Human immunodeficiency virus type 2 (HIV-2) gene expression is regulated by upstream promoter elements, including the peri-Ets (pets) site, which mediate enhancer stimulation following treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). We previously showed that the oncprotein DEK binds to the pets site in a site-specific manner. In this report, we show that binding to the HIV-2 pets site is modulated by treatment of U937 monocytic cells with TPA, an activator of protein kinase C. TPA treatment resulted in a reduction in the levels of DEK and the formation of a faster migrating pets complex in gel shift assays. We show further that the actions of TPA on pets binding can be duplicated by phosphatase treatment of nuclear proteins and is blocked with okadaic acid, a protein phosphatase-2A (PP2A) inhibitor. Finally, we demonstrate that ectopic expression of the catalytic domain of PP2A can activate the HIV-2 enhancer/promoter alone or in synergy with TPA, an effect mediated in part through the pets site. These results suggest that, through an interaction with the protein kinase C pathway, PP2A is strongly involved in regulating HIV-2 enhancer-mediated transcription. This is a consequence of its effects on DEK expression and binding to the pets site, as well as its effects on other promoter elements. These findings have implications not only for HIV-2 transcription but also for multiple cellular processes involving DEK or PP2A.

Transcriptional regulation of the genome of human immunodeficiency virus type 2 (HIV-2) is mediated by cellular factors acting via response elements located in the 5′ long terminal repeat (LTR) (1–5). One such response element in the HIV-2 LTR, denoted pets (peri-Ets), is a TG-rich site found between two Elf-1 binding sites (PuB1 and PuB2) (2, 4, 5). Recently, our laboratory demonstrated that the oncprotein DEK binds to the HIV-2 pets response element in a site-specific manner (6). DEK was first identified as part of a fusion protein with the nucleoporin CAN (Nup214) seen in a subtype of acute myelogenous leukemia (7, 8). The ubiquitously expressed dek gene encodes a 50-kDa nuclear phosphoprotein and is transcribed at high levels, especially in hematopoietic tissues (9, 10). Since its initial cloning, DEK or the immune response to DEK has been associated with several different cellular and pathologic processes. These include ataxia telangiectasia (11), pauciarticular onset juvenile rheumatoid arthritis (12), systemic lupus erythematosus (13–16), sarcoidosis (13, 15), idiopathic uveitis (15), and Kikuchi’s disease (18).

The pets site mediates transcriptional activation in response to 12-O-tetradecanoylphorbol-13-acetate/ phorbol 12-myristate 13-acetate (TPA/PMA), phytohemagglutinin, PMA + phytohemagglutinin, T-cell receptor activation by soluble or immobilized antibodies, and antigen (4, 5). Although the roles of various kinases activated by these agents, such as protein kinase C (PKC) and IκB kinase, in regulation of the HIV-1 LTR have been well documented, little is known about the actions of specific phosphatases on LTR activity. A significant portion of the intracellular serine/threonine-specific protein phosphatase activity of many cells is due to phosphoprotein phosphatase-2A (PP2A) activity (19). The PP2A holoenzyme is composed of three subunits denoted A (65-kDa scaffold subunit), B (55-kDa regulatory subunit), and C (37-kDa catalytic subunit) (19, 20). These subunits associate to form an AC dimer (the core enzyme) or an ABC trimer (holoenzyme), each of which has different substrate specificities. Direct regulation of PP2A activity occurs in the replication cycle of some DNA tumor viruses such as simian virus 40 (SV40) and polyoma virus, which both encode antigens, small-t and middle-T, respectively, that form complexes with PP2A core enzyme (21). These complexes, although not transforming on their own, facilitate or enhance the cellular transformation processes caused by these viruses. Recently, a report by Reudiger et al. (22) provided direct evidence for the involvement of PP2A in HIV-1 LTR regulation. In these experiments, increasing the ratio of PP2A core enzyme to holoenzyme by using an N-terminal mutant of the A subunit of PP2A inhibited Tat-stimulated HIV-1 transcription and virus production.

Additional evidence that PP2A functions in regulating HIV LTR activity is provided by studies with okadaic acid (OKA), a marine sponge toxin that specifically inhibits protein phosphatase-1 (PP1) and PP2A. OKA has been reported to activate the HIV-1 LTR by inducing NF-κB nuclear translocation and binding to its cognate sites (23–26) or via an NF-κB-independent mechanism involving the Sp1 response elements (26). OKA-induced Sp1-mediated transcription is further enhanced by the
PP2A Enhances Activation of the HIV-2 Promoter

Presence of Tat (26, 27). These studies with OKA suggest the involvement of one or more phosphatases (PP2A or PP1) in regulating transcriptional activation of the HIV-1 LTR.

Despite the observation by our laboratory that the HIV-2 pets site mediates activation of the promoter in response to several mitogenic and differentiating agents, DEK binding appears to be constitutive. This leads to questions of how activation through this HIV-2 element is regulated. In this paper, we demonstrate that TPA activation of the monocytic cell line U937 alters DEK protein levels and binding to the pets response element through a signaling cascade involving PKC and PP2A. We also show that ectopic expression of the catalytic domain of PP2A can increase the basal activity of the HIV-2 enhancer and enhances TPA-mediated activation of the promoter, acting largely through the pets response element. Finally, we show that the effects of TPA or PP2Ac on the HIV-2 enhancer can be blocked by treatment with the PP2A inhibitor OKA. Thus, PP2A plays an integral role in modulating transcription of the AIDS-causing retrovirus HIV-2.

Experimental Procedures

Cell Culture—Human U937 monocytic cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (Life Technologies, Inc.), penicillin (50 units/ml, streptomycin (50 μg/ml), and 1-glutamine (2 mm).

Nuclear Protein Extraction—Nuclear protein extracts from U937 monocytic cells were prepared using a modification of the method of Dignam (28). All steps were performed at 4°C. Cells were washed once with 10 ml of phosphate-buffered saline and twice with 2 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 1× Complete protease inhibitor solution (Roche Molecular Biochemicals)). The cells were resuspended in lysis buffer (15 μl of buffer A/107 cells + 0.01% Nonidet P-40) for 5 min. The nuclei were pelleted and the supernatant discarded. The nuclear membrane was disrupted by resuspension of the pellet in 10 μl of buffer C/107 cells (20 μM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 0.5 mM DTT, 1× Complete protease inhibitor, Roche Molecular Biochemicals) and gentle rocking of the tubes for 10 min followed by centrifugation at 12,000 rpm for 10 min. The supernatant containing the nuclear proteins was removed and diluted by the addition of 5 μl of modified buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 1× Complete protease inhibitor, Roche Molecular Biochemicals) and 5% milk at room temperature. After the membrane was washed as described above, the secondary antibody was detected using Supersignal chemiluminescent substrate (Pierce) as instructed. For some Western blots, the anti-DEK primary antibody was preincubated for 1 h with a 1:1000 dilution of goat anti-phosphatase-conjugated IgG (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Sigma).

Shift-Western Blotting—The shift-Western assay was performed as described previously (30) with modifications. Briefly, 30 μg of nuclear extract was used in binding reactions followed by EMSA on a 5% native acrylamide gel. Following separation of the nucleoprotein complexes, the gel and nitrocellulose membrane were soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 20% (v/v) methanol) with 0.1% SDS for 30 min at room temperature. The gel, Whatman 3MM filter papers, and membranes were stacked as described (30). Proteins were transferred to nitrocellulose membrane (Schlier & Schuell), and probe was removed and replaced with PAP solution (125 mM Tris-HCl, 1.25 mM chloramphenicol, 0.1 mM 7-mercaptoethanol). The gel was dried and autoradiographed. The blots and autoradiographs were aligned, and the positions of the bands in each were compared.

Phosphatase Inhibition—U937 cells were treated with 5 or 50 nM OKA (Calbiochem) only or pretreated for 30–45 min with OKA prior to stimulation with 32 nM TPA (Sigma). EMSA and DEK Western blots were performed 24 h post-treatment.

PKC Inhibition—U937 cells were treated with only 2 μM staurosporine or 10 μM Gö6983 (Calbiochem) or pretreated for 30–45 min prior to stimulation with 32 nM TPA. 24 h post-treatment, nuclear extract was prepared and EMSA was performed.

Plasmids—HIV-2-chloramphenicol acetyltransferase (HIV-2/CAT) and HIV-2-luciferase (HIV-2-luc) plasmids contain enhancer/promoter sequences (−556/+156) from the HIV-2 ROD strain (31). Δpets (2), Δpets/ΔPuB2 (2), −107 (2), ΔS1p, and ΔB HIV-2 (1-reporter plasmids have been described previously (29). HIV-2 site-specific mutant reporter plasmids were generated by the gap-heteroduplex method from wild-type HIV-2 enhancer (2). pCMV5 PP2Ac (gift of Marc Mumber) constitutively expresses the catalytic domain of PP2A under the control of the CMV promoter. All plasmids were prepared using the Concert Maxiprep kit (Life Technologies, Inc.).

Transfections—5 μg of reporter plasmid, alone or in combination with 5–10 μg of empty pcMV5 vector, was combined with empty pcMV5 vector for a total of 25 μg of total DNA. U937 undergoing logarithmic growth were harvested at 5 × 106 cells/ml, washed once with 50 ml of serum-free RPMI 1640 medium, and resuspended to a concentration of 25 × 106 cells/ml in serum-free RPMI medium. 107 cells (0.4 ml) were aliquoted to 0.4-cm electroporation cuvettes (Invitrogen) and incubated for 5 min at room temperature with the DNA mixture to be transfected. The cells were electroporated using an Invitrogen Electroporator II at a setting of 330 V, 1000 microfarads, and infinite resistance with an input voltage of −325 V. Following electroporation, the cells from each co-transfection were resuspended in 10 ml of RPMI + 10% fetal bovine serum and split evenly (5 × 105 cells/pool) into control and treatment groups. 12–18 h post-transfection, the treatment group was stimulated with 32 nM TPA. Any pretreatment with phosphatase inhibitors occurred 30 min prior to TPA stimulation. 24 h post-stimulation, the cells were harvested, pelleted, washed once with 10 ml of phosphate-buffered saline, and resuspended in 100 μl of 0.25 M Tris-HCl (pH 7.5) (CAT assays) or 1× passive lysis buffer (luciferase assays; Promega). The protein concentration of the lysates was determined by the Bradford reagent (Bio-Rad) method.

Chloramphenicol Acetyltransferase (CAT) Assay—for CAT assays, 5–30 μl of cell lysate was combined with 20 μl of 100 mM Tris-HCl (pH 7.5). The final volume was increased to 50 μl with lysis buffer, if necessary, and the mixture was incubated for 15 min at 65°C to inactivate endogenous acetyltransferases. The lysates were then transferred to 7-ml scintillation vials, and 200 μl of CAT reaction mixture (125 mM Tris-HCl, 1.25 mM chloramphenolin, 0.1 μg/ml of [3H]acetyl-CoA) were added and the blot incubated for 45 min in TBS-T + 5% milk at room temperature. After the membrane was washed as described above, the secondary antibody was detected using Supersignal chemiluminescent substrate (Pierce) as instructed. For some Western blots, the anti-DEK primary antibody was preincubated for 1 h with a 1:1000 dilution of goat anti-phosphatase-conjugated IgG (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Sigma).

PP2A Enhances Activation of the HIV-2 Promoter

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was added. 5 ml of Econofluor-2 (PerkinElmer Life Sciences) scintillation fluid was overlaid and the reaction incubated for 15–90 min at 37 °C before counting. Background and total counts were determined by mixing 200 μl of CAT reaction mixture with 5 ml of Econofluor-2 or Scintiverse (Fisher Scientific), respectively. CAT activity was background-subtracted and normalized for the total amount of protein used.

Luciferase Assays—Luciferase assays were performed using the Promega luciferase assay system according to the manufacturer’s instructions.

RESULTS

Stimulation of U937 Cells Alters the Pattern of Binding to the pets Site and Reduces DEK Protein Expression—TPA is a biologically active diterpene with pleiotropic effects that binds to and activates PKC. Although TPA has been shown to activate the HIV-2 promoter, an effect mediated in part by the pets site (Fig. 1A), the effect of TPA treatment on binding to the pets site has not been investigated. To determine whether binding to the pets site could be modulated by cell activation, we treated U937 monocytic cells for at least 24 h with 32 nM TPA or the carrier dimethyl sulfoxide (control). Nuclear extracts were prepared and EMSA performed using a radiolabeled probe containing a pets site. The protein-DNA complexes were transferred from the native gel to membranes by electroblotting. The protein-DNA complexes were then dried and autoradiographed. Both membranes were ground-subtracted and normalized for the total amount of protein used. The inability to detect DEK protein by Western blotting localizes DEK to the upper but not lower TPA-induced bands seen in the absence (open) or presence (solid) of TPA, respectively. C, TPA and 4β-PDD, but not the inactive phorbol ester 4α-PDD, alter binding to the pets site of HIV-2. Binding reactions and EMSA were carried out using nuclear extract from U937 cells treated for 24 h with the carrier dimethyl sulfoxide (lane 1), 50 nM 4β-PDD (lane 2), 50 nM TPA (lane 3), or 50 nM 4β-PDD (lane 4). D, TPA and 4β-PDD but not 4α-PDD treatment of U937 cells leads to a marked reduction of DEK levels. 40 μg of lyate from U937 cells treated with 50 nM 4α-PDD, 50 nM TPA, or 50 nM 4β-PDD was used for DEK Western blot. E, shift-Western blotting localizes DEK to the upper but not lower TPA-induced pets band seen on EMSA. 30 μg of nuclear extract from untreated (lanes 1 and 3) or 32 nM TPA-treated (lanes 2 and 4) U937 cells was used in binding reactions and EMSA. The protein-DNA complexes were transferred from the native gel to membranes by electroblotting. The protein was immobilized on nitrocellulose and a DEK Western blot performed (lanes 3 and 4). The protocol was transferred to DE-81 paper (lanes 1 and 2), which was then dried and autoradiographed. Both membranes were aligned, and the bands on each were compared.
nuclear factor detected by immunoblot is localized to the gel shift band. A gel shift assay using nuclear extract from untreated or 32 nM TPA-treated U937 was performed using a radiolabeled pets probe followed by shift-Western assay using α-DEK antiserum (Fig. 1E). An overlap can be clearly seen between the DEK protein band on the Western blot (Fig. 1E, lane 3) and the band on the DE-81 paper autoradiograph from the samples that received no treatment (Fig. 1E, lane 1). This finding was not unexpected given that DEK has already been shown capable of binding to the pets site (6). However, no overlap between the DEK protein bands and the TPA-induced pets band was observed (Fig. 1E, lanes 2 and 4). This observation correlates with the loss of DEK seen with TPA treatment (Fig. 1D) and suggests the possibility of a second pets factor. Another interpretation of these results is that there are alternatively spliced or post-translationally modified forms of DEK induced by TPA that the anti-DEK antiserum could not detect. To test this possibility, we performed Western blots using total cell lysates from U937 or 293 cells transfected with a FLAG-DEK expression vector. Both the FLAG monoclonal and DEK rabbit polyclonal antibodies detected identical sets of bands on Western blots (data not shown).

The PKC Inhibitors Staurosporine and Gö6983 Block the Effect of TPA—To determine whether PKC activation was required for the effect of TPA on pets site binding, we utilized the PKC inhibitors staurosporine and Gö6983. Gö6983 is a staurosporine derivative capable of inhibiting the α, β, γ, δ, ζ, and µ PKC isoforms (32) without affecting other intracellular kinases. The α, β, γ, δ, and ζ isoforms of PKC have all been implicated in the TPA-induced signaling cascade leading to the differentiation of monocytes into macrophages (33–39). As is seen in Fig. 2, A and B, pretreatment of U937 cells with 2 μM staurosporine or 10 μM Gö6983 for 30–45 min prior to the addition of 32 nM TPA is able to block the TPA-elicted changes in pets site binding. Gö6983 completely blocked the TPA-induced changes, and staurosporine clearly diminished but did not completely block the TPA-induced changes in pets site binding (Fig. 2, A and B). Staurosporine also appears capable of inducing the complex seen with TPA treatment (Fig. 2A, lane 7), perhaps due in part to the pleiotropic inhibitory actions of staurosporine, which block a variety of kinases in addition to PKC. The more specific PKC inhibitor, Gö6983, does not induce the faster migrating complex either alone or in combination with TPA (Fig. 2B). Both staurosporine and Gö6983 were also able to partially block the effects of TPA at lower concentrations in a dose-dependent manner (data not shown). Additional specific bands besides those indicated by arrowheads are observed in Fig. 2, A and B. The identity of the proteins in these bands is unknown. However, these bands are not always observed on binding gels using the pets probe (compare Fig. 1, B and E with Fig. 2, A and B) and may represent intermediates in the transition from one complex to another. Another possibility is that the observed bands may also result from the partial degradation of one or more of the factors in the specific complexes seen in the binding gels. Given the sporadic appearances of these additional bands, we have chosen not to pursue identification of the DNA-binding factors within them.

**PP2A Enhances Activation of the HIV-2 Promoter**

Potato Acid Phosphatase Treatment of Nuclear Extract from U937 Cells Leads to an Increase in the Electrophoretic Mobility of the pets Complex—Our observation that PKC activation by TPA results in the formation of the faster migrating complex within minutes post-treatment (data not shown) suggests that post-translational modifications such as phosphorylation/dephosphorylation may be responsible, in part, for the alterations seen in the electrophoretic mobility of the pets complex. To further address this hypothesis, we treated nuclear extract from unstimulated U937 cells with active or heat-inactivated Type II PAP for 20 min at room temperature and then performed gel shift assays using a [32P]-labeled pets probe. Active PAP treatment led to the formation of a new band on gel shift at the same position as the band seen with TPA treatment of U937 (Fig. 3A, lane 4). The formation of this new band was specific to PAP treatment, as heat-inactivated PAP had no effect (Fig. 3A, lane 1). PAP treatment also had no effect on the binding pattern when nuclear extract from TPA-treated U937 cells was used (Fig. 3A, lane 5). Because 20 min of PAP treatment resulted in a partial transition to the TPA-induced pets band, U937 nuclear extract was subjected to longer PAP treatment (15, 30, or 60 min) followed by gel shift assay. Extended PAP treatment effectively led to the loss of much of the uninduced pets complex (Fig. 3B, lane 1) and the formation of a band (Fig. 3B, lanes 2–4) with identical mobility in gel shift assay to the TPA-induced complex (Fig. 3B, lane 5). These results suggest that the TPA-induced complex is the result of dephosphorylation of the protein(s) bound to the pets site.

OKA Blocks the TPA-induced Loss of DEK and Formation of the TPA-induced pets Band in Gel Shift Assays—OKA is a potent and specific inhibitor of the catalytic activity of PP1 and PP2A (19, 40, 41). To determine whether the change in mobility of the pets complex in response to TPA treatment of U937 was due to PP1/PP2A activity, U937 cells were pretreated for 30–45 min with 5 or 50 nM OKA prior to treatment with TPA for 24 h. EMSA was performed using either a radiolabeled pets probe or, as a positive control, an NF-κB probe, because OKA-mediated inhibition of PP2A induces NF-κB binding. 50 nM OKA was able to both induce NF-κB binding (Fig. 4A, lane 18) and block the effects of TPA (Fig. 4A, lane 12) on the mobility of the pets complex. 5 nM OKA proved to be a sub-optimal dose, unable to elicit activation of NF-κB (Fig. 4A, lane 17) or to block the effect of TPA (Fig. 4A, lane 9). Neither 5 nor 50 nM OKA (Fig. 4A, lanes 3 and 6) alone had any effect on binding to the pets site when compared with untreated controls.

DEK Western blots were performed with equal amounts of the nuclear extract used for EMSA in Fig. 4A. Concomitant with the TPA-induced change in pets site binding, a loss of DEK protein was also observed (Fig. 4B, lane 2). The loss of
PP2A Enhances Activation of the HIV-2 Promoter

FIG. 3. PAP treatment of nuclear extract from U937 cells induces a transition from the upper to lower pets complex. A, potato acid phosphatase type II (1 unit) was used to treat 10 μg of nuclear extract (NE) from control or TPA-treated U937 cells for 20 min at 25 °C prior to EMSA with a pets probe labeled using Klenow polymerase. Untreated U937 nuclear extract was treated with: lane 1, heat-inactivated PAP (INA); lane 2, PAP buffer; or lane 4, PAP. 24-h TPA-treated U937 nuclear extract was treated with: lane 3, PAP buffer; or lane 5, PAP. A band can be seen at the level of the TPA-induced complex (lane 5) with active (lane 4) but not heat-inactivated (lane 1) PAP treatment of U937 nuclear extract. B, nuclear extract from untreated U937 cells was incubated with 1 unit of PAP Type II for 15, 30, or 60 min (lanes 2–4) at 25 °C prior to EMSA. Binding reactions using nuclear extract from untreated (lane 1) or 32 nM TPA-treated U937 cells (lane 5) were also included as controls. The arrowheads in panels A and B indicate the positions of the specific bands.

PP2Ac Activates the HIV-2 Enhancer—The above findings suggested the involvement of PP2A in the activation of the HIV-2 LTR and the possibility that PP2A may act on the HIV-2 LTR, at least in part, through the pets site. To test this intriguing possibility, we transfected U937 cells with an HIV-2 enhancer-CAT reporter and pCMV5 PP2Ac, a plasmid encoding the catalytic domain of PP2A (gift of Marc Mumby) or empty pCMV5 vector. Transfected cells from each condition received 32 nM TPA treatment or went untreated for 24 h. Increasing levels of ectopic PP2Ac enhanced both the base-line (Figs. 5A and 6B) and TPA-induced (Fig. 5A) activity of the HIV-2-CAT reporter significantly over no treatment or TPA alone, respectively. To further assure that the effects that we observed were indeed due to increased levels of PP2A activity, we performed PP2A enzymatic activity assays (Life Technologies). As seen in Fig. 5B, ectopic expression of PP2A resulted in an increase in intracellular PP2A activity over base line, which was further augmented by the addition of TPA. Similar results were obtained with a Promega PP2A activity assay (data not shown). Furthermore, the increased HIV-2 reporter activity seen in U937 cells transfected with pCMV5 PP2Ac and treated with TPA (750-fold over base line) could be reduced in a dose-responsive manner by increasing levels of OKA (Fig. 5C). Although OKA had a similar effect on U937 cells treated with TPA alone, much greater levels of OKA were required to achieve the same degree of CAT activity reduction in cells expressing ectopic PP2Ac. This antagonistic action of OKA on TPA activation of the HIV-2-CAT reporter has also been observed for the HIV-1 enhancer in Jurkat, a T-lymphocyte cell line (25).

In contrast to the antagonistic effect of OKA on TPA and DEK due to TPA treatment could be blocked by 50 nM of OKA (Fig. 4B, lane 6), whereas 5 nM OKA had no effect (Fig. 4B, lane 5). The DEK band also appeared to be more heavily stained when 50 nM OKA was used (Fig. 4B, lanes 4 and 6) as compared with 5 nM OKA alone (Fig. 4B, lane 3) or no treatment (Fig. 4B, lane 1). Taken together these data suggest that TPA treatment of U937 cells results in activation of a phosphatase, likely PP2A, that dephosphorylates the DEK protein bound to the HIV-2 LTR pets site, with subsequent loss of DEK from the nucleus and binding of a second, unknown factor to the pets site.

FIG. 4. OKA treatment of U937 cells blocks the TPA-induced loss of DEK and changes pets site binding on EMSA. A, EMSA was performed using nuclear extract from U937 cells subjected to the following treatments for 24 h: lane 1, no treatment; lane 2, 32 nM TPA; lanes 3–5, 5 μM OKA; lanes 6–8, 50 μM OKA; lanes 9–11, 5 μM OKA + TPA; lanes 12–14, 50 μM OKA + TPA. Competitive binding reactions utilized 200 ng of cold probe (S) or an oligonucleotide with a mutated pets site (M). Because OKA has been shown to induce NF-κB binding, the same nuclear extract was used in binding reactions with a NF-κB probe (lanes 15–20) as a positive control. The arrowheads indicate the positions of specific bands with the pets probe. B, okadaic acid treatment prevents the TPA-induced loss of DEK. A DEK Western blot of the nuclear extract preparations used for EMSA is shown. Equal amounts of nuclear extract (40 μg) were used in each lane as confirmed by Ponceau S staining. The positions of full-length DEK (50 kDa) and the DEK breakdown product (35 kDa) are indicated. OKA at levels capable of inducing NF-κB binding (50 μM) but not at a sub-optimal dose (5 nM) is capable of blocking the TPA-induced loss of DEK and the concomitant loss of the specific upper band seen on EMSA with pets probe.
PP2A activity, OKA alone enhanced CAT reporter activity in a dose-dependent fashion, with the maximal activity (a 150-fold increase) occurring at 200 nM (data not shown). This is most likely because of activation of NF-κB and Sp1 by OKA as has been reported previously (23, 25–27). These data support and extend the observations that PP2A phosphatase activity appears to be important in the signaling cascade culminating at the HIV-2 LTR.

**Activation of the HIV-2 Promoter by PP2A Is Mediated by the pets, PuB2, and Sp1 Sites**—Because binding to the pets site of HIV-2 is modulated by phosphatase activity, we sought to determine whether the activation of the HIV-2 promoter by PP2A is mediated in part by the pets response element. Various site-directed deletion or mutant HIV-2-reporter gene constructs were used in these experiments (Fig. 6A). Because PP2Ac increases the activity of the HIV-2-CAT reporter construct (Fig. 5A), we assessed the role of the pets site in the response of the HIV-2 promoter to ectopic PP2Ac. Mutation of the pets site led to a significant decrease in the PP2Ac activation of the HIV-2 LTR ($p < 0.05$) and a further decrease ($p < 0.02$) could be observed with an additional mutation in the PU2 site (Fig. 6B). To address concerns that the deletions in the enhancer region of the CAT reporters had a nonspecific adverse effect on the promoters, we cotransfected U937 cells with a plasmid expressing the Tat transactivating gene from HIV-2 (Tat-2) and the mutant constructs. As seen in Fig. 6C, Tat-2 results in a marked increase in CAT activity over base line for all of the constructs. This demonstrates that the mutant reporters are still transcriptionally active in U937 cells, consistent with previously published reports from our laboratory utilizing these reporter constructs (2, 4, 5).

PP2A has been shown previously to regulate expression through Sp1 and NF-κB sites (23, 25–27). To test whether these sites play a role in the response of the HIV-2 promoter to PP2A, we transfected U937 cells with plasmids in which the reporter gene (luciferase) was under control of the intact HIV-2 promoter or promoters in which the pets sequence, NF-κB site, all upstream enhancer elements, or the 3′ Sp1 site were altered or deleted. Although mutation of the NF-κB site had no effect on the response to PP2A, loss of the pets site, all upstream enhancer elements (~107), or the 3′ Sp1 site were altered or deleted. Although mutation of the NF-κB site had no effect on the response to PP2A, loss of the pets site, all upstream enhancer elements, or the 3′ Sp1 site diminished the PP2A-induced activation (Fig. 6D). The Sp1 mutant actually showed decreased activity in response to PP2A (Fig. 6D). Taken together, the data from Fig. 6 show that the pets, PuB2, and

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**FIG. 5. PP2A enhances activation of the HIV-2 promoter.** A, U937 cells were cotransfected with HIV-2-CAT reporter and 1, 5, or 10 μg of pCMV5 PP2Ac. Cells were treated with 32 nM TPA 24 h post-transfection or left untreated. B, PP2A enzymatic assay was performed using whole cell lysates from U937 cells transfected with 2.5, 5, or 10 μg of pCMV5 PP2Ac and treated with 50 nM TPA (black bars) or left untreated (white bars) for at least 24 h. C, U937 cells were cotransfected with HIV-2-CAT reporter and 5 μg of pCMV5 PP2Ac. 24 h post-transfection, the cells received no treatment, 32 nM TPA only, or 50, 100, 200, or 400 nM OKA (30–45 min pretreatment) + TPA. For all transfections, the total amount of transfected DNA was normalized using pCMV5 vector. CAT assays were performed 18–24 h later. The CAT activity values shown in panel C are normalized relative to the CAT activity in the absence of TPA and pCMV5 PP2Ac.
Sp1 sites contribute to the activation of the HIV-2 promoter by PP2A and that the xB site is not necessary for this effect.

**DISCUSSION**

In this report, we have made the following observations. 1) TPA treatment of U937 monocyctic cells results in a marked reduction in the levels of the DEK oncoprotein, with a concomitant change in the mobility of the pets complex in gel shift assays. 2) The change in the electrophoretic mobility of the pets complex is phorbol ester-dependent and can be blocked with PKC inhibitors. 3) Dephosphorylation of the nuclear extract from U937 cells resulted in a pattern of binding to the pets site similar to that seen with TPA treatment, implicating a phosphatase in the signaling pathway. 4) Okadaic acid, a potent inhibitor of PP2A/PP1, is able to block the effects of TPA on pets binding and DEK protein levels. OKA also suppresses HIV-2 LTR activation in response to TPA. 5) The catalytic domain of PP2A synergizes with TPA to activate the HIV-2 LTR, an effect mediated in part by the pets response element, which can be antagonized by treatment with OKA. These findings are summarized in Fig. 7 and demonstrate that PP2A plays a significant role in one or more of the pathways activated by PMA, culminating at the HIV-2 promoter. By overexpressing the catalytic domain of PP2A, the transcriptional activity induced by these pathways is enhanced. Additionally, as PP2A can also activate the promoter in the absence of PMA (Fig. 5A), PP2A overexpression may be acting on signaling pathways not normally activated by TPA, which in concert with PMA-induced signaling pathways lead to synergistic activation of the HIV-2 enhancer.

We have demonstrated in this paper that binding to the pets site is responsive to signaling by the phorbol ester TPA (an agent known to activate the HIV-2 LTR). TPA activation of U937 cells appears to trigger an exchange of DEK for another pets factor, because DEK protein levels are significantly reduced post-TPA treatment and the DEK protein is not observed in the TPA-induced pets complex by shift-Western assay. The reduction in DEK levels following TPA treatment has also been observed in other monocytic cell lines (HL60 and THP-1), suggesting that this is not a cell line-specific phenomenon (data not shown). These observations, and the fact that DEK is expressed constitutively at high levels, suggest that post-translational modifications to DEK and alteration of its intracellular levels following induction of specific cellular signal transduction pathways allow for greater control of the DEK effect on
transcription versus only modulating basal expression levels.

The identity of the second pets factor remains unknown, but in the course of purifying pets-binding proteins, we noted that a 65-kDa nuclear factor also appears to bind to the pets site specifically (42). However, as it was not the dominant pets-binding protein under the conditions used, we did not pursue its identification further. In light of the current data, we are now attempting to identify this second factor, as it may be the protein that activates the HIV-2 LTR through the pets site in response to antigenic stimulation.

Although TPA activates PKC (Fig. 2, A and B), further observations implicate a phosphatase, likely PP2A (Figs. 3, A and B and 4A), in the mediation of the transition from the slower to faster mobility complex seen on EMSA; this suggests that PKC potentially up-regulates, directly or indirectly, intracellular PP2A activity. If this is the case, then ectopic PP2Ac expression may serve to accelerate the rate at which the transition from one pets complex to another takes place in the presence of TPA stimulation. We have observed that the pets complex transition in response to TPA stimulation of various monocytic cell lines (U937, HL60, and THP-1) begins as early as 30 min post-TPA treatment and is completed within 24–48 h (data not shown). Recent studies suggest that PP2A exists in complexes with various kinases, allowing for rapid modifications of the phosphorylation state of the kinases or substrates (19, 43). Additionally, PP2A regulates and to compare it with HIV-1. Our results implicate the interaction of DEK and PP2A in the activation of HIV-2. In addition, this study also suggests that PP2A may act synergistically with other activating signals to boost transcription regulated by the HIV-2 LTR. This finding was unexpected, in view of the observations of several groups that OKA activates HIV-1 transcription, leading to the assumption that PP2A suppresses HIV-1 transcription (25, 26, 27). Thus, to ascertain whether this represents one reason for the difference in pathogenic potential or a shared mechanism of activation between the two viruses, our future studies will directly compare the effect of PP2A on HIV-1 and HIV-2 transcription and replication.

**Acknowledgments**—We thank Marc Mumby for the gift of pCMV5 PP2Ac vector, Gerard Grosvidel for antibodies to DEK, and David Pallas for the PP2Ac mutant expression vectors.

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2. B. Adams and D. Markovitz, manuscript in preparation.
REFERENCES

1. Markovitz, D. M., Hannibal, M., Perez, V. L., Gauntt, C., Folks, T. M., and Nabel, G. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5098–5102
2. Markovitz, D. M., Smith, M. J., Hilfinger, J., Hannibal, M. C., Petryniak, B., and Nabel, G. J. (1992) J. Virol. 66, 5479–5484
3. Clark, N. M., Hannibal, M. C., and Markovitz, D. M. (1995) J. Virol. 69, 4854–4862
4. Hannibal, M. C., Markovitz, D. M., van Baal, S., Jaegle, M., de Wit, T., and Grosveld, G. (1992) Mol. Cell. Biol. 12, 1687–1697
5. Robin, M., van Baal, S., Jaegle, M., van Lindern, M., Murti, K. G., Davis, D., and Grosveld, G. (1995) Oncogene 10, 1739–1748
6. Fu, G. K., and Markovitz, D. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1811–1815
7. von Lindern, M., Fornerod, M., van Baal, S., Jaegle, M., Hagemeijer, A., and Grosveld, G. (1992) Baillieres Clin. Haematol. 5, 857–879
8. von Lindern, M., Fornerod, M., van Baal, S., Jaegle, M., de Wit, T., and Grosveld, G. (1992) EMBO J. 11, 53–60
9. von Lindern, M., Fornerod, M., van Baal, S., Jaegle, M., de Wit, T., Buijs, A., and Grosveld, G. (1992) Mol. Cell. Biol. 12, 1687–1697
10. von Lindern, M., Fornerod, M., van Baal, S., Jaegle, M., von Lindern, M., Murti, K. G., Davis, D., and Grosveld, G. (1995) Oncogene 10, 1739–1748
11. Meyn, M. S., Lu-Kuo, J. M., and Herzing, L. B. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1023–1030
12. Thevenin, C., Kim, S. J., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., Fauci, A. S., and Kehrl, J. H. (1990) New Biol. 2, 793–800
13. Schonthal, A. H. (1998) Mol. Carcinog. 26, 603–609
14. Tussawad, D., Ebi, S., and Kehrl, J. H. (1992) Front. Biosci. 3, D1262–D1273
15. Markovitz, D. M. (1993) Annu. Rev. Biochem. 62, 299–327
16. Chiu, S. J., Suter, W., and Jones, D. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9491–9495
17. Arnaudo, J. P., Deibener, J., and Kaminsky, P. (1998) J. Rheumatol. 25, 1861–1862
18. jeder, M. and Keene, J. R. (1999) Oncogene 18, 3554–3559
19. Markovitz, D. M. (1993) Mol. Cell. Biol. 13, 1206–1216
20. Murray, K. J., Stier, W., Grom, A. A., Donnelly, P., Levinson, J. E., Giannini, E. H., Glass, D. N., and Sier, I. S. (1997) J. Rheumatol. 24, 576–580
21. Dong, X., Wang, J., and Grosveld, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 90, 1203–1208
22. Wooten, M. W., and Seibenhener, M. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5755–5760
23. Chun, R. F., Semmes, O. J., Neveuvel, C., and Jeang, K. T. (1998) Science 280, 1622–1625
24. Kiley, S. C., and Parker, P. J. (1995) J. Virol. 69, 1116–1121
25. Rieckmann, P., Thevenin, C., and Holland, J. H. (1992) J. Virol. 66, 5479–5484
26. Vlach, A. B., Garcia, A., Jaque, J. M., et al. (1998) J. Biol. Chem. 273, 1035–1016
27. Chun, R. F., Semmes, O. J., Neveuvel, C., and Jeang, K. T. (1998) Science 280, 1622–1625
28. Vlach, A. B., Garcia, A., Jaque, J. M., et al. (1998) J. Biol. Chem. 273, 1035–1016
29. Rieuken, P., Thevenin, C., and Kehrl, J. H. (1992) J. Virol. 66, 5479–5484
30. Davis, D., Bonten, J., Buijs, A., and Grosveld, G. (1995) Mol. Cell. Biol. 15, 3554–3559
31. Markovitz, D. M. (1993) Mol. Cell. Biol. 13, 1206–1216
32. Markovitz, D. M. (1993) Mol. Cell. Biol. 13, 1206–1216
33. Markovitz, D. M. (1993) Mol. Cell. Biol. 13, 1206–1216
34. Markovitz, D. M. (1993) Mol. Cell. Biol. 13, 1206–1216
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*J. Biol. Chem.* 2001, 276:25804-25812. doi: 10.1074/jbc.M006454200 originally published online April 24, 2001

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