, arguing for constant, unaltered expression of the housekeeping genes. The relative viral mRNA changes were nearly identically regulated with respect to both markers (GAPDH and HPRT). The level of latently expressed orf1 mRNA strongly decreased after HDAC inhibitor treatment, in contrast to the level observed after mitogen stimulation (14). This decrease was already prevalent after 8 h of TSA incubation. The levels of immediate-early orf14, orf50, and orf57 mRNAs clearly increased after 8 to 16 h (on average 23-fold, 15-fold, and 18-fold, respectively). orf25 mRNA levels were slightly increased after 16 h, whereas orf6 transcription was not induced during the 16-h period. This finding is in accordance with its regulation by the transactivator protein (orf50). With kinetics similar to that of the immediate-early genes, orf73 mRNA transcription increased on average 32-fold after 16 h of TSA treatment. Taken together, these findings reveal a transcriptional profile of HVS with clear differences before and after treatment with TSA. Induction of immediate-early genes and orf73/lana was detected, while the delayed-early gene orf6 was not activated during a period of 16 h.

Further detailed investigation of protein amounts after TSA induction was not possible, as our specific antibodies that reproducibly allowed detection of strain C488 orf50- and orf57-encoded proteins in lytic infection in OMK cells consistently...
failed to detect the respective proteins in lymphocytes. Only the small protein encoded by the viral superantigen transcript (orf14) was found to be considerably increased after TSA treatment (see Fig. S4 in the supplemental material).

**DISCUSSION**

Herpesviruses of all subgroups have been shown to regulate latency and the transition to lytic replication on the chromatin level (2, 5). Tight regulation of potentially immunogenic genes is thought to enable herpesvirus persistence, as clearance by the host’s immune system is avoided. Here, the acetylation status of the complete *Herpesvirus saimiri* genome both during latency and after experimental induction of lytic replication was shown for the first time at high resolution. Gene expression of HVS is similarly regulated at the epigenetic level; this became obvious by the hypoacetylation of almost all viral genes during latency. The oncogenes *stpc* and *tip* and the RNA polymerase III-dependent U RNAs are the only genes with considerable levels of expression/transcription, and they show a corresponding active chromatin state. By disturbing the balance between HDACs and histone acetyl transferases (HAT) with a small-molecule HDAC inhibitor, TSA converts this latency phenotype into a phenotype reminiscent of early lytic replication. Inhibition of the HDACs, which permanently act to remove acetyl groups from the histones at sites of lytic genes, reveals the activities of the counteracting HATs (30). We demonstrated previously that even on-treatment of HVS *orf73* promoter acetylation and enhanced transcription, similar to those of HVS *orf50*, were also detected at the homologous KSHV *orf50* locus following butyrate treatment. ChIP analysis followed by semiquantitative PCR analysis had previously revealed that the acetylation level of the KSHV *orf73* promoter remains unaltered in BCBL-1 cells after 4 h of incubation with butyrate (31), while the overall acetylation level of the KSHV *orf73* promoter region was found to be low (41). In contrast, the HVS *orf73* promoter became markedly acetylated, depending on TSA (Fig. 2, green arrow), and was accompanied by an increase in *orf73* mRNA expression. KSHV can be reactivated to complete the lytic replication cycle and to produce viral particles in BCBL-1 cells (36). This was not possible for HVS in human T cells. KSHV and HVS maintain latency by direct binding of the LANA protein to the *orf50* promoter region and the resulting repression of *orf50* expression. It has been shown that small interfering RNA knockdown of KSHV *orf73/lana* in latently infected BCBL-1 cells derepresses *orf50* expression. Furthermore, transfection of 293T cells with recombinant *lana*-deficient KSHV bacmids resulted in elevated *orf50* mRNA levels (30). We demonstrated previously that even ongoing lytic HVS replication in permissive OMK cells could be blocked by the overexpression of *orf73/lana* in a recombinant viral system (38); on the other hand, deregulated low expression of HVS *orf73* did not increase lytic replication in this system. Similar results were also obtained with murine herpesvirus 68; *orf73* has been shown to be critical for latency in splenocytes in vivo (16). However, an increase in lytic replication has not been observed after *orf73* deletion. In other gamma-2-herpesviruses, like the rhesus monkey rhadinovirus (RRV), exogenously expressed LANA also strongly inhibited RRV lytic replication (10). In RRV, lytic replication was enhanced when LANA was deleted from the viral genome (46), albeit with a loss of genome persistence in latency. The mechanism proposed for the reactivation of KSHV by HDAC inhibitors involves the acetylation and dissociation of KSHV LANA protein, thus allowing for *orf50* transcription (30). In addition, the KSHV *orf50* gene product RTA directly affects the *orf73/lana* promoter (32). In the case of HVS, we speculate that higher amounts of LANA became available after TSA treatment (which also led to an initial but insufficient induction of *orf50*) and contributed to permanent repression of the *orf50* promoter and blocking of lytic replication. The HVS *orf6* promoter can be stimulated by the R transactivator protein encoded by *orf50* (43, 47). An increase in *orf6* transcription was not observed for up to 16 h after TSA treatment, further supporting the explanation that there were in-
sufficient amounts of the R transactivator protein at that time. Interestingly, histone acetylation was already observable 4 h after the addition of TSA at orf6; if one assumes that the number of mRNA molecules was not too low for detection by sensitive RT-PCR, our data argue for the occurrence of acetylation independently of transcription.

Studies on the induction of KSHV lytic replication by TSA have revealed that most of the infected cells rapidly undergo apoptosis, and only a minority, 3 to 7%, of cells produces viral particles. In this population, the maximum mRNA levels of KSHV late genes are reached 48 h to 72 h after TSA treatment (36). Two recent, detailed studies on KSHV epigenetics provide additional in-depth insight into the regulation of latent and early KSHV promoters and the putative mechanisms of reactivation (20, 44). They describe the mutually exclusive presence of markers for active and inactive chromatin, similar to what we found in HVS (Fig. 1b) and the poised state of viral lytic gene promoters during latency, enabling a rapid proliferative response and thus reactivation. We did not investigate the mRNA levels of TSA-induced T cells with regard to HVS late genes for longer than 24 h, since most of the cells had undergone apoptosis at 24 h after TSA treatment, too early for considerable amounts of lytic gene transcripts to be produced. There was no lytic virus detectable from any cell as late as 96 h after TSA or butyrate induction, as tested by sensitive culture method. There was no lytic virus detectable from any cell as late as 96 h after TSA treatment, too early for considerable amounts of lytic gene transcripts to be produced. We did not investigate the mRNA levels of TSA-induced T cells with regard to HVS late genes for longer than 24 h, since most of the cells had undergone apoptosis at 24 h after TSA treatment, too early for considerable amounts of lytic gene transcripts to be produced.

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