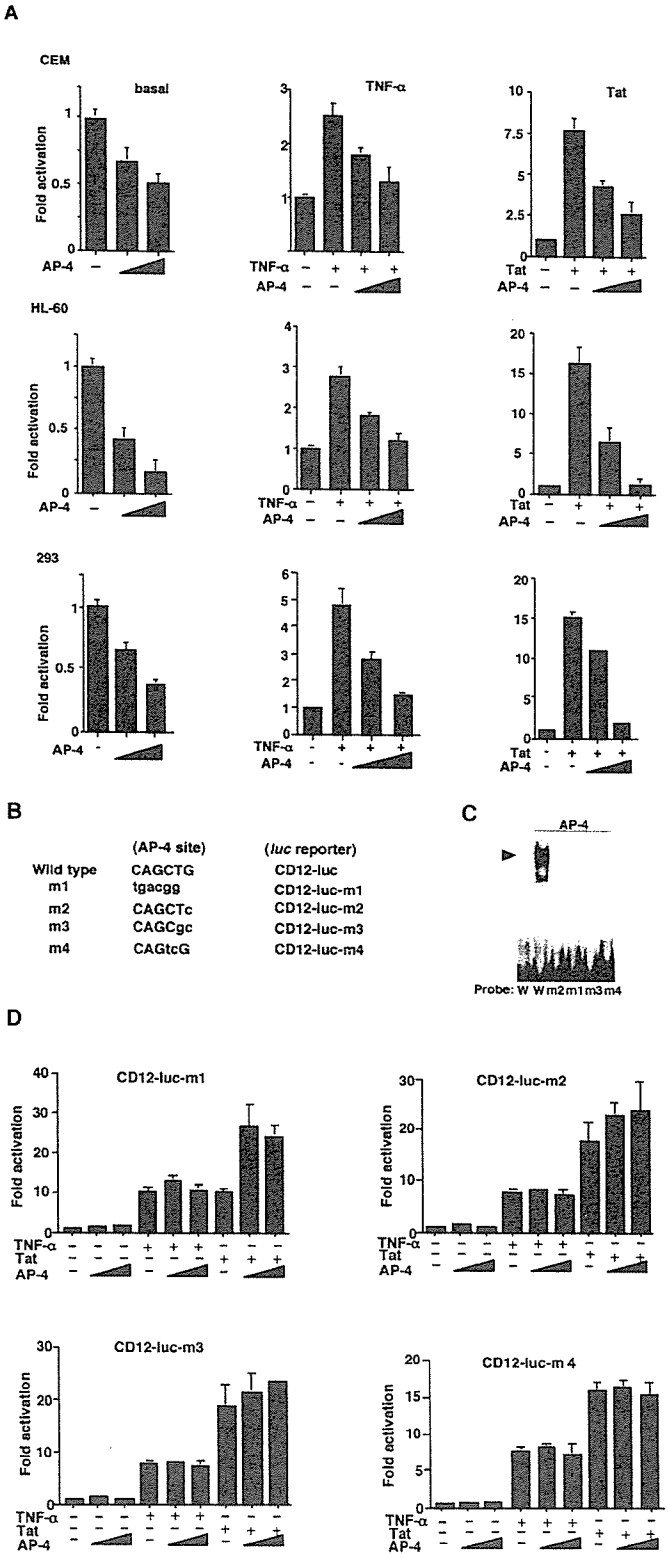

FIGURE 2. **Repression of HIV-1 gene expression by AP-4.** *A*, AP-4-mediated repression of HIV-1 gene expression. AP-4 expression plasmid pMyc-AP-4 was cotransfected with CD12-luc reporter construct, expressing luciferase gene under the control of HIV-1 LTR, into CEM, HL-60 or 293 cells. Extents of HIV-1 gene expression and the effects of AP-4 were evaluated at the basal level (*left panels*), upon TNF-α stimulation (*middle panels*), or upon Tat-mediated transactivation by cotransfecting pCMV-Tat at 2 and 0.1 mg per transfection for CEM/HL60 and 293 cells, respectively (*right panels*). pMyc-AP-4 were cotransfected at 2 and 12 μg for CEM and HL60 cells, and 0.1 and 0.4 μg for 293 cells per transfection. In the TNF-α experiments, cells were stimulated with TNF-α (3 ng/ml) after 24 h of transfection and incubated for additional 24 h. The cells were harvested and the luciferase activity was measured. Each value shown is the fold increase in the luciferase activity (means ± S.D.) relative to the control transfection for three independent experiments. Luciferase activity was measured as above. The values shown are the means ± S.D. for three independent experiments. *B*, the sequences of wild-type and AP-4 binding site mutants. These mutant sequences (m1 to m4) were used in EMSA and luciferase assays by replacing the authentic AP-4 site within CD12-luc (named as CD12-luc-m1 to CD12-luc-m4). *C*, lack of AP-4 DNA binding to mutant AP-4 sequences. The DNA binding activities of purified recombinant AP-4 protein was analyzed by EMSA with the wild-type and mutant AP-4 probes. *Arrowheads* indicate the positions of specific DNA-protein complex. *D*, effects of AP-4 binding site mutation on the AP-4-mediated repression. 293 cells were transfected with mutant CD12-luc containing mutations in the AP-4-site together with various amounts of pMyc-AP-4 (0.1 and 0.4 μg per transfection) with or without stimulation of TNF-α (3 ng/ml) or cotransfection of pCMV-Tat. The luciferase activity was measured as in *A*.

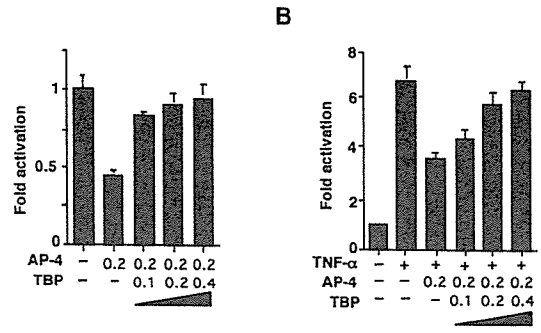

FIGURE 3. **Overexpression of TBP overcomes the repressive effect of AP-4 on HIV-1.** 293 cells were transfected with CD12-luc in combination with pMyc-AP-4 and pCMV-TBP expressing AP-4 and TBP, respectively. After 24 h, cells were untreated (*A*) or treated (*B*) with TNF-α (3 ng/ml) and incubated for additional 24 h. Cells were harvested, and the luciferase activity was measured as described in the legend to Fig. 2.

The Effect of AP-4 Knock-down—To examine the effect of endogenous AP-4, we adopted siRNA technique to specifically knock-down AP-4 mRNA and examined the HIV-1 gene expression when the endogenous AP-4 was depleted. Transduction of AP-4 siRNA caused the depletion of AP-4 protein (Fig. 4*A*), which resulted in significant increase in the basal transcriptional level from HIV-1 LTR (8.2-fold as compared with control siRNA) (Fig. 4*B*). In addition, TNF-α-stimulated LTR gene expression was greatly elevated by AP-4 depletion (5.7-fold). These results indicate that endogenous AP-4 acts as a negative regulator of HIV-1 gene expression.

The DNA Binding Activity of AP-4 Is Essential for the Repression of HIV-1 Gene Expression—AP-4 contains three functional domains, a basic HLH (bHLH) motif (48–99 aa) and two distinct leucine repeat elements, leucine repeat (LR) 1 (99–120 aa) and LR2 (151–179 aa) (Fig. 5*A*). A previous study (20) showed that the HLH motif and an adjacent basic domain are necessary and sufficient to direct sequence-specific DNA binding to its target DNA. Unlike other HLH proteins, AP-4 contains two additional protein dimerization motifs LR1 and LR2. Although both LRs contribute to the formation of AP-4 homodimers, AP-4 requires LR2 to form a stable homodimer (20). The C-terminal half of AP-4 contains a Gln/Pro-rich domain and an acidic region.

To investigate the role of functional domains of AP-4 in down-regulating HIV-1 gene expression, we examined the effects of deletion mutants of AP-4 (shown in Fig. 5*A*). Deletion of the N-terminal regions (ΔN100, ΔN143, and ΔN180) of AP-4 abolished the repressive action on both basal and TNF-α-stimulated HIV-1 expression (Fig. 5*B*). In contrast, deletion of the C-terminal region of AP-4 (ΔC179, retaining the bHLH and two LR domains) repressed HIV-1 gene expression similarly to the full-length AP-4. These results indicate that bHLH domain is indispensable for the repression of HIV-1 gene expression. Because inhibitory effect of ΔC130 (excluding LR2 domain from the ΔC179) was weaker than ΔC179, AP-4 dimerization is important for its effect through stabilization of AP-4 homodimer (20).

Effect of the Location of AP-4 Site Within HIV-1 LTR on the Repressive Effect of AP-4—To further examine whether the repressive effect of AP-4 depends on its location relative to the TATA box within HIV-1 LTR, we created mutant HIV-1 LTR reporter constructs in which AP-4 binding sites were inserted into various positions of the HIV-1 LTR. Thus, AP-4 binding site were inserted at nucleotide positions −400, −15, −79, and +55 (Fig. 6*A*) within HIV-1 LTR into CD12-luc-m2 in which the authentic AP-4 site was mutated. Basal promoter activities of these promoter constructs were not significantly changed as compared with the original construct (data not shown). As shown in Fig. 6*B*, even when AP-4 sites were distantly located from TATA box, AP-4 could still exert repressive action irrespective of the stimulation by TNF-α. The greatest repressive effect of AP-4 was observed with CD12-luc-m2(−79) although it was less than that with the wild-type promoter. Other AP-4 site mutants exhibited less susceptibility to AP-4-mediated transcriptional repression. These findings indicate that AP-4 could repress HIV-1 transcription even from the distant locations from TATA box although the maximal repressive effect of AP-4 was observed when AP-4 was located in close proximity to the TATA box.

Interaction of HDAC with AP-4—Cumulative evidence has demonstrated that chromatin modification by HDAC complex plays a significant role in transcriptional repression (reviewed in Refs. 10 and 48) and many transcriptional repressors, such as YY-1 (8), silencing mediator of retinoic acid and thyroid hormone receptor (28), nuclear receptor corepressor (29), and special AT-rich sequence-binding protein 1 (30) have been shown to tether HDACs to the promoter. Because AP-4 could still exert repressive action even when AP-4 sites were distantly located from TATA box (Fig. 6*B*), we examined whether HDAC was involved in the HIV-1 gene repression by AP-4. To address this possibility we first examined whether AP-4

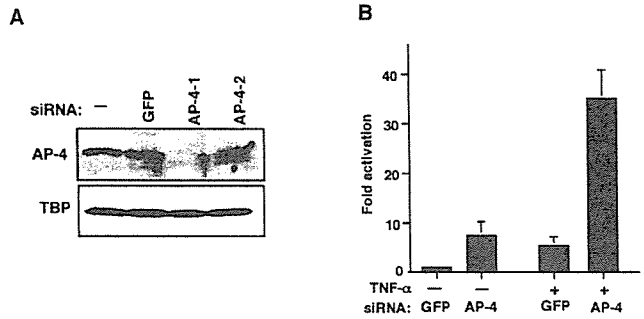

FIGURE 4. **Effect of AP-4 knock-down.** *A*, confirmation of the siRNA-mediated knock-down of AP-4. 293 cells were transfected with 100 nM siRNAs directed against various portions of AP-4 or GFP (control) mRNAs. After 36 h of transfection, cells were lysed, and AP-4 and TBP (control) protein levels were assessed by immunoblotting using specific antibodies. The blot was stripped and reprobed with anti-α-tubulin antibody. *B*, augmentation of HIV-1 gene expression by AP-4 depletion. 293 cells were transfected with CD12-luc together with AP-4 siRNA-1 or its control. After 24 h of transfection, cells were untreated or treated with 3 ng/ml of TNF-α and incubated for an additional 24 h. Cells were harvested, and the luciferase activity was measured as described in the legend to Fig. 2.

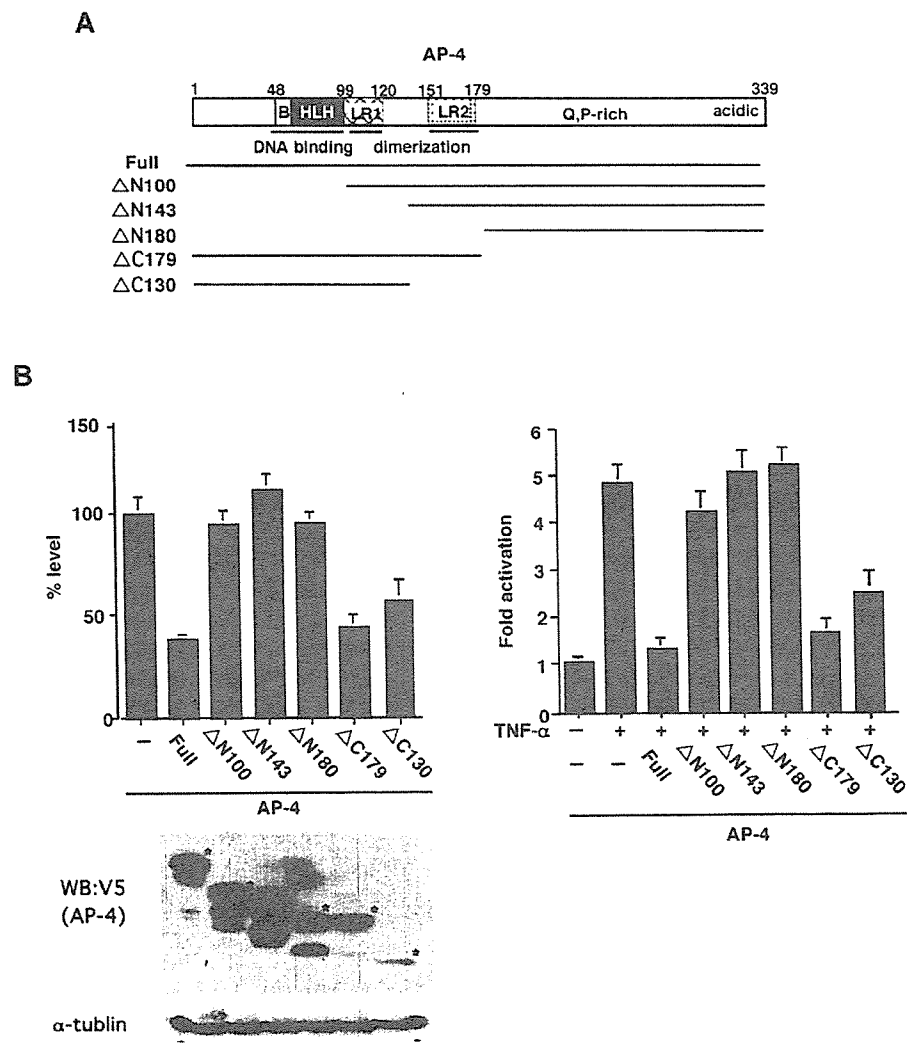

FIGURE 5. **Effects of AP-4 deletion mutants on the HIV-1 gene expression.** *A*, schematic representation of the AP-4 protein and constructs of AP-4 mutants. *Hatched box*, conserved basic stretch; *closed box*, HLH motif; *LR1*, first leucine repeat; *LR2*, second leucine repeat. The amino acid positions of AP-4 are marked on the *top*. *B*, effect of AP-4 mutants on HIV-1 gene expression. Effect of AP-4 was evaluated at the basal level (*left panel*) and upon stimulation with TNF-α (3 ng/ml) (*right panel*). 293 cells were transfected with CD12-luc together with the plasmid expression of wild-type or mutant AP-4. Luciferase assays were performed as described in the legend to Fig. 2. The expression level of each protein was assessed by immunoblotting of cell lysates with anti-V5 antibodies (detecting AP-4 and its mutants). *Asterisks* indicate the positions of specific bands of AP-4 proteins. The low protein level of AP-4 mutant ΔC130, lacking the LR2 domain responsible for protein homodimerization, is considered because of destabilization of the protein.

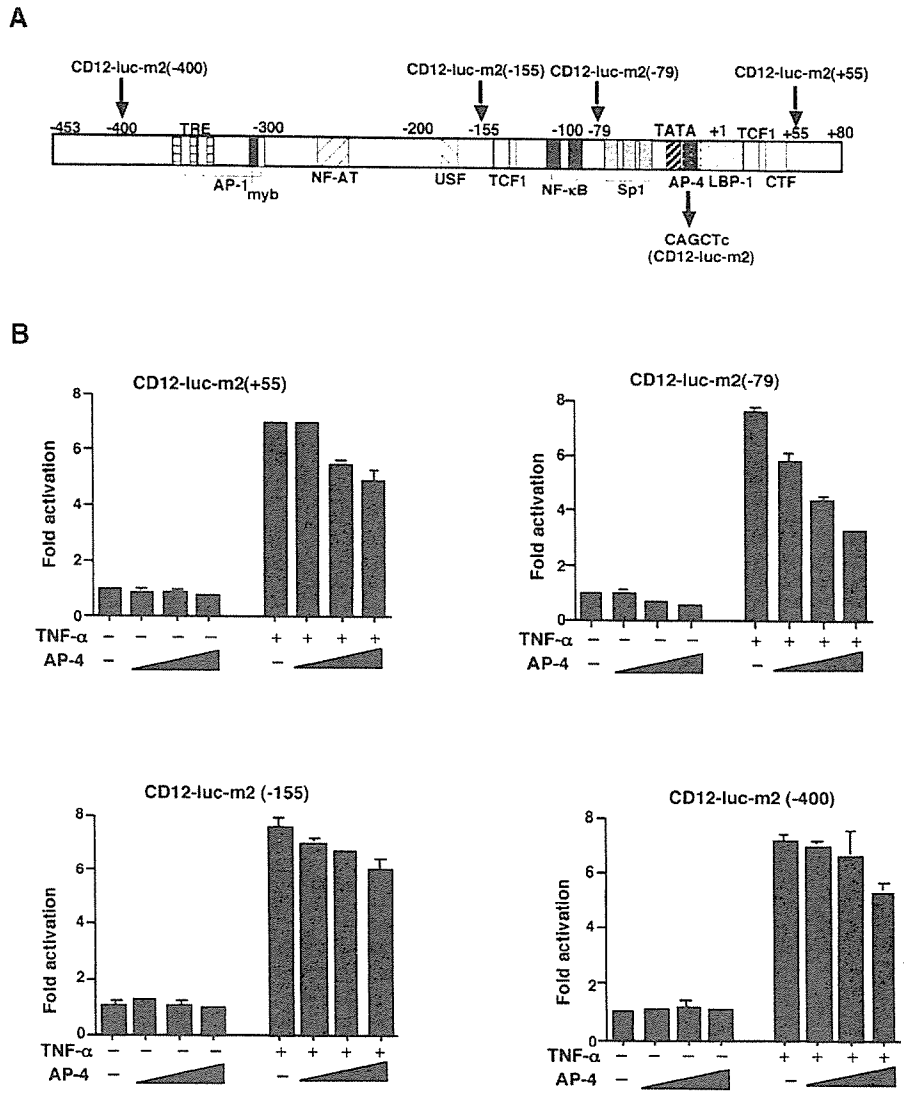

FIGURE 6. **Effects of AP-4 site location on AP-4-mediated repression of HIV-1.** *A*, schematic map of the U3 and R regions of the HIV-1 LTR and positions of aberrant AP-4 sites in mutant constructs. AP-4 site is inserted into various locations (nucleotide positions: −400, −15, −79, and +55) within CD12-luc-m2 (Fig. 2*B*), in which the authentic AP-4 site is abolished. *B*, effect of the AP-4 site on susceptibility to AP-4-mediated repression. 293 cells were transfected with reporter constructs and various amounts of pMyc-AP-4 (0.05, 0.2, and 0.4 μg per transfection). After 24 h, cells were untreated (*left side of each panel*) or treated (*right side*) with TNF-α (3 ng/ml) and incubated for an additional 24 h. The luciferase activity was measured as described in the legend to Fig. 2.

interacts with HDACs in culture cells. In Fig. 7, 293 cells were transfected with FLAG- or Myc- (used as a negative control for immunoprecipitation) tagged AP-4 expression plasmids, and cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Immune complexes were collected and subjected to SDS-PAGE followed by immunoblotting for detection of the class I HDACs using antibodies to HDAC1, 2, and 3. As shown in Fig. 7*A*, AP-4 interacted with HDAC1, and to a much lesser extent HDAC2, but not detectably with HDAC3. In Fig. 7*B*, similar experiments with untransfected cells showed the interaction of endogenous AP-4 with endogenous HDAC1. The interaction of AP-4 with HDAC2 was observed but much less than that with HDAC1. No interaction between AP-4 and HDAC3 was observed (data not shown). To examine whether AP-4 directly binds to HDAC1, we performed *in vitro* protein-protein interaction assay using GST-AP-4 fusion protein, radiolabeled HDAC1, and luciferase (as a control). The radiolabeled HDAC1 directly bound GST-AP-4 but not GST and no binding was observed between GST-AP-4 and luciferase (Fig. 7*C*). These results demonstrated that AP-4 directly interacts with HDAC1. To further confirm the involvement of HDACs in transcriptional repression of HIV-1 by AP-4, trichostatin A (TSA), a specific inhibitor of HDACs, was added to the cells transfected with AP-4, and the luciferase assay was performed. As shown in Fig. 7*D*, TSA abrogated the repressive effect of AP-4 on HIV-1 gene expression in a dose-dependent manner. These findings suggest that HDACs are involved in the AP-4-mediated repression of HIV-1 gene expression.

ChIP Assays Detecting AP-4 and HDAC1 on HIV-1 LTR—Together with the results demonstrated above, it was suggested that AP-4-mediated HDAC recruitment to and elimination of TBP (TFIID) from the HIV-1 promoter might play a role in the cellular maintenance of HIV-1 latency. We thus examined the presence of AP-4 and HDAC1 on the HIV-1 promoter in latently infected cells, ACH2 (T-cell line latently infected with HIV-1) and U1 (promyelocytic cell line latently infected with HIV-1) (31). As shown in Fig. 8*A*, the newly raised anti-AP-4 antibody could specifically precipitate AP-4. Although the nature and/or the extent of chromatin formation on transiently transfected DNA templates likely differ from that of chromosomal genes, previous reports of ChIP assays using plasmids containing HIV-1 LTR have validated ChIP studies in transiently transfected cells (32–35). In Fig. 8*B*, 293 cells were transiently transfected with CD12-luc or its mutant CD12-luc-m2 followed by ChIP analysis using the anti-AP-4 antibody. We amplified the HIV-1 LTR DNA fragment (−109/+79) containing binding sites for NF-κB, Sp1, TBP, AP-4, LBP-1 (YY-1), TCF1, and CTF (Fig. 6*A*) in the AP-4 and HDAC1 immune complexes. We were able to detect the binding of both AP-4 and HDAC1 to the HIV-1 promoter by ChIP assay in 293 cells transfected with CD12-luc (Fig. 8*B*). The binding

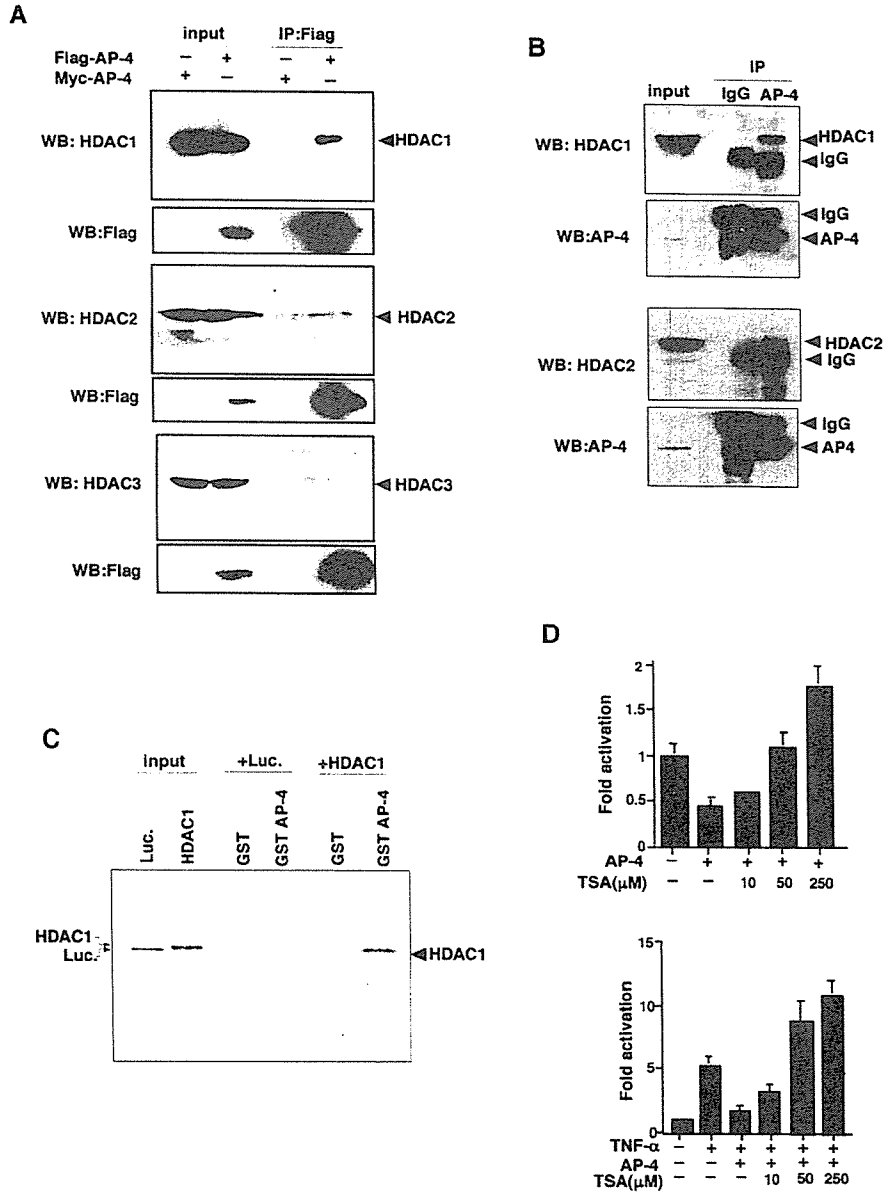

FIGURE 7. **Interaction of HDAC with AP-4.** *A*, interaction between AP-4 and HDAC proteins *in vivo*. 293 cells were transfected with FLAG-(pFLAG-AP-4) or Myc-tagged (pMyc-AP-4) AP-4 expression plasmids. After 48 h of transfection, the cell lysates were prepared, immunoprecipitated with anti-FLAG antibody, and subsequently separated on SDS-PAGE followed by immunoblotting with anti-HDAC antibodies. One-tenth of each protein lysate was loaded as input control. *B*, endogenous AP-4 interacts with HDACs. 293 cell lysates were immunoprecipitated with anti-AP-4 antibody, and the immune complex was analyzed by immunoblotting with anti-HDAC antibodies. *C*, AP-4 binds to HDAC1 *in vitro*. HDAC1 and luciferase (negative control) proteins were synthesized and labeled with [^{35}S]methionine *in vitro*. These radiolabeled HDAC1 and luciferase proteins were incubated with GST-AP-4 or GST (control) immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by SDS-PAGE and subjected to autoradiography. *D*, effect of TSA on the repressive activity of AP-4. 293 cells were transfected with CD12-luc together with pMyc-AP-4. After 24 h of transfection, cells were untreated (*upper panel*) or treated (*lower panel*) with TNF-α (3 ng/ml), incubated for additional 24 h, and various amounts of TSA were added to the culture. After 8 h of additional incubation, cell lysates were prepared, and the luciferase activity was measured as described in the legend to Fig. 2.

with AP-4 or HDAC1 was detectably reduced when CD12-luc-m2, in which AP-4 site was mutated, was transfected. The trace amount of HDAC1 recruitment was detected even with the mutant, presumably due to the presence of binding region (from −10 to +27) of LBP-1 and YY-1, known to recruit HDAC1 (8).

In Fig. 8C, ChIP assays were similarly performed with ACH2 and U1 cells using antibodies to AP-4, HDAC1, acetylated histone H3 (Ac-H3), TBP, and RNAPII. AP-4 and HDAC1, but only traceable amounts of TBP (TFIID) or RNAP II, were detected on the HIV-1 promoter when these cells maintained the latency (without any stimulation). However, when ACH2 and U1 cells were treated with TNF-α to stimulate HIV-1 replication, AP-4 and HDAC1 were readily dissociated from the HIV-1 promoter, and TBP and RNAPII became clearly detectable on the HIV-1 promoter over time (Fig. 8C). Moreover, the disappearance of AP-4 from the HIV-1 LTR correlated with dissociation of HDAC1 and appearance of the acetylated form of histone H3 (Ac-H3). These results, together with the findings described above, suggest that AP-4 acts as a negative regulator of HIV-1 gene expression by recruitment of HDAC1 as well as by preventing the TBP (TFIID) binding to the TATA box in latently infected cells.

Repression of HIV-1 Production by AP-4—To assess the biological relevance of the repressive action of AP-4, we examined the effect of AP-4 on HIV-1 production. 293 cells were transfected with a replication-competent full-length HIV-1 clone (pNL4-3) together with various amounts of AP-4-expression plasmid pFLAG-AP-4, and virus production was evaluated by measuring HIV-1 p24 antigen levels in the culture supernatant. In Fig. 9*A*, transduction of AP-4 resulted in dose-dependent decrease in the HIV-1 p24 level by 3.7-fold (Fig. 9*A*, *left panel*). Inhibition of viral protein synthesis was also observed in these cells when AP-4 was overexpressed (Fig. 9*A*, *right panel*). In Fig. 9*B*, the effect of AP-4 on the TNF-α-stimulated HIV-1 production was examined. When pNL4-3 was transfected and cells were subsequently stimulated with TNF-α, 7.4-fold increase of HIV-1 production was observed (Fig. 9*B*, *right panel*). When AP-4 was overexpressed, a dramatic inhibition of HIV-1 production was observed in a dose-dependent manner, almost to the basal unstimulated level (Fig. 9*B*). In Fig. 9*C*, effects of AP-4 mutants were examined. Although an AP-4 mutant ΔC179, retaining the AP-4 DNA binding and dimerization domains, could suppress HIV-1 production as well as wild-type AP-4, another mutant ΔN143, lacking these two functional domains, showed no suppressive effect on the HIV-1 pro-

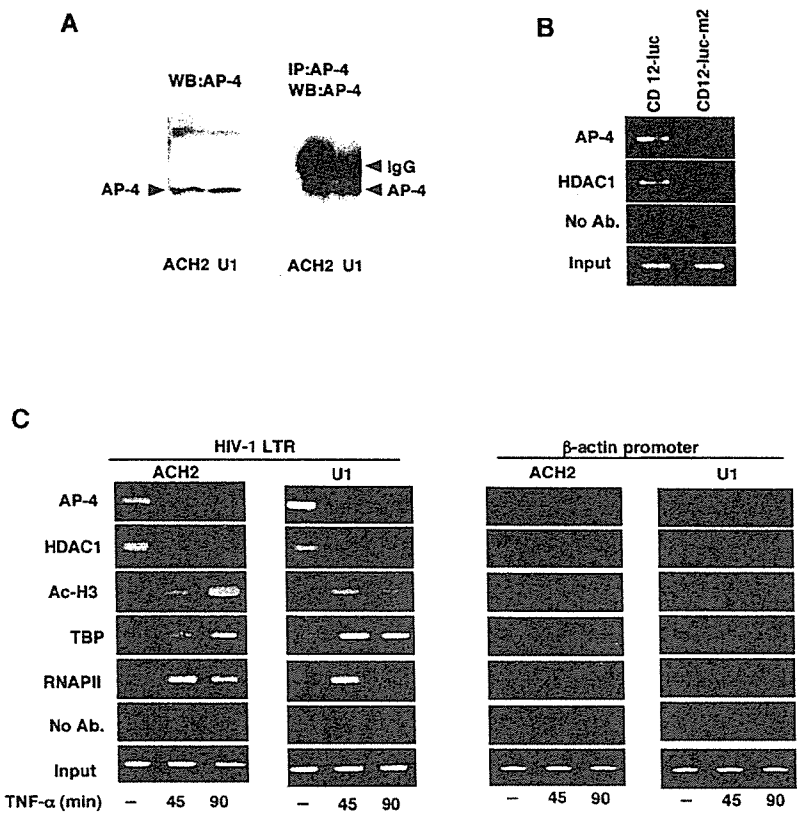

FIGURE 8. **ChIP assays detecting AP-4 and HDAC1 on HIV-1 LTR.** *A*, immunoreactivity of anti-AP-4 antibody. To assess the immunoreactivity of anti-AP-4 antibody for immunoblotting (*left panel*) and immunoprecipitation (*right panel*), ACH2 and U1 cell lysates were reacted with the rabbit anti-AP-4 antibody. *Arrowheads* indicate the locations endogenous AP-4. Position of IgG was also indicated (*right*). The AP-4 immune complex was separated on SDS-PAGE followed by immunoblot with anti-AP-4 antibody. *B*, AP-4 recruits HDAC1 to the HIV-1 LTR. 293 cells were transfected with CD12-luc or CD12-luc-m2, incubated for 48 h, and ChIP assay was performed. Cross-linked chromatin fragments were prepared, and the association of AP-4 and HDAC1 was analyzed by ChIP assay using antibodies to AP-4 and HDAC1. The recovered DNA was amplified by PCR with promoter-specific primers (spanning from −109 to +79 of HIV-1 LTR) and analyzed on a 2% agarose gel. Input DNA represents total input chromatin (1%) while immunoprecipitation with no antibody (*No Ab.*) serves as negative control. *C*, dynamic association/dissociation of AP-4, HDAC1, acetylated histone H3, TBP, and RNAP II. ChIP assays were performed with latently infected cell lines. ACH2 and U1 cells were either untreated or treated with TNF-α (3 ng/ml) for indicated times and subjected to ChIP assays. Cross-linked chromatin fragments were prepared, and the association of AP-4, TBP, RNAPII, HDAC1, Ac-H3, and HIV-1 LTR DNA (−109/+79) was analyzed by ChIP assay as described in *B*. The β-actin promoter DNA (−980/−915) was similarly analyzed as a control. The experiments were repeated performed with reproducible results, and the representative results are shown.

duction (Fig. 9*C*). These results indicate that AP-4 can inhibit HIV-1 production and that the DNA binding activity of AP-4 is crucial for its inhibitory action.

In Fig. 9*D*, we examined the effect of depleting endogenous AP-4 on HIV-1 production using siRNA for AP-4. AP-4 depletion resulted in significant increase in the basal HIV-1 production (3.2-fold as compared with control siRNA (GFP)). Similarly, TNF-α-stimulated HIV-1 production was elevated by the treatment with AP-4 siRNA (5.7-fold as compared with control siRNA). We also observed the elevation of viral protein synthesis in the transfected cells by AP-4 depletion (Fig. 9*D*, *right panel*).

Finally, we examined the effect of AP-4 on HIV-1 replication in Jurkat CD4+T cells. To examine the effect of AP-4 binding site within HIV-1 LTR, we created a mutant pNL4-3 lacking the AP-4 binding and quantified the amounts of HIV-1 virions in the culture supernatant of Jurkat cells transfected with either the wild-type or the mutant pNL4-3. As shown in Fig. 9*E*, the amounts of HIV-1 production were not significantly changed over time between the wild-type and the mutant HIV-1 clones. In Fig. 9*F*, the repressive effect of AP-4 on HIV-1 production was examined with or without TNF-α stimulation. When the mutant pNL4-3, containing mutation in the AP-4 binding, was cotransfected with AP-4, the inhibitory effect of AP-4 was abolished irrespective of the TNF-α stimulation (Fig. 9*F*, *right panel*).

DISCUSSION

The extent of HIV-1 replication is controlled at the step of transcription and the level of transcription is governed by the coordinated actions of viral and cellular transcription factors acting on LTR both in positive and negative fashions (1, 2, 9). In contrast to the abundant literatures reporting actions of positive transcription factors, mostly NF-κB (1, 2), little is known about the action of negative transcription regulators. Here, we demonstrate evidences demonstrating that AP-4 is a negative regulator of HIV-1 transcription and its production. Although a previous study reported that AP-4 blocks TBP binding to TATA box *in vitro* (10) and suggested that AP-4 negatively regulates HIV-1 transcription, no evidence has been presented supporting the repressive action of AP-4 on HIV-1 transcription and viral production. In this study, we were able to demonstrate that AP-4 acts as a negative transcription factor for HIV-1 gene expression by recruitment of HDAC1, as well as by preventing the TBP (TFIID) binding to the TATA box, and that AP-4 is actively involved in the transcriptional silencing of HIV-1 gene expression in latently infected cell lines.

The AP-4 binding site in the HIV-1 LTR is located immediately downstream of TATA box (2, 10, 13). Among various HIV/SIV isolates AP-4 sites are conserved among HIV-1 subtypes A, B, C, D, and CRF02-AG, HIV-2, and SIVcpz-gab. However, although most (92%) of the clinical HIV-1 isolates had functional AP-4 binding site (11), no clear cor-

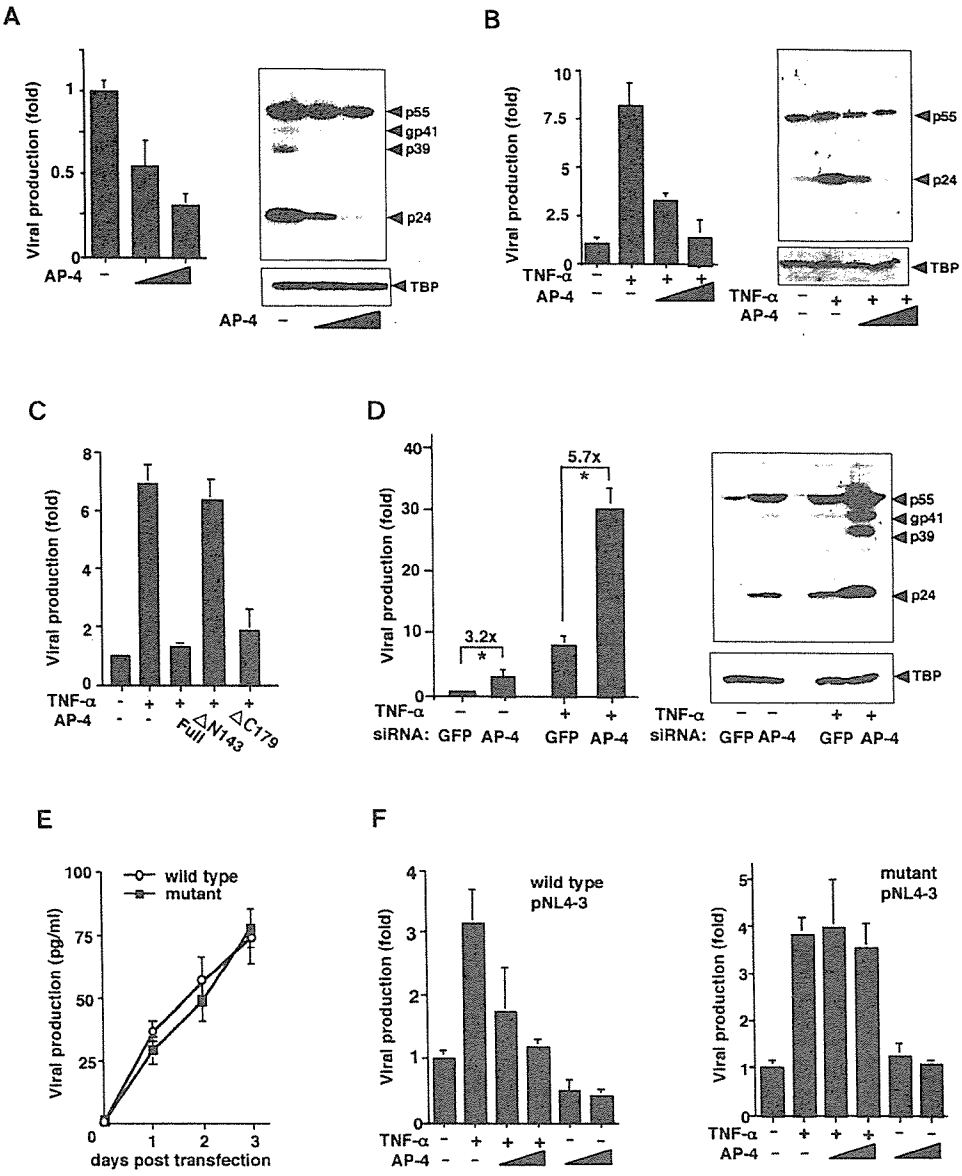

FIGURE 9. **Repression of HIV-1 production by AP-4.** *A* and *B*, HIV-1 production and AP-4-mediated repression in transfected cells. 293 cells were transfected with pNL4-3 and pFLAG-AP-4. After 36 h of transfection, cells were either untreated (*A*) or treated (*B*) with TNF-α (3 ng/ml) and incubated for additional 24 h. The culture supernatants and cell lysates were collected and subjected to the determination of p24 antigen level by ELISA and detection of virus proteins by immunoblot with AIDS patient serum, respectively. *C*, effects of AP-4 mutants on HIV-1 production. 293 cells were transfected with pNL4-3 together with expression vectors for wild-type or mutant AP-4. After 24 h of transfection, cells were untreated or treated with TNF-α (3 ng/ml) and incubated for additional 24 h. The p24 antigen level in the culture supernatant was determined as in *A*. *D*, effects of AP-4 knockdown on HIV-1 production. 293 cells were transfected with pNL4-3, and AP-4 siRNA-1 (Fig. 4) or control siRNA (GFP). After 36 h of transfection, cells were untreated or treated with 3 ng/ml of TNF-α and incubated for an additional 24 h. The culture supernatants and cell lysates were collected and analyzed the p24 antigen level and viral protein expression, respectively, as in *B*. *, $p < 0.01$. *E*, HIV-1 production of the pNL4-3 mutant lacking the AP-4 sites within both LTR. Either the wild-type or the mutant pNL4-3 was transfected into Jurkat CD4+T cells, and the amounts of HIV-1 virion production were quantified. The culture supernatants were collected after 1, 2, and 3 days post-transfection, and the p24 antigen levels were measured as in *A*. *F*, repression of HIV-1 production by AP-4 in Jurkat CD4+T cells. Jurkat cells were transfected either with the wild-type (*left panel*) or the mutant (*right panel*) pNL4-3 lacking the AP-4 sites in both 5′- and 3′-LTR, together with various amounts of pFLAG-AP-4 (0.2 and 0.8 μg per transfection). After 36 h of transfection, cells were either untreated or treated with TNF-α (3 ng/ml) and incubated for an additional 24 h. The culture supernatants were prepared, and the p24 antigen levels were measured. Experiments were repeated at least three times, and reproducible results were obtained. The representative data are shown.

relation with the clinical stage was observed. Thus, the biological significance of the presence of AP-4 site in HIV-1 LTR in determining the virulence awaits further *in vivo* investigations.

There are a number of transcription factors acting as repressors. However, the mechanisms of their actions are not uniform and multiple modes of action are reported including: 1) inhibition of transcriptional activators upon its DNA binding or interaction with co-activators (36–38), 2) prevention of the binding of general transcription factors such as TBP (TFIID) and TFIIB to the promoter (39–43), 3) direct repression of promoter activity by recruiting co-repressors (8, 28, 30), 4) alteration of chromatin structure (19, 40), and 5) inhibition of transcriptional elongation (44, 45). AP-4 appears to exert transcriptional repression of HIV-1 promoter through bimodal mechanisms: 1) masking the HIV-1 TATA element from TBP binding and 2) recruiting HDAC1. In this context, it is noted that HIV-1 does not appear to use the TATA box but instead use the CATA box motif located two nucleotides upstream of the conventional TATA box (46). It is possible that AP-4 may preclude the usage of TATA box by physical masking. However, since we observed that AP-4 blocked the TBP binding to CATATA box in EMSA (Fig. 1*D*), AP-4 may also block the CATA box.

Like other members of bHLH family to which AP-4 belongs, the AP-4 HLH motif and the adjacent basic domain are necessary to confer site-specific DNA binding (20). Unlike other HLH proteins, AP-4 also contains two additional protein dimerization motifs consisting of leucine repeat elements LR1 and LR2, through which AP-4 forms a homodimer (20). We found that both bHLH motif and two LR elements were necessary to exert transcriptional repression, presumably by masking the TATA box. However, unlike HIV-1 LTR, most other promoters where AP-4 has negative role have AP-4 sites at distant locations from the TATA box and even some promoters are TATA-less (22–24) and the mechanism by which AP-4 represses transcription other than masking the TATA box has not been elucidated. In addition, we found that AP-4 could exert transcriptional repression of HIV-1 even when the AP-4 site was located distant from the TATA box. Subsequent experiments have revealed that AP-4 could recruit HDAC1 to the promoter and that the AP-4-mediated repression could be restored by the treatment with a histone deacetylase inhibitor TSA. These bimodal actions of AP-4 make this factor a strong negative regulator for HIV-1 transcription. However, further studies are needed such as to determine where AP-4 binds to HDAC and to clarify whether AP-4 binds other transcriptional reg-

ulators. Interestingly, AP-4 was reported to be a transcriptional activator of transforming growth factor β (47), immunoglobulin κ chain (48), and SV40 (late promoter) (21) where AP-4 binding sites were found in the enhancer elements of these genes although no direct evidence is thus far available to show that AP-4 actually act as a transcriptional activator for these genes. Mermod *et al.* (21) showed that AP-4 acts in concert with AP-1, binding to the adjacent site of AP-4, in stimulating SV40 transcription *in vitro*. Thus, it is possible that the effect of AP-4 binding on transcription may be modified by other transcription factors recruited to the promoter and depend on the promoter context through combinatorial interaction with other transcription factors.

Our finding that AP-4 is constitutively present on the silent HIV-1 promoter in latently infected cells may have significant biological implications. We also found that TNF-α stimulation abrogated the AP-4-mediated repression of HIV-1 promoter. It is conceivable that nuclear translocation of NF-κB and its binding to the HIV-1 promoter might induce local chromatin remodeling, thus eliminating AP-4 and its repressor complex. It appears that the interplay among various transcription factors on the HIV-1 promoter determines the transcriptional competence of the latent HIV-1 provirus.

The ability of HIV-1 to establish a latent infection is considered crucial for the pathogenesis of AIDS (49, 50). Whereas HIV-1 entry into activated $CD4^+$ lymphocytes leads to a productive infection, the virus remains latent in resting $CD4^+$ lymphocytes (51). For many HIV-infected patients, although current anti-HIV treatment can reduce viral loads to undetectable levels, infected cells persist in a long term and harbor integrated proviruses capable of reseeding virus production after cessation of therapy. Our observation of AP-4 in the negative regulation of HIV-1 gene expression could give us a clue to understand how the latency is maintained at least in cells. Moreover, it is proposed that breakdown of viral latency during the early clinical stage where potent anti-viral cytotoxic T lymphocyte is still present is considered to benefit the outcome of HIV-1 infection by eliminating the otherwise long lasting chronically and latently infected cells (52, 53). Interestingly, Lehrman *et al.* (54) have recently reported a clinical study that combination therapy of conventional anti-HIV therapy with an HDAC inhibitor, valproic acid, could successfully accelerate the clearance of HIV-1 from resting CD4+T cells. It is plausible that HDAC inhibitors have clinical benefit in preventing the clinical development of AIDS. Further studies are needed to clarify the role of AP-4 and other repressor proteins in the maintenance of HIV-1 latency *in vivo* and to determine the clinical benefit of HDAC inhibitors.

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Inhibition of Human Immunodeficiency Virus Type 1 Replication in Latently Infected Cells by a Novel IκB Kinase Inhibitor

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In human immunodeficiency virus type 1 (HIV-1) latently infected cells, NF-κB plays a major role in the transcriptional induction of HIV-1 replication. Hence, downregulation of NF-κB activation has long been sought for effective anti-HIV therapy. Tumor necrosis factor alpha (TNF-α) stimulates IκB kinase (IKK) complex, a critical regulator in the NF-κB signaling pathway. A novel IKK inhibitor, ACHP {2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl-nicotinonitrile}, was developed and evaluated as a potent and specific inhibitor for IKK-α and IKK-β. In this study, we examined the ability of this compound to inhibit HIV-1 replication in OM10.1 cells latently infected with HIV. When these cells were pretreated with ACHP, TNF-α-induced HIV-1 replication was dramatically inhibited, as measured by the HIV p24 antigen levels in the culture supernatants. Its 50% effective concentration was approximately 0.56 μM, whereas its 50% cytotoxic concentration was about 15 μM. Western blot analysis revealed inhibition of IκBα phosphorylation, IκBα degradation, p65 nuclear translocation, and p65 phosphorylation. ACHP was also found to suppress HIV-1 long terminal repeat (LTR)-driven gene expression through the inhibition of NF-κB activation. Furthermore, ACHP inhibited TNF-α-induced NF-κB (p65) recruitment to the HIV-1 LTR, as assessed by chromatin immunoprecipitation assay. These findings suggest that ACHP acts as a potent suppressor of TNF-α-induced HIV replication in latently infected cells and that this inhibition is mediated through suppression of IKK activity.

Although the recent progress in combination therapy against viral reverse transcriptase and protease has achieved considerable reduction of the viral load in human immunodeficiency virus type 1 (HIV-1)-infected individuals and significant improvement in survival, chemotherapy could not be terminated unless chronically infected cell populations, such as resting memory T cells and monocytes/macrophages, could be eradicated (15, 51, 53). Thus, it is crucial to inhibit HIV-1 replication in the latently infected cells. Molecular analyses of HIV-1 replication have revealed a concerted complexity that regulates the viral life cycle (52). Among the various steps of the viral life cycle, the step of transcription from HIV-1 provirus is conceived to be crucial for viral replication, since amplification of the viral genetic information is attainable only through transcription. It is through this step that HIV acquires genetic variation, thus enabling the emergence of HIV quasispecies containing clones resistant to host immune responses and anti-HIV drugs. In addition to the virus-encoded transcriptional transactivator Tat, several cellular factors are known to regulate HIV-1 transcription (29, 52). Among these host factors, nuclear factor κB (NF-κB) is known to play a major role in regulated HIV-1 gene expression (44, 48, 52).

NF-κB is an inducible cellular transcription factor that regulates a wide variety of cellular and viral gene expression, including that of HIV (6, 7, 22, 44, 48, 50, 65). Recently, two major signaling pathways leading to receptor-mediated NF-κB activation have been classified: the canonical and noncanonical (alternative) pathways. In the canonical pathway, a diverse range of stimuli, such as tumor necrosis factor alpha (TNF-α), viral and bacterial pathogens, and stress-inducing agents (24), stimulate the signal transduction pathways that lead to the activation of NF-κB. In cells, NF-κB, a hetero- or homodimer consisting of the Rel family proteins p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100, resides in the cytoplasm and is complexed with an inhibitory molecule, IκB (6). Stimulation activates the IκB kinase (IKK) complex composed of two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ (22). IKK rapidly phosphorylates IκBα on its two NH_2-terminal serine residues (Ser32 and Ser36) (21, 41, 71). Phosphorylation targets IκBα for its ubiquitination and degradation by the β-transducin repeat-containing protein ubiquitin ligase and 26S proteasome, respectively, thus allowing free NF-κB to translocate to the nucleus to activate gene expression (22). In this event, IKK-β and IKK-γ mainly regulate IκB degradation, while IKK-α is dispensable (22), although its nuclear function remains essential for the transcriptional activity of NF-κB (1, 70). The noncanonical pathway, however, is strictly dependent on the NF-κB-inducing kinase (NIK)-mediated activation of IKK-α, which phosphorylates p100, causing its inducible processing into p52 (60, 68). This IKK-β/IKK-γ-independent pathway is induced in response to stimuli, such as lymphotoxin B (18), B-cell-activating factor (16), and CD40 ligand (17). Moreover, recent reports by us and others have shown that IKK-α also phosphorylates p65 at Ser536, which is pivotal for the transcriptional competence of NF-κB when it is bound to the promoter sequence of target genes in the nucleus (27, 28, 56).

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The role of NF-κB in activating HIV transcription has been extensively studied. HIV-1 replication is positively regulated by several cytokines, or T-cell activators, most of which act either completely or partially via NF-κB (4, 8, 38). NF-κB has been shown to regulate viral transcription via the two NF-κB sites located in the HIV-1 long terminal repeat (LTR) enhancer region (44) and is further enhanced through synergism with Sp1 (34, 49). In HIV-1 latently infected cells, activation of NF-κB could trigger the transcription of viral genes, including the transactivator Tat, resulting in an explosive increase in HIV replication (29, 47, 52). Treatment with compounds that block NF-κB activation inhibits HIV-1 gene expression and viral replication (3, 20, 33, 45, 59, 63, 64). Hence, downregulation of NF-κB activity by suppressing NF-κB or the signaling proteins involved in the NF-κB activation pathway, such as the IKKs (23, 30), is considered a feasible target for future anti-HIV therapy.

To control HIV-1 expression from latently infected cells, we examined the effect of a novel IKK inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-ylnicotinonitrile (ACHP). ACHP was found on a massive screening to have specific inhibitory action on IKK-β and IKK-α (42, 43). The 50% inhibitory concentrations for IKK-β and IKK-α are 8.5 and 250 nmol/liter, respectively, measured by in vitro kinase assays, and those for other kinases, such as IKK-γ, Syk, and mitogen-activated protein kinase kinase kinase 4, were greater than 20 μmol/liter (42). ACHP also showed good aqueous solubility and cell permeability, thus demonstrating high bioavailability in mice and rats (43).

In this study, we demonstrate the inhibitory action of ACHP on IκBα phosphorylation and its degradation, as well as the nuclear translocation and phosphorylation of p65, resulting in the reduction of HIV production in HIV-1 latently infected cells. Furthermore, NF-κB (p65) binding to the HIV-1 LTR was also abolished by this compound. From these findings, this compound and its derivatives appear to be feasible candidates for novel anti-HIV therapy.

MATERIALS AND METHODS

Reagents. An IKK inhibitor, ACHP, was a kind gift from T. Murata of Bayer Yakuhin Inc. (Kyoto, Japan). Human recombinant TNF-α was purchased from Roche and used at 1 ng/ml for NF-κB stimulation. Antibodies for IκBα, p65 (RelA), and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies for phospho-IκBα (Ser32) and phospho-p65 (Ser536) were purchased from Cell Signaling Technology (Beverly, MA). The pooled sera from HIV-1-infected individuals were kindly given by T. Hamano (National Institute of Infectious Diseases, Tokyo, Japan). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, United Kingdom) (rabbit and mouse) and from DAKO (DAKO A/S, Denmark) (goat).

Cell lines. OM10.1 cells (13), a human macrophage/monocytic cell line latently infected with HIV-1, and MOLT4/III_B cells (3), a T-cell line chronically infected with HIV-1 (III_B strain), were used in the antiviral assays. The OM10.1 cells and MOLT4/III_B cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a 5% CO_2 incubator. To maintain the latency in OM10.1 cells, 20 μM of AZT was added to culture media and was removed prior to experiments (3, 58, 59, 64).

Antiviral assay. The antiviral activity of ACHP was evaluated based on the extent of inhibition of p24 antigen production in OM10.1 cells as previously described (59, 64). Cells (2×10^5/ml) were incubated with or without ACHP for 1 h and then stimulated with TNF-α (1 ng/ml) for 24 h at 37°C. A time course experiment (until 72 h) was also conducted. On the other hand, MOLT4/III_B cells (2×10^5/ml) were cultured in the absence or presence of ACHP with or without TNF-α stimulation. The culture supernatants were then collected and assayed for viral p24 antigen. Experiments were carried out in triplicate and repeated at least twice. The cytotoxicity of the test compound was also determined by the WST-1 method (Roche) (58).

Quantitation of HIV-1 replication. Viral p24 antigen levels in the cell supernatants of OM10.1 and MOLT4/III_B cells were determined using the commercial Retrotek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Cellular Products, Buffalo, NY) according to the manufacturer's instructions. Assays were performed in triplicate and repeated at least twice.

Transient luciferase assay. 293 cells (1×10^5/well) were transfected with reporter plasmids using FUGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. For each transfection, 0.3 μg of reporter plasmid, CD12wt-Luc containing wild-type HIV-1 LTR (47) or CD12mut-Luc containing mutated NF-κB binding sites (59), and 0.1 μg of the internal control plasmid, phRL-TK, expressing *Renilla* luciferase, were used (57). Twenty-four hours after transfection, the cells were treated with ACHP for 30 min and stimulated with TNF-α (5 ng/ml) for 4 h. The transfected cells were then harvested, and the extracts were subjected to luciferase assay using the Luciferase Assay System (Promega). The luciferase activity was normalized with *Renilla* luciferase activity as an internal control to assess the transfection efficiency. The data are presented as the increase in luciferase activities (means ± standard deviations) relative to the control from triplicate transfections.

Preparation of whole-cell and nuclear extracts. OM10.1 cells (1×10^6/ml) were treated with or without ACHP for 1 h and stimulated with or without TNF-α (1 ng/ml) at various times. The cells were then washed with cold phosphate-buffered saline and resuspended in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% Triton X-100, and protease inhibitors [Roche Diagnostics GmbH, Mannheim, Germany]), incubated on ice for 10 min, and centrifuged at 15,000 rpm for 15 min. The supernatant was then collected (whole-cell extract) and stored at −80°C until it was used. In order to prepare the nuclear extract, sedimented cells were resuspended in cytoplasmic lysis buffer (Chemicon International, Tenecula, CA) and incubated for 15 min on ice. The cells were vortexed and then centrifuged at 15,000 rpm for 10 min, and the supernatant was removed. The cell pellets were washed twice with cytoplasmic buffer to remove any trace of proteins from the cytoplasmic extracts, resuspended in 20 μl of nuclear lysis buffer (Chemicon International, Tenecula, CA), and incubated on ice for 15 min. The cell suspensions were then sonicated for 10 s and centrifuged at 15,000 rpm for 10 min, and the supernatant fractions were stored at −80°C until they were used. The protein content was measured by a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA).

Western blotting. Equal amounts of the proteins (14 μg) were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, MA), and reacted with specific antibodies for various proteins. Expression of HIV-1 viral proteins was examined by reaction with pooled sera from HIV-1-infected individuals. Detection of immunoreactive bands was visualized by chemiluminescence using SuperSignal (Pierce, Rockford, IL).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed with a previously described protocol with some modifications (25). In brief, cells either with or without ACHP treatment and with or without TNF-α stimulation were cross-linked by adding formaldehyde to the medium (1% final concentration) and incubated at 37°C for 10 min. The cells were then washed with cold phosphate-buffered saline containing protease inhibitors and PMSF and lysed in SDS-lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], 16.7 mM NaCl, PMSF, and protease inhibitors), and the chromatin was sheared by sonication 20 times for 30 s each time at one-third of the maximum power with 1 min of cooling on ice between each pair of pulses (Bioruptor; COSMO Bio, Tokyo, Japan). The lysates were then collected after centrifugation at 15,000 rpm for 15 min, followed by the addition of specific antibodies, and the mixture was rotated at room temperature for 2 h and further incubated for another hour at 4°C. DNA samples were then precipitated with salmon sperm DNA and protein G-agarose beads (Upstate Biotechnology, Lake Placid, NY), and cross-linking of the immunoprecipitates and input DNAs was reversed by incubation at 65°C for 6 h. The DNAs were then purified using Qiaquick spin columns (QIAGEN), and PCR was performed with a HotStar*Taq* Master Mix kit (QIAGEN). The PCR primers used for amplifying promoters containing the NF-κB binding sites included HIV-1 LTR promoter (−176 to +61), 5′-CGA GAG CTG CAT CCG GAG TA-3′ and 5′-AGC TTT ATT GAG GCT TAA GC-3′ (37); human IκBα promoter (−316 to −15), 5′-GAC GAC CCC AAT TCA AAT CG-3′ and 5′-TCA GGC TCG GGG AAT TTC C-3′ (70); and human β-actin promoter (−980 to −915), 5′-TGC ACT GTG CGG CGA AGC-3′ and 5′-TCG AGC CAT AAA AGG CAA-3′ (70).

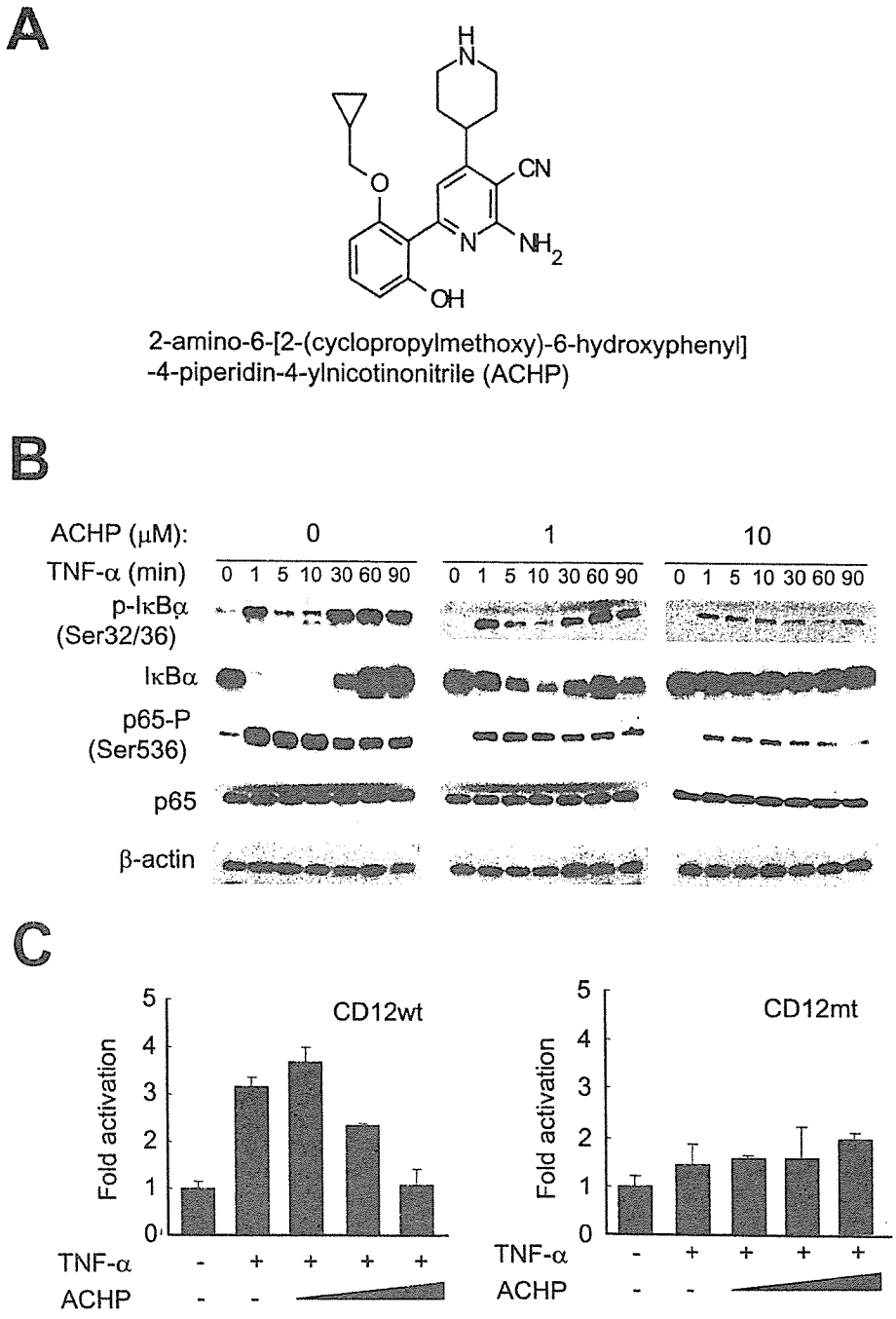

FIG. 1. Effect of ACHP on IκBα phosphorylation and degradation and p65 phosphorylation induced by TNF-α. (A) Chemical structure of ACHP. (B) Inhibition of phosphorylation of IκBα and p65 by ACHP. OM10.1 cells (1×10^6/ml) were pretreated with or without ACHP for an hour and then stimulated with TNF-α (1 ng/ml) for the indicated time periods. Whole-cell extracts were fractionated on 10% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with the specified antibodies. Immunoblotting of β-actin indicated that equal amounts of protein were applied in each lane. (C) Inhibitory effect of ACHP on HIV-1 LTR-driven gene expression in 293 cells. A luciferase reporter plasmid containing wild-type NF-κB binding sequence (CD12wt, left) or its mutant (CD12mt, right) was transfected into 293 cells under the control of the HIV-1 LTR. After transfection, the cells were incubated in the absence or presence of ACHP for 30 min, stimulated with TNF-α (5 ng/ml) for 4 h, and harvested for luciferase assay, as described in Materials and Methods. The luciferase activity is indicated as increase relative to the untreated control (lane 1). The data are mean values plus standard deviations of triplicate experiments.

RESULTS

ACHP inhibited TNF-α-induced IκBα phosphorylation and degradation and p65 phosphorylation. Activated IKKs target the IκBα inhibitor of NF-κB for phosphorylation on Ser32 and Ser36, leading to the rapid ubiquitylation and degradation of the inhibitor by the 26S proteasome (44). To assess whether ACHP (Fig. 1A) modulates this cascade, OM10.1 cells, a macrophage/monocyte cell model widely used in studying thera-

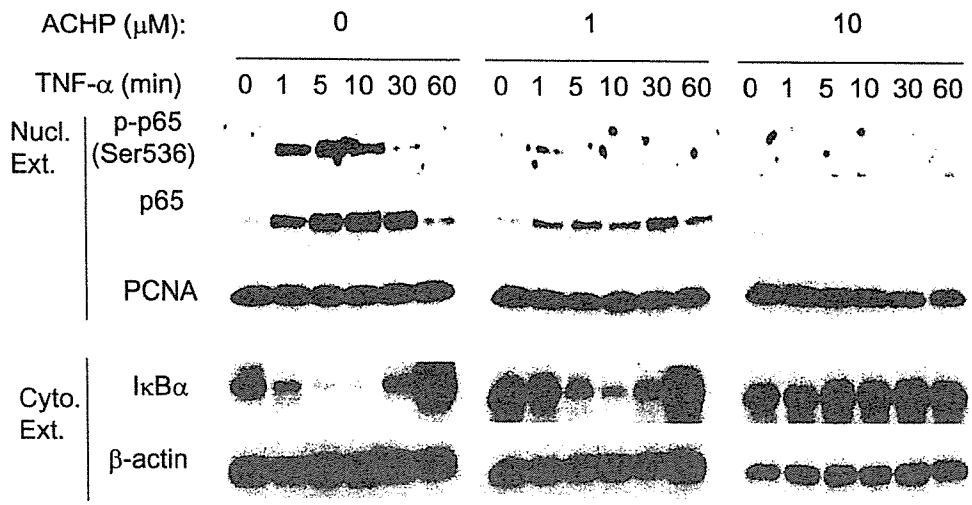

FIG. 2. Effect of ACHP on TNF-α-induced p65 translocation and phosphorylation in the nucleus. OM10.1 cells (1×10^6/ml) were pretreated with or without ACHP for an hour and then stimulated with TNF-α (1 ng/ml) for the indicated time periods. Nuclear extracts (Nucl. Ext.) were fractionated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with the anti-phospho-p65 (Ser536) antibody (top). The membrane was reprobed with anti-p65 (upper middle) and anti-PCNA (lower middle) antibodies. Cytoplasmic extracts (Cyto. Ext.) were immunoblotted with the anti-IκBα and β-actin antibodies (bottom). The purity of the nuclear and cytoplasmic extracts was confirmed with antibodies specific for PCNA, a known nuclear protein (lower middle) and β-actin, a cytoplasmic protein (bottom), respectively.

peutic interventions (3, 13, 58, 64), were stimulated with TNF-α for the indicated times in the absence or presence of ACHP and analyzed for IκBα and NF-κB (p65) expression and for their phosphorylation status by Western blot analysis.

As shown in Fig. 1B, immunoblotting analysis showed that IκBα was readily phosphorylated at Ser32 and Ser36 as early as 1 min after TNF-α stimulation. Following its degradation, a massive increase was observed, indicating the activation of the IκBα gene by NF-κB (Fig. 1B, first gel, first row) (12, 14, 37, 61). However, treatment with ACHP reduced IκBα phosphorylation in a dose-dependent manner (second and third gels, first row). ACHP (10 μM) maintained the phosphorylation approximately at basal levels throughout the time course experiment (third gel, first row). Because IκBα normally undergoes degradation following phosphorylation, we proceeded to ascertain whether ACHP blocked its degradation. Remarkably, IκBα expression levels were apparently sustained at basal levels, and no degradation was observed when cells were treated with 10 μM ACHP, confirming that the preceding step, IκBα phosphorylation, was inhibited (third gel, second row).

To further explore the status of NF-κB activation in these cells, we also investigated the phosphorylation levels of the p65 subunit of NF-κB. Figure 1B (third row of each gel) shows constitutive phosphorylation of p65 at Ser536, which was markedly induced a minute after TNF-α treatment and gradually decreased in a time-dependent manner. In contrast, pretreatment with ACHP reduced this TNF-α-stimulated phosphorylation of p65 (third gel, third row), whereas the total protein level of p65, like that of IκBα, was maintained at basal levels in the absence or presence of TNF-α (fourth row of each gel). β-Actin levels were unchanged (bottom panels), indicating equal loading of proteins in the gel. These results indicate that ACHP promoted a substantial defect in the TNF-α-induction of NF-κB activation.

We then examined the effect of ACHP on HIV-1 gene expression using a transient luciferase assay. As shown in Fig. 1C, TNF-α stimulated the gene expression from CD12wt containing the wild type HIV-1 LTR (47) by approximately threefold. When cells were pretreated with ACHP, the gene expression was inhibited in a dose-dependent manner (Fig. 1C, left). However, when the NF-κB sites were mutated (CD12mt), no such activation by TNF-α or inhibitory effects of ACHP were observed ((Fig. 1C), right).

ACHP repressed TNF-α-induced nuclear translocation and phosphorylation of p65. Because IκBα phosphorylation and its degradation were inhibited by treatment with ACHP, and since both are prerequisite steps for the release and transport of NF-κB to the nucleus, we also examined whether ACHP modulated this event. Following TNF-α stimulation at 1, 5, 10, 30, and 60 min, in either the absence or the presence of ACHP, nuclear extracts from OM10.1 cells were isolated and NF-κB (p65) was examined by Western blotting analysis. TNF-α treatment resulted in a rapid nuclear accumulation of p65 as early as 1 min, followed by increase until 30 min, and then drastically decreased at 60 min (Fig. 2, "Nucl. Ext.," first gel, second row). Concurrently, IκBα appeared, indicating its nuclear-export function (data not shown). Consistent with previous studies using OM10.1 cells (3, 58, 64), a low level of NF-κB activation, i.e., nuclear localization of p65, was observed in unstimulated cells, which was notably reduced by the presence of ACHP alone (Fig. 2, lanes at 0 min, second row). Whereas TNF-α induced the accumulation of p65 in the nucleus, ACHP almost totally abolished this effect (Fig. 2, "Nucl. Ext.," third gel, second row), suggesting that the nuclear transport of NF-κB was inhibited. Immunoblots of the cytoplasmic extracts also revealed that 10 μM ACHP maintained IκBα protein levels at basal levels, reaffirming the persistence of latent NF-κB–IκB complexes (Fig. 2, "Cyto. Ext.," first row of each gel). In addition to nuclear translocation, p65 phosphorylation is also essential for its maximum transcriptional activity (28, 66, 72).

Concomitant with the appearance of p65 in the nucleus, rapid phosphorylation of p65 at Ser536 was detected (Fig. 2, "Nucl. Ext.," first row). This phosphorylation, however, occurred only transiently, followed by a marked reduction after 10 min of TNF-α stimulation, suggesting dephosphorylation of p65. In contrast, no phosphorylation was observed in the presence of 10 μM ACHP, implying that ACHP efficiently blocked the phosphorylation of p65 in the nucleus (Fig. 2, "Nucl. Ext.," third gel, first row). These observations further strengthen the view that ACHP impairs the IKK activity that enables p65 to translocate to the nucleus and phosphorylates p65.

Inhibition of TNF-α-induced HIV-1 production by ACHP from OM10.1 cells. To examine the effect of ACHP on TNF-α-induced HIV replication in latently infected OM10.1 cells, the cells were treated with various concentrations of TNF-α in the absence or presence of ACHP. After 24 h of incubation, the culture supernatants were collected and assayed for HIV-1 p24 antigen levels. In agreement with previous studies (3, 58, 64), HIV-1 production was dose-dependently induced by TNF-α (Fig. 3A). However, in the presence of ACHP, a dose-dependent inhibition of virus production was observed. A similar effect was also observed in the time course experiment (Fig. 3B). This strongly suggests that ACHP compelled a defect in the TNF-α-induced NF-κB activation in HIV. No significant induction was observed with ACHP alone. An effective concentration for 50% reduction of HIV production was estimated to be approximately 0.56 μM. Meanwhile, the 50% cytotoxic concentration (CC_{50}) was approximately 15 μM, and thus, the estimated therapeutic window of ACHP was approximately 27 (Fig. 3D). Moreover, immunoblotting of whole-cell extracts (Fig. 3C) with the pooled sera from individuals infected with HIV-1 revealed inhibition of the envelope (*env*) gene products gp160 and gp41 and the *gag* gene products p66, Pr55, and p39 in the presence of 10 μM ACHP, thus confirming the effect of ACHP on the viral-production step.

We also explored the effect of ACHP on viral production from MOLT4/III_B cells chronically infected with HIV-1 (Fig. 4). Whereas ACHP efficiently suppressed viral production in latently infected cells, no significant inhibition was observed in chronically infected cells. A slight decrease in HIV production at 10 μM ACHP, although not statistically significant, was presumably due to a nonspecific cytotoxic effect of ACHP (Fig. 4A, left). The presence of constitutive viral production in the absence of TNF-α suggests the involvement of multiple mechanisms in the regulation of HIV-1 replication (24). From the cytotoxicity profile of ACHP, a CC_{50} value of 11 μM was obtained for these cells (Fig. 4A, right). There was no effect of ACHP on the level of HIV production without TNF-α (Fig. 4B). These results also imply that the anti-HIV-1 activity of ACHP in chronic infection is cell type specific, as previously reported by others (46).

ACHP suppressed NF-κB binding to HIV-1 LTR. HIV-1 replication, particularly in latently infected cells, such as OM10.1, is promoted by NF-κB binding to the HIV-1 LTR upon induction with TNF-α (37, 44, 55). To assess whether NF-κB binding to the HIV-1 LTR is inhibited by ACHP, ChIP assays were performed. OM10.1 cells were preincubated with ACHP and stimulated with TNF-α for 10 min, the time at which maximum NF-κB binding was observed in repeated experiments. Following stimulation, the cells were cross-linked

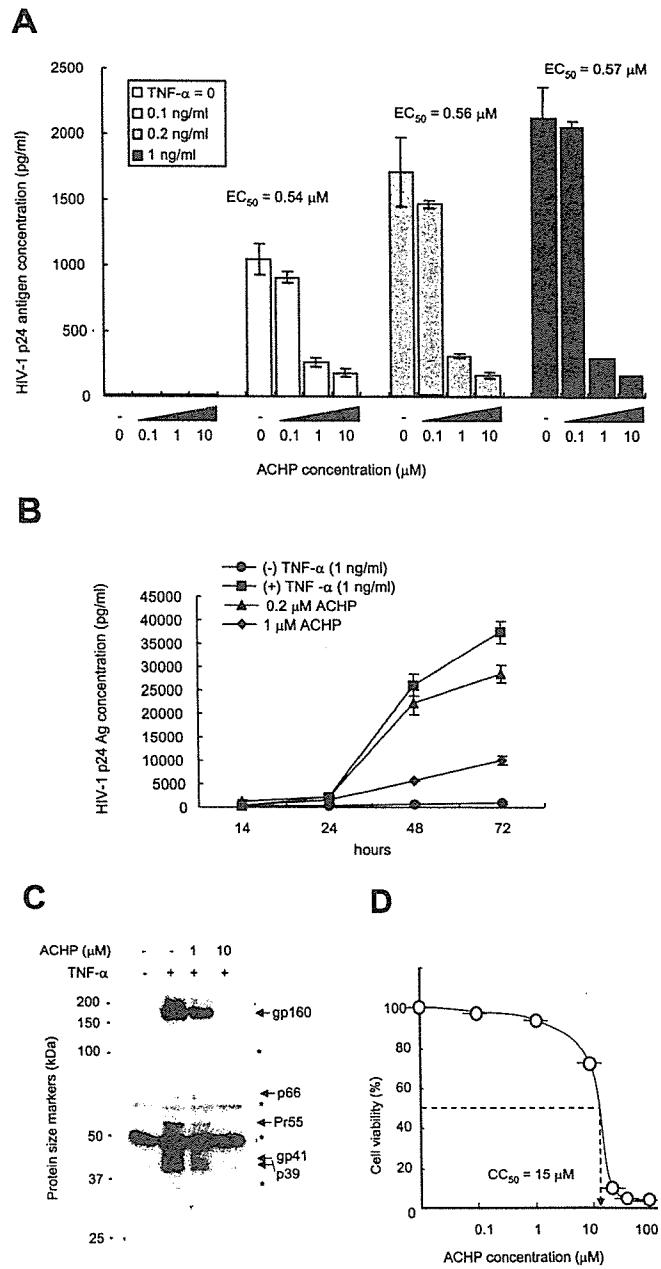

FIG. 3. Effect of ACHP on TNF-α-induced HIV-1 production in latently infected cells. (A) OM10.1 cells (2×10^5/ml) were pretreated with or without ACHP for 1 hour and then stimulated with TNF-α (1 ng/ml) for 24 h. The cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels using a commercial ELISA kit. The data are mean values ± standard deviations of triplicate experiments. EC_{50}, 50% effective concentration. (B) Time course experiment showing the effect of ACHP in OM10.1 cells. Cells (2×10^5/ml) were pretreated in the absence or presence of ACHP for 1 hour and then stimulated with or without TNF-α (1 ng/ml) for 24 h. The cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels using a commercial ELISA kit. The data are mean values ± standard deviations of triplicate experiments. (C) Whole-cell extracts were prepared and immunoblotted with human HIV-1-infected serum for the analysis of HIV-1 viral proteins (whole-cell extracts). The location of each viral product is indicated by an arrow. *, nonspecific bands. (D) Cytotoxicity of ACHP on OM10.1 cells. Cell viability was determined by the WST-1 method, and the CC_{50} value was extrapolated from this measurement.

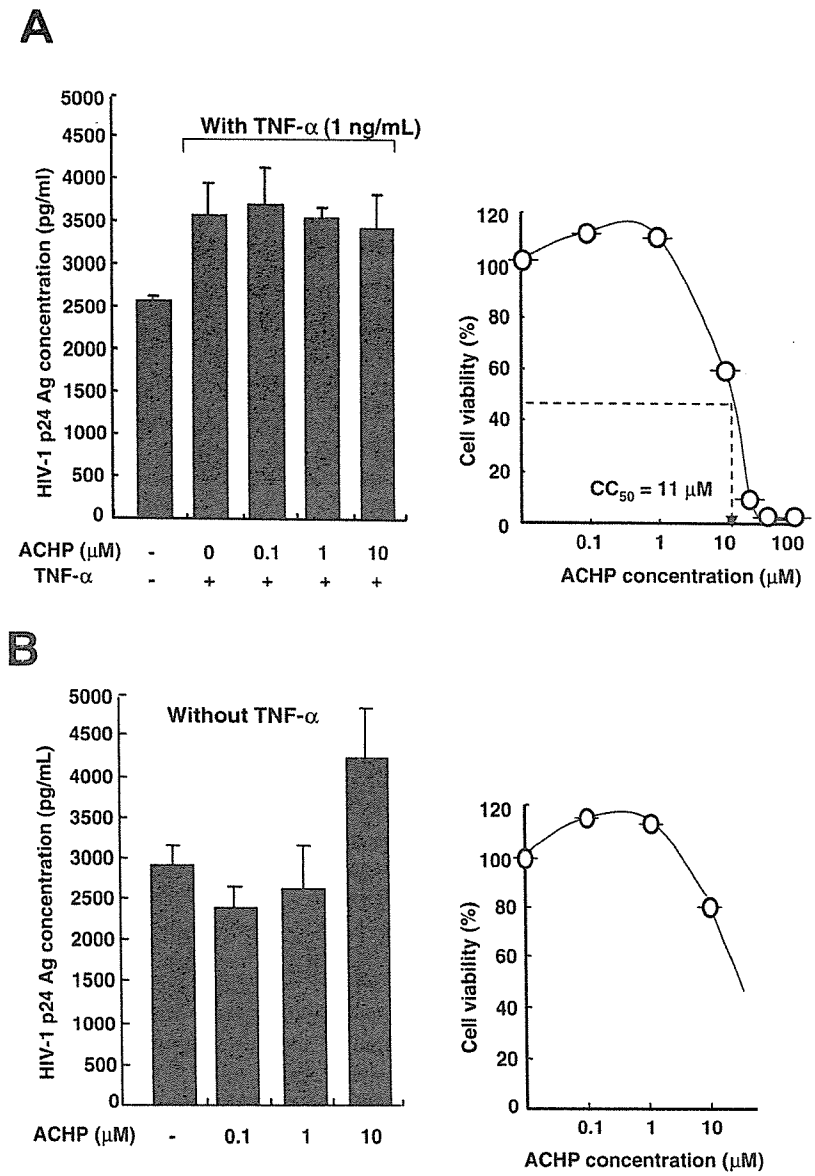

FIG. 4. Effect of ACHP on chronically HIV-1 infected cells. (A and B, left) Effect of ACHP on HIV-1 production. MOLT4/IIIB cells (2×10^5/ml) were pretreated with or without ACHP for 1 hour and then stimulated with or without TNF-α for 24 h. Cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels by ELISA. (A and B, right) Cytotoxicity of ACHP on MOLT4/IIIB cells. Cell viability was determined by the WST-1 assay. The CC_{50} value was extrapolated from this measurement. The data are mean values plus standard deviations of triplicate experiments.

and lysed, and DNA was sheared by sonication. The lysates were immunoprecipitated with anti-p65 antibody and agarose beads, and DNA-protein cross-links were reversed. The p65-immunoprecipitated samples and controls were probed by PCR for HIV-1 LTR sequence containing NF-κB binding sites. In agreement with our Western blotting results, the kinetics of NF-κB binding to the HIV-1 LTR showed a rapid recruitment of NF-κB (p65) to the promoter in response to TNF-α (Fig. 5A, first gel, first row). A remarkable association was observed until 30 min and thereafter decreased to an almost undetectable level, suggesting the cytoplasmic export of p65. The presence of constitutive, almost undetectable NF-κB (p65) binding in the HIV-1 LTR in unstimulated cells again correlated with our Western blot analysis of nuclear p65, thus conforming to previous studies (19, 26). Similarly, NF-κB (p65) binding in the IκBα promoter was also detected, although with a slower onset of association (Fig. 5A, second gel, first row).

In contrast, in the presence of ACHP, both basal and TNF-α-induced p65 recruitment in both promoters was abolished in a dose-dependent manner (Fig. 5B, first and second gels, first row). No amplification was detected with the β-actin promoter (internal control) or in the absence of anti-p65 antibody, confirming the specificity of the DNA immunoprecipitation (Fig.

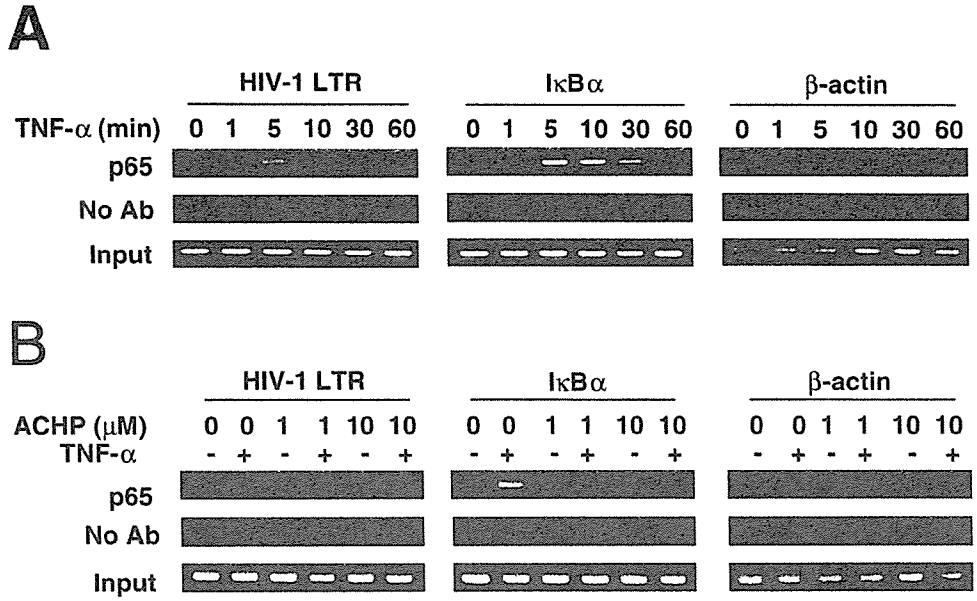

FIG. 5. ChIP assays for NF-κB (p65) binding to promoters. (A) Kinetics of TNF-α-induced NF-κB binding to the HIV-1 LTR. Ab, antibody. (B) ACHP-mediated inhibition of TNF-α-induced NF-κB binding to the HIV-1 LTR. OM10.1 cells (1×10^5/ml) were treated with TNF-α (1 ng/ml) for the indicated time periods, and ChIP assays were performed with anti-p65 antibody or no antibody (negative control). The detection of the immunoprecipitated DNAs in the HIV-1 LTR (first gel), IκBα (second gel), or β-actin (third gel) promoter was analyzed by PCR with promoter-specific primers. Input DNA represents total input chromatin (1%).

5B, second row of each gel). Collectively, these findings suggest that ACHP can efficiently block the binding of NF-κB to the HIV-1 LTR promoter.

DISCUSSION

In this study, we have addressed the question of whether a novel IKK inhibitor, ACHP, can inhibit HIV-1 replication in a macrophage/monocyte cell line latently infected with the virus. We observed that TNF-α-mediated NF-κB signaling could efficiently induce HIV-1 replication, which was subsequently blocked by ACHP. IκBα phosphorylation and degradation, p65 nuclear translocation, and p65 phosphorylation at Ser536 were effectively inhibited by ACHP. Moreover, ACHP suppressed HIV-1 LTR-driven gene expression through the inhibition of the NF-κB activation pathway. We also found, using a ChIP assay, that TNF-α could activate NF-κB (p65)–DNA binding to the HIV-1 LTR in OM10.1 cells and that treatment with ACHP could abolish its binding.

Although NF-κB plays a central role in mediating inducible HIV-1 gene expression (44, 48, 52), the coordinated HIV-1 replication with the cellular activation is partially ascribed to the ability of HIV to assimilate host signaling pathways to activate viral transcription (24, 55). An essential step in the stimulus-induced activation of the canonical NF-κB pathway is the phosphorylation of IκB proteins by the IKK complex (69). In addition, Asin et al. (2) demonstrated that HIV infection itself could induce NF-κB activation through the canonical pathway involving activation, that is, the Ser32 and Ser36 phosphorylation of IκBα by IKK-β, thus highlighting the role of NF-κB in HIV latent infection (11). In fact, transdominant mutants of IκBα that block NF-κB induction inhibited de novo HIV-1 infection in T cells by interfering with viral transcription (32, 54). Furthermore, CD4 engagement with gp120 selectively enhanced IKK activity and mediated the phosphorylation of IκBα, while dominant-negative forms of IKKs inhibited gp120-induced NF-κB activation (11). Moreover, recruitment of NF-κB to the HIV promoter is considered essential for the action of Tat and efficient transcriptional elongation (9, 67). Recent studies using ChIP assays have indicated the importance of IKK-α for induction of NF-κB-mediated gene expression by forming a complex with p65 or CBP in these promoter regions and thus regulating histone H3 phosphorylation, followed by acetylation of CBP (1, 70).

Whereas IKK-β is largely responsible for cytokine-induced IκBα phosphorylation and NF-κB activation (22, 35, 36), IKK-α was initially implicated in more specified biological actions, such as formation of secondary lymphoid tissues (39, 62). However, recent studies have revealed an essential role of IKK-α in the noncanonical/alternative pathway of NF-κB activation, such as lymphotoxin β receptor signaling (28) and NIK-induced p100 processing (16, 17, 18, 22, 60). Moreover, IKK-α has been implicated in the phosphorylation of p65 at Ser536 (7, 28, 66, 72). Of note, the point mutation of Ser536 eventually resulted in the failure of nuclear translocation of NF-κB (40). In addition, since Ser536 is located in the carboxyl-terminal transactivation domain of p65, it is postulated that the phosphorylated NF-κB (p65 at Ser536), once bound in the target DNA, might further recruit basal transcription factors and transcriptional coactivators, thereby increasing the transcriptional competence of NF-κB (p65) (28).

In spite of a robust effect in latently infected cells, ACHP did not show a significant inhibitory effect in chronically infected

cells in which HIV-1 was actively replicating. The reason for the unresponsiveness of these cells to the compound is unclear. A previous study of the effects of an NF-κB inhibitor (4, 46) against chronic HIV infection reported it to be ineffective in suppressing constitutive HIV-1 production, which is consistent with what we observed in this study. Hence, it is also possible that the cellular uptake or intracellular metabolism of ACHP is different in OM10.1 and MOLT4/III$_B$ cells. Another plausible explanation is that the constitutive activation of NF-κB in chronically infected T cells (24, 31, 55) perpetuates the production of other viral proteins, such as Tat and Nef, yielding more important roles for such proteins in activating and regulating the expression of HIV-1 than for NF-κB in these cells. Thus, NF-κB-independent mechanisms appear to be operating in chronic HIV-1 infection.

Although it is evident that ACHP is not potent in actively replicating cells, considering the presence of latent reservoirs that are sources of viral rebounds and its contribution to disease progression (24), a rationale exists for the use of the compound in HIV-1 infection. Apparently, given its mechanism, use of ACHP might cause aberrant regulation of inflammatory cytokines (5, 10, 43). Therefore, further studies are needed to further evaluate its feasibility as a potential drug candidate for novel anti-HIV therapy.

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Induction of *OGG1* Gene Expression by HIV-1 Tat*

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To identify the cellular gene target for Tat, we performed gene expression profile analysis and found that Tat up-regulates the expression of the *OGG1* (8-oxoguanine-DNA glycosylase-1) gene, which encodes an enzyme responsible for repairing the oxidatively damaged guanosine, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG). We observed that Tat induced *OGG1* gene expression by enhancing its promoter activity without changing its mRNA stability. We found that the upstream AP-4 site within the *OGG1* promoter is responsible and that Tat interacted with AP-4 and removed AP-4 from the *OGG1* promoter by *in vivo* chromatin immunoprecipitation assay. Thus, Tat appears to activate *OGG1* expression by sequestrating AP-4. Interestingly, although Tat induces oxidative stress known to generate 8-oxo-dG, which causes the G:C to T:A transversion, we observed that the amount of 8-oxo-dG was reduced by Tat. When *OGG1* was knocked down by small interfering RNA, Tat increased the amount of 8-oxo-dG, thus confirming the role of OGG1 in preventing the formation of 8-oxo-dG. These findings collectively indicate the possibility that Tat may play a role in maintenance of the genetic integrity of the proviral and host cellular genomes by up-regulating OGG1 as a feed-forward mechanism.

Tat is an essential transactivator of human immunodeficiency virus (HIV)[1] gene expression and viral replication (1). Tat stimulates viral gene expression by directly binding to the characteristic RNA stem-loop-bulge structure called the transactivation response region located within the long terminal repeat (2, 3) and enhancing the processivity of RNA polymerase II (4, 5). The transcriptional activity of Tat is supported by interaction with cellular factors such as positive transcription elongation factor-b (6–8) and histone acetyltransferase (9). Cyclin T1, a regulatory subunit of the positive transcription elongation factor-b complex, binds to the activation domain of Tat and facilitates the hyperphosphorylation of the C-terminal domain of RNA polymerase II at the vicinity of HIV genes. Thus, Tat makes RNA polymerase II highly competent for the transcription elongation and productive expression of HIV genes (10).

Although much of the efforts in Tat studies have focused on its transcriptional activation from the HIV provirus, the actions of Tat on cellular genes have also been revealed. For example, Tat is known to promote cellular transformation (11), to induce oxidative stress (12, 13), and to elicit inflammatory reactions (14, 15). Choi *et al.* (16) observed that Tat transgenic mice exhibit decreased gene expression of the γ-glutamylcysteine synthetase regulatory subunit and decreased GSH content in tissues. These biological actions of Tat are considered to cause activation of nuclear factor-κB, AP-1 (activating protein-1), and mitogen-activated protein kinase (13, 17). These findings prompted us to search for cellular target genes of Tat, either up-regulated or down-regulated, using a gene expression profile analysis.

In addition to the very high efficiency of the viral replication rate that is mainly ascribable to Tat action, HIV owes its morbidity to its high mutation rate, leading to the emergence of drug resistance and escape from the host immune response. In fact, the high frequency of G:C to A:T and G:C to T:A mutations was previously observed in HIV-1 and other lentiviruses (18–21). Recent studies (22–25) have deciphered one such mechanism that involves the HIV-encoded virion infectivity factor blocking the enzymatic activity of cytidine deaminase CEM15 (also known as APOBEC3G for apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which induces G:C to A:T hypermutation in newly synthesized DNA. Another type of mutation, G:C to T:A transversion, is mediated by the generation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) by radical oxygen species (ROS) and occurs at the DNA level (26, 27). The oxidatively damaged guanosine, 8-oxo-dG, is widely accepted as a pre-mutagenic lesion because of its potential to mispair with adenine, thus generating the G:C to T:A transversion. This type of mutation is often found in tumor suppressor genes and oncogenes, such as p53 and K-*ras*, in mammalian cells (28, 29). The OGG1 (8-oxoguanine-DNA glycosylase-1) enzyme is responsible for the excision/repair of this oxidatively damaged DNA by excising 8-oxo-dG (30–32). In fact, *OGG1* gene knockout actually shows accumulation of such a mutation (33, 34).

In this study, we demonstrate the up-regulation of OGG1 by Tat and provide evidence that this effect of Tat is through the sequestration of the negative transcription factor AP-4 for the expression of *OGG1*. We examine the effect of Tat on the actual

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[1] The abbreviations used are: HIV, human immunodeficiency virus; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ROS, radical oxygen species; siRNA, small interfering RNA; mTat, mutant Tat; PonA, ponasterone A; PBMCs, peripheral blood mononuclear cells; RT, reverse transcription; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; HPLC, high performance liquid chromatography; ECD, electrochemical detector.

levels of 8-oxo-dG in the presence and absence of small interfering RNA (siRNA) against *OGG1* mRNA.

EXPERIMENTAL PROCEDURES

Plasmids—The cDNA of wild-type Tat (101 amino acids) originating from HIV-1 was amplified by PCR with the oligonucleotide primer pair 5′-CGC GGA TCC GCG CCA CCA TGG ATT ACA AGG ATG ACG ACG ATA AGA TGG AGC CAG TAG ATC CTA GAC TAG AGC CCT GG-3′ (forward; containing an EcoRI site and a FLAG epitope) and 5′-CCG GAA TTC CGG CTG ATG GAC CGG ATC TGT CTC-3′ (reverse; containing a BamHI site). The amplified DNA fragment was digested with EcoRI and BamHI and ligated in-frame into the pIND-V5 expression vector (Invitrogen), thus generating pIND-Tat. As a control, we employed mutant Tat (mTat) lacking transcriptional activity because of the absence of binding activity with cyclin T1 or the transactivation response region (6–8). The plasmid expressing mutant Tat (pIND-mTat) in which Cys^{30} and Lys^{41} were substituted with Ala was generated using a QuikChange site-directed mutagenesis kit (Stratagene) with the following mutagenic oligonucleotide primer pairs: 5′-CTA TTG TAA AAA GGC CTG CTT TCA TTG CC-3′ (forward) and 5′-GGC AAT GAA AGC AGG CCT TTT TAC AAT AG-3′ (reverse) or 5′-GTT TCA CAA CAG CCG CCT TAG GCA TC-3′ (forward) and 5′-GAT GCC TAA GGC GGC TGT TGT GAA AC-3′ (reverse). To generate the mammalian expression plasmid for AP-4, AP-4 gene was amplified by PCR with the oligonucleotide primer pair 5′-CGC GGA TCC GCG CCA CCA TGG ATT ACA AGG ATG ACG ACG ATA AGA TGG AGC CAG TAG ATC CTA GAC TAG AGC CCT GG-3′ (forward; containing an EcoRI site) and 5′-CCG GAA TTC CGG CTG ATG GAC CGG ATC TGT CTC-3′ (reverse; containing a BamHI site). The amplified DNA fragment was digested with EcoRI and BamHI and ligated in-frame into the pcDNA-Myc expression vector (Invitrogen). The construction of pCD12-luc, containing the HIV-1 long terminal repeats V3 and R linked to the luciferase gene, and pcDNA-Tat was described previously (35, 36). Human *OGG1* promoter-luciferase fusion constructs, including pPR116, pPR128, pPR130, and pPR143, were kindly provided by Dr. J. P. Radicella (Radiobiologie Moleculaire et Cellulaire, CNRS-CEA, Fontenay aux Roses, France) (37). The mutant pPR128-luc reporter constructs were generated using a QuikChange site-directed mutagenesis kit. The mutant sequences (sense strand) utilized were as follows: 5′-AP-4 site mutant (m5′AP-4), GAC GGC AGG CAG tcg cga TGG CGG CCG GCG; 3′-AP-4 site mutant (m3′AP-4), GGG AAA GGC GAG tcg cga GCA GAG AGC CCA G; GA TA site mutant (mGATA), CTT GCA GCC Tct TAG TTA AGA TAC AGC; and AP-2 site mutant (mAP-2), CAG CTG TGG CGG CCa ttC GGG ACG ACA ATC (with consensus binding sites underlined and mutated sequences in lowercase letters). The construct containing mutations in both the 5′- and 3′-AP-4 sites (mwAP-4) was generated by two successive PCRs using the m5′AP-4 and m3′AP-4 mutant sequences. The control luciferase reporter plasmid pGL3-Basic vector was purchased from Promega. All constructs were confirmed by dideoxynucleotide sequencing using an ABI PRISM™ dye terminator cycle sequencing ready kit (PerkinElmer Life Sciences) on an Applied Biosystems 313 Automated DNA Sequencer.

Cell Lines That Inducibly Express Tat, mTat, and LacZ—HEK293-EcR cells, stably transfected with pVgRXR expressing the ecdysone receptor, were purchased from Invitrogen and transfected with pIND-Tat and pIND-mTat to establish Tat/293 and mTat/293 cells, respectively. The control cell line (LacZ/293) was a gift from Dr. L. Naumovski (Stanford University, Stanford, CA) (38). Expression of these genes is under the stringent control of a homolog of the insect hormone 20-OH-ecdysone, ponasterone A (PonA; Invitrogen). Cells containing these plasmids were selected by 500 μg/ml G418 and 450 μg/ml Zeocin. Cell clones were singly isolated by two successive rounds of limiting dilution of cells and were screened for the expression and transcriptional activity of Tat proteins.

Cell Culture—Tat/293, mTat/293, and LacZ/293 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Sigma) with 10% heat-inactivated fetal bovine serum (Immuno-Biological Laboratories, Maebashi, Japan), 100 units/ml penicillin, and 100 μg/ml streptomycin. The Jurkat T cell line was maintained in RPMI 1640 medium (Sigma) with 10% fetal bovine serum, penicillin, and streptomycin. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors, stimulated with phytohemagglutinin for 48 h, and further cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 20 units/ml interleukin-2.

Preparation of mRNA—Total cellular RNA was prepared from each cell clone using RNeasy (Qiagen Inc.). Purification of polyadenylated mRNA was carried out using an Oligotex-dT30 super RNA purification kit (Takara, Ohtsu, Japan) as described previously (39, 40). The mRNA samples were digested with RNase-free DNase, ethanol-precipitated, and further purified through Microcon YM-100 columns (Amicon Inc.). The quantity and quality of mRNA were assessed by capillary electrophoresis using an Agilent 2100 bioanalyzer.

Generation of Fluorescently Tagged cDNA and Gene Expression Profile Analysis—Gene expression profiles were examined as described (39, 40) using the human 3K DNA CHIP™ (Takara) containing 2600 human genes of known functions. Briefly, fluorescently labeled cDNA was synthesized from 1-μg aliquots of purified mRNA by oligo(dT)-primed polymerization using SuperScript II reverse transcriptase (Invitrogen). The pool of nucleotides in the labeling reaction contained 0.5 mM each dGTP, dATP, and dTTP; 0.3 mM dCTP; and 0.1 mM fluorescent nucleotide (Cy3- or Cy5-labeled dCTP, Amersham Biosciences). Fluorescently labeled cDNA was purified by chromatography through Microcon YM-20 columns (Amicon Inc.). The microarray slide was hybridized to combined Cy5-dCTP- and Cy3-dCTP-labeled cDNA probes for 14 h in hybridization solution (6× SSC and 0.2% SDS with 5× Denhardt's solution and carrier DNA) at 65 °C under coverslips. After hybridization, the microarray slide was washed twice with 1.2× SSC and 0.2% SDS at 55 °C for 5 min, with 1.2× SSC and 0.2% SDS at 65 °C for 5 min, and with 0.05× SSC at room temperature as a final wash. The hybridized array was scanned at 10-μm resolution on an Affymetrix 428 array scanner. Analysis of differential expression of each gene was performed using ImaGene Version 4.2 computer software (Bio-Discovery Ltd.). Normalization of hybridized signals was performed by global scaling. These experiments were repeatedly performed: we performed comparative microarray analyses three times (24 h after PonA stimulation in 293/Tat and 293/LacZ cells) and two times (12 h after PonA stimulation in 293/Tat and 293/LacZ cells).

Co-immunoprecipitation and Immunoblot Assay—The experimental procedures have been described previously (41). Briefly, cells were harvested with lysis buffer (25 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation, and the supernatants were incubated overnight with the indicated antibodies at 4 °C. For immunoprecipitation with the FLAG epitope, anti-FLAG antibody M2 affinity gel beads (Sigma) were used. The immune complexes were washed three times with 1 ml of lysis buffer, and the antibody-bound proteins were dissolved by boiling in 2× Laemmli sample buffer. After centrifugation, the supernatant proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C, Amersham Biosciences). The membrane was probed with antibodies, including anti-cyclin T1 and anti-AP-4 (Santa Cruz Biotechnology Inc.), anti-FLAG (Sigma), and anti-Myc (Invitrogen) antibodies; and immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce). To evaluate the level of OGG1 protein, cells were similarly treated with lysis buffer, and the cell lysate was analyzed by Western blotting using anti-OGG1 antibody (Novus Biologicals, Inc.).

Transfection and Luciferase Assay—Cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) as described (36). Jurkat cells were transiently transfected by electroporation (42). Briefly, cells (2×10^7/ml) were electroporated in the presence of 2 μg of pcDNA-Tat or control plasmid (pcDNA3.0, Invitrogen) in 400 μl of serum-free RPMI 1640 medium using the Electro Cell Manipulator 600 apparatus (BTX) at 260 V/1050 microfarads. For the internal control, we employed pRL-TK, expressing *Renilla* luciferase, which is not modified by Tat action. The transfected cells were harvested, and the extracts were subjected to luciferase assay using the Luciferase Assay System™ (Promega). The luciferase activity was normalized to *Renilla* luciferase activity as an internal control to assess the transfection efficiency. The data are presented as the -fold increase in luciferase activities (means ± S.D.) relative to the control from three independent transfections.

Reverse Transcription (RT)-PCR—For cDNA synthesis, 1 μg of purified total RNA were reverse-transcribed using oligo(dT) primer and SuperScript II reverse transcriptase. The cDNA was then amplified from each RNA sample with *Taq* PCR Master Mix (Qiagen Inc.) and gene-specific primers designed using Oligo Version 4.0 software (Molecular Biology Insights). The primer sequences for each amplified gene were as follows: *TFPI2* (tissue factor pathway inhibitor-2), 5′-CAG GAG CCA ACA GGA AAT AAC-3′ (forward) and 5′-GAA TAC GAC CCC AAG AAA TGA-3′ (reverse); *OGG1*, 5′-GCG TGC GCA AGT ACT TCC AGC-3′ (forward) and 5′-CCA GTG ATG CGG GCG ATG TTG-3′ (reverse); *OGG1* type 1, 5′-GCG TGC GCA AGT ACT TCC AGC-3′ (forward) and 5′-TAA AGG GAA GAT AAA ACC ATC-3′ (reverse); *OGG1* type 2, 5′-GCG TGC GCA AGT ACT TCC AGC-3′ (forward) and 5′-GCA TCA CAT GAC CAA TTA CTG-3′ (reverse); *MEN1β2* homolog

FLJ23538/clone 137308, 5′-GAG AGG GTT GGT TAG AGA TAC-3′ (forward) and 5′-TGA TTT TAG GTG ATA GTT TCC-3′ (reverse); integrin α7, 5′-AAG ACC GAC AGC AGT TCA AGG-3′ (forward) and 5′-GAC GAA ACC ACG AAA CCA CTA-3′ (reverse); *SLC20A1* (solute carrier family 20, member 1), 5′-TAT GTT TGG TTC TGC TGT GTC-3′ (forward) and 5′-GCT ATC TAT GCT GGT TTC CTC-3′ (reverse); *ENPP2* (ectonucleotide pyrophosphatase/phosphodiesterase-2), 5′-TTC TTT TGG TCT GTG TCA TC-3′ (forward) and 5′-TTC TTC TGT TGT TGG CAT AGT-3′ (reverse); *SEPP1* (selenoprotein P, plasma, 1), 5′-GGA ACA GAG AGC CAG GAC CA-3′ (forward) and 5′-CCT ATG CTG ACC CTT GTG CTT-3′ (reverse); stanniocalcin-1, 5′-AAG AAA GAA AGA GGG AAA AAG-3′ (forward) and 5′-AAC CAA ATC ACA AGG AAA GAA-3′ (reverse); *ETV5* (Ets variant gene-5), 5′-TTG TGT TGT GCC TGA GAG ACT-3′ (forward) and 5′-TCT ATG GGT TTG TGA TTT TTC-3′ (reverse); *NDRG1* (N-Myc downstream regulated gene-1), 5′-GCG GTG GCT GAG AAA ATG TAA-3′ (forward) and 5′-CAA GGT GAT GGG CGG CAG GTA-3′ (reverse); *ARHE* (Ras homolog gene family, member E), 5′-ACT TCG GGT TCT CCT TAC TAT-3′ (forward) and 5′-TTC TCA TCA CTT GGT CTA CAT-3′ (reverse); *HSTF2* (heat shock transcription factor-2), 5′-CAG AAC CAA CCC AAA GTA AGC-3′ (forward) and 5′-ACA GCA TCA ACA GGA AAA CA-3′ (reverse); *NTHL1* (Nth endonuclease III-like 1 (*E. coli*)), 5′-CAG CAG AAG CGA GGA AAA GC-3′ (forward) and 5′-CGC GCA GAG GGC TTG GTT GAG-3′ (reverse); *RGS16* (regulator of G-protein signaling-16), 5′-TGA GAG TCC TGC TGA AAT CCA-3′ (forward) and 5′-CCA ACA ATA ACA AAA ACA ATG-3′ (reverse); hexokinase-2, 5′-GAA CTG GTG GAA GGA GAA GAG-3′ (forward) and 5′-AGG GAA GAA GGA GAG AAA GAG-3′ (reverse); *LTA1* (L-type amino acid transporter subunit-1), 5′-TCG GGG TCT GGT GGA AAA ACA-3′ (forward) and 5′-AAC AAA GGA GGG AAG GGA AAA-3′ (reverse); *SLC1A3* (solute carrier family 1, member 3), 5′-TAT GTT TGG TTC TGC TGT GTG-3′ (forward) and 5′-GCT ATC TAT GCT GGT TTC CTC-3′ (reverse); and β-actin, 5′-CAG CAA GCA GGA GTA TGA CGA-3′ (forward) and 5′-GTG GAC TTG GGA GAG GAC TGG-3′ (reverse). The number of cycles was selected to allow linear amplification of the cDNA under study. PCR products were separated on 1.5% agarose gels and visualized by EtBr staining.

Quantitative real-time RT-PCR was performed essentially as described (39). The oligonucleotide primers and probes for the *OGG1* and β-actin genes were purchased from Applied Biosystems (Assays-on-Demand™). Quantitative measurements of each mRNA level were performed in triplicate. The accuracy of mRNA quantitation for each gene was confirmed by measurement of serially diluted control mRNA samples and comparison of the fluorescent intensity from a standard carve of the mRNA levels. Amplification of β-actin mRNA was performed with all samples to control the variation in mRNA levels. The gene expression levels were normalized to β-actin levels for each mRNA preparation, and the -fold increase in an individual gene was calculated by comparison with the result obtained without PonA stimulation. The non-template control was incubated in each amplification reaction to exclude the contaminating template.

HIV Infection and p24 Determination—PBMCs were stimulated with phytohemagglutinin for 48 h and infected with HIV-1_{MN}. HIV-1_{MN} was challenged at 100 $TCID_{50}$ (where $TCID_{50}$ is the tissue culture infectious dose resulting in 50% infected cells)/2.0–2.5 × 10^6 PBMCs or Jurkat cells for 1 h (43). These cells were washed twice with phosphate-buffered saline (PBS), and HIV-1 p24 antigen concentration in the culture supernatant was measured using a commercial kit (ZeptoMetrix Corp., Buffalo, NY).

mRNA Stability Assay—Total cellular RNA was serially prepared from Tat/293 and mTat/293 cells treated with PonA for 24 h, followed by the treatment with 2 μg/ml actinomycin D (Sigma). The amount of *OGG1* mRNA was analyzed by real-time RT-PCR as described above.

Detection of ROS—The detection of ROS was measured by flow cytometry with a FACScan (BD Biosciences) equipped with argon ion laser delivering 200 megawatts of power at 488 nm, and the results were analyzed using CellQuest™ software (BD Biosciences). After treatment of cells with PonA (10 μM) to induce Tat or mTat, the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (Molecular Probes, Inc.) was added to the culture at a final concentration of 5 μM, followed by incubation at 37 °C for 30 min. The cells were washed twice with PBS, resuspended in PBS, and then subjected to the fluorescence-activated cell sorter detection of 5,6-carboxy-2′,7′-dichlorofluorescein (green) at 530 nm.

Measurement of Intracellular Reduced GSH and GSSG Contents—The total cellular GSH and GSSG concentrations were measured using a glutathione quantification kit (Dojindo). Briefly, each cell culture (5 × 10^6 cells) was centrifuged at 2000 × *g* for 5 min, and the cell pellet was resuspended in 100 μl of PBS. The cell suspension was treated with 80 μl of 10 mM HCl, crashed by freeze/thawing repeated twice, and further treated by adding 20 μl of 5% sulfosalicylic acid to precipitate the proteins. The supernatant was obtained by centrifugation at 10,000 × *g* for 10 min, and the total GSH and GSSG concentrations were determined. The sample was incubated with GSH reductase and NADPH at 30 °C for 5 min, and the total GSH concentration thus generated was measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) at 30 °C for 10 min. Spectrophotometric detection of 5-mercapto-2-nitrobenzoic acid was performed at 415 nm. The GSSG concentration was determined by performing the same procedure, but GSH was masked by treatment with 2-vinylpyridine and triethanolamine prior to the reaction with GSH reductase and NADPH. The concentration of reduced GSH was determined by subtracting the GSSG concentration from the total GSH concentration. Each determination was performed in triplicate, and experiments were repeated at least twice.

Measurement of Manganese Superoxide Dismutase Activity—The enzymatic activity of manganese superoxide dismutase was measured using a WST-1™ superoxide dismutase assay kit (Dojindo) with slight modifications. Briefly, equal numbers of cells (1.5 × 10^7) were washed twice with PBS, and the cell lysates were extracted by freeze/thawing. The manganese superoxide dismutase activity in the supernatant protein lysate was determined by incubation with WST-1, xanthine, and xanthine oxidase at 37 °C for 20 min. To mask the copper superoxide dismutase and zinc superoxide dismutase activities, the protein lysate was treated with 1 mM KCN. The inhibitory action of manganese superoxide dismutase contained in each cell lysate was assessed by the spectrophotometric determination of WST-1 formazan at 450 nm. Quantitation was achieved by comparison with the absorption of standard manganese superoxide dismutase (Sigma) with known concentrations.

Electrophoretic Mobility Shift Assay—The experimental procedure was carried out as described previously (36). The AP-4 consensus sequence was taken from the 5′-AP-4 site in the *OGG1* promoter. The wild-type and mutant oligonucleotide sequences (sense strand) utilized were as follows: wild-type, 5′-CGG CAG GCA GCA GCT GTG GCG G-3′; and mutant, 5′-CGG CAG GCA GTC GCG ATG GCG G-3′. These oligonucleotides were labeled using a 5′-end labeling kit (Takara) in the presence [γ-^{32}P]dATP (Amersham Biosciences). DNA binding reactions were performed at 4 °C for 20 min in a 10-μl reaction volume. Analysis of binding complexes was performed by electrophoresis on 6% polyacrylamide gels with 0.5× Tris borate/EDTA buffer, followed by autoradiography. The specificity of DNA binding was assessed by preincubating extracts with anti-AP-4 antibody and competitor at room temperature for 10 min.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed according to the recommendations of Upstate Biotechnology, Inc., with some modification. Briefly, cells were cross-linked with 1% formaldehyde for 10 min at room temperature, washed twice with ice-cold PBS, and lysed for 10 min at 2 × 10^6 cells in 200 μl of SDS lysis buffer. The chromatin was sheared by sonication 13 times for 10 s at one-third of the maximum power with 20 s of cooling on ice between each pulse. Cross-linked released chromatin fractions were precleared with salmon sperm DNA and protein A-agarose beads for 1 h, followed by immunoprecipitation overnight with the desired antibodies at 4 °C. The immunoprecipitates were sequentially washed once with lysis buffer, twice with high salt buffer, twice with low salt buffer, and twice with Tris/EDTA buffer. After the wash, immune complexes were collected with salmon sperm DNA and protein A-agarose beads at room temperature for 1 h and extracted with 1% SDS and 0.1 M $NaHCO_3$. The eluted samples were reverse-cross-linked with proteinase K at 65 °C for 6 h and treated with RNase at 37 °C for 1 h. DNA was recovered by phenol/chloroform and chloroform extraction and ethanol precipitation. Finally, DNA was dissolved in 30 μl of Tris/EDTA buffer and subjected to PCR. The primer sequences were as follows: *OGG1* promoter (−615 to −450), 5′-CAA ACG TCC CAT TCC GAG GAA AG-3′ (forward) and 5′-GGC CTT TAG GCG TCC TCT GAG A-3′ (reverse); and β-actin promoter (−980 to −915; used as a control), 5′-TGC ACT GTG CGG CGA AGC-3′ (forward) and 5′-TCG AGC CAT AAA AGG CAA-3′ (reverse). The number of PCR cycles was as follows: 33 PCR cycles for all ChIP experiments and 25 PCR cycles for the input samples, in which PCR amplification was performed under the linear range of AP-4 binding to the *OGG1* promoter. For each reaction, 10% of the cross-linked released chromatin was saved and reversed by proteinase K digestion at 65 °C for 6 h, followed by DNA extraction; and the recovered DNA was used as input control.

Measurement of 8-Oxo-dG—The amounts of 8-oxo-dG in the cellular DNA were measured using a high performance liquid chromatography (HPLC)-electrochemical detector (ECD) system, which is highly selec-

tive, with sensitivity at the femtomole level, as described previously (26, 44). Briefly, cellular DNA was isolated using a DNA extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan). The isolated DNA was digested with P1 nuclease (Wako Pure Chemical Industries) to obtain 8-oxo-dG in the nucleoside form (8-hydroxydeoxyguanosine). The nucleoside solution was filtered with an Ultrafree Probind filter (Millipore Corp.) and injected into an HPLC column (CAPCELL PAK C_{18} MG, Shiseido, Tokyo, Japan) equipped with an ECD (Coulochem II, ESA, Inc.) at a flow rate of 0.8 ml/min with the mobile phase consisting of 10 mM Na_2HPO_4 and 8% methanol. The 8-oxo-dG value in the DNA was calculated as the number of 8-oxo-dG residue/10^6 dG residues.

RNA Interference—siRNA with two thymidine residues (dTdT) at the 3′-end of the sequence was synthesized by Takara. The target sequences were as follows: OGG1 No. 1, 5′-GCC UUC UGG ACA AUC UUU C-3′; OGG1 No. 2, 5′-GCC UUC UGG ACA AUC UUU C-3′; OGG1 No. 3, 5′-GGC UCA GAA AUU CCA AGG U-3′; and green fluorescent protein, 5′-GGC UAC GUC CAG GAG CGC ACC-3′. Transfection of siRNA was performed using Lipofectamine 2000 reagent (Invitrogen). Cells were incubated for 72 h and harvested for the analysis of 8-oxo-dG and OGG1 protein expression.

RESULTS

Establishment and Characterization of Ecdysone-inducible Cell Lines Expressing Tat and mTat—Because Tat is known to impair cell viability and its activity in long-term maintenance of cells expressing Tat may preselect certain cell types and preclude exploration of the Tat-mediated alteration of cellular functions, we adopted a stringent ecdysone-inducible system using PonA, a phytoecdysteroid that is inert in mammals (45). To generate Tat- or mTat-expressing cells upon treatment with PonA, we transfected the pIND-Tat or pIND-mTat plasmid into HEK293-EcR cells stably transfected with pVgRXR expressing the receptor for ecdysone (PonA). These cells were singly isolated by two successive rounds of limiting dilution and screened for expression of Tat proteins and their transcriptional activity in stimulating HIV gene expression.

As shown in Fig. 1*A*, the expression of Tat and mTat proteins was detected after 12 h of PonA treatment and was maintained for at least an additional 60 h. (PonA dose-dependent Tat expression is also shown.) In Fig. 1*B*, Tat-mediated HIV-1 transactivation was examined. As expected, Tat (but not mTat) augmented HIV-1 gene expression in a PonA dose-dependent manner. Fig. 1*C* shows that Tat (but not mTat) bound to endogenous cyclin T1 in cells as reported previously (6–8). These results indicate that both Tat/293 and mTat/293 cells inducibly express Tat proteins and that the functional integrity of Tat is maintained in Tat/293 cells. Thus, we explored the gene expression profile in these cells.

Gene Expression Profile Analysis of Cells Expressing Tat—To identify genes either up-regulated or down-regulated by Tat in the newly established ecdysone-inducible cell lines, the gene expression profiles were compared with and without Tat expression. The mRNA was isolated from Tat/293 and Lac Z/293 cells without (control) and with PonA treatment. The cDNA probes were synthesized from each mRNA, labeled with Cy5 (for Tat- or LacZ-expressing cells) or Cy3 (for non-expressing cells), and hybridized to a gene chip (human 3K DNA CHIP™). Representative results are shown in Fig. 2*A*, where we compared genes expressed in Tat-expressing (Cy5-labeled) and non-expressing control (Cy3-labeled) 293/Tat cells. Similar comparisons were made with LacZ-expressing cells (data not shown).

As shown in Fig. 2*A*, 12 genes, including *TFPI2*, stanniocalcin-1, *SEPP1*, and *OGG1*, were up-regulated by >2-fold by Tat after 24 h of induction upon PonA treatment. Five of these 12 genes were up-regulated by >2-fold even after 12 h of Tat induction. The details of these genes are summarized in Table I. In control LacZ/293 cells, expression of the *TFPI2* gene was up-regulated by 2.0-fold when cells were treated with PonA, suggesting nonspecific stimulation by PonA (data not shown). On the other hand, eight genes were down-regulated to <60% after 24 h of Tat induction (summarized in Table II). Down-regulation of these genes was not observed in LacZ-expressing cells (data not shown).

To confirm these results, we carried out RT-PCR analysis to examine the mRNA levels of Tat-regulated genes before and after Tat induction. We also examined the effect of mTat to further confirm the specificity of Tat action. Fig. 2*B* shows the results of eight genes up-regulated by Tat by >2.3-fold (stanniocalcin-1, *SEPP1*, *OGG1*, *MEN1β2* homolog FLJ23538/clone 137308, *ETV5*, *SLC20A1*, integrin α7, and *ENPP2*; excluding *TFPI2*). Among these genes, stanniocalcin-1, *SEPP1*, and *ETV5* were also up-regulated by induction of mTat or even LacZ, suggesting a nonspecific effect of PonA. Thus, we concluded that five genes (*OGG1*, *MEN1β2* homolog FLJ23538/clone 137308, integrin α7, *SLC20A1*, and *ENPP2*) are specifically up-regulated by Tat because these genes were not up-regulated by either mTat or LacZ. Whereas *ENPP2* was up-regulated after 12–24 h of Tat induction and subsequently down-regulated, the other four genes were constitutively up-regulated.

Similarly, RT-PCR analysis was performed with the eight genes down-regulated by Tat (Fig. 2*C*). Two of the genes (*SLC1A3* and *LTA1*) were down-regulated by mTat or LacZ. The other six genes were down-regulated by Tat, but not by mTat or LacZ. Among the genes down-regulated by Tat, *NDRG1*, *RGS16*, and hexokinase-2 are known to be under the transcriptional control of p53 (46–48), an observation consistent with previous reports of Tat down-regulating the action of p53 (49, 50).

Induction of OGG1 Gene Expression by Tat—Because Tat up-regulated the *OGG1* gene the most, we further analyzed the effect of Tat on *OGG1* mRNA expression and stability. The human *OGG1* gene encodes two isoforms, type 1 (a and b) and type 2 (a, b, and c), resulting from alternative splicing of the single *OGG1* precursor mRNA (51). As shown in Fig. 3*A*, Tat induced all types of *OGG1* mRNA. We carried out real-time RT-PCR to determine more precisely the mRNA levels of *OGG1* before and after Tat induction. As shown in Fig. 3*B*, *OGG1* gene expression was up-regulated by 3.6-, 6.7-, 9.8-, and 8.2-fold upon Tat expression after 6, 12, 24, and 48 h of PonA treatment, respectively. mTat did not affect *OGG1* gene expression (Fig. 3*B*). A similar extent of stimulation was observed for OGG1 protein levels as revealed by Western blotting (Fig. 3*C*). No induction of OGG1 protein was observed when mTat was induced. Induction of OGG1 protein by Tat (but not mTat) was confirmed in Jurkat T cells, a natural host of HIV-1 infection (Fig. 3*C*, *right panels*). Furthermore, the effect of HIV-1 infection on *OGG1* gene expression was examined with PBMCs isolated from two individuals and Jurkat cells. These cells were infected with $HIV\text{-}1_{MN}$, and the *OGG1* mRNA level was quantified by real-time RT-PCR together with the amounts of HIV-1 produced in the culture supernatant. As shown in Fig. 3*D*, up-regulation of *OGG1* mRNA levels upon HIV-1 infection was observed and was associated with elevation of viral p24 antigen levels. Mock infection did not induce *OGG1* expression (data not shown).

Furthermore, we examined the effect of Tat on the stability of *OGG1* mRNA using PonA-inducible cells. After 24 h of Tat or mTat induction, cells were treated with actinomycin D, and total RNA samples were obtained after 1, 3, 5, and 7 h of actinomycin D treatment to determine the level of *OGG1* mRNA. As shown in Fig. 3*E*, the decay profiles of *OGG1* mRNA were similar in cells expressing Tat and mTat (control), with half-lives of 5.0 and 4.3 h, respectively. These findings indicate that the positive effects of Tat on *OGG1* gene expression are at the level of transcription.