Acetylated Histone H3 and H4 Mark the Upregulated LMP2A Promoter of Epstein-Barr Virus in Lymphoid Cells[▽]

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We analyzed the levels of acetylated histones and histone H3 dimethylated on lysine 4 (H3K4me2) at the LMP2A promoter (LMP2Ap) of Epstein-Barr virus in well-characterized type I and type III lymphoid cell line pairs and additionally in the nasopharyngeal carcinoma cell line C666-1 by using chromatin immunoprecipitation. We found that enhanced levels of acetylated histones marked the upregulated LMP2Ap in lymphoid cells. In contrast, in C666-1 cells, the highly DNA-methylated, inactive LMP2Ap was also enriched in acetylated histones and H3K4me2. Our results suggest that the combinatorial effects of DNA methylation, histone acetylation, and H3K4me2 modulate the activity of LMP2Ap.

Epstein-Barr virus (EBV) is closely associated with a variety of neoplasms. In latently infected cells, depending on the gene expression pattern, three main classes of latency have been described (17). Latent membrane protein 2A (LMP2A) is transcribed from the LMP2A promoter (LMP2Ap) in large quantities in type III latency, at variable levels in type II latency, and at low levels or not at all in type I latency (15, 20, 32).

Previous *in vitro* binding and reporter gene experiments charted two CBF1 sites and other elements in the regulatory region of LMP2Ap (10, 15, 18, 30–32). Our previous *in vivo* analysis of LMP2Ap showed that, in lymphoid cell lines, the characteristic footprints on two CBF1 and further binding sites, together with the overall hypomethylation of CpG dinucleotides, correlate well with promoter activity. In contrast, the absence of several genomic footprints, as well as the presence of patches of highly methylated CpG dinucleotides, is characteristic of silent LMP2Ap's in lymphoid cells (20). However, in addition to DNA methylation and protein-DNA interactions, the acetylation of histone H3 and H4 and methylation on the lysine 4 residue of histone H3 may play an important role in the regulation of LMP2Ap, as they lead to chromatin relaxation and subsequent modulation of gene expression (4, 7, 16, 24, 26). It was also shown previously that the binding of EBNA2 to CBF1 directs the p300, CBP, and P/CAF histone acetyltransferase coactivators to the LMP1 promoter (28). On the other hand, CBF1 in the absence of EBNA2 or activated Notch1C represses transcription, in part by tethering a histone deacetylase corepressor complex (11–13, 29). Furthermore, previous studies have shown good correlation between the levels of acetylated histones and histone H3 dimethylated at the lysine 4 residue (H3K4me2) and the activity of the CBF1-

regulated C-promoter of EBV (1, 5; G. Fejer et al., submitted for publication). Therefore, we wished to analyze the levels of acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), and H3K4me2 at LMP2Ap in well-characterized type I (Mutu-BL-I-CI-216 and Rael) and type III (Mutu-BL-III-CI-99 and CB-M1-Ral-STO) lymphoid cell line pairs carrying the same viral strains (20, 21) and additionally in the only available EBV-positive nasopharyngeal carcinoma (NPC) cell line, C666-1, representative of latency type II (6). These cell lines contain only tightly latent episomal EBV genomes, as tested by terminal repeat analysis (3, 25; J. Minarovits, unpublished data). Furthermore, Western blot analysis revealed that early antigens associated with productive EBV replication could not be detected in the above-mentioned cell lines and clones throughout our experiments.

Upregulated LMP2Ap is enriched in AcH3 and AcH4 in lymphoid cell lines. First, we quantified the relative levels of LMP2A expression with real-time PCR (Fig. 1). This procedure showed high levels of LMP2A expression in Mutu-BL-III-CI-99 cells, moderately elevated levels in cells of CB-M1-Ral-STO (a lymphoid cell line carrying the EBV strain recovered from Rael cells), and low levels in Mutu-BL-I-CI-216, Rael, and C666-1 cells.

Next, we analyzed the levels of AcH3 and AcH4 in the BALF2 coding region (used as a control) and at LMP2Ap in the four lymphoid cell lines by chromatin immunoprecipitation (ChIP) (8) combined with real-time PCR using antibodies directed to diacetylated histone H3 and tetra-acetylated histone H4 (Fig. 2A and B). We found that the coding region of the BALF2 gene (a lytic cycle gene inactive during latent infection), approximately 3.5 kb upstream from LMP2Ap, contained only low levels of AcH3 and low (Mutu-BL-I-CI-216 cells) or only slightly elevated (Mutu-BL-III-CI-99, Rael, and CB-M1-Ral-STO cells) levels of AcH4 in the lymphoid cell lines. On the other hand, LMP2Ap's with strong (Mutu-BL-III-CI-99 cells) or moderate (CB-M1-Ral-STO cells) activity were highly enriched in AcH3 and AcH4 compared to the low-level-activity LMP2Ap's (Mutu-BL-I-CI-216 and Rael

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FIG. 1. Expression of LMP2A mRNA relative to the expression of β -actin mRNA. After RNA purification, the reverse transcription reaction was initiated with 1 μ g of DNase-treated RNA by using a primer corresponding to nucleotides 239 to 222 of the B95-8 prototype EBV genome (2) and an oligonucleotide (5'-TGTAACGCAACTAAGTCATAG-3') complementary to the human β -actin mRNA. The levels of LMP2Ap-initiated transcripts and β -actin mRNA were determined with the LC FastStart DNA Master SYBR Green I kit in a LightCycler instrument (Roche) with primers corresponding to nucleotides 166698 to 166720 and 80 to 61 of the prototype B95-8 EBV genome (2) and oligonucleotides (5'-GGCGGCACCACCATGTACCCT-3' and 5'-AGGGGCCGGACTCGTCATACT-3') amplifying a region of the human β -actin cDNA. LMP2A mRNA expression was quantified relative to the expression of the human β -actin mRNA, and the results of triplicate experiments are expressed as percentages of the average relative level of Mutu-BL-III-CI-99 LMP2A mRNA. Abbreviations: Mutu-I, Mutu-BL-I-CI-216 cells; Mutu-III, Mutu-BL-III-CI-99 cells; and CBM1, CB-M1-Ral-STO cells. The first and second exons of LMP2A mRNA, containing the primer binding sites, were sequenced, and the primers were chosen to bind to sequences without polymorphisms compared to the prototype B95-8 EBV genome (2).

cells) and the BALF2 coding region. It should be noted, however, that we observed a slight increase in the levels of AcH3 at LMP2Ap compared to those in the coding region of the BALF2 gene in Mutu-BL-I-CI-216 and Rael cells also, which fits well with the low-level expression of LMP2A in these two cell lines.

Variable levels of H3K4me2 at LMP2Ap in lymphoid cell lines. Enrichment in H3K4me2 correlates with the activity of certain promoters (4, 7), including the C-promoter of EBV (5; Fejer et al., submitted). Therefore, H3K4me2 levels in the BALF2 coding region and at LMP2Ap were analyzed by ChIP (8) combined with real-time PCR using antibody directed to H3K4me2 (Fig. 2C). The results showed low levels of H3K4me2 in the coding region of the BALF2 gene in all lymphoid cell lines. In contrast, we found high levels of H3K4me2 at LMP2Ap in Mutu-BL-III-CI-99 cells, moderate levels in Mutu-BL-I-CI-216 and CB-M1-Ral-STO cells, and only slightly elevated levels in Rael cells compared to the levels in the BALF2 coding region. The observation that H3K4me2 is moderately elevated at LMP2Ap in Mutu-BL-I-CI-216 and CB-M1-Ral-STO cells is remarkable. This elevation may contribute to the inducibility of Mutu-BL-I-CI-216 cells with trichostatin A (TSA) (see below) and may explain the difference in LMP2A expression in CB-M1-Ral-STO and Mutu-BL-III-CI-99 cells (Fig. 1).

TSA treatment activates LMP2Ap in Mutu-BL-I-cl-216 cells but not in Rael cells. To evaluate the functional significance of histone acetylation, we measured the levels of LMP2Ap-initiated transcripts in Mutu-BL-I-CI-216 and Rael cells treated with TSA (an inhibitor of histone deacetylases) or left un-

treated. The efficiency of TSA treatments was monitored by measuring AcH4 levels at LMP2Ap by ChIP. This monitoring showed enrichment with AcH4 at LMP2Ap in TSA-treated cells to levels comparable to those of AcH4 observed in type III cell lines (Fig. 2B). As TSA treatment upregulates the activity of the C-promoter in Mutu-BL-I-CI-216 cells (Fejer et al., submitted), we also combined TSA with the protein synthesis inhibitor cycloheximide (CHX) to rule out possible transactivation effects.

The results showed that, in Rael cells, LMP2Ap activity decreased slightly after TSA treatment (a finding similar to previous observations at the LMP1 promoter [22]), was up-regulated more than twofold after CHX treatment, and was nearly unchanged after the combined action of TSA and CHX (Fig. 3). In contrast, in Mutu-BL-I-CI-216 cells, the activity of LMP2Ap increased threefold after TSA or CHX treatment alone and more than sevenfold after combined treatment. The activating effect of CHX alone (also observed by others [23, 32]) on both cell lines was remarkable and may support the role of repressor complexes in the regulation of LMP2Ap in type I Burkitt's lymphoma cells (13, 27, 29) and/or simply reflect an increased LMP2A mRNA half-life. The observation that combined CHX and TSA treatment activated LMP2Ap more than two times more effectively than CHX alone in Mutu-BL-I-CI-216 cells excludes any transactivation effect of TSA treatment and supports the activating role of histone acetylation in the regulation of LMP2Ap. These results also show the importance of the combinatorial effects of DNA methylation, histone acetylation, and H3K4me2 on the regulation of LMP2Ap, as LMP2A could be induced with TSA or

FIG. 3. Effects of TSA and CHX treatment on LMP2A expression in type I Burkitt's lymphoma cell lines. Mutu-BL-I-Cl-216 (Mutu-I) and Rael cells were untreated or treated for 16 h with 600 nM TSA, either alone or combined with 10 μ g of CHX/ml, or with CHX only. LMP2A mRNA expression was quantified relative to the expression of the human β -actin mRNA as described in the legend to Fig. 1. The results are expressed as percentages of the average relative level of uninduced Mutu-BL-III-Cl-99 LMP2A mRNA.

TSA combined with CHX only in Mutu-BL-I-Cl-216 cells, containing a broad DNA region without methylated cytosines and moderate levels of H3K4me2, and not in Rael cells, containing patches of highly methylated cytosines and low levels of H3K4me2 at LMP2Ap (Fig. 4).

High levels of Ach3, Ach4, H3K4me2, and DNA methylation at LMP2Ap in C666-1 cells expressing low levels of LMP2A mRNA. C666-1 cells express low levels of LMP2A mRNA, comparable to those in type I cells (Fig. 1). Surprisingly, however, our ChIP analysis detected highly elevated levels of Ach3, Ach4, and H3K4me2 at LMP2Ap and low or only slightly elevated levels in the BALF2 coding region in C666-1 cells (Fig. 2). Because of this apparently contradictory result, we hypothesized that DNA methylation may antagonize the presence of these activating histone modifications at LMP2Ap. Therefore, we analyzed the methylation state of LMP2Ap in C666-1 cells by bisulfite genomic sequencing as described previously (20). We found highly methylated CpGs at the upstream regulatory region containing the two Sp1 and CBF1 sites and also downstream around the site of transcription initiation and only a small region with hypomethylated CpGs around the 3' Sp1 site and two CAAT boxes (Fig. 4). This hypomethylated region at LMP2Ap is much smaller than the

one we found previously in Mutu-BL-I-cl-216 cells, and similar to that in Rael cells, it does not extend into the region of transcription initiation like the region in the Mutu-BL-I-Cl-216 cell line (20). As TSA treatment could induce LMP2A transcription only in Mutu-BL-I-Cl-216 cells, with a broad unmethylated region at LMP2Ap, and not in Rael cells, with predominantly highly methylated CpGs, we hypothesize that the patch of highly methylated CpGs at the transcription initiation site in C666-1 cells may overcome the activating effects of the high levels of histone acetylation and H3K4me2.

It has been shown previously that the levels of Ach3, Ach4, and H3K4me2 correlate with promoter activity (4, 7, 16, 24, 26). Furthermore, highly methylated DNA regions may recruit histone deacetylase to repress transcription (9, 14, 19). Therefore, the association of high levels of activating histone modifications with LMP2Ap containing highly methylated CpGs and low levels of LMP2A expression in C666-1 cells is remarkable. To elucidate if these observations are specific for only C666-1 cells or are a feature of NPC cells, both activating and repressive histone modifications, together with DNA methylation, need to be analyzed in a larger number of NPC tumor tissues.

As LMP2Ap contains two CBF1 sites, with an essential role

FIG. 2. Levels of Ach3, Ach4, and H3K4me2 at LMP2Ap. Formaldehyde-cross-linked chromatin was prepared from 2×10^7 of the indicated cells and immunoprecipitated with specific antibodies (8) directed to diacetylated histone H3 (reference no. 06-599; Upstate Biotechnology) (A), tetra-acetylated histone H4 (reference no. 06-598; Upstate Biotechnology) (B), and H3K4me2 (reference no. 07-030; Upstate Biotechnology) (C) or mock-precipitated with nonspecific antibody (D). Recovered DNA aliquots were quantified with real-time PCR using the LC FastStart DNA Master SYBR Green I kit in a LightCycler instrument (Roche) with primers specific for the 5' regulatory region of LMP2Ap, corresponding to nucleotides 166128 to 166152 and 166410 to 166387, and with primers specific for the coding region of BALF2, corresponding to nucleotides 162475 to 162496 and 162621 to 162599 of the B95-8 prototype EBV genome (2). The results of triplicate experiments are expressed as the percentage of input DNA (TIC, total input chromatin). Abbreviations: Mutu-I, Mutu-BL-I-Cl-216 cells; Mutu-III, Mutu-BL-III-Cl-99 cells; and CBM1, CB-M1-Ral-STO cells. TSA, cells treated with TSA; TSA+CHX, cells treated with the combination of TSA and CHX.

FIG. 4. Methylation pattern in the sequenced region of LMP2Ap in C666-1 cells. Numbers and vertical lines indicate positions of cytosines within CpG dinucleotides based on the prototype B95-8 sequence (2). Underlined numbers mark additional CpG dinucleotides that are absent from the B95-8 sequence. The degree of methylation of cytosines is indicated by the height of the lines as follows: line only, 0%; line with one horizontal bar, 0 to 25%; line with two horizontal bars, 25 to 50%; line with three horizontal bars, 50 to 75%; and line with four horizontal bars, 75 to 100%. Gray columns represent previously described or hypothetical *in vitro* binding sites. The transcription initiation site of LMP2A is shown by a thick arrow. The methylation maps for Rael and Mutu-BL-I-CI-216 (Mutu-BL-I) cells were published previously (20) and are shown together with that for C666-1 cells only for the better comparability of methylation patterns. Abbreviation: Mutu-BL-I, Mutu-BL-I-CI-216 cells.

in the regulation of the promoter (10, 15, 18, 30–32), and previous *in vitro* results showed that histone acetylation is a key component of CBF1 regulatory mechanisms (13, 28, 29), our *in vivo* results strongly support the role of histone acetylation and H3K4me2 in the regulation of LMP2Ap in lymphoid cells. To our knowledge, this is the first study demonstrating that histone modifications contribute to the regulation of LMP2Ap activity.

Nucleotide sequence accession numbers. The sequences of the first and second exons of LMP2A mRNA from the analyzed cell lines have been deposited in GenBank under the following accession numbers: C666-1 cells, AM746938; Mutu-BL-I-CI-216 cells, AM746939; Mutu-BL-III-CI-99 cells, AM746940; Rael cells, AM746941; and CB-M1-Ral-STO cells, AM746942.

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