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Deacetylase Inhibitors and the Viral Transactivator Tax_{BLV} Synergistically Activate Bovine Leukemia Virus Gene Expression via a cAMP-responsive Element- and cAMP-responsive Element-binding Protein-dependent Mechanism*

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Efficient bovine leukemia virus (BLV) transcription requires the virus-encoded transactivator Tax_{BLV}, which acts through three Tax_{BLV}-responsive elements located in the 5′ long terminal repeat. It has been proposed that the binding of the CRE-binding protein (CREB) and the activating transcription factor (ATF) to the three imperfect cAMP-responsive elements (CREs) located in each Tax_{BLV}-responsive element mediates Tax_{BLV} transactivation. Here we demonstrated that deacetylase inhibitors (HDACis) synergistically enhanced the transcriptional activation of the BLV promoter by Tax_{BLV} in a CRE-dependent manner. Tax_{BLV} was acetylated *in vivo* at its N^{α} terminus but not at internal lysine residues. Rather, HDACi potentiation of Tax_{BLV} transactivation was mediated by an HDACi indirect action that requires new protein synthesis. Mechanistically, using a dominant-negative form of CREB, we showed that Tax_{BLV} and HDACi synergistically activated BLV gene expression via a CREB-dependent mechanism. Moreover, electrophoretic mobility shift assay and Western blot experiments revealed that HDACi increased the *in vitro* DNA binding activity of CREB/ATF but did not alter CREB/ATF intranuclear presence. Remarkably, chromatin immunoprecipitation assays demonstrated that HDACi treatment increased the level of CREB bound to the BLV promoter *in vivo*. Our results together suggest that an increase in CREB/ATF occupancy of the viral CREs in response to HDACi potentiates Tax_{BLV} transactivation of the BLV promoter.

Bovine leukemia virus (BLV)[1] is a B-lymphotropic retrovirus associated with enzootic bovine leukosis, a disease characterized by an increased number of B-lymphocytes and, in some cases, after a long latency period, by the subsequent development of B-cell leukemia or lymphosarcoma (1). BLV is closely related structurally and biologically to the human T-lymphotropic viruses HTLV-I and HTLV-II (1). Expression of BLV is regulated at the transcriptional level by the virus-encoded transactivator Tax_{BLV} (2, 3). The molecular mechanism by which Tax_{BLV} activates viral transcription is not fully understood. Transactivation by Tax_{BLV} requires three 21-bp imperfect repeats located in the U3 region of the 5′ long terminal repeat (LTR) (2, 4). These Tax_{BLV}-responsive elements (called TxREs) contain a core octanucleotide sequence with similarity to the cAMP-responsive element (CRE) consensus (TGACGTCA) (5). The BLV CRE-like motifs located in the middle of each TxRE have been shown to serve as binding sites for three members of the basic leucine zipper (bZIP) family of cellular transcription factors: the CRE-binding protein (CREB) and the activating transcription factors-1 and −2 (ATF-1 and ATF-2) (4, 6). Because there is no evidence for direct binding of Tax_{BLV} to DNA, it has been proposed that Tax_{BLV} transactivation of the BLV promoter could be mediated, as reported for the HTLV-I system, through protein-protein interactions with CREB/ATF (4, 6, 7). The formation of this promoter-bound Tax_{BLV}-CREB/ATF complex could then serve for the recruitment of the multifunctional cellular coactivators CBP (CREB-binding protein) and p300.

There is now strong evidence that both transcriptional activation and silencing are mediated through the recruitment of enzymes that control protein acetylation: the histone deacetylases (HDACs) and the histone acetyltransferases (HATs). Acetylation of specific lysine residues within the amino-terminal tails of nucleosomal histones is generally linked to chroma-

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[1] The abbreviations used are: BLV, bovine leukemia virus; HTLV, human T-lymphotropic virus; LTR, long terminal repeat; TxRE, Tax-responsive element; CRE, cAMP-responsive element; bZIP, basic leucine zipper; CREB, CRE-binding protein; ATF, activating transcription factor; CBP, CREB-binding protein; HDAC, histone deacetylase; HAT, histone acetyltransferase; HDACi, HDAC inhibitor; TSA, trichostatin A; NaBut, sodium butyrate; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; CREM, cAMP-responsive element modulator; CH, cycloheximide; actD, actinomycin D; A-CREB, acidic CRE-binding protein; HIV-1, human immunodeficiency virus-1; luc, luciferase; p300, protein 300.

tin disruption and transcriptional activation of genes (8, 9). Reversible acetylation has also been identified as a critical posttranslational modification of non-histone proteins, including general and specific transcription factors, non-histone structural chromosomal proteins, acetyltransferases themselves, the human immunodeficiency virus-1 (HIV-1) Tat protein, non-nuclear proteins (α-tubulin), and nuclear import factors (such as human importin-α) (10–12). Depending on the functional domain that is modified, acetylation can regulate different functions of these non-histone proteins, such as DNA recognition, protein stability, protein-protein interaction, and subcellular localization.

In the case of BLV, we have reported previously that treatment with deacetylase inhibitors (HDACi) increases viral expression in peripheral blood mononuclear cells from BLV-infected sheep or cows (13), in BLV-infected cell lines, and in stably transfected cells containing a LTR_{BLV}-luciferase construct.[2] Indeed, several of the nuclear factors that are known to bind to the BLV LTR have been shown to interact with HATs (CREB/ATF, PU.1/Spi-B, USF2, the glucocorticoid receptor, IRF1, and IRF2) (14–23) and/or with HDACs (rat CREB-1, PU.1, glucocorticoid receptor) (24–26), and/or to be directly acetylated (rat CREB-1, human CREB-2, IRF2) (21, 27–29). Among these factors binding to the BLV LTR, the members of CREB/ATF family are of particular interest because they have been demonstrated to play a critical role in BLV gene expression and because they are known to interact with the transcriptional coactivators CBP/p300. CBP/p300 act as bridging factors between cellular activators and the general transcription factors (30). The best characterized coactivator property of CBP and p300 is their intrinsic HAT activity (31–33). CBP and p300 also bind to other coactivator complexes such as p300/CBP-associated factor, SRC-1/NcoA-1, TIF-2/GRIP-1/NcoA-2, and pCIP/ACTR, which also possess intrinsic HAT activity (10, 34). Moreover, ATF-2 is so far the only transcription factor that possesses its own HAT activity (35, 36).

In this study, we have investigated the potential link between protein acetylation and transactivation of the BLV promoter by Tax_{BLV}. We demonstrated that HDACi (such as trichostatin A (TSA) and sodium butyrate (NaBut)) markedly potentiated Tax_{BLV} transactivation of the BLV LTR in a CRE-dependent manner, leading to a strong synergism between Tax_{BLV} and HDACi. These data suggested that Tax_{BLV} transactivation could be functionally regulated by post-translational acetylation *in vivo*. However, additional studies showed that Tax_{BLV} and its mediator bovine CREB-2 were not subjected to direct acetylation at internal lysine residues *in vivo*. Nevertheless, Tax_{BLV} was acetylated *in vivo* at its N^{α} terminus. The synergistic activation of the BLV LTR by Tax_{BLV} and HDACi required neither intact internal lysine residues in Tax_{BLV} nor the N^{α}-terminal acetylation of Tax_{BLV}. Rather, the effect of TSA on Tax_{BLV} transactivation of the BLV LTR was indirect through the activation of cellular genes because it was sensitive to protein synthesis inhibition. Mechanistically, overexpression of a dominant-negative acidic CREB (A-CREB) polypeptide markedly inhibited the Tax_{BLV}/HDACi synergism, indicating that CREB/ATF are required for HDACi to synergize maximally with Tax_{BLV} on the BLV promoter. Moreover, electrophoretic mobility shift assays (EMSAs) and Western blot experiments revealed that HDACi increased the DNA binding activity of the CREB/ATF factors in both the absence and the presence of Tax_{BLV} but did not alter their intranuclear presence. Remarkably, chromatin immunoprecipitation (ChIP) assays using the BLV-infected YR2 cell line demonstrated the *in vivo* relevance of our EMSAs in the context of a chromosomally integrated BLV provirus. Our results support a role of CREB/ATF in the synergistic activation of the BLV promoter by Tax_{BLV} and inhibitors of deacetylases.

[2] C. Calomme, A. Dekoninck, S. Nizet, E. Adam, T. L.-A. Nguyên, A. van Den Broeke, L., Willems, R. Kettmann, A. Burny, and C. Van Lint, submitted for publication.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The pLTRwt-luc, containing the luciferase gene under the control of the complete 5′LTR of the 344 BLV provirus, was described previously (37). This construct was used as a substrate for mutagenesis by the QuikChange site-directed mutagenesis method (Stratagene). Mutation of the three viral CRE-like motifs was generated by combining the following pairs of mutagenic oligonucleotide primers (mutations are in bold and the three CRE-like motifs are underlined on the coding strand primer): CV 409/410, 5′-CGTAAACCAGACAGAG**TG**GTCAGCTGCCAGAAAAGCTGGTG**TG**GGCAGCTGGTGGCTAGAATC-C-3′ and CV 415/416, 5′-CCACACCCCGAGCTGCTG**TG**CTCACCTGCT-GATAAAAC-3′. The mutated construct was fully resequenced after identification by cycle sequencing using the Thermosequenase DNA sequencing kit (Amersham Biosciences). The resulting plasmid was designated pLTR-mut3CRE-luc.

The eukaryotic expression vectors pSG-WT-Tax_{BLV} and pSG-bCREB2 were gifts from Drs. Luc Willems and Richard Kettmann (5). A mutant pSG-WT-Tax_{BLV} construct containing combinations of mutations in the three Tax_{BLV} lysine residues (Lys-149, Lys-288, and Lys-296) was generated by site-directed mutagenesis (Stratagene) using simultaneously the following three mutagenic oligonucleotide pairs (mutations are highlighted in bold and the three lysine codons are underlined on the coding strand primer): CV 202/203, 5′-CCTGTCCT-CGGA**GC**AGTCCTTATATTAAATC-3′; CV 204/205, 5′-GGTTGCTA-GCAGA**GC**AATAGGACTTGATTCC-3′; and CV 206/207, 5′-CTT-GATTCCCCCTTA**GC**ATTACAACTGCTAG-3′. The mutated resulting plasmid was designated pSG-Tax_{BLV}-mut3K. In addition, a Tax_{BLV} expression vector with an HA epitope at the Tax_{BLV} N terminus was generated with the mutagenic oligonucleotide primers CV503/504, 5′-CGACTCTAGAGGATCATCAGATGTACCCATACGACGTCCCAGAC-TACGCTGGAGGC**GCA**^{aa1}AGTGTTGTTGGTTGG-3′ (the HA tag is underlined on the coding strand primer and the Tax_{BLV} open reading frame is indicated in bold by the Tax_{BLV} N-terminal alanine codon (*aa1*)). The mutated resulting plasmid was designated pSG-HA-Tax_{BLV}. Mutated constructs were fully resequenced after identification.

The $pLTR_{HIV-1}$-luc containing the luciferase gene under the control of the HIV-1_{LAI} 5′LTR (nt 345–531) was described previously (38). The eukaryotic expression vector pTat-wt was described previously (39). The p53 expression vector was kindly provided by Dr. Bert Volgestein. The pGEM-LTR_{BLV} used in RNase protection analysis contains a 201-bp fragment from the BLV 5′LTR (nt −118 to +83) and was described previously (37). The dominant-negative inhibitor A-CREB cytomegalovirus expression vector was a kind gift of Dr. Charles Vinson (40).

Cell Lines and Cell Culture—The Raji cell line is a human B-lymphoid Epstein Barr virus-positive cell line derived from a Burkitt's lymphoma. The human T-lymphoid cell line SupT1 was obtained from J. Hoxie through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health). Raji and Sup T1 cell lines were grown as reported previously (37, 41). The adherent cell line COS-7, a simian fibroblastic cell line, was cultured in Dulbecco's modified Eagle's-Glutamax I medium containing 5% fetal bovine serum, 50 units of penicillin/ml, and 50 μg of streptomycin/ml. The YR2 B-cell line is derived from leukemic cells of a BLV-infected sheep and contains a single, monoclonally integrated silent provirus with two mutations in Tax_{BLV} that impair the infectious potential of the integrated provirus. The $YR2_{LTaxSN}$ cell line is derived from YR2 cells and contains a retroviral vector gene encoding for a transactivation-competent Tax_{BLV} (42). YR2 and $YR2_{LTaxSN}$ cell lines were maintained in Opti-MEM medium supplemented with 10% fetal bovine serum, 50 units of penicillin/ml, and 50 μg of streptomycin/ml. All cells were grown at 37 °C in an atmosphere of 5% CO_2.

Transient Transfection and Luciferase Assays—Raji and SupT1 cells were transfected by using the DEAE-dextran procedure as described previously (41). At 22 h after transfection, the cells were mock-treated or treated with TSA (500 nM) or NaBut (5 mM) (Sigma). At 42 h after transfection, cells were lysed and assayed for luciferase activity (Promega). Luciferase activities were normalized with respect to protein concentrations using the detergent-compatible protein assay (Bio-Rad).

RNase Protection Assays—RNase protection assays were performed as described previously (37). The BLV-specific ^{32}P-labeled antisense

riboprobe was synthesized *in vitro* by transcription of XbaI-restricted pGEM-LTR_{BLV} with SP6 polymerase according to the protocol provided with the Riboprobe *in vitro* Transcription Systems (Promega). The luciferase antisense riboprobe was similarly synthesized by transcription of SgrAI-restricted pSP-*luc*+ vector (Promega) with T7 polymerase. As control, a glyceraldehyde-3-phosphate dehydrogenase-specific antisense riboprobe was synthesized by the same method and used on the same RNA samples.

In Vivo Acetylation Assays—*In vivo* acetylation assays were performed as described previously (43). In brief, COS-7 cells were transfected, using FuGENE-6 (Roche Molecular Biochemicals) according to the manufacturer's protocol, with expression vectors for WT-Tax_{BLV}, Tax_{BLV}-mut3K, HA-Tax_{BLV}, bovine CREB2, or p53 (500 ng). Experiments were performed in duplicate. Twenty-four hours after transfection, cells were pulsed for 1 h with 1 mCi/ml sodium [^{3}H]acetate (20 Ci/mmol; Amersham Biosciences) in the presence of 500 nM TSA in complete culture medium or, as a control for expression and immunoprecipitation of the proteins, for 4 h with 0.2 mCi/ml [^{35}S]methionine/cysteine (10 mCi/ml; ICN Corp.) in the presence of TSA in methionine/cysteine-free medium. Tax_{BLV}, bCREB2 or p53 were immunoprecipitated from cellular lysates with either an anti-Tax_{BLV} (monoclonal antibody termed 5A5 provided by Daniel Portetelle), an anti-hCREB1, or an anti-p53 antibody (all from Santa Cruz Biotechnology). The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared and EMSAs and supershift assays were performed as described previously (37). The DNA sequence of the coding strand of the double-stranded TxRE2 probe (nt −139 to −133) is 5′-AAGCTGGTGACGGCAGCTGGT-3′ (the CRE-like site is underlined). For supershift assays, monoclonal antibodies against ATF-1 and ATF-2 and polyclonal antibodies against human CREB-1 and CREM (from Santa Cruz Biotechnology) were added at a final concentration of 2 μg/reaction mixture at the beginning of the binding reaction for 20 min before adding the DNA probe (blocking conditions). As loading controls, the same nuclear extracts were tested for binding of Sp1 to an Sp1 consensus probe, 5′-ATTCGATCGGGGCGGGGCGAGC-3′ (Promega).

ChIP Assays—The ChIP assays were performed with the ChIP assay kit (Upstate Biotechnology) according to the manufacturer's recommendations. Formaldehyde cross-linking reactions from 10^7 BLV-infected YR2 cells, mock-treated or treated with TSA (500 nM) for 20 h, were quenched with 125 mM glycine. Cells were lysed, and chromatin was sonicated to obtain an average DNA length of 500 bp. After centrifugation, the chromatin was diluted 10-fold and precleared with a protein A-agarose slurry containing salmon sperm DNA and bovine serum albumin (Upstate Biotechnology). Precleared chromatin (2 ml) was incubated or not overnight at 4 °C with 5 μg of anti-hCREB-1 antibody or a purified rabbit IgG as control, (from Santa Cruz Biotechnology), followed by immunoprecipitation with protein A-agarose. Immunoprecipitated complexes were washed and eluted twice with 200 μl of elution buffer. The protein-DNA cross-links were reversed by heating at 65 °C overnight, and 10% of the recovered DNA was used for radioactive PCR amplification (35 cycles) with a primer set amplifying the BLV promoter TxRE region (nt −172 to 29): 5′-C^{nt-172}CGTAAACCAGACAGAGACGTCAG-3′/5′-C^{nt+29}ACGAGGGTCTCAGGAGGAGAAC-3′. PCR products were analyzed by polyacrylamide gel electrophoresis, and bands were visualized by autoradiography.

RESULTS

Synergistic Activation of BLV Promoter Activity by Tax_{BLV} and HDACi—To examine the potential link between protein acetylation and Tax_{BLV} transactivation, we tested the effect of two HDACi (TSA and NaBut) on both basal and Tax_{BLV}-induced BLV LTR activity. To this end, human B-lymphoid Raji cells were transiently cotransfected with a LTRwt-luciferase reporter construct (pLTRwt-luc) and increasing amounts of a Tax_{BLV} expression vector, pSG-WT-Tax_{BLV}. Transfected cells were either mock-treated or treated with TSA or NaBut and assayed for luciferase activity (Fig. 1). As expected, in the absence of TSA or NaBut, Tax_{BLV} transactivated the BLV promoter in a dose-dependent manner up to 402-fold (Fig. 1, Tax_{BLV} -fold activation, *lanes 2–7*). Treatment of cells with TSA or NaBut alone resulted in a 51.6- or 51.4-fold activation of transcription, respectively (Fig. 1, *lane 1*). Remarkably, when cells were both cotransfected with increasing amounts of the Tax_{BLV} expression vector and treated with TSA (or NaBut), a strong synergism was observed between Tax_{BLV} and these HDACi, resulting in transactivations ranging from 421- to 4578-fold in the presence of TSA (Fig. 1, Tax_{BLV} + TSA -fold activation, *lanes 2–7*) and from 409- to 6522-fold in the presence of NaBut (Fig. 1, Tax_{BLV}+NaBut -fold activation, *lanes 2–7*). Transcriptional activators synergize when their combination produces a transcriptional rate that is greater than the sum of the effects produced by the individual activators (44). Transfection of 0.5 ng of pSG-WT-Tax_{BLV} led to an 88.7-fold stimulation of transcription in absence of HDACi, whereas, in the presence of TSA or NaBut, it led to a 915- or 833-fold stimulation, respectively (Fig. 1, *lane 3*). This amount of transcription is 6.5- or 5.9-fold greater (-fold synergism) than the sum of the effect produced by each activator separately (88.7 + 51.6 or 88.7 + 51.4) (Fig. 1, *lanes 1* and *3*, respectively). Likewise, transfection of 8 ng of pSG-WT-Tax_{BLV} in the presence of TSA (or NaBut) stimulated transcription 4578- (or 6522-)fold, corresponding to a 10- (or 14-) fold synergism (Fig. 1, compare *lanes 1* and *7*). This synergism between Tax_{BLV} and TSA (or NaBut) persisted even at saturating amounts of the Tax_{BLV} transactivator (data not shown), indicating that the observed effect was not the consequence of increased Tax_{BLV} expression because of activation of the simian virus 40 promoter by TSA or NaBut. Similar results were observed using other B-lymphoid cell lines (such as the DG75 and Daudi cell lines) or non-B-lymphoid cell lines (such as the T-lymphoid cell line SupT1 and the epithelial cell line Hela) (data not shown).

Synergistic activation by ectopically expressed Tax_{BLV} and by HDACi required intact CRE binding sites in the three BLV TxREs, because point mutations in these three CRE-like motifs abrogated the synergistic effect (Fig. 1, pLTR-mut3CRE-luc, *lanes 8–14*). This implied that the synergistic effect was mediated by interactions at the CRE-like motifs and not at the otherwise intact LTR_{BLV} DNA sequences.

In conclusion, these results demonstrate that HDACi TSA and NaBut synergistically enhance transcriptional activity of the BLV promoter by Tax_{BLV} in a CRE-dependent manner, suggesting that Tax_{BLV} transactivation could be functionally regulated by posttranslational acetylation *in vivo*.

The Tax_{BLV}/TSA Synergism Occurs at the Transcriptional Level—To demonstrate that the synergistic activation by Tax_{BLV} and TSA can be observed when the amount of transcription (*i.e.* RNA levels) is analyzed, transcript levels in transiently transfected Raji cells were measured by RNase protection assays using probes proximal and distal to the BLV promoter (Fig. 2*A*). The proximal probe, which overlaps the start of transcription in the BLV reporter plasmid, stretches from nt −118 to +83 and therefore hybridizes to all transcripts that initiate at the BLV LTR to produce a protected species of 83 nt. The distal probe, producing a 225-nt protected luciferase product, can only detect transcripts that have extended into the luciferase gene and it therefore provides a measure of transcriptional elongation. We performed RNase protection assays using RNAs extracted from Raji cells transiently cotransfected with pLTRwt-luc and with the Tax_{BLV} expression vector pSG-WT-Tax_{BLV} in the presence or absence of TSA (Fig. 2*B*). Quantification of the RNA bands was carried out by densitometric scanning of the autoradiographs (Fig. 2*C*). We detected weak amounts of reporter transcripts in the absence of both Tax_{BLV} and TSA and in the presence of TSA alone with both the BLV promoter-specific riboprobe and the luciferase gene-specific riboprobe (Fig. 2, *B* and *C*, *lanes 1* and *2*, respectively), probably as a consequence of the weak BLV promoter activity in the absence of Tax_{BLV} and of the weak transfection efficiency of the DEAE-dextran procedure. Activation of the LTR activity by

pLTRwt-luc

	pSG-Tax_{BLV} (ng)	RLU (mock)	RLU (+TSA)	RLU (+NaBut)	Tax_{BLV} fold activation	Tax_{BLV} + TSA fold activation	Tax_{BLV} + NaBut fold activation	Tax_{BLV} + TSA fold synergism	Tax_{BLV} + NaBut fold synergism
1	0	2.32	120	119	1	51.6	51.4	-	-
2	0.025	68.7	979	951	29.6	421	409	**5.2**	**5.1**
3	0.5	206	2127	1936	88.7	915	833	**6.5**	**5.9**
4	1	210	2878	2654	90.3	1239	1142	**8.7**	**8.1**
5	2	322	4233	4104	138	1822	1766	**9.6**	**9.3**
6	4	496	6573	6483	213	2828	2790	**11**	**11**
7	8	934	10638	15155	402	4578	6522	**10**	**14**

pLTR-mut3CRE-luc

	pSG-Tax_{BLV} (ng)	RLU (mock)	RLU (+TSA)	RLU (+NaBut)	Tax_{BLV} fold activation	Tax_{BLV} + TSA fold activation	Tax_{BLV} + NaBut fold activation	Tax_{BLV} + TSA fold synergism	Tax_{BLV} + NaBut fold synergism
8	0	0.85	128	82.8	1	151.8	98.0	-	-
9	0.025	0.85	112	66.5	1.01	133	78.7	**0.87**	**0.79**
10	0.5	0.71	86.7	55.6	0.84	103	65.8	**0.67**	**0.67**
11	1	0.74	114	91.3	0.88	135	96.2	**0.88**	**0.97**
12	2	0.74	97.2	49.2	0.87	115	58.2	**0.75**	**0.59**
13	4	0.98	111	70.0	1.16	132	82.8	**0.86**	**0.84**
14	8	1.76	93.7	62.4	2.09	111	73.8	**0.72**	**0.74**

FIG. 1. **Synergistic activation of BLV promoter activity by Tax_{BLV} and HDACi.** Raji cells were transiently cotransfected with 500 ng of either pLTRwt-luc (*lanes 1–7*) or pLTR-mut3CRE-luc (*lanes 8–14*) and with increasing amounts of pSG-WT-Tax_{BLV} (from 0.025 to 8 ng of plasmid DNA). Cells were mock-treated or treated with TSA (500 nM) or NaBut (5 mM) for 20 h. Luciferase activities were measured in cell lysates 42 h after transfection and were normalized with respect to protein concentrations of the lysates. Results are presented as relative light units (*RLU*), as Tax_{BLV} fold activation, as Tax_{BLV}+TSA -fold activation, and as Tax_{BLV}+NaBut -fold activation of the reporter constructs (pLTRwt-luc and pLTR-mut3CRE-luc) with respect to their respective basal activity, which was arbitrarily set at a value of 1. The Tax_{BLV}+TSA (or Tax_{BLV}+NaBut) -fold synergism was determined as described previously (44) using the following formula: -fold activation by (Tax_{BLV}+TSA)/-fold activation by Tax_{BLV} alone + -fold activation by TSA alone. Values represent the means of duplicate samples. An experiment representative of four repeated transfections is shown. Variation for a given plasmid between different experiments was <15% in most cases.

Tax_{BLV} alone led to a higher amount of reporter transcripts with both the luciferase probe and the 5′LTR probe (Fig. 2, *B* and *C*, *lane 3*) as a consequence of the important increase of BLV transcriptional activity by Tax_{BLV}. Importantly, when cells were both transfected with pSG-WT-Tax_{BLV} and treated with TSA, analysis of the steady-state mRNA level showed that Tax_{BLV} and TSA synergistically increased transcript production, as detected with the proximal BLV promoter-specific probe and with the distal luciferase gene-specific probe (Fig. 2, *B* and *C*, *lane 4*). As an internal control, RNase protection analysis of the same RNA samples using an antisense riboprobe corresponding to the glyceraldehyde-3-phosphate dehydrogenase gene showed no change in the level of mRNA.

We thus demonstrate that Tax_{BLV} and TSA synergistically increase the amount of transcription directed by the BLV promoter. These results are consistent with those of the LTRwt-luciferase assays and show that the synergistic activation of BLV promoter activity by Tax_{BLV} and TSA occurs at the level of transcription.

Tax_{BLV} Is Acetylated in Vivo at Its N^{α} Terminus but Not at Internal Lysine Residues—It has become clear that the HATs and HDACs modify not only histones but also a variety of non-histone proteins, including general and specific transcription factors (10). For some of these transcription factors, such as p53 (45) and GATA-1 (46), acetylation has been shown to lead to increased DNA binding and transactivation capacities. Because our results above showed that TSA enhanced transcriptional activation of the BLV promoter by Tax_{BLV} in a CRE-dependent manner, we considered that these effects could be mediated by direct acetylation of Tax_{BLV} or of its mediator bovine CREB-2 (bCREB-2). To test Tax_{BLV} and bCREB-2 acetylation *in vivo*, COS-7 cells were transiently transfected with either the Tax_{BLV} expression vector (pSG-WT-Tax_{BLV}), a bCREB-2 expression vector (pSG-bCREB-2), or, as a positive control, a p53 expression vector (p-p53). Transfected cells were metabolically labeled for 1 h with [^{3}H]sodium acetate in the presence of TSA and were lysed. Whole-cell extracts were immunoprecipitated with either an anti-Tax_{BLV}, an anti-human CREB-1 (anti-hCREB-1), or an anti-p53 antibody. The anti-hCREB-1 antibody we used has been demonstrated by our laboratory to recognize bCREB-2.[3] Fig. 3 shows that intracellularly expressed p53 and Tax_{BLV} were acetylated *in vivo* (Fig. 3, *top*, *lanes 1* and *2*), whereas intracellularly expressed bovine CREB-2 was not (*lane 3*). As a control, metabolic labeling using [^{35}S]methionine+cysteine demonstrated similar levels of expression for all the proteins tested (Fig. 3, *bottom*). Moreover, Western blot analysis (Fig. 3, *bottom*) was performed either with the anti-Tax_{BLV} antibody, the anti-hCREB-1 antibody, or the anti-p53 antibody to confirm the identity of the ^{35}S-labeled immunoprecipitated proteins (data not shown). Taken together, our results show that acetylation of Tax_{BLV} occurs *in vivo*, whereas the Tax_{BLV} mediator bovine CREB-2 is not acetylated under the same experimental conditions.

To test whether Tax_{BLV} is acetylated at internal lysine residues, we mutated Tax_{BLV} simultaneously at the three potential acceptor residues at positions Lys-149, Lys-288, and Lys-296 by site-directed mutagenesis of the pSG-WT-Tax_{BLV} expression vector, thereby generating pSG-Tax_{BLV}-mut3K. These lysine residues were substituted with arginine residues, thereby conserving the positive charge of the lysine and mimicking a non-acetylated and non-acetylable lysine. Western blot

[3] T. L. A. Nguyên and C. Van Lint, unpublished results.

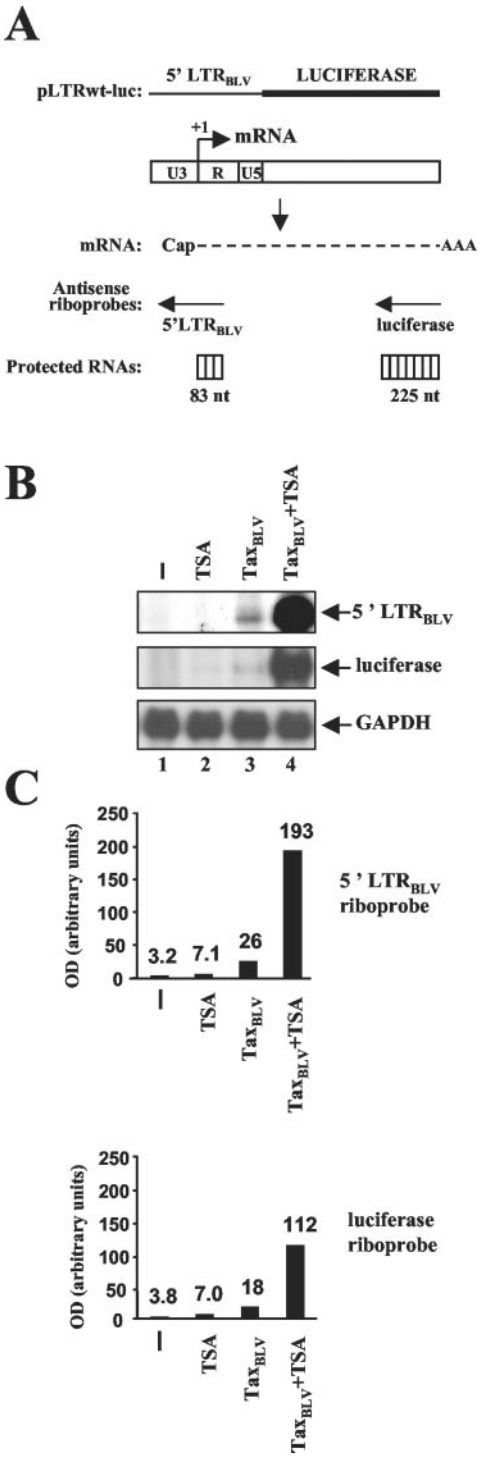

FIG. 2. **Synergistic activation of BLV promoter by Tax_{BLV} and TSA at the mRNA level.** *A*, diagram illustrating the riboprobes used to detect transcript production from the pLTRwt-luc reporter construct. The transcription initiation site at the U3-R junction (+1) is indicated by an *arrow*. The reporter transcripts contain the R and U5 regions of the BLV 5′LTR plus the sequence encoding the luciferase gene. The proximal and distal antisense RNA probes used in RNase protection assays are indicated. The 5′LTR_{BLV} riboprobe is BLV-specific and overlaps the start of transcription, whereas the luciferase-specific riboprobe hybridizes at the 3′ end of the reporter transcript. *B*, Raji cells were transiently transfected with 500 ng of pLTRwt-luc in absence (*lanes 1* and *2*) or presence (*lanes 3* and *4*) of pSG-WT-Tax_{BLV} (5 ng). Cells were mock-treated (*lanes 1* and *3*) or treated with TSA (*lanes 2* and *4*) for 20 h. Total RNA samples were prepared from the transfected cells and were incubated either with the 5′LTR_{BLV} riboprobe or with the luciferase-specific riboprobe. *B*, autoradiographs of the RNase protection gels with the 83-nt LTR_{BLV} protected band (*top*) and the 225-nt luciferase protected band (*middle*). As control, the same RNA samples were incubated with a specific riboprobe corresponding to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (*bottom*). *C*, quantification of the RNA bands shown in *B* was performed by densitometric scanning of the autoradiographs. The results are expressed as optical density (*OD*) arbitrary units. A representative experiment of three independent RNase protection assays is shown.

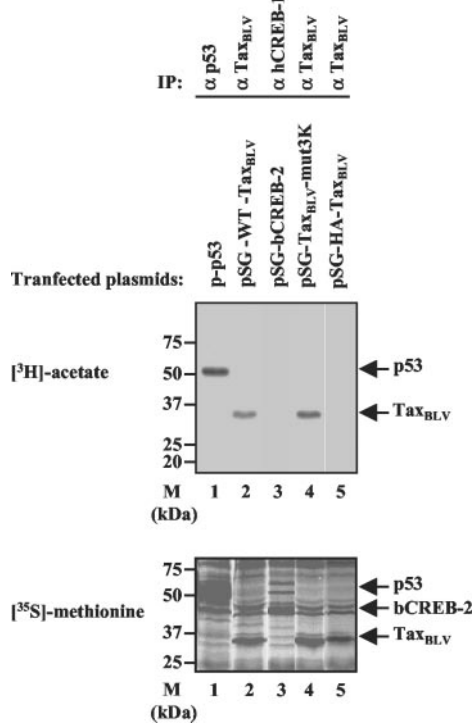

FIG. 3. **Tax_{BLV} is acetylated *in vivo* at its N terminus but not at internal lysine residues.** COS-7 cells were transfected with expression vectors for p53, Tax_{BLV}-WT, bovine CREB-2, Tax_{BLV}-mut3K, or HA-Tax_{BLV} (*lanes 1–5*, respectively) and biosynthetically labeled either for 1 h with [^{3}H]sodium acetate or for 3 h with [^{35}S]methionine-cysteine. Whole-cell extracts from transfected cells were immunoprecipitated with an anti-p53 (*lane 1*), an anti-human CREB-1 (*lane 3*), or an anti-Tax_{BLV} antibody (*lanes 2*, *4*, and *5*). The immunoprecipitated proteins were analyzed by SDS-PAGE followed by autoradiography. Molecular mass markers (*lane M*) (indicated in kilodaltons) are shown on the *left* for reference. *IP*, immunoprecipitation.

analysis of nuclear extracts prepared from COS-7 cells transiently transfected with either pSG-WT-Tax_{BLV} or pSG-Tax_{BLV}-mut3K showed that the Lys to Arg changes did not affect cellular localization and/or level of expression of Tax_{BLV} (data not shown). COS-7 cells were transiently transfected with either pSG-WT-Tax_{BLV} or pSG-Tax_{BLV}-mut3K and labeled with [^{3}H]acetate or [^{35}S]methionine+cysteine. Whole-cell extracts were immunoprecipitated using the anti-Tax_{BLV} antiserum. We found that Tax_{BLV}-mut3K was acetylated *in vivo* at a level comparable with that observed for the wild-type Tax_{BLV} (Fig. 3, *top*, compare *lanes 2* and *4*). This indicated that Tax_{BLV} is acetylated at residue(s) other than the internal lysines and prompted us to test the potential acetylation of Tax_{BLV} at its N^{α} terminus. Indeed, N^{α}-terminal acetylation is one of the most common modifications occurring on the vast majority of eukaryotic proteins (47). N-terminal acetylation of proteins is catalyzed by *N*-acetyltransferases, which transfer acetyl groups from acetyl-CoA to termini of α-amino groups. N-terminal acetylation of eukaryotic proteins occurs when the nascent chain begins to emerge from the ribosome at a length of about 25 residues, or somewhat later if the initiator methionine has to be removed first (47). The biological significance of this post-translational modification varies with the particular proteins; some proteins require N-terminal acetylation for

pSG-Tax_{BLV} (ng)	Tax_{BLV} fold activation		Tax_{BLV} + TSA fold activation		Tax_{BLV} + TSA fold synergism		Tax_{BLV} + NaBut fold activation		Tax_{BLV} + NaBut fold synergism	
	WT	mut 3K	WT	mut 3K	WT	mut 3K	WT	mut 3K	WT	mut 3K
0	1	1	51.6	37.8	-	-	51.4	36.3	-	-
0.025	29.6	5.30	421	106	**5.2**	**2.5**	409	84.8	**5.1**	**2.1**
0.5	88.7	27.0	915	248	**6.5**	**3.8**	833	200	**5.9**	**3.2**
1	90.3	10.2	1239	279	**8.7**	**5.8**	1142	208	**8.1**	**4.5**
2	138	61.6	1822	899	**9.6**	**9.1**	1766	613	**9.3**	**6.3**
4	213	65.7	2828	1514	**11**	**15**	2790	1210	**11**	**12**
8	402	157	4578	2389	**10**	**12**	6522	2179	**14**	**11**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

FIG. 4. **Mutations in the Tax_{BLV} internal lysine residues does not affect the BLV promoter synergistic activation by Tax_{BLV} and HDACi.** Raji cells were transiently transfected with 500 ng of pLTRwt-luc and with increasing amounts (from 0.025 to 8 ng of plasmid DNA) of either pSG-WT-Tax_{BLV} or pSG-Tax_{BLV}-mut3K. Cells were mock-treated or treated with TSA (500 nM) or NaBut (5 mM) for 20 h. Luciferase activities were measured in cell lysates 42 h after transfection and were normalized to protein concentration. Results are presented as Tax_{BLV} -fold activation, Tax_{BLV}+TSA -fold activation, and Tax_{BLV}+NaBut -fold activation with respect to basal activity of pLTRwt-luc in absence of Tax_{BLV} and in absence of HDACi, which was assigned a value of 1. The Tax_{BLV}+HDACi fold synergism was determined as described previously (44). Values represent the means of duplicate samples. An experiment representative of three repeated transfections is shown. Variation for a given plasmid between different experiments was < 15% in most cases.

function, whereas others do not. A review published by Driessen *et al.* (1985) has reported that functional roles for N-terminal acetylation include stabilization of the protein or protection against proteolysis by aminopeptidases (48). However, N-terminal acetylation has so far not been reported to be associated with transcriptional control.

Cleavage of the N-terminal methionine residue is by far the most common modification occurring on the vast majority of proteins. Methionine excision occurs before completion of the nascent amino acid chain and before other N-terminal processing events, such as N-terminal acetylation (49). The two methionine aminopeptidases, Map1p and Map2p, cleave N-terminal methionine residues that are in an environment of amino acids with small side chains (glycine, alanine, serine, cysteine, threonine, proline, and valine) (47). In the case of BLV, the N-terminal sequence of Tax_{BLV} (Met-Ala-Ser-Val-Val-) is probably subjected to cleavage of its N-terminal methionine residue by Maps because the Tax_{BLV} methionine residue is in an environment of amino acids with small side chains. After methionine cleavage, the N-terminal alanine of Tax_{BLV} will become a good substrate for N^{α}-terminal acetylation by NATs because proteins with serine and alanine termini are known to be the most frequently *N*-acetylated proteins. To discriminate between internal lysine acetylation and N^{α}-terminal alanine acetylation, the N-terminal sequence of Tax_{BLV} was fused with an HA tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) sequence, thereby generating the pSG-HA-Tax_{BLV} expression vector. The presence of HA tag, which cannot be subjected to N^{α}-terminal acetylation, masked the N-terminal sequence of Tax_{BLV} that is normally subjected to N-terminal methionine cleavage and alanine N^{α}-terminal acetylation. As shown in Fig. 3, complete abrogation of *in vivo* acetylation was observed in cells transfected with pSG-HA-Tax_{BLV} compared with cells transfected with the wild-type vector pSG-WT-Tax_{BLV} (Fig. 3, *top*, compare *lanes 2* and *5*), indicating that the alanine N-terminal residue of Tax_{BLV} is the sole acetylation site in the viral protein. Metabolic labeling using [^{35}S]methionine+cysteine confirmed that the mutant HA-Tax_{BLV} and the wild-type Tax_{BLV} were expressed to equivalent levels in transfected cells (Fig. 3, *bottom*, compare *lanes 2* and *5*).

In conclusion, Tax_{BLV} is not acetylated at internal lysine residues, only at its N^{α}-terminal residue, whereas bovine CREB-2 is not acetylated, suggesting that the role of protein acetylation during Tax_{BLV} transactivation of the BLV promoter cannot be explained by the direct acetylation of Tax_{BLV} or of bovine CREB-2 but could be explained by other acetylation/deacetylation events.

The BLV LTR Synergistic Activation by Tax_{BLV} and HDACi Does not Require Intact Internal Lysine Residues in Tax_{BLV}—To further investigate the Tax_{BLV}/TSA synergistic activation of the BLV promoter, we compared the effect of TSA and NaBut on the intracellular transactivating activity of the wild-type Tax_{BLV} and of the Tax_{BLV}-mut3K. Raji cells were cotransfected with pLTRwt-luc and with increasing amounts of either pSG-WT-Tax_{BLV} or pSG-Tax_{BLV}-mut3K (Fig. 4). Transfected cells were mock-treated or treated with TSA or NaBut and were assayed for luciferase activity. TSA or NaBut strongly synergized with wild-type Tax_{BLV} to levels of 5.2- to 10-fold synergism or of 5.1- to 14-fold synergism, respectively (Fig. 4, *columns 5* or *9*, respectively). It is noteworthy that whereas the substitution of the three Tax_{BLV} lysine residues into arginines resulted in a >3-fold reduction of Tax_{BLV} transactivation (Fig. 4, compare *columns 1* and *2*), no effect was observed on the -fold synergism with TSA or NaBut (Fig. 4, compare *columns 5* and *6* or *columns 9* and *10*). Thus, mutation of the three internal lysines of Tax_{BLV} resulted in a decreased Tax_{BLV} transactivation but did not result in a decreased Tax_{BLV}/HDACi synergism. These results support the notion that the functional synergism between Tax_{BLV} and TSA does not occur because of Tax_{BLV} internal acetylation, thereby corroborating our *in vivo* acetylation results.

The BLV LTR Synergistic Activation by Tax_{BLV} and HDACi Does not Require N^{α}-terminal Acetylation of Tax_{BLV}—To examine whether the N^{α}-terminal acetylation of Tax_{BLV} could play a role in the Tax_{BLV}/TSA synergistic activation of the BLV promoter, we compared the transactivation of the BLV promoter by the HA-tagged Tax_{BLV} to its transactivation by the wild-type Tax_{BLV}. To this end, Raji cells were cotransfected with the pLTRwt-luc reporter construct and with increasing amounts of either pSG-WT-Tax_{BLV} or pSG-HA-Tax_{BLV} in the presence or absence of TSA (or NaBut). Results in Fig. 5 showed that the transactivation level of the HA-tagged Tax_{BLV} was significantly weaker than that of the wild-type Tax_{BLV} (Fig. 5, compare *columns 1* and *2*), suggesting an important role for Tax_{BLV} function of its N-terminal acetylation. In contrast, the presence of the HA-Tag did not decrease but even reproducibly increased the synergism between Tax_{BLV} and TSA (or NaBut) (Fig. 5, compare *columns 5* and *6* or *columns 9* and *10*). Therefore, we conclude that the N-terminal acetylation of Tax_{BLV} is not implicated in the Tax_{BLV}/HDACi synergistic activation of the BLV promoter but could play a role in Tax_{BLV} function and/or stability.

The Effect of TSA on Tax_{BLV} Transactivation of the BLV Promoter Is Mediated by a TSA Indirect Action That Requires New Protein Synthesis—We next considered the possibility

pSG-Tax_{BLV} (ng)	Tax_{BLV} fold activation		Tax_{BLV} + TSA fold activation		Tax_{BLV} + TSA fold synergism		Tax_{BLV} + NaBut fold activation		Tax_{BLV} + NaBut fold synergism	
	WT	HA-Tax	WT	HA-Tax	WT	HA-Tax	WT	HA-Tax	WT	HA-Tax
0	1	1	50.7	51.8	-	-	37.6	37.1	-	-
0.025	7.76	5.82	121	274	**2.1**	**4.8**	91.7	266	**2.0**	**6.2**
0.5	17.0	8.87	224	358	**3.3**	**5.9**	195	326	**3.6**	**7.1**
1	36.0	15.7	621	651	**7.2**	**9.6**	514	457	**7.0**	**8.7**
2	81.0	23.7	1272	634	**9.7**	**8.4**	1114	630	**9.4**	**10**
4	61.8	43.4	1365	1911	**12**	**20**	1240	1632	**12**	**20**
8	188	70.6	3136	2561	**13**	**21**	2374	2548	**11**	**24**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

FIG. 5. **Tax_{BLV} N^{α}-terminal acetylation does not affect the BLV promoter synergistic activation by Tax_{BLV} and HDACi.** Raji cells were transiently transfected with 500 ng of pLTRwt-luc and with increasing amounts (from 0.025 to 8 ng of plasmid DNA) of either pSG-WT-Tax_{BLV} or pSG-HA-Tax_{BLV}. Cells were mock-treated or treated with TSA (500 nM) or NaBut (5 mM) for 20 h. Luciferase activities were measured in cell lysates 42 h after transfection and were normalized to protein concentration. Results are presented as Tax_{BLV} -fold activation, Tax_{BLV}+TSA -fold activation, and as Tax_{BLV}+NaBut -fold activation with respect to basal activity of pLTRwt-luc in absence of Tax_{BLV} and in absence of HDACi, which was assigned a value of 1. The Tax_{BLV}+HDACi -fold synergism was determined as described previously (44). Values represent the means of duplicate samples. An experiment representative of four independent transfections is shown. Variation for a given plasmid between different experiments was <15% in most cases.

that the effect of HDACi on Tax_{BLV}-mediated LTR activation might be indirect, through the activation of cellular genes. To address this question, the effect of TSA was tested in transient expression assays performed on cells treated with the protein synthesis inhibitor cycloheximide (CH) (Fig. 6). Raji cells were transiently cotransfected with $pLTR_{BLV}$wt-luc and with increasing amounts of the Tax_{BLV} expression vector. Twenty-two hours post-transfection, cells were mock-treated or treated with TSA, in the absence or presence of CH. CH was withdrawn and the cells were incubated for 4 h in fresh complete medium ± TSA to allow the synthesis of luciferase enzyme from the accumulated mRNAs. During this 4-h period, actinomycin D (actD) was added to the CH-treated cells to inhibit transcription. Cells were then lysed and assayed for luciferase activity. Results presented in Fig. 6A showed that Tax_{BLV} transactivation of the BLV LTR was clearly observed when cells were treated with the protein synthesis inhibitor but was reduced by ~2-fold compared with the absence of treatment (Fig. 6*A*, compare *columns 1* and *2*). This probably resulted from the inhibition of Tax_{BLV} synthesis after CH treatment. Activation of the BLV promoter by TSA alone in absence of Tax_{BLV} was strongly reduced by CH + actD treatment from 36- to 1.23-fold (Fig. 6*A*, 0 ng of pSG-WT-Tax, compare *columns 3* and *4*). This reduction could be explained by the suppression of the TSA indirect effect after CH + actD treatment. In the presence of both Tax_{BLV} and TSA, we observed that the LTR_{BLV} synergistic activation was completely abolished by the CH + actD treatment (Fig. 6*A*, compare *columns 5* and *6*), indicating that this synergism was mediated by an indirect mechanism, because it was sensitive to protein synthesis inhibition. As control for a direct effect of TSA on a transcriptional synergistic activation, we used in a parallel experiment the HIV-1 system (Fig. 6*B*). Indeed, our laboratory has previously demonstrated the synergistic activation of the HIV-1 promoter activity by the viral Tat_{HIV-1} transactivator and by HDACi, as well as the direct acetylation of Tat_{HIV-1} at internal lysine residues Lys-28 and Lys-50 (50). As shown in Fig. 6*B* and as observed for the BLV promoter, CH + actD treatment reduced HIV-1 Tat transactivation of the HIV-1 promoter compared with the absence of treatment (Fig. 6*B*, compare *columns 1* and *2*). Moreover, activation of the HIV-1 promoter by TSA alone was strongly reduced by CH + actD treatment (Fig. 6*B*, 0 ng of pTat-wt, compare *columns 3* and *4*), similarly to what we observed in the Tax_{BLV}/TSA system (Fig. 6*A*, 0 ng of pSG-WT-Tax_{BLV}, *columns 3* and *4*). However, in marked contrast to what we observed with the BLV promoter, CH + actD treatment did not affect the synergistic activation of the HIV-1 promoter by Tat_{HIV-1} and TSA (Fig. 6*B*, compare *columns 5* and *6*), in good agreement with the fact that the Tat_{HIV-1}/TSA synergism is caused, at least in part, by the direct acetylation of the Tat_{HIV-1} protein after TSA treatment.

These results indicate that the effect of TSA on Tax_{BLV} transactivation of the BLV LTR is caused by a TSA-mediated indirect mechanism, which requires *de novo* protein synthesis. Indeed, the Tax_{BLV}/TSA synergism is abolished by cycloheximide treatment, indicating that it is mediated by the activation of cellular genes by TSA. In fact, TSA is known to increase the global acetylation level of histones and of non-histone proteins through inhibition of HDACs. Acetylation of histones generally increases the accessibility of transcription factors to nucleosomal DNA and correlates with transcriptional activity *in vivo* (51, 52). Here, TSA could activate the expression of proteins, which are involved in the mechanism of Tax_{BLV} transactivation, thereby potentiating the activation of the BLV promoter by Tax_{BLV}.

CREB/ATF Are Involved in the Synergistic Activation of the BLV Promoter by Tax_{BLV} and HDACi—Based on the above results, the synergism between Tax_{BLV} and TSA seems to be mediated by an indirect action of TSA on the expression of cellular genes involved in Tax_{BLV} transactivation. Therefore, we next investigated whether the CREB/ATF transcription factors (known to mediate Tax_{BLV} transactivation) could play a role in the synergistic activation of the BLV promoter. To this end, we used the dominant-negative CREB inhibitor A-CREB. A-CREB consists of the CREB leucine zipper domain with an acidic amphipathic extension on its N terminus. Functionally, A-CREB heterodimerizes with CREB/ATF and block binding of CREB/ATF to CRE sites (40). We carried out cotransfection experiments of Raji cells with pLTRwt-luc and increasing amounts of pSG-WT-Tax_{BLV}, either in the presence or in absence of the pCMV-A-CREB expression vector. As shown in Fig. 7, overexpression of the dominant negative A-CREB polypeptide potently inhibited the transactivation of the BLV promoter by Tax_{BLV} (Fig. 7, compare *columns 1* and *2*), in agreement with the role of bCREB-2 in Tax_{BLV} transactivation. A-CREB slightly increased the activation of the BLV LTR by TSA alone and by NaBut alone in absence of Tax_{BLV} (1.28-fold and 1.3-fold increase, respectively) (Fig. 7, 0 ng of pSG-WT-Tax_{BLV}, compare *columns 3* and *4* and *columns 7* and *8*, respectively). However, the Tax_{BLV}+TSA or Tax_{BLV}+NaBut -fold synergisms were decreased in the presence of A-CREB (Fig. 7, compare *columns 5* and *6* or columns *9* and *10*, respectively). These results indicate that CREB/ATF are required in order for HDACi to synergize maximally with Tax_{BLV} on the BLV promoter.

A **Raji, pLTR $_{BLV}$wt-luc**

pSG-Tax_{BLV} (ng)	Tax_{BLV} fold activation		Tax_{BLV} + TSA fold activation		Tax_{BLV} + TSA fold synergism	
	no treatment	CH + actD	no treatment	CH + actD	no treatment	CH + actD
0	1	1	36	1.23	-	-
500	110	78	2122	74	14	0.93
700	21	98	1372	72	5.5	0.73
	1	2	3	4	5	6

B **SupT1, pLTR $_{HIV-1}$-luc**

pTat-wt (ng)	Tat_{HIV-1} fold activation		Tat_{HIV-1} + TSA fold activation		Tat_{HIV-1} + TSA fold synergism	
	no treatment	CH + actD	no treatment	CH + actD	no treatment	CH + actD
0	1	1	66	2.6	-	-
500	30	8.6	981	167	11	15
700	36	7.9	1672	181	14	17
	1	2	3	4	5	6

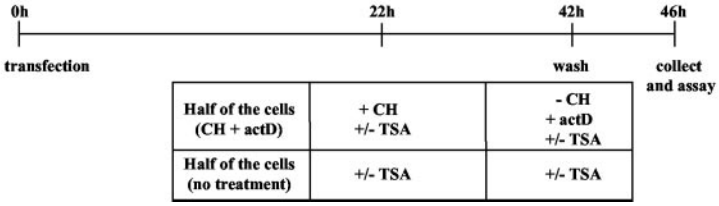

FIG. 6. **Potentiation by TSA of Tax_{BLV} transactivation depends on *de novo* protein synthesis.** *A*, Raji cells were transiently cotransfected with 500 ng of the pLTR$_{BLV}$wt-luc and with two different doses (500 ng or 700 ng) of pSG-WT-Tax_{BLV}. Transfected cells were divided into two equal points. Twenty-two hours after transfection, cycloheximide (*CH*; 10 μg/ml) was added to half of the cells, and all the transfected cells were treated or not with TSA (500 nM) for 20 h. After this 20-h incubation period, cells were washed twice and resuspended for 4 h in fresh medium containing (for half of the cells treated with CH) or not actD (10 μg/ml), in absence or presence of TSA (see diagram at the bottom). Luciferase activities were measured in cell lysates and were normalized with respect to protein concentrations of the lysates. Results are presented as Tax_{BLV} -fold activation and as Tax_{BLV}+TSA -fold activation with respect to the activities of pLTR$_{BLV}$wt-luc either in the presence (CH + actD) or in absence (no treatment) of CH + actD treatment. The measurements of the pLTRwt-luc activity in the presence or absence of CH + actD were very similar and were both assigned a value of 1. The Tax_{BLV}+TSA fold synergism was determined as described previously (44). Values represent the means of duplicate samples. An experiment representative of three repeated transfections is shown. Variation for a given plasmid between different experiments was <15% in most cases. *B*, as a positive control, the synergistic activation of the HIV-1 promoter by Tat_{HIV-1} and TSA, which is caused by the direct acetylation of Tat_{HIV-1}, was studied in parallel. The same experiment as in *A* was carried out in $CD4^+$ T-lymphoid SupT1 cells cotransfected with a pLTR$_{HIV-1}$-luc reporter plasmid and with an expression vector for the Tat_{HIV-1} protein.

pSG-Tax_{BLV} (ng)	Tax_{BLV} fold activation		Tax_{BLV} + TSA fold activation		Tax_{BLV} + TSA fold synergism		Tax_{BLV} + NaBut fold activation		Tax_{BLV} + NaBut fold synergism	
	control	A-CREB	control	A-CREB	control	A-CREB	control	A-CREB	control	A-CREB
0	1	1	62.7	80.6	-	-	57.0	74.1	-	-
0.025	10.3	2.70	241	98.1	3.3	1.2	204	116	3.0	1.5
0.5	30.0	6.14	372	126	4.0	1.5	351	128	4.0	1.6
1	49.3	11.2	838	212	7.5	2.3	759	269	7.1	3.1
2	71.5	14.7	1580	262	12	2.7	1352	317	11	3.6
4	148	28.7	2443	498	12	4.6	2316	589	11	5.7
8	238	63.2	4169	1067	14	7.4	3844	1366	13	10
	1	2	3	4	5	6	7	8	9	10

FIG. 7. **Tax_{BLV} and HDACi synergistically activate BLV gene expression via a CREB-dependent mechanism.** Raji cells were transiently transfected with 500 ng of pLTRwt-luc, with increasing amounts of the Tax_{BLV} expression vector (from 0.025 to 8 ng of pSG-WT-Tax_{BLV}), and with either a dominant-negative A-CREB cytomegalovirus expression vector (500 ng of plasmid DNA) or its corresponding empty control vector. Cells were mock-treated or treated with TSA (500 nM) or NaBut (5 mM) for 20 h. Luciferase activities were measured in cell lysates 42 h after transfection and were normalized to protein concentration. Results are presented as Tax_{BLV} -fold activation, Tax_{BLV}+TSA -fold activation, and Tax_{BLV}+NaBut -fold activation in absence (control) or presence of A-CREB. The Tax_{BLV} fold activation, Tax_{BLV}+TSA fold activation, and Tax_{BLV} +NaBut fold activation of the pLTRwt-luc were obtained with respect to the basal activities of pLTRwt-luc in absence or in the presence of A-CREB. Although these two pLTRwt-luc activity measurements were not identical (presence of A-CREB had a 0.47-fold inhibitory effect on the basal LTR-directed luciferase expression), both were assigned a value of 1. The Tax_{BLV}+HDACi fold synergism was determined as described previously (44). Values represent the means of duplicate samples. An experiment representative of three independent transfections is shown. Variation for a given plasmid between different experiments was < 15% in most cases.

Thus, our results show a positive correlation between the inhibition of CREB/ATF DNA binding and the inhibition of the Tax_{BLV}/HDACi synergism. This indicates that CREB/ATF play a critical role in the synergistic activation of the BLV promoter by Tax_{BLV} and HDACi. Because the effect of TSA is indirect and requires new protein synthesis (see Fig. 6 above), the results obtained with A-CREB suggest that TSA could mediate its action through activation of CREB/ATF expression or

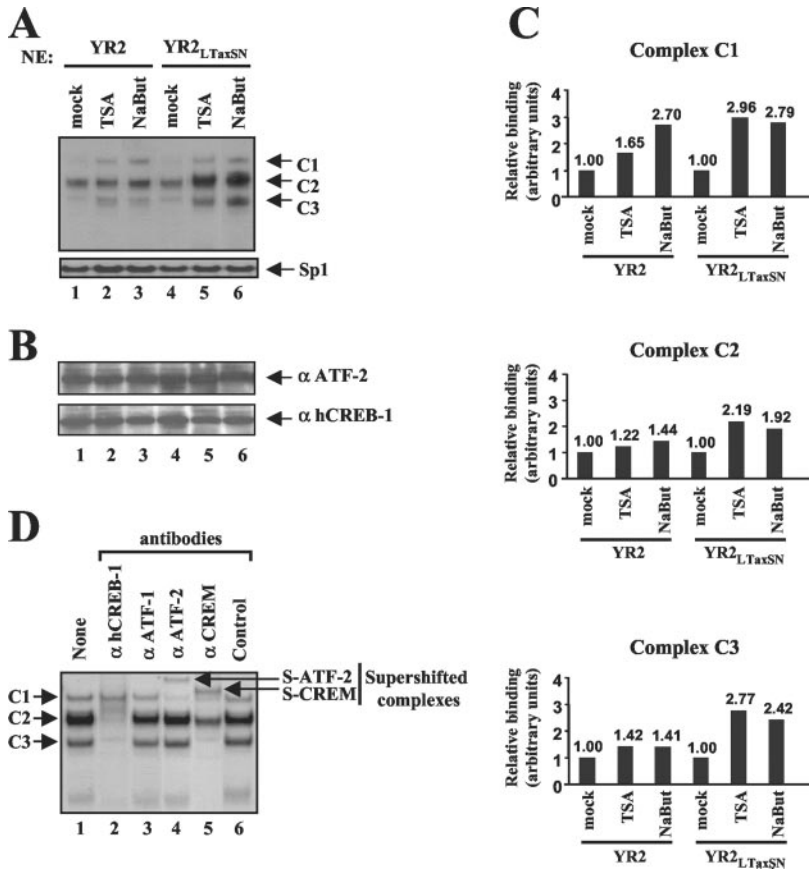

FIG. 8. **Inhibition of deacetylases increases CREB/ATF DNA binding activity *in vitro*.** *A*, EMSA analysis of CREB/ATF binding activity. An oligonucleotide corresponding to the BLV TxRE2 was used as probe and was incubated with 10 μg of nuclear extracts (*NE*) from YR2 or $YR2_{LTaxSN}$ cells either mock-treated or treated with TSA (500 nM) or NaBut (5 mM) for 20 h. The three major DNA-protein complexes C1, C2, and C3 are indicated by *arrows*. As a control for equal loading, the lower shows comparability of the various nuclear extracts assessed by EMSA with a Sp1 consensus probe. *B*, Western blot analysis of nuclear level of CREB/ATF proteins after HDACi treatment. The same nuclear extracts used in *A* were fractionated by electrophoresis, and Western blots were probed with an anti-ATF-2 (top) or an anti-hCREB-1 (lower) antibody. *C*, Quantification of levels of complex formation. The gel shown in *A* was quantified by radioimaging analysis using an Instant Imager (PerkinElmer Life and Analytical Sciences). Formation of complexes C1, C2, and C3 in absence of any treatment were all assigned a value of 1. *D*, supershift assays. Before the addition of the TxRE2 probe in the binding reaction, nuclear extracts from TSA-treated $YR2_{LTaxSN}$ cells were incubated in the absence of antibody (*lane 1*), or in the presence of anti-hCREB1 antibody (*lane 2*), of anti-ATF-1 antibody (*lane 3*), of anti-ATF-2 antibody (*lane 4*), of anti-CREM antibody (*lane 5*), or of a purified rabbit IgG as negative control (*lane 6*). The major DNA-protein complexes C1, C2, and C3 are indicated by *arrows*. The supershifted complexes are also indicated.

through activation of proteins involved in the CREB/ATF signaling pathway.

Inhibition of Deacetylase Activity Increases in Vitro CREB/ATF Binding Activity to the BLV TxREs Both in the Absence and Presence of Tax_{BLV} but Does Not Alter the Intranuclear Presence of CREB/ATF—To substantiate further the involvement of the CREB/ATF transcription factors in the synergistic activation of the BLV promoter by Tax_{BLV} and HDACi, we studied by EMSA the binding activity of CREB/ATF complexes to the BLV CREs in response to TSA. These experiments were carried out with the BLV-infected YR2 and $YR2_{LTaxSN}$ B-cell lines. Nuclear extracts from YR2 and $YR2_{LTaxSN}$ cells either mock-treated or treated with TSA (or NaBut) were analyzed by EMSAs for their ability to interact with the BLV TxRE2. Three major retarded protein-DNA complexes (designated C1, C2, and C3) were detected (Fig. 8*A*). These complexes corresponded to the specific binding of members of the CREB/ATF family, as shown by supershift assays using antibodies directed against hCREB-1, ATF-1, ATF-2, and CREM (Fig. 8*D*, *lanes 1–6*). Using both the YR2 and $YR2_{LtaxSN}$ cell lines, treatment of cells with TSA or NaBut increased the DNA binding activity of the CREB/ATF proteins to the BLV TxRE2, both in the absence and presence of Tax_{BLV}, compared with mock-treated cells (Fig. 8, *A* and *C*, *lanes 1–3* and *4-6*). It was interesting that the increase in CREB/ATF binding activity after treatment with HDACi was more important in the presence of the transduced functional Tax_{BLV} protein expressed in the $YR2_{LTaxSN}$ than the increase observed in the presence of the mutated Tax_{BLV} protein expressed in the YR2 cells, especially for complexes C2 and C3 (Fig. 8, *A* and *C*, compare *lanes 1–3* with *lanes 4–6*). Of note, TSA and NaBut did not alter the binding of the constitutively expressed Sp1 transcription factor (Fig. 8*A*, *lower*). These data reinforce the role of CREB/ATF in the synergistic activation of the BLV promoter by Tax_{BLV} and HDACi. Because the recruitment of CREB/ATF to the BLV CREs is essential for Tax_{BLV} transactivation, the potentiating effect of HDACi on CREB/ATF binding could explain the Tax_{BLV}/HDACi synergism.

The same nuclear extracts used in EMSAs were also examined by Western blotting with an anti-hCREB-1 or anti-ATF-2 antibody to monitor the presence of CREB/ATF in the nucleus after treatment with HDACi. Immunoblotting revealed that TSA or NaBut did not alter the nuclear presence of CREB/ATF (Fig. 8*B*), indicating that the increased DNA binding activity of CREB/ATF in the presence of HDACi did not correlate with an increased intranuclear presence of these factors. Similar results were obtained using nuclear extracts from the Raji B-cell cell line and using TxRE 1 and TxRE 3 as probes (data not shown).

Taken together, our EMSA and Western blot experiments demonstrate that HDACi increase the DNA binding activity of the CREB/ATF factors in both the absence and the presence of Tax_{BLV} but do not alter their intranuclear presence. These results suggest that TSA does not activate CREB/ATF expression but rather modulates the expression of other proteins involved in the CREB/ATF signaling pathway.

HDACi Treatment Enhances the Level of CREB Bound in Vivo to the BLV CRE-like Motifs—To demonstrate *in vivo* in the context of chromatin the relevance of our *in vitro* binding studies, we performed ChIP assays using the BLV-infected YR2 cell line. ChIP assays were carried out on either untreated cells or TSA-treated cells, using an anti-hCREB1 antibody or a purified rabbit IgG as negative control. After immunoprecipitation and reverse of the cross-link, the purified DNA was subjected to PCR analysis using a set of primers flanking the three TxREs of the BLV promoter (Fig. 9). Fig. 9 shows amplification of the input DNA from the BLV LTR region used in the

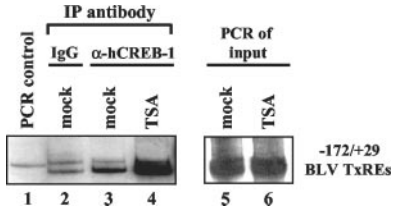

FIG. 9. **TSA enhances the level of CREB bound to the BLV promoter *in vivo* in the chromosomal context of integrated proviruses.** ChIP assays were used to detect binding of CREB to the BLV promoter TxREs. YR2 cells were mock-treated or treated with TSA (500 nM) for 20 h. DNA and protein were cross-linked with formaldehyde for 10 min, and DNA was sheared. The cross-linked protein/DNA complexes were immunoprecipitated with an anti-hCREB1 antibody (*lanes 3* and *4*) or with a purified rabbit IgG as negative control (*lane 2*). The protein-DNA cross-links were reversed and the purified DNA was amplified by radioactive PCR using primers amplifying a BLV promoter region (nt −172 to +29) containing the three TxREs. Equal amounts of the TxRE region in TSA-treated and untreated nucleosomal samples were determined by radioactive PCR from the input chromatin (*lanes 5* and *6*). The PCR control represents the PCR amplification in absence of DNA (*lane 1*).

ChIP assay in absence and in the presence of TSA (Fig. 9, *lanes 5* and *6*, respectively) and of the DNA after immunoprecipitation (Fig. 9, *lanes 2–4*). The PCR control represents the PCR amplification in absence of DNA (Fig. 9, *lane 1*). Comparison of PCR products from immunoprecipitated DNA showed an enrichment of the TxRE region when immunoprecipitation was carried out with the anti-hCREB-1 antibody compared with the purified rabbit IgG (Fig. 9, compare *lanes 2* and *3*). It is noteworthy that these data demonstrated for the first time the binding of a CREB/ATF member to the BLV TxREs *in vivo*. It was remarkable that treatment of cells with TSA resulted in an increased level of CREB bound to the TxRE region compared with the samples in absence of TSA (Fig. 9, compare *lanes 3* and *4*).

We thus demonstrate an increase in the occupancy of the BLV CREs by CREB/ATF in response to HDACi. These results could explain, at least in part, the synergistic activation of BLV promoter activity by Tax_{BLV} and HDACi.

DISCUSSION

In this report, we have demonstrated that Tax_{BLV} and HDACi synergistically activated BLV gene expression in a CRE- and CREB-dependent manner. Tax_{BLV} was acetylated *in vivo* at its N^{α} terminus but not at internal lysine residues. Synergistic activation of the BLV LTR by Tax_{BLV} and HDACi did not require either intact internal lysine residues or N^{α}-terminal acetylation. Rather, the effect of TSA on Tax_{BLV} transactivation of the BLV promoter was caused by a TSA indirect action that requires new protein synthesis. Mechanistically, overexpression of a dominant-negative inhibitor A-CREB markedly inhibited the Tax_{BLV}/HDACi synergism, indicating that CREB/ATF are required for HDACi to synergize maximally with Tax_{BLV} on the BLV promoter. Moreover, EMSA and Western blot experiments revealed that HDACi increased the DNA binding activity of the CREB/ATF factors in both the absence and the presence of Tax_{BLV} but did not alter their intranuclear presence. Finally, ChIP assays using the BLV-infected YR2 cell line confirmed *in vivo* in the context of chromatin that treatment of cells with TSA resulted in an increased level of CREB bound to the BLV TxREs region. Altogether, our results suggest that the enhancing effect of HDACi on CREB/ATF binding activity potentiates Tax_{BLV} transactivation of the BLV-promoter, leading to a strong transcriptional synergism between Tax_{BLV} and HDACi.

The CREB/ATF members belong to a large family of structurally related transcription factors that bind to their DNA target sequences (CRE motifs) as homo- or heterodimers via a C-terminal-conserved region called the bZIP domain. The bZIP domain is characterized by two regions; the first region, which is involved in dimerization, is a leucine zipper consisting of periodic heptad repeats of leucine residues, and the second region, which participates in DNA binding, is a basic helix region just amino-terminal to the leucine zipper (14, 53). The N-terminal transactivation domain of CREB consists of a central inducible activation domain (KID for kinase-inducible domain) flanked by two glutamine-rich constitutive activation domains Q1 and Q2. Phosphorylation of a particular serine residue within the KID domain (Ser-133 in CREB, Ser-121 in ATF-2, and Ser-63 in ATF-1), in response to activation of a variety of signaling pathways, leads to association of CREB/ATF with the coactivator proteins CBP and p300 (14, 54). The mechanism by which Tax_{HTLV-I} transactivates the HTLV-I promoter has been studied extensively. The HTLV-I TxREs are composed of a central CRE-like motif, flanked by guanine- and cytosine-rich domains. Tax_{HTLV-I} is unable to interact directly with the CRE sites but associates with the HTLV-I LTR through interactions with CRE-binding proteins such as hCREB-1, CREM, and ATF-1 (55). However, the Tax_{BLV} transactivation mechanism in the BLV system is far less understood than that of Tax_{HTLV-I} in the HTLV-I system. Because there is no evidence for direct binding of Tax_{BLV} to the BLV promoter and because CREB/ATF have been demonstrated to bind to the BLV CRE-like elements *in vitro* and to transactivate the BLV promoter in transient cotransfection experiments, it has been proposed that transcriptional activation by Tax_{BLV} could be mediated, as reported for the HTLV-I system, through the binding of CREB, ATF-1, and ATF-2 to the BLV TxREs (3, 4, 6, 7, 56, 57). Moreover, so far, the direct binding of a CREB/ATF member *in vivo* to the BLV TxREs has never been reported. In this report, we provide new insights into the involvement of the CREB/ATF transcription factors in the Tax_{BLV} transactivation mechanism. First, we reinforced the role of CREB/ATF recruitment in Tax_{BLV} transactivation by using a dominant-negative inhibitor of CREB/ATF. Indeed, overexpression of the A-CREB polypeptide, which blocks binding of CREB/ATF to CRE sites, potently inhibited transactivation of the BLV promoter by Tax_{BLV}. Second, and significantly, our ChIP assays provide the first direct demonstration of an interaction between CREB/ATF and the BLV promoter *in vivo* under physiological conditions.

In the present study, we have also demonstrated both *in vitro* and *in vivo* an increased binding activity of CREB/ATF to the BLV TxREs after HDACi treatment. This could be explained either 1) by direct acetylation of CREB/ATF, 2) by modulation of their nuclear presence, 3) by activation of proteins involved in the CREB/ATF signaling pathway, or 4) by changes in the redox state of the proteins.

Regarding the first possibility, we have found in this study no evidence for acetylation of bovine CREB-2, neither *in vitro* nor *in vivo*. Indeed, intracellularly expressed bovine CREB-2 (the homolog of human CREB-1) was not acetylated, whereas the acetylation of Tax_{BLV} and p53 was easily detectable under the same experimental conditions (Fig. 3). Moreover, despite multiple attempts, we could not demonstrate the acetylation of bovine CREB-2 *in vitro* by using a series of purified known acetyltransferases (*i.e.* CBP, p300, p300/CBP-associated factor, and GCN5) (data not shown), a result supported by previous studies that have failed to demonstrate acetylation of several CREB/ATF members (45, 58–60). However, in contrast to these above findings, a recent report indicates that rat CREB-1 can be acetylated at three lysines (Lys-91, Lys-94, Lys-136) within its activation domain (27). In this latter study, CREB-1 acetylation seems to enhance its transactivation potential independently of the CREB-1 DNA binding domain and of

CREB-1 interaction with the CRE (27). It is noteworthy that another member of the CREB/ATF family, ATF-4 (also called human CREB-2), was shown to be acetylated both *in vivo* and *in vitro*. This latter report demonstrates ATF-4 acetylation in two regions: the basic domain of the bZIP (from amino acids 270 to 300) and the short basic domain (from amino acids 342 to 351) located downstream of the bZIP, suggesting that acetylation of ATF-4 could influence its DNA affinity for the CRE motif (29). Thus, the acetylation status of the different CREB/ATF members and the functional role of their modifications should be investigated further to be fully elucidated.

Regarding the second possibility, our Western blot analysis of nuclear extracts from YR2 and Raji cells treated or not with TSA showed that HDACi did not alter the nuclear presence of CREB (bovine CREB-2 and human CREB-1) and ATF-2 (Fig. 8*B*). We therefore postulate that the increased occupancy of the BLV promoter by CREB/ATF in response to HDACi does not result from an increased presence of these factors in the nucleus.

Regarding the modulation by HDACi of the CREB/ATF signaling pathway (third possibility), it is possible that HDACi modulate the phosphorylation status of CREB/ATF members and consequently modulate their DNA binding activity. Importantly, Michael et al. (2000) have reported that HDACi potentiate CREB activity by prolonging CREB Ser-133 phosphorylation in response to cAMP stimulus, thereby extending the ability of CREB to engage the transcriptional machinery via its association with CBP/p300 (61). More recent studies of the same group have shown that HDAC1 associates with CREB (rat CREB-1) and blocks Ser-133 phosphorylation of CREB during prestimulus and attenuation phases of the cAMP response. Mechanistically, HDAC1 promotes CREB Ser-133 dephosphorylation via a stable interaction with protein phosphatase-1, thereby attenuating CREB-dependent transcription (24). These results indicate that HDACi can influence the phosphorylation status of CREB. But does the phosphorylation status of CREB influence its DNA binding activity? It is well established that Ser-133 phosphorylation of CREB promotes recruitment of the coactivator CBP and its paralog p300, HATs that have been proposed to mediate target gene activation, in part, by destabilizing promoter-bound nucleosomes and thereby allowing assembly of the transcriptional apparatus. However, the role of phosphorylation in regulating DNA binding activity of CREB remains controversial (53, 62–65). Several studies have failed to detect phosphorylation-induced changes in the CREB/DNA interactions, suggesting that CREB phosphorylation does not regulate CREB DNA binding activity (64, 66, 67). However, it is theoretically possible that, in the case of CRE sites of weak affinity that have much less CREB bound, Ser-133 phosphorylation could stimulate transcription by increasing the CRE frequency of occupancy (62, 67). Therefore, in the case of the BLV promoter imperfect CRE-like motifs studied here, we cannot exclude a role for Ser-133 phosphorylation in regulating DNA binding activity of CREB. It is noteworthy that studies on the imperfect CRE sites of the HTLV-I promoter TxREs have failed to detect phosphorylation-induced changes in the CREB/DNA interactions (53, 67).

Finally, regarding the fourth possibility, HDACi could influence CREB/ATF DNA binding activity by a redox-mechanism. Indeed, Honigman and colleagues (2001) have shown that reduction of two cysteine residues, located in the DNA binding basic domain of CREB, enhances the binding efficiency of CREB to DNA and regulates CRE-mediated gene expression. Substitution of these cysteine residues to serines renders insensitivity to reduction and enhances the binding of CREB to its cognate DNA sites under oxidative conditions (68). Another study has shown that the ubiquitous nuclear redox factor-1 (Ref-1), which possesses a redox activity known to influence the DNA binding activity of the bZIP transcription factors Fos and Jun, stimulates the DNA binding activity of CREB, ATF-1, and ATF-2 (69). So far, however, there are no reported data in the literature for potential effects of HDACi either on changes in the cellular redox state or on changes in the function of Ref-1. Therefore, it would be interesting to examine in future experiments the potential regulatory link between, on one side, HDACi and the redox state of CREB/ATF and, on the other side, HDACi and Ref-1 function.

Although the exact mechanism involved in potentiation by HDACi of CREB/ATF DNA binding activity must be further investigated, we suggest in this report that acetylation/deacetylation events indirectly influence transactivation of the BLV promoter by Tax_{BLV}, through regulation of CREB/ATF recruitment to the BLV TxREs. It is now well established that expression of numerous viral and cellular genes are regulated by acetylation/deacetylation phenomenons. In the HTLV-I system, the implication of coactivators possessing HAT activities in the process of transcriptional activation by Tax_{HTLV-I} has been clearly established. Efficient transcription of the HTLV-I promoter requires the formation of a transactivation complex containing Tax_{HTLV-I}, CREB, and CBP/p300 (55). Studies reported by Lu *et al.* (2002) (70) and Georges *et al.* (2002) (59) have demonstrated a connection between p300 HAT activity, histone tail acetylation, and HTLV-I transcriptional activation. However, studies reported by Gachard et al. (2002) have failed to detect any acetylation of Tax_{HTLV-I} by CBP/p300, suggesting that the main function of the CBP/p300 recruitment to the HTLV-I promoter could be to induce local nucleosome modifications by histone acetylation and to facilitate stable binding of components of the basal transcription machinery (29). Regulation of HTLV-I promoter transcriptional activity is also controlled by the recruitment of HDACs at the viral promoter. Indeed, Lemasson *et al.* (2002) have performed ChIP assays on HTLV-I-infected T cells and have demonstrated the presence of Tax_{HTLV-I}, a variety of ATF/CREB and AP-1 family members, p300, and CBP and class I HDACs at the integrated HTLV-I promoter (71). Moreover, Ego *et al.* (2002) have demonstrated both *in vitro* and *in vivo* a physical interaction between Tax_{HTLV-I} and HDAC1 as well as the involvement of HDAC1 in the repression of the Tax_{HTLV-I} transactivation function (72). Functionally, these two latter reports have shown the downregulation by HDACs of basal and Tax_{HTLV-I}-activated HTLV-I transcription. Another retrovirus using acetylation/deacetylation events to regulate its expression is HIV-1. Our laboratory has previously demonstrated that histone hyperacetylation induces HIV-1 expression by specifically disrupting a single nucleosome positioned immediately downstream of the transcription start site (73–75). Moreover, HIV-1 transcription can be synergistically activated either by the viral transactivator Tat_{HIV-1} and HDACi through direct acetylation of Tat_{HIV-1} (50, 76–78) or by NF-κB and HDACi through a persistent degradation of the NF-κB inhibitor IκB-α (38, 43).

Taken together, our results represent a major advance in our understanding of Tax_{BLV}- and HDACi-mediated transcriptional activation of the BLV promoter and in our understanding of the role of the CREB/ATF transcription factors in mediating Tax_{BLV} transactivation. More generally, we also uncover a prominent role for protein acetylation in CREB-activated transcription by demonstrating that HDACi increase, both *in vitro* and *in vivo*, the level of CREB bound to DNA. Finally, our results may help to understand how Tax_{BLV} in collaboration with CREB/ATF can influence the transcription of cellular promoters containing CRE-motifs and may therefore help to

further understand the mechanism(s) by which BLV infection can induce oncogenicity.

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