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Human Immunodeficiency Virus Type 1 (HIV-1) Accessory Protein Vpr Induces Transcription of the HIV-1 and Glucocorticoid-Responsive Promoters by Binding Directly to p300/CBP Coactivators

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The accessory Vpr protein of human immunodeficiency virus type 1 (HIV-1) is a promiscuous activator of viral and cellular promoters. We report that Vpr enhances expression of the glucocorticoid receptor-induced mouse mammary tumor virus (MMTV) promoter and of the Tat-induced HIV-1 long terminal repeat promoter by directly binding to p300/CBP coactivators. In contrast, Vpr does not bind to p/CAF or to members of the p160 family of nuclear receptor coactivators, such as steroid receptor coactivator 1a and glucocorticoid receptor (GR)-interacting protein 1. Vpr forms a stable complex with p300 and also interacts with the ligand-bound glucocorticoid receptor in vivo. Mutation analysis showed that the C-terminal part of Vpr binds to the C-terminal portion of p300/CBP within amino acids 2045 to 2191. The same p300 region interacts with the p160 coactivators and with the adenovirus E1A protein. Accordingly, E1A competed for binding to p300 in vitro. Coexpression of E1A or of small fragments of p300 containing the Vpr binding site resulted in inhibition of Vpr's transcriptional effects. The C-terminal part of p300 containing the transactivating region is required for Vpr transactivation, whereas the histone acetyltransferase enzymatic region is dispensable. Vpr mutants that bind p300 but not the GR did not activate expression of the MMTV promoter and had dominant-negative effects. These results indicate that Vpr activates transcription by acting as an adapter linking transcription components and coactivators.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vpr affects both viral replication and integration and cellular transcription, proliferation, differentiation, and apoptosis (4, 11, 18, 51, 63). Vpr is a 96-amino-acid virion-associated protein that has been shown to be important for virus propagation in vivo (23, 26). Vpr is packaged in significant quantities into viral particles (10, 52) and is imported into the nucleus soon after infection. These observations suggest that Vpr plays a role in early events in the viral cycle (35). Vpr was proposed to participate in the nuclear translocation of the HIV-1 preintegration complex (31, 54, 64). In addition, Vpr efficiently arrests cells at the G_2/M phase of the cell cycle (29, 36, 56, 57). Cyclin B/p34^{cdc2} kinase activity is suppressed in Vpr-expressing cells (29, 36, 56). Although the effects of this arrest on the virus life cycle are not fully understood, it has been proposed to enhance HIV-1 replication (26). Using a genetic approach in the fission yeast *Schizosaccharomyces pombe*, Vpr cell cycle activity was found to be associated with gene products of *wee1*, *ppa2*, and *rad24*, all of which function upstream of p34^{cdc2}, modulating its phosphorylation status (46).

A third function of Vpr is its action as a promiscuous transcriptional activator of several viral and cellular promoters. Although this was the first function assigned for Vpr (11, 12), its mechanism has remained unclear. It is apparent that Vpr has many interactions with cellular and viral proteins, and two-hybrid system analysis of Vpr interactors shows many potential candidates. It has therefore been challenging to identify among Vpr's multiple interactions those necessary for in vivo function.

Recent results have indicated that the function of Vpr as a transcriptional activator is affected by nuclear coactivators. It has been suggested that the transcriptional effects of Vpr on the HIV-1 long terminal repeat (LTR) promoter are mediated by the p300 coactivator (20, 34). The mechanism of transcriptional activation has not been well understood, and it has been suggested to be indirect (20, 34). We previously found that Vpr showed functional cooperation with p300/CREB-binding protein (CBP) and steroid receptor coactivator 1 (SRC-1) in enhancing transcription of another promoter, the glucocorticoid-activated mouse mammary tumor virus (MMTV) LTR promoter (38). We showed that Vpr markedly potentiates glucocorticoid receptor (GR) action on its responsive promoters, acting as a nuclear receptor coactivator. Like host nuclear receptor coactivators p300/CBP and the p160s, Vpr contains a

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coactivator signature motif, LXXLL, at amino acids 64 to 68. We also showed that Vpr is a general coregulator of nuclear receptors that influences not only the GR but also the progesterone and estrogen receptors. In contrast, Vpr did not affect transcription of the cyclic AMP-responsive somatostatin promoter. In addition to binding to the GR, Vpr was also reported to interact with the general transcription factor TFIIB, thus providing a link between nuclear receptors and the transcriptional machinery (1, 2).

The ligand-bound GR interacts with several recently described nuclear coactivators, which help transform the glucocorticoid complex signal to the transcription initiation complex. Several coactivators have histone acetyltransferase (HAT) activity, through which they alter the chromatin structure and facilitate access and/or binding of transcription machinery components to DNA (25, 47). Moreover, HAT activity modulates the binding of transcription factors to specific binding elements on their responsive promoters (8, 28) and the dissociation (9) of coactivators from nuclear receptors or other transcription factors. One family of these coactivator molecules, consisting of the homologous p300 and CBP, may serve as macromolecular docking platforms for transcription factors from several signal transduction cascades, including, in addition to nuclear receptors, CREB, AP-1, NF-κB, p53, Ras-dependent growth factor, and STATs (3, 5, 22, 25, 33, 37, 42, 47, 67). Because of their central position in many signal transduction cascades, the p300/CBP coactivators have been also called cointegrators. p/CAF, originally reported as a human homologue of yeast GCN5 that interacts with p300/CBP, is also a broad coactivator with HAT activity (6, 25, 66). Coactivator molecules interacting preferentially with nuclear receptors have also been described and include members of the p160 family of proteins: SRC-1; TIF-II or GRIP-1, also called SRC-2; the p300/CBP/cointegrator-associated protein; ACTR or RAC3, also called SRC-3 (25, 44, 47); and the recently reported riboprotein steroid receptor coactivator (43). These different classes of proteins form complexes with the ligand-activated nuclear receptors that interact with components of the transcription machinery on the promoter regions of responsive genes. p300/CBP and the members of the p160 family of coactivators contain one or more copies of the coactivator signature motif sequence LXXLL, which is necessary for interaction with the nuclear hormone receptors (25, 30, 44, 47).

In this study, we analyze the interactions of Vpr with the host coactivators p300 and CBP in an attempt to further understand the mechanisms by which Vpr exerts its transcriptional effects. It has been proposed that transcriptional effects of Vpr are a direct consequence of cell cycle arrest at G_2. In contrast, we have concluded that the cell cycle and transcriptional effects of Vpr are distinct, based on analysis of function of Vpr mutants (27, 38). This conclusion has been supported by other investigators (61). In agreement with our previous work, we show that Vpr acts as an adapter, bringing together different components of the transcription machinery. In addition to its GR binding (38), we show that Vpr also binds directly to p300/CBP. Our results are consistent with a function of Vpr as an adapter molecule, efficiently attracting coactivators to the site of transcription.

MATERIALS AND METHODS

Plasmids. Plasmids pCDNA3-VPR, pCDNA3-VPRL64A, pCDNA3-VPRR80A, pGEX-4T3-VPR, and pCMV-FLAG-VPR, used for expression of the Vpr wild type, L64A and R80A mutants, glutathione *S*-transferase (GST)-fused Vpr, and FLAG epitope-tagged Vpr, were reported previously (38). pCDNA3-VPRL64,67,68A, pCDNA3-VPRI70A,H71A, pCDNA3-VPRF72A,R73A, pCDNA3-VPRI74A,G75A, pCDNA3-VPRC76A,R77A, and pCDNA3-VPRH78A,S79A were constructed by replacing indicated amino acid codons of Vpr with alanine codons in pCDNA3-VPR, using a PCR-assisted in vitro mutagenesis reaction. In vitro Vpr expression vectors for Vpr(1-84), Vpr(1-64), and Vpr(44-96) were constructed by subcloning PCR-amplified Vpr fragments, using pCDNA3-VPR as a template, into pCDNA3 (Invitrogen, Carlsbad, Calif.). pM-VPR and pVP16-VPR were constructed by subcloning full-length Vpr cDNA into pM and pVP16, respectively (Clontech, Palo Alto, Calif.). BS-Tat and pL3-Tat express two-exon and one-exon Tat, respectively, under the control of the HIV-1 LTR. L3-Luc contains a full-length HIV-1 LTR promoter, which drives a luciferase reporter gene. $pNL_{4\text{-}3}\Delta$Tat, an HIV-1 molecular clone that does not express Tat protein, was constructed by introduction of six stop codons in the open reading frame of the first exon of the two-exon Tat. $pNL_{4\text{-}3}\Delta$VprΔTat was created by replacing a fragment of $pNL_{4\text{-}3}\Delta$Tat with one carrying a 115-bp deletion in a Vpr gene which originated from the plasmid $pNL_{4\text{-}3}$p210-19 5′ (24). Expression vectors for GST-p300(1925-2191), GST-p300(2184-2414), GST-p300(1925-2045), and GST-p300(2045-2191) were constructed by introducing PCR fragments of corresponding portions of p300 cDNA, amplified from pCDNA3-GAL4-p300(1-2414) (a gift from A. Giordano, Jefferson Medical College, Philadelphia, Pa.), into pGEX-4T3 (Amersham Pharmacia Biotech, Piscataway, N.J.). pGEX-4T3-GRα, which expresses GST-GRα fusion protein, was constructed by inserting the human GRα coding region into pGEX-4T3. pCDNA3.1-His/A-p300(2045-2191) was constructed by subcloning a PCR-amplified cDNA fragment corresponding to p300(2045-2191) from pCDNA3-GAL4-p300(1-2414) into pCDNA3.1-His/A (Invitrogen). pM-SRC-1a was constructed by subcloning SRC-1a cDNA from pCR3.1-SRC1a (a gift from B. W. O'Malley, Baylor College of Medicine, Houston, Tex.) into pM (Clontech). The other plasmids used were pFLAG-CMV2 (Eastman Kodak Co., Rochester, N.Y.), pSV40-β-Gal (Promega, Madison, Wis.), pCMV-Luc (38), Bluescript SK(+) (Stratagene, La Jolla, Calif.), pHook-1 (Invitrogen), pCMVβ-p300-CHA (a gift from D. Livingston), pCDNA3-GAL4-p300(1945-2414) (a gift from A. Giordano), GAL-CBP(1687-2441) (a gift from R. H. Goodman, Oregon Health Science Center), GST-CBP(1990-2441) (a gift from R. G. Roeder, The Rockefeller University, New York, N.Y.), GST-p300(10-596) (a gift from D. M. Livingston, Dana Farber Cancer Institute, Boston, Mass.), GST-p300(744-1571) and GST-p300(1572-2371) (gifts from R. Eckner, University of Zürich, Zürich, Switzerland), GAL4-p/CAF (a gift from B. W. O'Malley), GAL-CREB and pCMV-12S-E1A (gifts from Y. Shi, Harvard Medical School, Boston, Mass.), and MMTV-LTR-Luc (a gift from G. Hager, National Institutes of Health, Bethesda, Md.).

In vitro binding assay. ^{35}S-labeled Vpr and its mutants, p300 and SRC-1a, were generated by in vitro translation from pCDNA3-VPR wild-type and mutant plasmids [pCDNA3-GAL4-p300(1-2414) and pCR3.1-SRC1a, respectively], using wheat germ extract. They were tested for interaction with GST-p300 or CBP fragments and GST-VPR immobilized on glutathione-Sepharose beads in buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, and 0.1 mg of bovine serum albumin/ml at 4°C for 1.5 h. Chemically synthesized, purified, 96-amino-acid-long wild-type Vpr polypeptide from the HIV-1 $NL_{4\text{-}3}$ strain and anti-Vpr antibody, which were kindly provided by J. B. Kopp (National Institutes of Health, Bethesda, Md.), were also used in the assays whose results are presented in Fig. 1D (32). After vigorous washing with the buffer, proteins were eluted and separated on sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis (14% SDS-PAGE) gels for Vpr and its mutants or on 6% gels for p300 and SRC-1a. Three to five percent of the total input of in vitro-translated and -labeled proteins or Vpr peptide was loaded as a marker. Gels were fixed and exposed to film.

Cell transfections and reporter assays. A204 and CV-1 cells were transfected using Lipofectin (Life Technologies, Gaithersburg, Md.), as described previously (38). HeLa cells were transfected, using a $CaPO_4$ method, by culturing cells with precipitates for 18 h (19). In experiments using viral molecular clones, HeLa cells were transfected with Effectene reagent (Qiagen Inc., Valencia, Calif.). Jurkat cells were transfected using electroporation, as described previously (38). For the experiments using L3-Luc or MMTV-LTR-Luc as a reporter plasmid for A204 and HeLa cells, 0.5 to 1.0 μg of Vpr wild-type- and/or mutant-expressing plasmids/well and 1.0 to 2.0 μg of p300 fragment- and/or E1A-expressing plasmids/well were used with 0.5 μg of L3-Luc/well or 1.5 μg of MMTV-LTR-Luc/well. Empty vectors were used to maintain the same amount of transfected DNA.

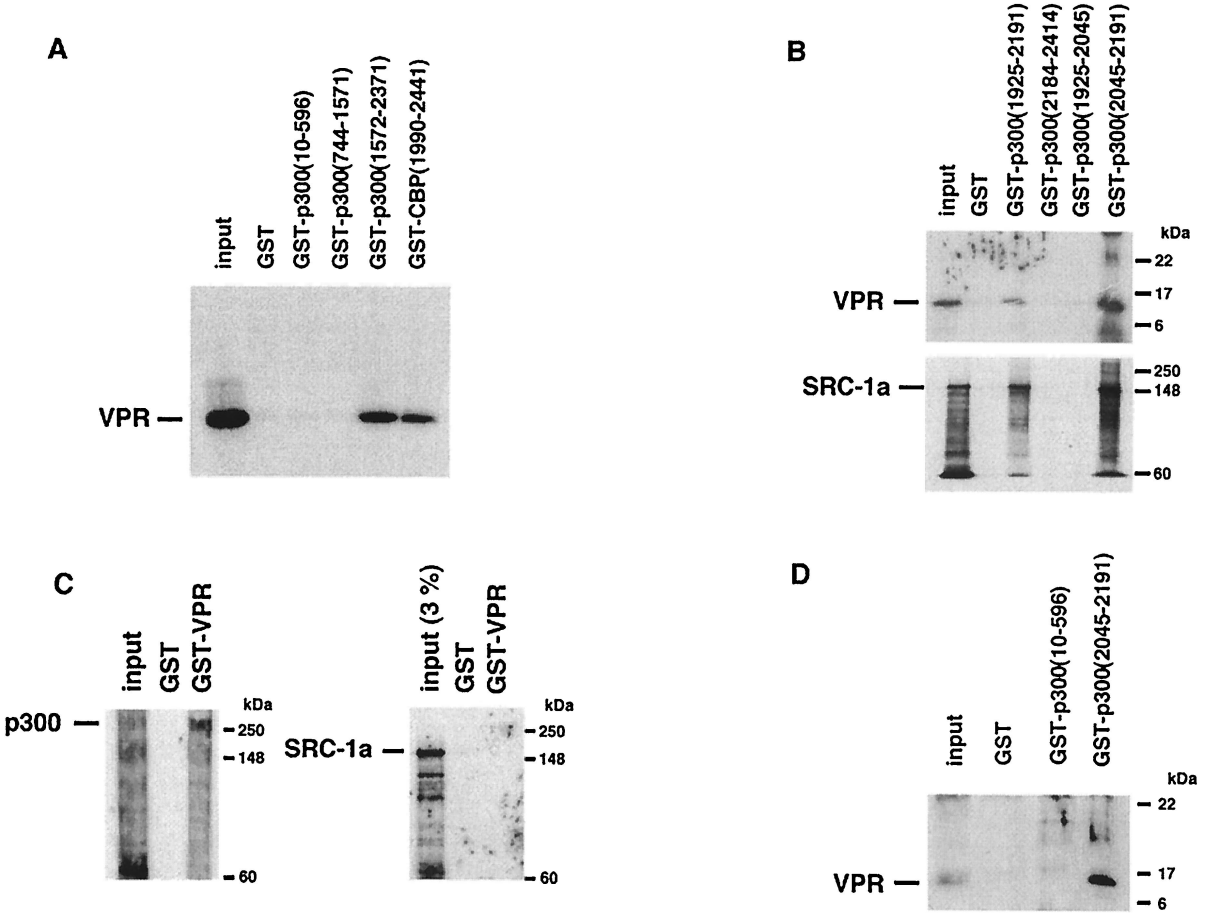

FIG. 1. In a GST pull-down assay, Vpr binds directly to the C terminus of p300/CBP but not to SRC-1a. (A) Vpr binds to p300(1572-2371) and CBP(1990-2441). In vitro-translated and -labeled Vpr was incubated with bacterially produced GST fusions to p300 or CBP, as indicated at the top of the panel. Numbers in parentheses indicate the amino acids expressed by each fusion. Samples were run on a 14% SDS-PAGE gel and detected by autoradiography. The input control lane contains approximately 10% of the amount of Vpr added in the pull-down reactions. (B) Both Vpr and SRC-1a bind to p300(2045-2191). In vitro-translated and -labeled Vpr or SRC-1a was incubated with bacterially produced GST-p300 fragment fusions. Samples were run on a 14% SDS-PAGE gel for Vpr or a 6% SDS-PAGE gel for SRC-1a and detected by autoradiography. (C) p300 but not SRC-1a binds to Vpr. In vitro-translated and -labeled p300 or SRC-1a was incubated with bacterially produced GST-Vpr fusion or GST as the control. Samples were run on a 6% SDS-PAGE gel and detected by autoradiography. (D) Synthetic Vpr binds to p300(2045-2191) but not to p300(10-596). Synthetic Vpr peptide was incubated with bacterially produced GST fusions to p300 fragments of amino acids 10 to 596 or 2045 to 2191. Samples were run on a 14% SDS-PAGE gel and detected with anti-Vpr antibody after blotting.

pSV40-β-Gal (0.5 μg/well) was also included to normalize luciferase activity in all transfections. BS-Tat (1.0 μg/well) was used for L3-Luc at transfection, and 10^{-6} M dexamethasone was added into the culture medium 24 h after transfection for MMTV-LTR-Luc. For electroporation of Jurkat cells, the amount of plasmids used was 10 times larger. Cell lysates were collected 48 h after transfection, and luciferase and β-galactosidase activities were determined as described previously (38).

Mammalian two-hybrid assay. Using Lipofectin (Life Technologies), CV-1 cells were transfected with 0.3 μg of vector expressing GAL4-DNA binding domain (DBD) fusion protein/well, a VP16-activation domain (AD) fusion protein expression vector, pGAL4-E1B-Luc, and 0.1 μg of pSV40-β-Gal/well. Cells were harvested 48 h after replacing the transfection medium, and luciferase and β-galactosidase activities were measured. The effect of Vp16-Vpr on the GAL4-protein fusions was measured as severalfold activation compared to their activity using VP16.

Coimmunoprecipitation experiments. HeLa cells were grown in 175-cm^2 flasks and transfected with 15 μg of pFLAG-CMV2 or pCMV-FLAG-VPR/flask and 45 μg of pCDNA3, pCMV-12S-E1A, or pCDNA3.1-His/A-p300(2045-2191)/flask, together with 60 μg of pHook-1/flask. Dexamethasone (10^{-5} M) was added to the medium 5 h before harvest (see Fig. 4C). Transfected cells were sorted by the pHook-1 method according to the company's recommendations. Cell lysis and coimmunoprecipitation were carried out using lysis buffer (50 mM Tris-HCl [pH 7.4], 400 mM NaCl, 0.2% NP-40, Complete tablets [1 tab/50 ml; Roche Molecular Biochemicals, Indianapolis, Ind.]). Proteins were precipitated by anti-human p300 antibody, anti-GR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) or anti-FLAG (M2) antibody (Eastman Kodak Co.) bound to protein A Trisacryl (Pierce, Rockford, Ill.). After separation of proteins in SDS-polyacrylamide gels and blotting on nitrocellulose membrane, FLAG-tagged Vpr, p300, or GR was detected by anti-FLAG (M2) antibody, anti-p300 antibody, or anti-GR antibody, respectively. Anti-His antibody, anti-GAL4 antibody (Santa Cruz Biotechnology), and polyclonal anti-Vpr antibody (a gift of J. B. Kopp) were used to detect His-p300(2045-2191), GAL4-p300(1945-2414), and Vpr mutants, respectively.

Viral experiments using HIV-1 molecular clone. HeLa cells were plated in 60-mm-diameter dishes and were transfected with 0.15 μg of $pNL_{4-3}\Delta Tat$ or $pNL_{4-3}\Delta Vpr\Delta Tat$/dish, 0.5 μg of pCMVβ-300-CHA/dish, 0.55 μg of pL3-Tat/dish, and pCMV-Luc reporter plasmid. Empty vector was added to maintain the same amount of total DNA. Cell culture medium and cell lysates were collected 38 h after transfection, and $p24^{gag}$ protein production was determined by enzyme-linked immunosorbent assay using a ZeptoMetrix kit (ZeptoMetrix Corporation, Buffalo, N.Y.). $p24^{gag}$ values were normalized to luciferase reporter values, and the total amount of synthesized $p24^{gag}$ was calculated.

RESULTS

Vpr binds directly to p300/CBP. To determine whether Vpr binds directly to p300 and the related coactivator CBP, we employed a GST pull-down assay using in vitro-translated and -labeled Vpr and bacterially produced GST protein (Fig. 1A), which was fused to different fragments of the p300 or CBP proteins as indicated by the numbers in parentheses. Vpr was associated with p300(1572-2371) and CBP(1990-2441) fragments but not with p300(10-596) or p300(744-1571). To identify the shortest fragment of p300 interacting with Vpr, we constructed a series of GST-p300 expression vectors having smaller fragments of the Vpr-interacting region p300(1925-2414) (Fig. 1B). Vpr interacted with p300(1925-2191) and p300(2045-2191), whereas p300(2184-2414) and p300(1925-2045) returned negative scores in this assay. Since SRC-1a is known to bind to a similar domain of CBP (37, 41), we examined the association of Vpr or SRC-1a with these p300 fragments in the same experiment. We verified that Vpr and SRC-1a bound to the same fragments of p300, demonstrating that Vpr and SRC-1a interact with the same region of p300. To support these conclusions, we also tested the binding of in vitro-translated full-length p300 and SRC-1a to a GST-Vpr fusion in the same GST pull-down assay (Fig. 1C). p300 bound efficiently to Vpr, whereas SRC-1a did not. Since the proteins used for the GST pull-down assay were produced using wheat germ extract, we wished to exclude the possibility that any wheat germ factors contributed to the Vpr-p300 association. We therefore used a preparation of chemically synthesized, purified Vpr to repeat the GST pull-down assays. Similar results were obtained with the purified Vpr, demonstrating a direct binding of Vpr to p300 and CBP (Fig. 1D).

Results supporting the interaction of Vpr with p300 and CBP were also obtained using a mammalian two-hybrid assay (Fig. 2). We first showed that GAL4-Vpr fusion alone did not stimulate the GAL4-E1B promoter, whereas a positive control, GAL4-p300(1945-2414), strongly activated this promoter (Fig. 2A). This indicated that Vpr does not have intrinsic transactivation activity. We next examined the interaction of Vpr fused to the VP16 activation domain with several GAL4 fusion constructs. VP16-Vpr enhanced GAL4-p300(1-2414) activity but not that of GAL4-CREB, GAL4-SRC-1a, or GAL4-p/CAF (Fig. 2B). This suggested that Vpr interacts with p300 but not with CREB, SRC-1a, or p/CAF. We obtained similar results in a yeast two-hybrid system (data not shown). To map the interaction, we studied p300 deletions in the same assay. Vpr interacted with p300(Δ244-1973) (Fig. 2C). A similar fragment of CBP protein also interacted with Vpr, in agreement with the results shown in Figure 1A.

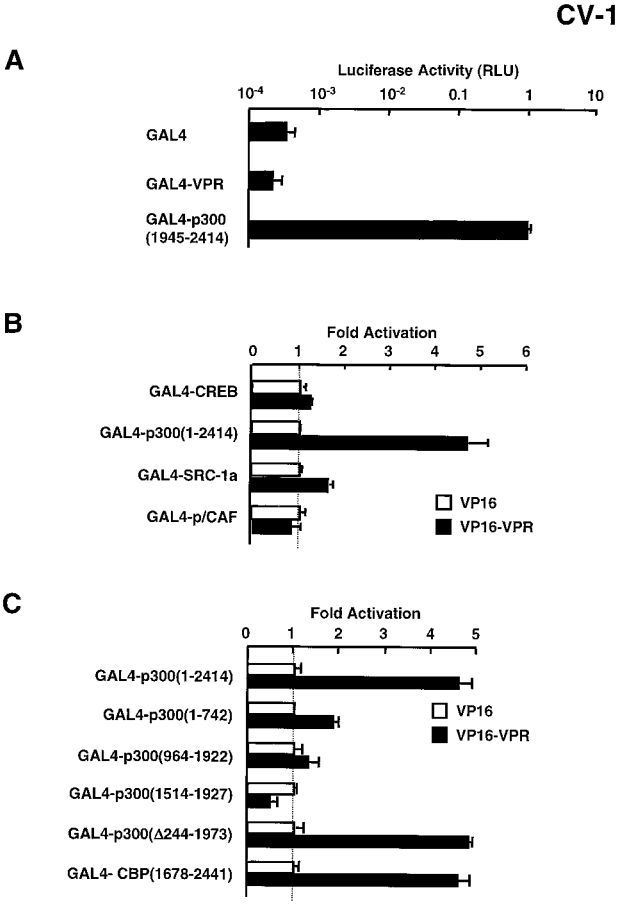

FIG. 2. Interaction of Vpr with p300 in a mammalian two-hybrid assay. (A) CV-1 cells were transfected with pM, pM-VPR, or pCDNA3-p300(1945-2414), together with pGAL4-E1B-Luc and pSV40-β-Gal. Bars show the means ± standard errors (SE). (B) CV-1 cells were transfected with plasmids expressing GAL4-DBD fusions to CREB, p300, SRC-1a, or p/CAF, together with pGAL4-E1B-Luc and pSV40-β-Gal. pVP16 or pVP16-VPR (open or filled bars, respectively) were also cotransfected. Severalfold induction was determined by calculating the ratio of normalized luciferase values of cells transfected with VP16-VPR to those of cells transfected with VP16. Bars show the mean ± SE. (C) CV-1 cells were transfected with plasmids for GAL4-p300 full-length or fragments together with pGAL4-E1B-Luc and pSV40-β-Gal. pVP16 or pVP16-VPR was also cotransfected (open or filled bars, respectively). Severalfold induction was calculated by the ratio of normalized luciferase values of cells transfected with VP16-VPR to those of cells transfected with VP16. Bars show the means ± SE.

The C-terminal part of Vpr is required for p300/CBP binding. To map the region of Vpr that interacts with p300, we examined the association of a series of Vpr mutants with p300 or with the related coactivator CBP in a GST pull-down assay, using in vitro-translated and -labeled Vpr mutants and bacterially produced GST-p300(1572-2371) and GST-CBP(1990-2441) (Fig. 3A). VprL64A, VprL64,67,68A, VprR80A, and Vpr(44-96) bound to these p300 and CBP fragments. Vpr(1-84) interacted weakly, whereas Vpr(1-64) completely lost its ability to bind to the coactivators. Vpr(1-44) also did not bind p300/CBP (data not shown). This result suggests that the C-terminal part of Vpr (amino acids 64-84) is essential for p300/CBP binding. To define this region more precisely, we constructed a series of Vpr point mutants in which two neighboring amino acids in the region between amino acids 70 and 80 of Vpr were replaced with alanines. We tested the binding of these mutants to p300(2045-2191) (Fig. 3B). VprF72A,R73A and VprI74A,G75A did not bind to the p300 fragment, whereas all other Vpr mutants retained binding. We also confirmed that these two mutants lost interaction with p300 in a mammalian two-hybrid assay, performed as described above (data not shown). An additional mutant, VprL64,67,68A, with a mutated LXXLL motif (38) and no GR

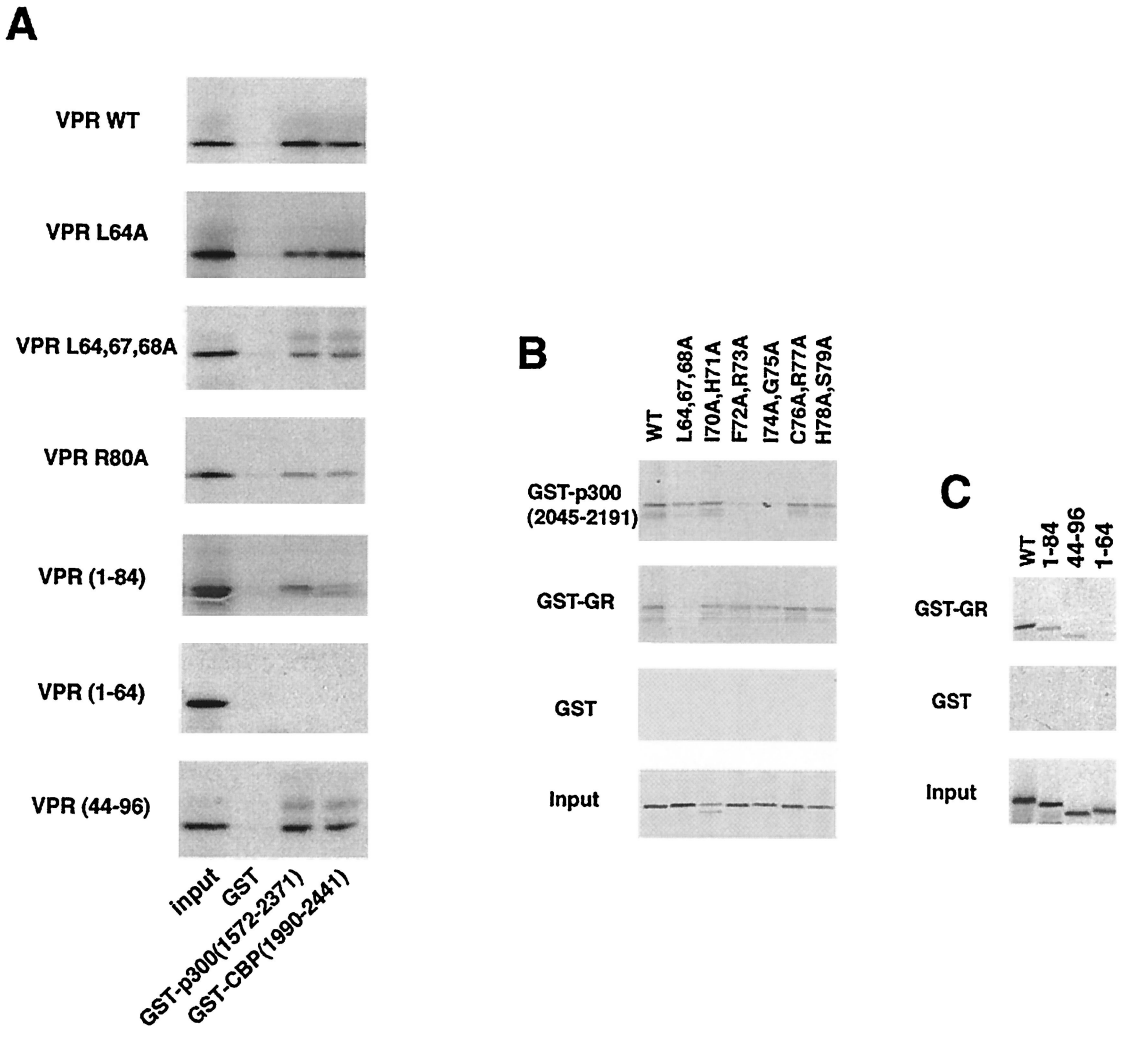

FIG. 3. (A) The C-terminal part of Vpr is necessary and sufficient for binding to p300. In vitro-translated and -labeled Vpr, VprL64A, VprL64,67,68A, VprR80A, Vpr(1-84), Vpr(1-64), or Vpr(44-96) was incubated with bacterially produced GST-p300(1572-2371), GST-CBP(1990-2441), or GST and tested for binding by the pull-down assay. Samples were run on a 14% SDS-PAGE gel and detected by autoradiography. (B) Vpr F72A,R73A and VprI74A,G75A lose binding to p300 while retaining binding to GRα. In vitro-translated and -labeled Vpr or the indicated mutants was incubated with bacterially produced GST-p300(2045-2191), GST-GRα, or GST and tested for binding by the pull-down assay. Dexamethasone (10^{-5} M) was added to the reaction mixture for GST-GRα. Samples were run on a 14% SDS-PAGE gel and detected by autoradiography. (C) Vpr(1-84) and Vpr(44-96) retained weak binding to GRα, whereas Vpr(1-64) lost binding. The indicated in vitro-translated and -labeled Vpr mutants were incubated with bacterially produced GST-GRα or GST and tested for binding by the pull-down assay. Dexamethasone (10^{-5} M) was added to the reaction mixture. Samples were run on a 14% SDS-PAGE gel and detected by autoradiography.

coactivator activity (unpublished data), was also tested for binding to p300 or GR. This mutant did not bind to GST-GRα, whereas it interacted with p300. All other Vpr mutants harboring mutations between amino acids 70 and 80 bound to GST-GRα. Vpr(1-84) and Vpr(44-96) weakly interacted with GST-GRα. These results suggest that Vpr uses two distinct sites within the same region to bind p300/CBP or GRα.

Vpr-p300 complexes are found in cells. To address whether Vpr-p300 complexes are formed in human cells, we transfected a vector expressing FLAG-tagged Vpr in HeLa cells and precipitated potential complexes, using specific antibodies against p300 or FLAG. The precipitated proteins were separated in SDS-polyacrylamide gels, and Vpr was visualized by probing with anti-FLAG(M2) antibody (Fig. 4A). Vpr was coimmunoprecipitated by a specific anti-human p300 antibody, indicating that it forms complexes with p300 in HeLa cells (Fig. 4A, lane 4). Since the Vpr binding site on p300 within the region of amino acids 2045-2191 is one of the sites of interaction of the adenovirus E1A protein with p300/CBP (41) and since E1A suppresses Vpr's transcriptional activity (20), we tested whether E1A 12S product or excess p300(2045-2191) fragment interfered with the binding of Vpr to p300 in the coimmunoprecipitation experiment. The presence of E1A or p300(2045-2191) fragment reduced coprecipitation of p300 and Vpr (Fig. 4A, lanes 5 and 6). Stable expression of the p300 fragment from pCDNA3-His/A-p300(2045-2191) was confirmed by Western blotting (Fig. 4B). These results demonstrated a direct interaction of Vpr with the region between amino acids

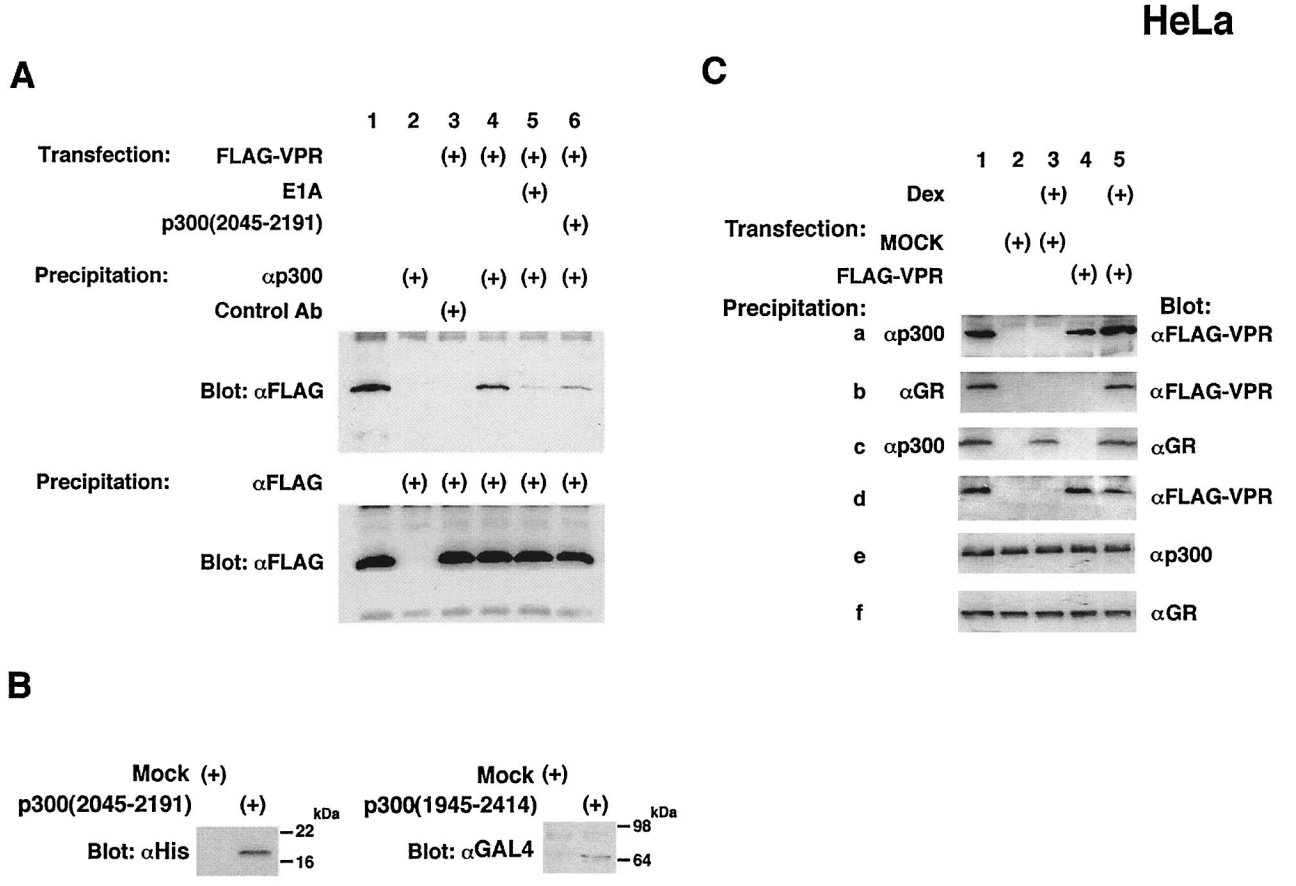

FIG. 4. (A) Vpr is coprecipitated with p300 from HeLa cell extracts. Coprecipitation is suppressed by E1A or p300(2045-2191). HeLa cells were transfected with pFLAG-CMV2 (lane 2) or pCMV-FLAG-VPR (lanes 3, 4, 5, and 6). pCMV-12S-E1A or pCDNA3.1-His/A-p300(2045-2191) was cotransfected in lanes 5 and 6, respectively. Vpr-p300 complexes were precipitated with anti-human p300 antibody and separated on a 14% SDS-PAGE gel. To determine the levels of expressed Vpr protein, Vpr was precipitated with anti-FLAG antibody from the same sample and separated on a 14% SDS-PAGE gel. Proteins were transferred to membranes and probed with anti-FLAG (M2) antibody. Five percent of the total lysate from cells transfected with pCMV-FLAG-VPR was run as a marker (lane 1). (B) His-p300(2045-2191) and GAL4-p300(1945-2414) are expressed in HeLa cells. HeLa cells were transfected with pCDNA3.1-His/A or pCDNA3-His/A-p300(2045-2191) or with pCDNA3 or pCDNA3-GAL4-p300(1945-2414), and cell lysates were analyzed on 14% or 10% SDS-PAGE gels, respectively. Transferred protein was blotted with anti-His or anti-GAL4 antibody. (C) Vpr forms a complex with p300 and GR in the presence of dexamethasone. HeLa cells were transfected with pFLAG-CMV2 (lanes 2 and 3) or pCMV-FLAG-VPR (lanes 4 and 5). Dexamethasone (10^{-5} M) was added to the medium 5 h before harvest. The Vpr/p300/GR complex was precipitated with anti-human p300 antibody or anti-GR antibody and separated on 14% or 8% SDS-PAGE gels, respectively. Proteins were transferred to membranes and probed with anti-FLAG(M2) antibody (gels a and b) or anti-GR antibody (gel c). Five percent of the total lysate from cells transfected with pCMV-FLAG-VPR was run as a marker (lane 1). To determine the levels of expressed Vpr, p300, or GR proteins, the same amounts of cell lysates were separated on 14%, 6%, or 8% SDS-PAGE gels and blotted with anti-FLAG(M2), anti-p300, or anti-GR antibody, respectively (gels d, e, and f).

2045 and 2191 of p300 coactivator in human cells. This interaction was inhibited by E1A, which binds to the same region of p300.

We have reported that Vpr formed complexes with the glucocorticoid-activated GRα in mammalian cells (38). To examine the Vpr complexes formed in cells in more detail, we studied the interaction of Vpr to p300 or GR in HeLa cells in the presence or absence of dexamethasone by coprecipitations (Fig. 4C). The result indicated that the three proteins formed a complex (Fig. 4Ca, b, and c, lanes 4 and 5) in a dexamethasone-dependent fashion. Vpr was precipitated with p300 in a dexamethasone-independent fashion (Fig. 4Ca, lanes 4 and 5), whereas the GR-Vpr complex was only found in the presence of dexamethasone (Fig. 4Cb, lanes 4 and 5). GR and p300 were coprecipitated in a dexamethasone-dependent fashion (Fig. 4Cc, lanes 3 and 5), as reported previously (59). This suggests that Vpr may be constitutively bound in the transcriptional complex containing p300. In response to dexamethasone, GR may then recruit the Vpr-p300 complex to the promoter region.

Functional significance. What is the functional significance of the Vpr-p300/CBP interaction? To answer this, we studied the expression and induction of two promoters affected by Vpr, namely, the HIV-1 LTR promoter and the MMTV glucocorticoid-inducible promoter, in the presence and absence of Vpr and/or p300. Transfection of either Vpr- or p300-expressing plasmids in A204 or Jurkat cells increased expression of these promoters above the induced levels achieved by Tat and dexamethasone, respectively (Fig. 5). In the absence of Tat or dexamethasone, the Vpr effects were increased up to twofold

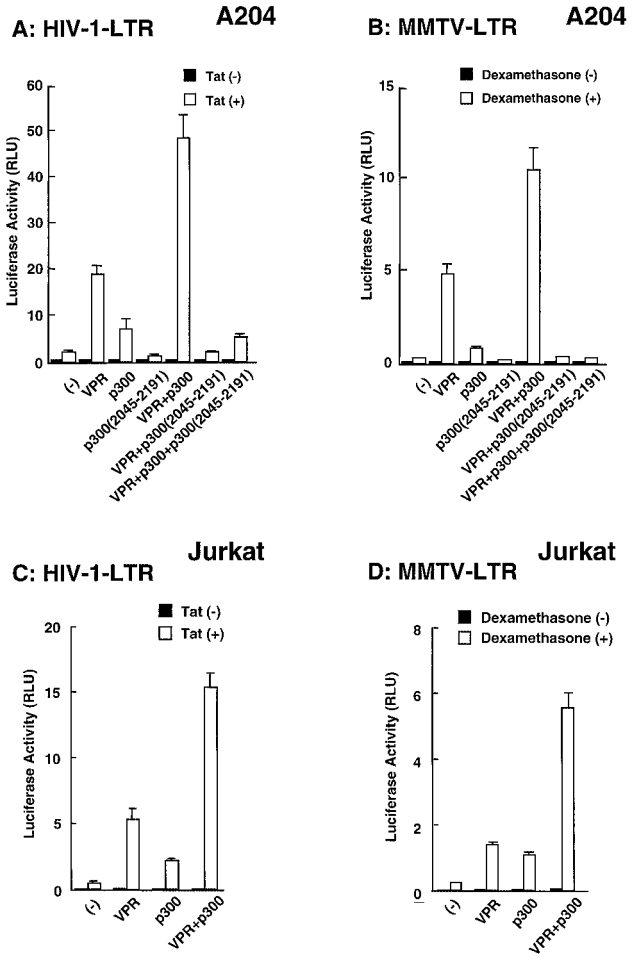

FIG. 5. Vpr and p300 synergistically enhance the HIV-1 and MMTV LTRs in both A204 (A and B) and Jurkat (C and D) cells. Overexpression of the p300(2045-2191) fragment abolishes Vpr's transcriptional activity and synergy with p300 on both the HIV-1 LTR and the MMTV LTR in A204 cells (A and B). Cells were transfected with L3-Luc or MMTV-LTR-Luc, together with pCDNA3-VPR, pCMVβ-p300-CHA, and pCDNA3.1-His/A-p300(2045-2191), as indicated. pSV40-β-Gal was included in all transfections. Bars represent mean ± SE values of the luciferase activity normalized for β-galactosidase activity in the absence or presence of BS-Tat (for the HIV-1 LTR) or in the absence or presence of 10^{-6} M dexamethasone (for the MMTV LTR).

TABLE 1. Effect of E1A on Vpr and p300 or p300(1945–2414) synergism on MMTV LTR in A204 cells[a]

E1A-expressing plasmid	Protein-expressing plasmid(s)	Luciferase activity (RLU)[b]	
		Dex (−)	Dex (+)
Absent	None	0.028 ± 0.0029	0.54 ± 0.037
	Vpr	0.018 ± 0.0044	5.25 ± 0.394
	p300	0.037 ± 0.0016	1.12 ± 0.265
	p300 (1945–2414)	0.031 ± 0.0065	0.58 ± 0.106
	Vpr + p300	0.025 ± 0.0038	15.92 ± 0.946
	Vpr + p300 (1945–2414)	0.029 ± 0.0123	14.05 ± 1.825
Present	None	0.027 ± 0.0055	0.28 ± 0.032
	Vpr	0.028 ± 0.0048	0.78 ± 0.295
	p300	0.031 ± 0.0138	0.32 ± 0.211
	p300 (1945–2414)	0.041 ± 0.0015	0.22 ± 0.141
	Vpr + p300	0.024 ± 0.0052	1.25 ± 0.231
	Vpr + p300 (1945–2414)	0.024 ± 0.0100	1.34 ± 0.381

[a] A204 cells were transfected with the indicated protein-expressing plasmids, MMTV-LTR-Luc, and pSV40-β-Gal in the absence or presence of the E1A-expressing plasmid, pCMV-12S-E1A. Cells were then exposed to 10^{-6} M dexamethasone [Dex (+)] or vehicle without dexamethasone [Dex (−)].

[b] Values are expressed as means ± SE.

and were not statistically different than those of basal levels. Similar results were obtained in other human cell lines, such as HeLa. Cotransfection of both p300 and Vpr resulted in strong synergistic effects on both promoters. Interestingly, the transactivation-inactive p300 fragment 2045-2191 containing the Vpr binding site did not activate promoter expression, but it inhibited the Vpr/p300 effects. Therefore, the Vpr-p300 interaction stimulates the expression of these promoters, and blocking of p300-Vpr interaction prevents activation. These results indicate that Vpr interaction with p300 has important consequences for promoter activation.

To define more details of Vpr and p300 interaction, we studied the effect of the presence of a set of p300 fragments on Vpr, using MMTV-LTR-Luc and L3-Luc in A204 cells (data not shown). In the presence of Vpr, fragment p300(1945-2414), containing the Vpr/SRC-1a binding site and the transactivation domain, was able to activate MMTV LTR promoter expression in the presence of dexamethasone to a level comparable to that of full-length p300, while this fragment could not enhance MMTV LTR in the absence of Vpr (Table 1). Since E1A and Vpr have one common binding region on p300 and since we showed that E1A reduced the binding of Vpr to p300 (Fig. 3), we further studied whether adenovirus E1A was able to affect Vpr-induced expression of the MMTV promoter and whether E1A abolished further enhancement of the Vpr action by p300 or p300(1945-2414) (Table 1). E1A suppressed dexamethasone-stimulated MMTV LTR activity approximately twofold. It also inhibited Vpr enhancing effect by seven- to eightfold. E1A abolished further enhancement of the Vpr action by p300 or p300(1945-2414). These results suggest that Vpr binds to the promoter through interaction with the GR via the LXXLL domain and efficiently recruits p300 through another interaction mediated by the region between amino acids 1945 and 2414 of p300, which is consistent with a model based on the hypothesis that Vpr acts as an adapter molecule, connecting different molecules required for high promoter activity.

We next tested the cooperative action of Vpr and p300 on the HIV-1 LTR in the context of the entire viral genome. It has been shown that absence of Vpr does not impede HIV or SIV propagation in cultured cells (14, 24). In agreement with this, a mutant HIV-1 molecular clone lacking Vpr replicated only about twofold less efficiently than the wild type after transfection of HeLa or Jurkat cells (data not shown). This indicated that Vpr function is not necessary under optimal in vitro viral growth conditions. Nevertheless, Vpr is known to be advantageous for viral replication in vivo and may be important for pathogenesis (23, 26, 65). Since Vpr is incorporated in the virion, it may be required for the stimulation of HIV-1 LTR at an early stage of the viral life cycle in which Tat may not be optimal (35). Therefore, we examined Vpr's effect on a Tat-

TABLE 2. p300 induction of $p24^{gag}$ from HIV-1 mutant molecular clones[a]

Clone	Activator	p24 (ng/plate)/ luciferase	SE	Fold induction
$pNL_{4-3}\Delta Tat$		0.26	0.03	1
	p300	1.72	0.32	6.61
	Tat	57.42	1.86	220.85
$pNL_{4-3}\Delta Vpr\Delta Tat$		1.68	0.10	1
	p300	4.2	0.00	2.5
	Tat	105.34	9.89	62.7

[a] HeLa cells were transfected with $pNL_{4-3}\Delta Tat$ or $pNL_{4-3}\Delta Vpr\Delta Tat$, together with pCMVβ-300-CHA or pL3-Tat, and cell-associated $p24^{gag}$ levels were determined by enzyme-linked immunosorbent assay. $p24^{gag}$ values were normalized for luciferase activity.

deficient viral mutant, $pNL_{4-3}\Delta Tat$, to simulate such a condition. This mutant produced an amount of $p24^{gag}$ 2 orders of magnitude smaller than that produced in the presence of Tat in *trans* (Table 2). Expression of exogenous p300 enhanced $p24^{gag}$ production sixfold compared to that produced in the presence of $pNL_{4-3}\Delta Tat$ alone. p300 had a small effect on $pNL_{4-3}\Delta Vpr\Delta Tat$, which does not express either Vpr or Tat (Table 2). These results indicate that Vpr is important for the enhancement of viral production by p300 in the absence of Tat and are consistent with the interpretation that Vpr leads to a better utilization of p300 on HIV promoter.

A p300 binding site on Vpr: a possible cause of transdominance of Vpr mutants. To gain further insights on Vpr function, we tested the activities of Vpr mutants on Tat-stimulated HIV-1 LTR and dexamethasone-stimulated MMTV LTR in A204 cells. Of these mutants, VprL64A, VprL64,67,68A, VprF72A,R73A, VprI74A,G75A, Vpr(44-96), Vpr(1-84), and Vpr(1-64) lost their enhancing effect on both HIV-1 LTR and MMTV LTR (Table 3). We next examined the effects of the mutants on the activities of the two promoters in the presence of wild-type Vpr. VprL64A efficiently suppressed Vpr activity on both promoters, showing a dominant-negative effect, as previously described for MMTV promoter (38). VprL64,67,68A, Vpr(1-84), and Vpr(44-96) also showed weak dominant-negative activities. VprR80A showed a small enhancement in the presence of Vpr. VprF72A,R73A, VprI74A, G75A, and Vpr(1-64) did not affect wild-type Vpr activity (Table 3). Results of the GST pull-down assay (Fig. 3) and the reporter assay of Table 3 are summarized and compared in Table 4. Vpr molecules retaining the ability to bind both GR and p300 were functional. Molecules that bound only to p300 showed a dominant-negative phenotype. Vpr(1-84) and Vpr (44-96), which contains both binding sites, could not enhance promoter activities but behaved as transdominant mutants. This lack of enhancement may be due to their relatively weak binding to GR, a level of binding which is not strong enough to support their enhancing effect in vivo. Our data are consistent with the hypothesis that Vpr uses two binding sites to bind GR (through LXXLL) and p300 (through the region between amino acids 65 and 84). Vpr mutants not binding to GR (L64A) show dominant-negative activity, possibly through a squelching mechanism. We note that L64A is also transdominant for the activation of the HIV-1 promoter, suggesting the presence of similar Vpr promoter interactions which are important for HIV-1 and MMTV activation. Further analysis is required to characterize in detail the interactions important for transactivation, but it is clear from Table 4 that transactivation through Vpr correlated with binding to both GR and p300.

TABLE 3. Transcriptional and transdominant activity of Vpr mutants on Tat-stimulated HIV-1 LTR and dexamethasone-stimulated MMTV LTR in A204 cells[b]

Promoter	Vpr mutant	Promoter activity (mean ± SE)	Transdominance against WT Vpr (mean ± SE)	Mutant type[a]
HIV-1 LTR	None	100.0 ± 2.2		
	WT	397.2 ± 16.5	100.0 ± 12.8	
	R80A	259.5 ± 7.6	140.0 ± 7.4	A
	L64A	35.3 ± 5.9	4.3 ± 2.2	T
	L64, 67, 68A	70.6 ± 1.3	20.0 ± 5.6	T
	F72A, R73A	101.2 ± 3.2	90.2 ± 8.1	I
	174A, G75A	94.1 ± 6.8	78.7 ± 12.8	I
	44–96	82.2 ± 5.5	19.1 ± 6.0	T
	1–84	111.8 ± 4.8	36.2 ± 4.1	T
	1–64	117.6 ± 32.3	95.6 ± 3.2	I
MMTV LTR	None	100.0 ± 37.3		
	WT	1,027.3 ± 112.5	100.0 ± 15.9	
	R80A	590.9 ± 12.6	128.0 ± 13.1	A
	L64A	50.9 ± 1.5	6.1 ± 1.3	T
	L64, 67, 68A	72.3 ± 15.3	36.7 ± 4.3	T
	F72A, R73A	96.3 ± 6.8	98.1 ± 5.1	I
	I74A, G75A	90.1 ± 18.6	85.5 ± 11.0	I
	44–96	114.1 ± 25.1	22.0 ± 4.3	T
	1–84	63.4 ± 8.6	64.6 ± 7.3	I
	1–64	102.6 ± 16.1	107.1 ± 9.7	I

[a] A, active mutant; T, transdominant mutant; I, inactive mutant.
[b] For the transactivation assay, A204 cells were transfected with the plasmid expressing the indicated Vpr mutants in the presence of L3-Luc or MMTV-LTR-Luc and pSV40-β-Gal. BS-Tat or 10^{-6} M dexamethasone was included to stimulate activities of HIV-1 LTR and MMTV LTR, respectively. Promoter activities in the presence of Tat or dexamethasone were assigned a value of 100. Shown are mean ± SE relative values of luciferase activity normalized for β-galactosidase activity. For the transdominance assay, A204 cells were transfected with 1.5 μg of plasmids/well expressing the indicated Vpr mutants in the presence of 0.3 μg of pCDNA3-VPR/well, together with L3-Luc or MMTV-LTR-Luc and pSV40-β-Gal. BS-Tat or 10^{-6} M dexamethasone were included to stimulate activities of the two promoters, respectively. Induced promoter activities in the presence of wild-type Vpr were assigned a value of 100. Mean ± SE values of relative luciferase activity normalized for β-galactosidase activity are shown. WT, wild type.

TABLE 4. Activities of Vpr mutants

Vpr mutant	Binding of:		Transactivation	Transdominance
	p300	GR		
WT[a]	++	+	++	
R80A	+	+	+	−
L64A[b]	++	−	−	++
L64, 67, 68A	++	−	−	+
44–96	++	±	−	+
1–84	+	±	−	+/−
F72A, R73A	−	+	−	−
174A, G75A	−	+	−	−
1–64	−	−	−	−

[a] WT, wild type.
[b] Binding result for VprL64A and GR is from our published study (38).

DISCUSSION

Importance of Vpr/p300 interaction for transcription activation. Vpr has been reported to have multiple roles and activities in the HIV-1 life cycle. It assists the viral preintegration complex to enter the nucleus (31, 54, 64) and stimulates transcriptional activation of the HIV-1 LTR (21, 53). Vpr efficiently arrests the host cell cycle at the G_2/M phase (29, 36, 56, 57). Although the effects of this arrest on the virus life cycle are not fully understood, it has been proposed to enhance HIV-1 replication (26). Vpr functions as a coactivator of nuclear receptors through its coactivator signature motif, LXXLL, and renders host cells more sensitive to several steroid hormones (38).

Since Vpr enhances the HIV-1 LTR in addition to the several other promoters (12), we studied the effects of Vpr on two promoters to elucidate the general mechanism of Vpr transactivation. Kino et al. have reported that Vpr cooperates with nuclear receptor coactivators p300/CBP and SRC-1a to activate the glucocorticoid-responsive promoter MMTV LTR (38). In addition, it has been reported that the transcriptional effects of Vpr on the HIV-1 LTR promoter are mediated by the p300 coactivator (20, 34). We therefore examined the interaction of Vpr with these cellular proteins and found, in contrast to previous suggestions (20, 34), that Vpr binds directly to p300/CBP but not to members of the p160 family of coactivators, such as SRC-1a. Vpr-p300/CBP interaction was demonstrated using different experimental approaches, including a GST pull-down assay, coimmunoprecipitations of cell extracts, and mammalian and yeast two-hybrid functional assays. Vpr interacts with p300 at the region between amino acids 2045 to 2191, which also contains the binding sites for p160 coactivators, adenovirus E1A protein, and several other cellular proteins. This association of Vpr and p300/CBP also takes place in vivo, as shown by the successful coprecipitation of Vpr with p300. A p300 fragment (2045 to 2191), which contains the Vpr binding site, suppressed coprecipitation of these proteins and also suppressed Vpr synergism with p300 for HIV-1 LTR and MMTV LTR expression. E1A showed effects similar to p300(2045-2191) in vitro and in vivo. After completion of this report, the crystal structure of a 46-amino acid domain in the region of p300/CBP that binds p160 coactivators, E1A, Ets-2, interferon regulatory factor 1 (IRF-1), and IRF-3 (called IBiD [IRF-3 binding domain]) was reported (15, 45). Since E1A competed with Vpr/p300 coprecipitation and transcriptional enhancement (Fig. 4A; Table 1), it will be of interest to study whether this smaller fragment can bind Vpr.

Our results suggest that Vpr efficiently recruits p300 on these two promoters and enhances their transcription activities. Vpr is necessary for the enhancement of HIV-1 viral replication by p300, suggesting that p300 is efficiently attracted to the HIV-1 LTR through Vpr (Table 2). Vpr enhanced Tat stimulation of the HIV-1 LTR by six- to eightfold in transfections, while deletion of Vpr caused a small twofold reduction of viral replication using an otherwise intact HIV molecular clone. A clear enhancement of $p24^{gag}$ production by Vpr and p300 was seen using a Tat-defective molecular clone (Table 2). Since Vpr is incorporated in the virion, Vpr/p300 interaction may be important in the early stages of viral replication or in a specific subset of infected cells in vivo when the amounts of other virus-encoding factors, including Tat, are limited. Vpr may act by recruiting p300/CBP to the HIV-1 LTR promoter and enhancing viral expression. In addition, Vpr may affect cellular promoters, including those of glucocorticoid-responsive genes that may play a role in the HIV-1 life cycle. Since limited amounts of p300/CBP are expressed in the cells, its redistribution and/or sequestration by Vpr could also modulate cellular activities through alternative mechanisms.

p300(1945-2414), which contains the Vpr/p160 binding region and the C-terminal transactivation domain, was able to activate MMTV LTR in the presence of Vpr, whereas it did not do so in the absence of Vpr, despite the presence of endogenous p160 (Table 1). This is an interesting result supporting the model of Vpr tethering p300 to the promoter, since it has been previously shown that almost all deletion fragments of p300 are inactive in coactivation of the estrogen receptor (40). Moreover, the HAT activity of p300/CBP is not required for Vpr function, at least in our assay system, since p300(1945-2414) does not contain the HAT domain and is able to cooperate with Vpr to activate both the HIV-1 and MMTV promoters (Table 1). Therefore, only the transactivation domain of p300/CBP, in addition to the Vpr binding region, is required for Vpr action. It is known that p300 is tethered to some promoters mainly through p160 coactivators, by their mutually interacting domains (59). However, p300 also contains a nuclear receptor binding site in the N-terminal region (37). Additional regions of p300 are essential for coactivation of estrogen-responsive promoters (40). These sites may be important for efficient tethering of p300 to GR-bound promoters where p160 resides. Vpr appears to bypass this process and to efficiently tether p300 to the promoter.

Vpr functions as an adapter using two distinct sites at the C-terminal part. The analysis of Vpr mutants suggested that Vpr uses two distinct sites at the C-terminal portion for transactivation, namely, a region containing the LXXLL motif localized on the second α-helical domain and a region approximately spanning amino acids 65 to 84, which overlaps with the second α-helix and its downstream acidic region. This activity of Vpr is reminiscent of that of the p160 coactivator, which requires only its nuclear receptor binding site and a p300/CBP interacting domain to stimulate estrogen receptor activity (60). Our model postulates that Vpr is tethered to the MMTV promoter through GR (38). Vpr was found complexed with p300 irrespective of the presence or absence of dexamethasone, while Vpr interacts with GR in a dexamethasone-dependent fashion (Fig. 4C). This evidence further suggests that Vpr is bound to p300/CBP and that upon glucocorticoid stimulation, the ligand-bound GR may recruit p300/CBP through Vpr. The nature of the interaction with transcriptional components in the HIV-1 LTR remains unclear. One possibility is that HIV-1 LTR also binds nuclear receptors, which in turn may attract Vpr. Several reports have described the association of nuclear receptors with the HIV-1 promoter (17, 48, 58). Putative responsive elements were also identified for some of these receptors in the LTR (13, 16, 39, 49, 55). Alternatively, a molecule(s) other than that of a nuclear receptor(s) may interact with Vpr. Our Vpr mutant analysis suggests that activation of the HIV-1 LTR also requires the LXXLL motif. Although no protein binding the LXXLL motif other than

nuclear receptors has been reported, it is possible that this is the case. According to one report, DAF16, one of the forkhead transcription factors, binds to the nuclear receptor binding domain of SRC-1 and uses it as a coactivator, although specific binding to LXXLL motif was not examined (50). The estrogen receptor activation function 2 uses a degenerate LXXLL motif to bind to LXXLL motifs of p160 coactivators (7, 62).

Mechanism of transdominant activity of Vpr mutants. The interaction of Vpr with p300/CBP also explains the dominant-negative activity of Vpr mutants, of which VprL64A showed the most prominent effect (38). The mutants that lost association with the GR but still interacted with p300/CBP showed dominant-negative activity, whereas VprF72A,R73A and VprI74A,G75A, which lost the ability to associate with p300/CBP, did not show transdominance. Notably, the effects of Vpr mutants on the HIV-1 LTR and MMTV LTR are similar, suggesting that similar mechanisms of Vpr function are responsible for the activation of both promoters.

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