The EMBO Journal vol.9 no.4 pp.1165-1170, 1990

Trans-activation of HIV-1 LTR-directed gene expression by *tat* requires protein kinase C

Aya Jakobovits[1,2], Arnon Rosenthal and Daniel J.Capon

Department of Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA

[1]Present address: Cell Genesys, Inc., 344A Lakeside Drive, Foster City, CA 94404, USA

[2]Corresponding author

Communicated by M.L.Birnstiel

Human immunodeficiency virus (HIV) spends a significant part of the viral life cycle as a latent provirus integrated into the host genome. Activation of latent HIV-1 requires mitogenic stimulation of the cell, which increases basal viral transcription, and the HIV-1 *tat* protein. As *tat* itself dramatically increases HIV-1 gene expression, it too is presumably regulated in the latent state, and may also be activated by mitogenic stimulation. We show here that depletion of protein kinase C (PKC), which is essential to the stimulation of T cells by several mitogens, dramatically reduces HIV-1 *trans*-activation without affecting synthesis of *tat* protein. *Trans*-activation in PKC-depleted cells can be restored by transfection with a PKC expression vector. The requirement for PKC in *trans*-activation does not involve the PMA-responsive enhancer elements responsible for the effect of mitogens on basal transcription. Our results indicate that PKC regulates the process of HIV-1 *trans*-activation, suggesting a key role for the mitogenic induction of *trans*-activation in the transition of HIV from latency to productive growth.

Key words: gene expression/HIV/protein kinase C/*tat*/*trans*-activation

Introduction

Infection with human immunodeficiency virus (HIV-1) is characterized by a prolonged latency period which precedes the clinical manifestation of the acquired immunodeficiency syndrome (AIDS) (Curran *et al.*, 1985). HIV-1 can establish a latent infection in T cells (Zagury *et al.*, 1986), which is believed to reflect a dormant state of the integrated virus. An understanding of how the HIV-1 provirus is triggered to begin viral replication may thus help to explain the progressive nature of the HIV-1 disease. Activation of latent HIV-1 requires both mitogenic stimulation of the infected cell (McDougal *et al.*, 1985; Folks *et al.*, 1986; Zagury *et al.*, 1986) and the HIV-1 *trans*-activator (*tat*) gene product (Dayton *et al.*, 1986). It has been shown that T cell mitogens such as phorbol 12-myristate-13-acetate (PMA) can induce transcription from the HIV-1 LTR (Harada *et al.*, 1986; Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starsken *et al.*, 1987). The element involved in the PMA response is a duplicated 10 nucleotide enhancer sequence found in the HIV-1 LTR and many other viral and cellular promoters (Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987). This element interacts with PMA-regulated DNA binding factors such as NF-κb (Nabel and Baltimore, 1987) an HIVEN86A (Bohnlein *et al.*, 1988).

As T cell activation enhances basal HIV-1 transcription, it has been suggested that it may be critical for the activation of latent HIV-1 (Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starsken *et al.*, 1987). The effect of mitogenic stimulation on HIV-1 *trans*-activation in infected cells is unknown, but since *tat* increases gene expression during productive infection by perhaps as much as several hundred-fold (Arya *et al.*, 1985; Sodroski *et al.*, 1985) it is likely that the regulation of *tat* activity is also crucial to the establishment and maintenance of latency. As protein kinase C (PKC) is essential to the induction of T cell proliferation by several mitogens (Manger *et al.*, 1987), we have investigated the involvement of PKC in HIV-1 *trans*-activation. We reasoned that, unlike in resting T cells *in vivo*, levels of activated PKC in proliferating T cell lines are sufficient to maximize *trans*-activation thus obscuring further induction of HIV-1 *trans*-activation by mitogenic stimulation (Siekevitz *et al.*, 1987; Tong-Starsken *et al.*, 1987). Therefore, the requirement for PKC to support HIV-1 *trans*-activation was investigated by determining *tat* activity in cells depleted of endogenous functional PKC. We show that the depletion of PKC from Jurkat and 293 cells leads to a dramatic reduction in HIV-1 *trans*-activation. The effect of PKC depletion on *trans*-activation is not due to decreased amounts of *tat* protein and is independent of the PMA-responsive enhancer element that mediates the effect on basal transcription. *Trans*-activation in PKC-deficient cells can be restored by transfection of a wild-type PKC expression vector, but not by a mutant PKC lacking a functional ATP binding site. Thus, our results suggest that activation of latent HIV-1 by mitogens involves induction of *tat*-mediated *trans*-activation as well as basal HIV-1 transcription, and suggest that selective inhibition of PKC activity may suppress both latent and actively replicating HIV-1.

Results

Depletion of functional PKC inhibits tat-mediated HIV-1 trans-activation

The phorbol ester tumor promoter PMA, a potent T cell mitogen, mediates its known cellular effects by activating protein kinase C, a family of calcium and phospholipid-dependent serine/threonine kinases (for review see Nishizuka, 1988; Knopf *et al.*, 1986; Ono *et al.*, 1986; Coussens *et al.*, 1986; Ohno *et al.*, 1987). To study the requirement for a functional PKC in HIV-1 *trans*-activation, cells were depleted of PKC activity either by prolonged exposure of cells to 100 ng/ml PMA (48-72 h), which

© Oxford University Press

results in down-regulation of PKC (Young *et al.*, 1987), or by blocking PKC activity with the isoquinolonesulfonamide derivative H-7, a selective inhibitor of PKC (Hikada *et al.*, 1984; Kawamoto and Hidaka, 1984). We investigated HIV-1 LTR-directed expression of the chloramphenicol acetyltransferase gene product (CAT) using the Jurkat T lymphoblastoid cell line in a transient expression assay. An expression vector containing the HIV-1 LTR (nucleotides −121/+232) linked to the CAT gene (pHIV-1CAT, Figure 1) was transfected into untreated or PKC-deficient cells in the absence or presence of the HIV-1 *tat* protein, which was provided by cotransfection of a *tat* cDNA expression plasmid (Muesing *et al.*, 1987; Jakobovits *et al.*, 1988). The level of CAT expression directed by pHIV-1CAT in Jurkat cells was stimulated ~200-fold by *tat* (Table I).

Exposure of Jarkut cells to PMA (100 ng/ml) for relatively short periods (up to 20 h) gave a 4- to 5-fold induction of the basal level of CAT expression directed by the HIV-1 LTR but did not affect its *trans*-activation by *tat* (data not shown), consistent with previous results obtained using T cell lines (Harada *et al.*, 1986; Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starsken *et al.*, 1987). Depletion of PKC by prolonged exposure (48 h) of the cells to PMA before transfection resulted in a 2.4-fold increase in the basal expression level (Table I). In addition, PKC depletion led to a dramatic decrease (52-fold) in *tat*-stimulated expression and to a complete abolition of HIV-1 *trans*-activation (Table I), indicating that PKC has a direct effect on *tat*-stimulated expression.

To investigate the PKC dependence of *tat* activation of the HIV-1 LTR further, we compared the effect of the selective PKC inhibitor H-7 with that of HA1004, an isoquiniamide derivative which preferentially inhibits cAMP- and cGMP-dependent protein kinases and is a relatively weak inhibitor of PKC (Hidaka *et al.*, 1984). Treatment with H-7 (35 μM) was comparable to PKC depletion by PMA overexposure in increasing basal LTR-directed expression (4-fold) and in its ability to inhibit *tat*-induced HIV-1 *trans*-activation (52-fold) (Table I). In contrast, HA1004 treatment did not significantly affect *tat* activity (2-fold decrease), indicating that the reduction in *trans*-activation by H-7 is due to the specific inhibition of PKC. Thus, depletion of cellular PKC activity in two different ways substantially decreases *trans*-activation of the HIV-1 LTR by *tat*.

The PKC effect on HIV-1 trans-activation is independent of the LTR enhancers

The element responsible for the induction by PMA of basal HIV-1 LTR-directed transcription is a duplicated 10 nucleotide enhancer element at nucleotides −105 to −86 (Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Tong-Starsken *et al.*, 1987). This element binds the PMA-regulated DNA binding proteins NF-κb (Nabel and Baltimore, 1987) and HIVEN86A (Bohnlein *et al.*, 1988). In contrast, *trans*-activation occurs efficiently in the absence of these enhancers (Muesing *et al.*, 1987, Table I, Table III); activation by *tat* involves a distinct element located 3′ of the RNA initiation site (Jakobovits *et al.*, 1988; Selby *et al.*, 1989). As PKC is thought to mediate the induction of basal transcription by PMA, we investigated whether the effect of PKC on *trans*-activation is independent of its effect on the HIV-1 enhancers, using a deletion mutant lacking both enhancer elements (Figure 1).

The level of basal expression directed by the enhancer deletion mutant was comparable to that of the wild type LTR

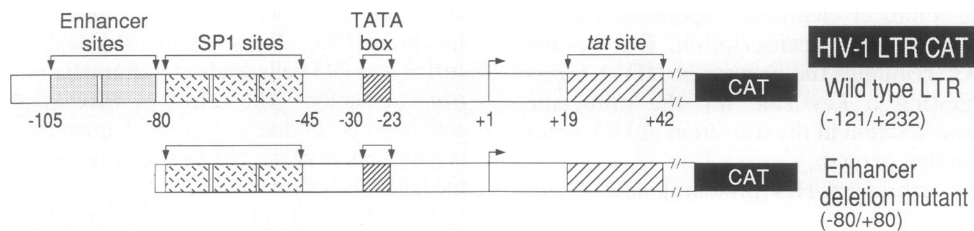

Fig. 1. HIV-LTR CAT constructs. Schematic representation of CAT expression plasmids containing the wild type HIV-1LTR (−121/+232) and the enhancer deletion mutant (−80/+80). *Tat* activation site, TATA box, SP1 binding sites and enhancer elements are indicated.

Table I. *Trans*-activation of wild type and mutant HIV-1 LTR in PKC-deficient Jurkat cells

LTR-CAT plasmid	Treatment	Relative CAT activity		*trans*-activation (fold)	Relative *trans*-activation (%)
		−*tat*	+*tat*		
HIV wild type HIV-1(−121/+232)	−	0.33 ± 0.10	67.6 ± 1.3	205	100
	PMA	0.78 ± 0.25	1.3 ± 0.5	1.6	0.8
	H-7	1.40 ± 0.20	5.5 ± 0.1	3.9	1.9
	HA1004	0.44 ± 0.15	39.9 ± 0.5	91	44.4
Enhancer deletion mutant HIV-1(−80/+80)	−	0.50 ± 0.05	29.2 ± 5.1	58	100
	PMA	1.10 ± 0.09	1.8 ± 0.4	1.6	2.8
	H-7	1.80 ± 0.24	3.4 ± 0.9	1.9	3.3
	HA1004	0.23 ± 0.07	14.2 ± 1.9	62	106

Wild type pLTRCAT(−121/+232) and the enhancer deletion mutant pLTRCAT(−80/+80) were transfected into Jurkat cells in the presence of HIV-1*tat* (cotransfection with plasmid expressing *tat* cDNA, pSVEtat or pCMVtat) or in the absence of *tat* (cotransfected with pUC13). The cells used for transfection were either untreated or treated with PMA, H-7 or HA1004 as described in Materials and methods. Relative CAT activity is normalized with respect to the duration of the CAT assay. The values shown are representative of three independent assays. Standard deviation values are indicated. Relative *trans*-activation represents the percentage of *trans*-activation detected for treated cells versus untreated cells; *trans*-activation of untreated cells corresponds to 100%.

(Table I), consistent with results obtained with enhancer base substitution mutants in Jurkat cells (Nabel and Baltimore, 1987). In contrast to the wild type LTR, basal expression directed by the enhancer deletion mutant LTR was not stimulated by short exposure to PMA (data not shown), confirming the role of the enhancers in mediating the effects of PMA on basal LTR-directed expression (Nabel and Baltimore, 1987; Kaufman *et al.*, 1987; Siekevitz *et al.*, 1987). However, as found for the wild type LTR, depletion of PKC by PMA overexposure increased the basal expression of the mutant by 2- to 3-fold (Table I). The enhancer deletion mutant supported high levels of *tat*-mediated *trans*-activation (Table I). Depletion of PKC activity by either PMA overexposure or H-7 treatment inhibited the response of the enhancer deletion mutant to *tat* stimulation to the same extent as that seen for the wild type LTR, resulting in abolition of HIV *trans*-activation (Table I). Thus, the requirement for PKC in *trans*-activation does not involve the PMA-responsive enhancers in the HIV-1 LTR.

To determine whether the decrease in basal and *tat*-stimulated LTR-directed expression resulting from PKC deficiency reflects a non-specific inhibition of cellular gene expression, we analysed the effect of PKC depletion or inhibition on CAT expression directed by other viral and cellular promoters. Inactivation of PKC did not reduce CAT expression directed by an SV40 early promoter lacking the 72 bp repeat elements, Rous sarcoma virus LTR or the human actin gene promoters (Table II). Thus, the observed decrease in *tat*-stimulation reflects a specific effect of PKC deficiency on HIV-1 *trans*-activation rather than a general effect on cellular gene expression.

Depletion of functional PKC does not affect synthesis of tat protein

To examine whether PKC deficiency affects the level of *tat* protein in the cell rather than its ability to *trans*-activate, we analysed *tat* protein levels after PKC depletion of cells. Due to the low transfection efficiency obtained in Jurkat cells, the level of *tat* protein expressed in these cells could be detected but it was too low to be quantitated reliably. Therefore, we used another cell line, the 293 human embryonic kidney cell line, which supports high levels of expression of HIV *trans*-activation and has been previously used to characterize the mechanism of HIV *trans*-activation (Jakobovits *et al.*, 1988). The requirement for functional PKC in *trans*-activation in 293 cells was investigated in transient transfection assays, similar to those described for the Jurkat cells, using the enhancer deletion mutant. Depletion of functional PKC from 293 cells, either by PMA overexposure or by H-7 treatment, did not affect the basal expression level but significantly inhibited *tat*-mediated *trans*-activation (Table III). These results indicate that the requirement for functional PKC to support *trans*-activation can be studied in 293 cells as well as in Jurkat cells.

Following PKC depletion, 293 cells expressing *tat* either transiently or stably were metabolically labelled with [^{35}S]cysteine, and *tat* protein was immunoprecipitated using rabbit anti-*tat* antibodies (Muesing *et al.*, 1987). Similar levels of the M_r 15 500 *tat* polypeptide were detected in untreated cells and in cells exposed for prolonged periods with PMA or treated with H-7, whether *tat* was expressed stably (Figure 2A) or transiently (Figure 2B), indicating that the decrease in *trans*-activation is not caused by a decrease in the level of the *tat* protein in the cell.

Transfection of a PKC expression vector restores trans-activation in PKC-deficient cells

To determine whether the restoration of PKC activity in cells depleted of PKC by PMA treatment can restore *trans*-activation, we introduced an expression vector encoding a PKC cDNA (hpkC-βII) isolated from human fetal brain (Coussens *et al.*, 1987) into PMA-treated Jurkat and 293 cells. In addition, a mutant cDNA encoding a PKC altered at the putative ATP binding site was also examined in 293 cells. Both vectors directed a high level of transcription of the predicted 3.5 kb PKC mRNA in both untreated and PMA-treated cells, while the endogenous pkC-βII mRNA was barely detectable (Figure 3A). The expression of endogenous PKC proteins in these cells was readily detected by measuring the specific binding of the phorbol ester analogue [^{3}H]phorbol dibutyrate (PDBu) (Figure 3B). PKC depletion by PMA treatment completely abolished [^{3}H]PDBu binding (Figure 3B). Transfection of cells depleted of PKC by PMA treatment with either the wild type or mutant PKC expression vectors restored PDBu binding

Table II. The effect of PKC-depletion on viral and cellular promoters

Treatment	Relative CAT activity		
	RSV	SV40ΔEN	Actin
–	1.62 ± 0.28	0.98 ± 0.42	1.09 ± 0.08
PMA	1.35 ± 0.27	0.75 ± 0.17	1.65 ± 0.25
H-7	4.40 ± 0.29	0.83 ± 0.28	4.60 ± 1.07

Plasmids expressing the CAT gene under the control of SV40ΔEN (SV40 early promoter lacking the 72 bp repeat enhancer elements), human actin or the Rous sarcoma virus (RSV) promoters were transfected into Jurkat cells, untreated or treated with PMA or H-7. The values shown are representative of three independent assays and are the average obtained from duplicate transfections.

Table III. *Trans*-activation of mutant HIV-1 LTR in PKC-deficient 293 cells

	Treatment	Relative CAT activity		*trans*-activation (fold)	Relative *trans*-activation (%)
		−*tat*	+*tat*		
Enhancer deletion mutant HIV-1(−80/+80)LTR-CAT	–	1.8 ± 0.4	435.8 ± 14.7	242	100
	PMA	2.5 ± 0.1	51.0 ± 1.4	20	8.3
	H-7	1.4 ± 0.1	26.2 ± 2.0	19	7.9
	HA1004	2.4 ± 0.7	205.0 ± 24.7	85	35.1

The enhancer deletion mutant pLTRCAT(−80/+80) was transfected into 293 cells in the presence of HIV-1*tat* (cotransfection with plasmid expressing *tat* cDNA, pSVEtat or pCMVtat) or in the absence of *tat* (cotransfected with pUC13). The cells used for transfection were either untreated or treated with PMA, H-7 or HA1004 as described in Materials and methods. The values shown are representative of three independent assays. Standard deviation values are indicated. Relative *trans*-activation represents the percentage of *trans*-activation detected for treated cells versus untreated cells; *trans*-activation of untreated cells corresponds to 100%.

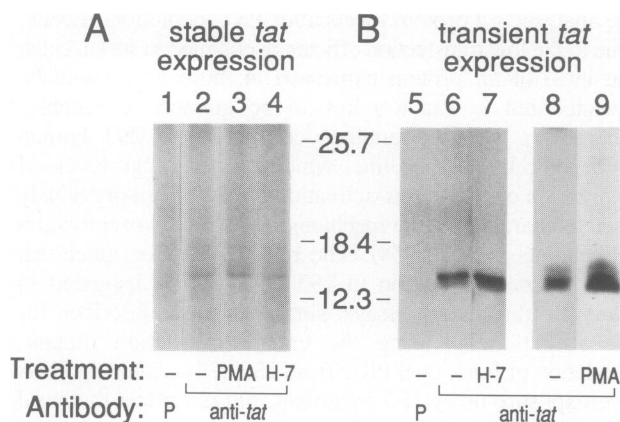

Fig. 2. Synthesis of *tat* protein is not affected in PKC-deficient cells. **A**. 293*tat* cells, which constitutively express the *tat* protein and **B**. 293 cells transfected with 2 μg pSVEtat, untreated (lanes 1, 2, 5, 6 and 8), treated wtih PMA (lanes 3 and 9) or with H-7 (lanes 4 and 7) were metabolically labelled for 4 h with [^{35}S]*L* cysteine (0.5 mCi/plate). PMA and H-7 were present throughout the labelling procedure. The 15.5 kd *tat* protein was analysed by immunoprecipitation of cell lysates with anti-*tat* antibodies (anti-*tat*; lanes 2, 3, 4, 6, 7, 8 and 9) or pre-immune serum (P; lanes 1 and 5) as described in Muesing *et al.* (1987). The immunoprecipitates were separated on a 15% SDS–polyacrylamide gel. The protein mol. wt markers (in kd) are indicated.

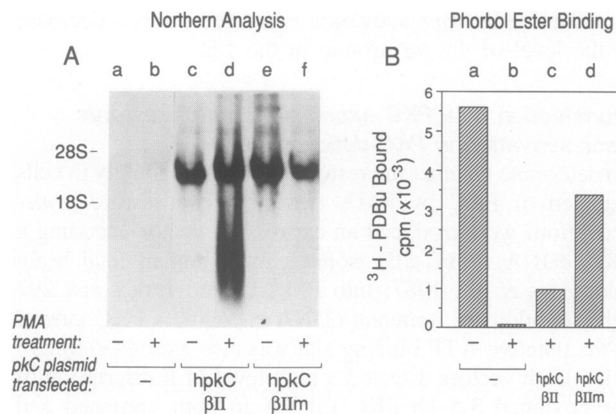

Fig. 3. Expression of hpkC-βII and hpkC-βIIm in transfected 293 cells. **A**. Northern blot analysis of pkC-βII transcripts in transfected and control 293 cells. Untreated (a,c,e) or PMA-treated (b,d,f) cells were transfected with 2 μg of: pU13 (a,b), hpkC-βII (c,d) or hpkC-βIIm (e,f). The blot was hybridized with ^{32}P-labelled hpkC-βII cDNA. **B**. Binding of [^{3}H]PDBu to untreated (a) or PMA-treated (b,c,d) 293 cells, transfected with hpkC-βII (c) or pkC-βIIm (d).

to ~18–60% of the level found in untreated cells, representing at least a 100-fold increase over the level of binding seen in untransfected, PMA-treated cells (Figure 3B).

To determine the effect of restoring PKC activity on *trans*-activation, PKC-downregulated Jurkat and 293 cells were cotransfected with the HIV-1 LTR enhancer deletion mutant and PKC expression vector, in the presence or absence of the *tat* expression vector (Table IV). *Trans*-activation was assessed 24 h post-transfection in cells continuously incubated with PMA. At this time endogenous PKC has been down-regulated, whereas the transfected PKC is activated by PMA (Figure 3B). As shown in Table IV, the wild type PKC expression vector restored *trans*-activation to levels comparable to those seen in untreated cells. In contrast, transfection of the expression plasmid encoding the mutant PKC did not restore the *trans*-activation (Table IV). That the restored *trans*-activation was due to the expression of PKC was confirmed by the ability of 100 μM H-7 to block restoration of *trans*-activation (data not shown). In contrast, no effect of the transfected PKC cDNAs on basal expression in the absence of *tat* was detected, as expected for the enhancerless HIV-1 LTR mutant (Table IV). Thus, in cells depleted of PKC by prolonged PMA exposure, the expression of functional PKC is sufficient to restore *trans*-activation.

Discussion

The involvement of mitogenic stimulation in the regulation of HIV-1 gene expression was previously studied by examining the ability of T cell mitogens to stimulate HIV-1 LTR expression (Harada *et al.*, 1986; Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Siekevitz *et al.*, 1987; Tong-Starsken *et al.*, 1987). These studies showed that the basal level of HIV-1 transcription is induced by the phorbol ester mitogen PMA, but failed to reveal any mitogenic effect on *trans*-activation. The activation of T cells by PMA is mediated, at least in part, by activation of protein kinase C. We reasoned that the levels of activated PKC in the transformed T cell lines tested, unlike in resting T cells *in vivo*, could be high enough to saturate the *trans*-activation response, obscuring further mitogenic induction of HIV-1 *trans*-activation. To test this hypothesis we have depleted PKC from cells, and restored it by transfection of PKC cDNA expression vectors. Depletion of functional PKC by down-regulation of the enzyme, due to prolonged exposure to PMA inhibits *tat*-mediated HIV-1 *trans*-activation in both Jurkat and 293 cells. Similarly, the PKC inhibitor H-7, strongly inhibits HIV-1 *trans*-activation, while HA1004, an isoquiniamide derivative with weak PKC inhibitory activity, does not affect *trans*-activation, indicating that inhibition of *trans*-activation by H-7 results from specific inhibition of PKC. In cells depleted of PKC by PMA overexposure, *trans*-activation can be restored by transfection with a normal PKC cDNA, confirming the requirement for PKC in *trans*-activation. Expression of a mutant PKC which binds PMA as efficiently as the wild type protein, but lacks a functional ATP binding site, does not restore *trans*-activation, demonstrating that the phosphorylation activity of PKC is necessary to support *trans*-activation.

We find that the inhibition of *trans*-activation by depletion of functional PKC is not due to a decrease in *tat* protein levels; however the mechanism by which PKC regulates HIV-1 *trans*-activation is not yet clear. Several mechanisms are plausible, but the possibility that *tat* protein is directly phosphorylated by PKC seems unlikely as efforts to detect phosphorylation of *tat* protein have been unsuccessful to date (Hauber *et al.*, 1988). Alternatively, PKC may modify other components involved in *trans*-activation. As a result, the activity or cellular localization of *tat*, or a cofactor that may act in concert with *tat*, may be altered, or alternatively, the interaction of *tat*, putative cofactor or the complex, with a nucleic acid recognition site may be changed, in a way similar to that already shown for other PKC-inducible nuclear *trans*-activators such as NF-κB (Baeuerle and Baltimore, 1988). Several cellular proteins which bind to DNA (Jones *et al.*, 1988; Wu *et al.*, 1988) or RNA (Gatignol *et al.*, 1989)

Table IV. Restoration of *trans*-activation in PKC-deficient cells by transfected PKC expression vector

	PMA treatment	PKC plasmid transfected	Relative CAT activity		*Trans*-activation (fold)	Relative *trans*-activation (%)
			−*tat*	+*tat*		
Jurkat T cells	−	−	0.5	29	58.3	100
	+	−	1.1	1.8	1.6	2.7
	+	hPKC-βII	1.1	24	22.0	37.7
293 cells	−	−	1.7	380	220	100
	+	−	2.5	49	20	9.1
	+	hPKC-βII	1.7	194	117	53.2
	+	hPKC-βIIM	1.7	51	30	13.6

Restoration of *tat*-mediated induction of CAT activity by transfected hpkC-βII into PKC-deficient Jurkat and 293 cells. The HIV-1 LTR enhancer deletion mutant (−80/+80) was cotransfected with pU13 or with *tat* expression plasmid into untreated or PMA-treated Jurkat or 293 cells in the absence or presence of hpkC-βII or hpkC-βIIm. Cells were harvested 24 h after transfection. The relative CAT activity and the corresponding standard deviation values are indicated. The values shown are representative of two independent assays and are the average obtained from duplicate transfections. Relative *trans*-activation represents the percentage of *trans*-activation detected for treated cells versus untreated cells; *trans*-activation of untreated cells corresponds to 100%.

sequences comprising the *tat*-responsive element have been suggested to be involved in *trans*-activation and therefore can be regarded as candidates for modification by PKC. Identification of one of these proteins as the target of PKC must await the establishment of their involvement in the process of *trans*-activation.

The requirement for PKC in *trans*-activation does not involve the PMA-responsive enhancers which mediate the mitogenic effect on basal transcription, as we have shown that HIV-1 LTR mutants with deleted enhancers still require PKC for *trans*-activation. These results indicate that the mechanisms by which PKC regulates *trans*-activation and basal HIV-1 transcription are distinct. The effect of PMA on the HIV-1 enhancers may not be specific for HIV-1, as similar elements are present in the promoters of many other viruses and cellular genes, including those of the IL-2 receptor (Bohnlein *et al.*, 1988), immunoglobulin light chains (Sen and Baltimore, 1986) and β2-microglobulin (Kimura *et al.*, 1986). In contrast, the effect of PKC on *trans*-activation may be highly specific for HIV-1, as *trans*-activation by *tat* is distinctive to HIV-1.

Protein kinase C has previously been implicated in several aspects of HIV-1 replication, including the regulation of HIV-1 transcription by factors that bind to the LTR enhancers (Dinter *et al.*, 1987; Kaufman *et al.*, 1987; Nabel and Baltimore, 1987; Bohnlein *et al.*, 1988), phosphorylation of the HIV-1 *nef* protein (Guy *et al.*, 1987) and *rev* protein (Hauber *et al.*, 1988) and phosphorylation of CD4, the receptor for HIV (Fields *et al.*, 1988). Indeed, the latter report showed that H-7 treatment decreases the amount of productive HIV infection of peripheral blood T cells. It was suggested that viral entry into the cells was affected as a result of decreased CD4 phosphorylation. But the recent demonstration that the cytoplasmic domain of CD4 is not required for HIV infection (Bedinger *et al.*, 1988; Maddon *et al.*, 1988) indicates that phopshorylation of this domain is not important to viral entry. Our results provide an alternative explanation for these observations, by showing that PKC is essential in the regulation of HIV-1 *trans*-activation. Thus, the inhibition of HIV-1 replication by H-7 may be due to decreased viral gene expression rather than diminished viral entry.

Our results indicate that T cell mitogens, possibly including pathogens, can activate HIV-1 replication by increasing *trans*-activation. While the possibility that immune activation may trigger latent virus has been previously suggested, it has been unclear how a relaitvely small effect, such as that of PKC on the enhancer region, could produce a major change. Here we have shown that the effect of PKC activation is in fact dramatic, as it increases *trans*-activation as well as the activity of the HIV-1 enhancers, so that activation of a resting lymphocyte could profoundly affect the state of the virus it harbours. Our results suggest that PKC inhibitors may suppress the activation of latent HIV-1 and ongoing viral replication; if PKC inhibitors specific for the PKC function which activates *tat* can be developed, they may eventually be useful in the treatment of HIV-1 disease.

Materials and methods

Cell culture and transfection assays

Jurkat T cells were grown in RPMI 1640 medium containing 10% fetal calf serum. Cells (4×10^6) were cotransfected with pHIV-LTR plasmids (6 μg) and pU13 or *tat* expression plasmid (0.5 μg) using DEAE-dextran method (Kaufman *et al.*, 1987). To deplete PKC, the cells were incubated in serum-supplemented medium containing 100 ng/ml PMA for 48 h before transfection. For PKC inactivation, following transfection, the cells were grown in complete medium containing 35 μM H-7 or HA1004. 293 cells were grown in DME/F12 medium containing 10% fetal calf serum. The cell line 293*tat* was obtained by cotransfecting 293 cells with pCMVtat plasmid and pRSVNEO. Stable transfectants were obtained upon selection with 0.5 mg/ml G418. The pHIV-LTR plasmids (3 μg) were cotransfected into 293 cells (60 mm plate) with 0.5 μg of pU13 or *tat* expression plasmid. To deplete PKC from cells by chronic exposure to 100 ng/ml PMA, 293 cells were grown in serum-supplemented medium to semi-confluency and then transferred to serum-free medium in the presence or absence of PMA. After 72 h, the cells were transfected by the calcium phosphate method (Gorman *et al.*, 1982). The cells were shocked with glycerol 4−6 h post-transfection and the medium was replaced by fresh serum-free medium containing PMA. For PKC inactivation, following the glycerol shock the medium was replaced by medium containing 100 μM H-7 or HA1004. The cells were harvested 48 h after transfection and CAT activity was assayed in the linear range of chloramphenicol conversion as described previously (Jakobovits *et al.*, 1988). Relative CAT activity is normalized with respect to the duration of the CAT assay. PMA was obtained from Sigma, 1[(5-isoquinolinylsulfonyl)]-2-methylpiperazine dihydrochloride (H-7) and *N*-(2-guanidinoethyl) 5-isoquinolinesulfonamide hydrochloride (HA1004) were obtained from Seikagaku America (St Petersburg, FL).

Plasmid construction

The plasmids expressing *tat* cDNA, pSVEtat and pCMVtat, were constructed as described by Jakobovits *et al.* (1988) and by Fisher *et al.* (1987), respectively. A 2.6 kb *Eco*RI cDNA fragment encoding human pkC-βII was cloned into CMV-based expression vector (Fisher *et al.*, 1987). The mutant, hpkC-βIIm, was generated by replacing the *Sac*I−*Eco*NI fragment

of the ATP binding domain with a synthetic fragment in which the codon for lysine 371 has been changed to tryptophan.

RNA analysis

Total cellular RNA (25 μg) was fractionated on 1% formaldehyde-agarose gel, transferred to nylon membranes, cross-linked by UV irradiation, and hybridized with [^{32}P]hpkC-βII probe as described by Jakobovits *et al.* (1988).

[^{3}H]PDBu binding assays

Specific [^{3}H]PDBu binding to transfected cells was determined by the method described (Knopf *et al.*, 1986). PMA was removed from PMA-treated cells by extensive washings 24 h before the binding assays to avoid PMA competition with the radiolabelled PDBu. Each 60 mm plate was labelled with 0.1 μCi (5 nM) [^{3}H]PDBu (12 Ci/mmol NEN) for 30 min at 37°C. The non-specific binding was determined by exposing the cells to 5 μM of unlabelled PDBu for 10 min before addition of the labelled PDBu. The non-specific binding comprises $<10\%$ of total binding in each case.

Acknowledgements

We thank Scot Marsters for technical assistance, Dr Rebecca Ward for critical comments on the manuscript, Dr Axel Ullrich for providing the hpkC-βII cDNA, Robert Klein for construction of CMV-based expression vectors, Dr David Goeddel for encouragement and Wayne Anstine and Kerrie Andow for preparation of the figures.

References

Baeurerle,P.A. and Baltimore,D. (1988) *Cell*, **53**, 211–217.

Bedinger,P., Moriarty,A., von Borstel,R.C., Donovan,N.J., Steimer,K.S. and Littman,D. (1988) *Nature*, **334**, 162–165.

Bohnlein,E., Lowenthal,J.W., Siekevitz,M., Ballard,D., Franza,B.R. and Greene,W.C. (1988) *Cell*, **53**, 827–836.

Coussens,L., Parker,P.J., Rhee,L., Yang-Feng,T.L., Chen,E., Waterfield,M.D., Francke,U. and Ullrich,A. (1986) *Science*, **233**, 859–866.

Coussens,L., Rhee,L., Paeker,P.J. and Ullrich,A. (1987) *DNA*, **6**, 389–394.

Curran,J.W., Meade Morgan,W., Hardy,A.M., Jaffe,H.W., Darrow,W.W. and Dowdle,W.R. (1985) *Science*, **229**, 1352–1357.

Dayton,A., Sodroski,J.G., Rosen,C.A., Goh,W.C. and Haseltine,W.A. (1986) *Cell*, **44**, 941–947.

Dinter,H., Chiu,R., Imagawa,M., Karin,M. and Jones,K. (1987) *EMBO J.*, **6**, 4067–4071.

Fields,A.P., Bednarik,D.P., Hess,A. and May,S.W. (1988) *Nature*, **333**, 278–280.

Fisher,K.L., Gorman,C.M., Vehar,G.A., O'Brien,D.P. and Lawn,R.M. (1987) *Thrombosis Res.*, **48**, 89–99.

Folks,T., Kelly,J., Benn,S., Kinter,A., Justement,J., Gold,J., Redfield,R., Sell,K.W. and Fauci,A.S. (1986) *J. Immunol.*, **136**, 4049–4053.

Gatignol,A., Kumar,A., Rabson,A. and Jeang,K.-T. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7828–7832.

Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.

Guy,B., Kieny,M.P., Riviere,Y., Le Peuch,C., Dott,K., Girard,M., Montagnier,L. and Lecocq,J.-P. (1987) *Nature*, **330**, 266–269.

Harada,S., Koyanagi,Y., Nakashima,H., Kobayashi,N. and Yamamoto,N. (1986) *Virology*, **154**, 249–258.

Hauber,J., Bouvier,M., Malim,M.H. and Cullen,B.R. (1988) *J. Virol.*, **62**, 4801–4804.

Hidaka,H., Inagaki,M., Kawamoto,S. and Sasaki,Y. (1984) *Biochemistry*, **23**, 5063–5041.

Jakobovits,A., Smith,D.H., Jakobovits,E.B. and Capon,D.J. (1988) *Mol. Cell. Biol.*, **8**, 2555–2561.

Jones,K.A., Luciw,P.A. and Duchange,N. (1988) *Genes Dev.*, **2**, 1101–1114.

Kaufman,J., Valandra,G., Roderiquez,G., Bushar,G., Giri,C. and Norcross,M.A. (1987) *Mol. Cell. Biol.*, **7**, 3759–3766.

Kawamoto,S. and Hidaka,H. (1984) *Biochem. Biophys. Res. Commun.*, **125**, 258–264.

Kimura,A., Israel,A., Le Bail,O. and Kourilsky,P. (1984) *Cell*, **44**, 261–272.

Knopf,J., Lee,M.-H., Sultzman,L.A., Kriz,R.W., Loomis,C.R., Hewick,R.M. and Bell,R.M. (1986) *Cell*, **46**, 491–502.

MacDougal,J.S., Mawle,A., Cort,S.P., Nicholson,J.K.A., Cross,G.D., Scheppler-Campbell,J.A., Hicks,D. and Sligh,J. (1985) *J. Immunol.*, **135**, 3151–3162.

Maddon,P.J., McDougal,J.S., Clapham,P.R., Dalgleish,A.G., Jamal,S., Weiss,R.A. and Axel,R. (1988) *Cell*, **54**, 865–874.

Manger,B., Weiss,A., Imboden,J., Laing,T. and Stobo,J.D. (1987) *J. Immunol.*, **139**, 2755–2760.

Muesing,M.A., Smith,D.H. and Capon,D.J. (1987) *Cell*, **48**, 691–701.

Nabel,G. and Baltimore,D. (1987) *Nature*, **326**, 711–713.

Nishizuka,Y. (1988) *Nature*, **334**, 661–665.

Ohno,S., Kawasaki,H., Imajoh,S. and Suzuki,K. (1987) *Nature*, **325**, 161–166.

Ono,Y., Kurokawa,T., Fujii,T., Kawahara,K., Igarashi,K., Kikkawa,U., Ogita,K. and Nishizuka,Y. (1986) *FEBS Lett.*, **206**,347–352.

Selby,M.J., Bain,E.S., Luciw,P.A. and Peterlin,B.M. (1989) *Genes Dev.*, **3**, 547–558.

Sen,R. and Baltimore,D. (1986) *Cell*, **41**, 921–928.

Siekevitz,M., Josephs,S.F., Dukovich,M., Peffer,N., Wong-Staal,F. and Greene,W.C. (1987) *Science*, **238**, 1576–1578.

Sodroski,J., Rosen,C., Wong-Staal,F., Salahuddin,S.Z., Popovic,M., Arya,S. and Gallo,R.C. (1985) *Science*, **227**, 171–173.

Tong-Starksen,S.E., Luciw,P.A. and Peterlin,B.M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6845–6849.

Wu,F.K., Garcia,J.A., Harrich,D. and Gaynor,R.B. (1988) *EMBO J.*, **7**, 2117–2129.

Young,S., Parker,P., Ullrich,A. and Stable,S. (1987) *Biochem. J.*, **224**, 775–779.

Zagury,D., Leonard,B.R., Cheynier,R., Feldman,M., Sarin,P.S. and Gallo,R.C. (1986) *Science*, **231**, 850–853.

Received November 17, 1989; revised January 26, 1990