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Human Immunodeficiency Virus Type 1 Tat-Mediated *trans* Activation Correlates with the Phosphorylation State of a Cellular TAR RNA Stem-Binding Factor

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Protein kinase C (PKC) is involved in the mitogenic stimulation of cell proliferation and has recently been reported to be essential for Tat-mediated *trans* activation. We have determined that RNA binding of a cellular factor which specifically interacts with the *trans*-activation response region (TAR) is blocked in cells depleted of PKC activity by chronic phorbol myristate acetate stimulation. We also show that nuclear extracts can be depleted of the cellular TAR-binding factor by in vitro treatment with purified protein phosphatase 2A. Furthermore, TAR RNA-binding activity can be partially restored to depleted nuclear extracts in vitro by addition of PKC. Chimeric constructs in which the Tat protein is artificially tethered to viral RNA show PKC independence for Tat-mediated *trans* activation. Specific mutations in the TAR RNA stem region which cause reduced binding of host cell factor in vitro also cause reduced Tat-mediated *trans* activation in vivo. Together, these results suggest that phosphorylation-dependent binding of a cellular cofactor to TAR RNA is an essential step in Tat-mediated *trans* activation. Deciphering the regulation of Tat-mediated *trans* activation by phosphorylation will be critical in fully understanding the regulation of human immunodeficiency virus type 1 activation.

Induction of latent human immunodeficiency virus type 1 (HIV-1) proviruses can be brought about by mitogenic stimulation of the infected cells (8, 12, 21, 40), which subsequently leads to the expression of *tat* gene product and active viral replication. Protein kinase C (PKC) is required for the induction of cell proliferation by several mitogens (23). Among these, phorbol 12-myristate-13-acetate (PMA) activation has been shown to enhance basal HIV-1 transcription, a critical step in the activation of latent HIV-1 (11, 20, 26, 34). On the other hand, depletion of PKC by chronic stimulation with PMA has been reported to markedly inhibit Tat-mediated *trans* activation without affecting the Tat protein levels (17). Although the precise mechanism of *tat* gene function remains unresolved, it is clear that Tat *trans* activation requires the TAR RNA, which is part of the 5′ untranslated leader sequence of all HIV-1 RNAs (3–5, 28). It has been argued that *trans* activation involves a direct interaction of Tat with the TAR RNA (10, 19, 30, 37). However, since Tat-TAR RNA interaction in vitro is still possible with TAR mutants lacking the loop sequences (30) required for *trans* activation, additional host cell cofactors may be required for a functional Tat-TAR interaction in vivo. Such a host cell factor mediating role has been suggested (19). We and others have shown specific host protein binding to the TAR RNA (13, 14, 24, 29, 33, 38). If the host cell Tat cofactor (TCF) plays an essential role in viral gene *trans* activation, we would argue that its functional interaction with TAR might reflect changes in the cellular transduction pathways that mediate mitogenic stimulation.

We show here that the phosphorylation state of the TCF correlates with the level of HIV-1 Tat-mediated *trans* activation. In cells depleted of PKC activity by chronic PMA stimulation, both Tat-mediated *trans* activation and TCF binding to TAR RNA are greatly diminished. TAR-binding activity can be partially restored to the depleted extracts in vitro by addition of PKC. Furthermore, nuclear extracts containing functional TCF can be depleted of TAR RNA-binding activity by in vitro treatment with purified protein phosphatase 2A. TAR mutants with specific alterations in the stem region show reduced binding of TCF in vitro and reductions in Tat-mediated *trans* activation in vivo. Finally, we show that the PKC-sensitive step in Tat-mediated *trans* activation is bypassed in constructs in which the Tat protein is artificially tethered near the 5′ end of the transcripts. These results suggest that the TCF is required for appropriate binding of Tat to TAR and that TCF RNA-binding activity is modulated by protein phosphorylation and dephosphorylation.

MATERIALS AND METHODS

Cell culture. To activate endogenous PKC, cells were treated with complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 ng of PMA per ml for 8 h before harvest. For PKC depletion, cells were grown in the same PMA-containing DMEM for 24 h prior to and 24 to 48 h after transfection.

Adherent cells (HeLa and HeLa-Tat) were transfected by the standard calcium phosphate method, with 2 to 10 μg of plasmid DNA per 3×10^5 to 5×10^5 cells. Transfected cells were harvested 36 to 48 h after transfection.

CAT assay. Chloramphenicol acetyltransferase (CAT) enzyme activity was determined by the procedure described previously (14, 19). The human growth hormone (hGH) transient-expression assay system (hGH kit; Nichols Insti-

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tute) was used as an internal transfection control. This system is based on immunological detection of hGH secreted by transfected cells (32). Following transfection (usually 48 h), the growth medium was removed and assayed for hGH activity. A 100-μl sample of medium was mixed with 100 μl of ^{125}I-antibody solution (^{125}I-labeled monoclonal hGH antibody [mouse Ab(1)] and biotin-coupled monoclonal hGH antibody [mouse Ab(2)]) and avidin-coated polystyrene beads and incubated on a horizontal rotator (170 rpm) at room temperature for 90 min. Beads were washed twice with 2 ml of wash solution (surfactant in phosphate-buffered saline [PBS] with 0.3% sodium azide), and the radioactivity in the test tubes was counted on a gamma counter for 1 min. (Standard solutions with 0, 0.5, 1.5, 5, 15, and 50 ng of hGH per ml and 100 ml of medium from mock-transfected cells were also assayed.) The counts per minute for ^{125}I-labeled anti-hGH antibody were corrected by subtracting the background counts from the mock-transfected medium, and the standard curve generated was used to calculate the amount of hGH secreted.

PKC assay. PKC activity was determined by measurement of the phosphorylation of N-terminally acetylated myelin basic protein peptide (Ac-MBP) (39) with a kit obtained from GIBCO BRL. Phosphorylation of this substrate is highly specific for PKC and preferentially detects the α, β, and γ subspecies (39). Exponentially growing cells (1×10^6 to 5×10^6) were washed with PBS and extracted in 0.6 ml of extraction buffer (20 mM Tris [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA [ethylene glycol tetraacetic acid], 0.5% Triton X-100, 10 mM β-mercaptoethanol, 25 μg of aprotinin per ml, 25 μg of leupeptin per ml). Cells were incubated on ice for 20 min, homogenized in a Dounce homogenizer, and incubated on ice for another 30 min. Cellular debris was removed by centrifuging for 2 min in a microcentrifuge. PKC was partially purified by DEAE-cellulose ion-exchange chromatography with 0.25 g of DEAE-cellulose (Sigma) in 1 ml of DEAE wash buffer (20 mM Tris [pH 7.5], 0.5 mM EGTA, 10 mM β-mercaptoethanol) and eluted in 2.5 ml of DEAE elution buffer (20 mM Tris [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2 M NaCl) per sample. Assay reaction mixes contained 1 to 25 μl of column eluate, 50 μM Ac-MBP (residues 4 through 14), 20 μM ATP, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 4 mM Tris (pH 7.5), and lipid preparation (1 mM PMA, 280 μg of phosphatidylserine per ml, Triton X-100-mixed micelles). [α-^{32}P]ATP (4,000 Ci/mmol) was added at 5×10^5 or 1×10^6 cpm per reaction mix (50-μl total reaction volume).

For negative controls, a PKC-specific pseudosubstrate inhibitor (20 μM PKC [residues 19 through 36], 4 mM Tris [pH 7.5]) was added to the reaction mix and the lipid preparation was omitted. Samples were incubated at room temperature for 30 min to allow binding of the inhibitor before addition of substrate and ATP. Reaction mixes were incubated at 30°C for 5 min, and 25 μl from each reaction mix was spotted on phosphocellulose disk and washed twice with 1% (vol/vol) phosphoric acid and twice with H_2O. The amount of peptide-incorporated ^{32}P was measured by scintillation counting.

Molecular clones. The chimeric *trans* activation system used was developed and generously provided by Selby and Peterlin (31). In pHIVSRCAT, the TAR region of the HIV-1 long terminal repeat (LTR)-CAT reporter construct was replaced by the operator region of bacteriophage R17, which is a stem-loop RNA target for the bacteriophage MS2 coat protein. pSVtat(1–67)CP, which expresses a chimeric Tat-coat protein (Tat-CP), was constructed by linking residues 1 to 67 of Tat to residues 2 to 129 of the MS2 coat protein. The wild-type reporter construct pHIVSCAT, pSVtat(1–67), and the construction of the chimeric reporters have been described previously (31).

TAR mutant. The recombinant plasmid pBCAcc, which contains an HIV-1 LTR (LAV strain) linked to the reporter gene for CAT, was derived from pBENNCAT (15) by eliminating the pBR322 sequences between the *Acc*I sites (positions 651 and 2246; pBR322 coordinates). A cassette vector, pBCKpnTAR, into which TAR mutant oligonucleotides were cloned, was made from pBCAcc by introducing a *Kpn*I site at position −3 and inserting a *Kpn*I-*Hin*dIII linker in place of the TAR region. The HIV-1 TAR region (nucleotide positions +1 through +57) and the TAR mutant (TM mutant shown below) were synthesized on an Applied Biosystems 380B DNA synthesizer and cloned into the *Kpn*I and *Hin*dIII sites of pBCKpnTAR as described before (29).

To generate TAR RNAs for in vitro RNA-protein interaction, the T7 RNA polymerase promoter was cloned into the *Eco*RI and *Kpn*I sites of pUC19, along with a *Kpn*I-*Hin*dIII linker. The wild-type TAR region (nucleotides +1 through +57) and the synthetic TAR mutant oligonucleotides were cloned into the *Kpn*I and *Hin*dIII sites as described above. The sequence of all TAR mutants was confirmed by dideoxy sequencing with Sequenase (United States Biochemical).

TAR RNAs. The wild-type and mutant TAR RNAs are shown below, with the changed nucleotide underlined:

```
           1        10         20        30         40        50
           .        .          .         .          .         .
Wild type  GGUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCUCUGGCUAACUAGGGAACC
Tm37       GGUCUCUCUGGUUAGACAAGAUCUGAGCCUGGGAGCUCUCUGGCUAACUAGGGAACC
                             -
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In vitro transcription. In vitro transcription reactions were performed in a 25-μl reaction volume containing 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 U of RNasin (Promega), 5 mM each ATP, CTP, and GTP, 0.012 mM UTP (Pharmacia), 50 μCi of [α-^{32}P]UTP (>400 Ci/mmol; Amersham), 1 μg of template DNA linearized with *Hin*dIII, and 30 U of T7 RNA polymerase (Ambion). The reaction mix was incubated at 37°C for 1 h, after which 2 U of RNase-free DNase I was added, and the incubation was continued for an additional 15 min. The volume was then brought up to 100 μl with sterile water; the mix was extracted once with phenol-chloroform and once with chloroform and then precipitated with ethanol in the presence of 0.3 M sodium acetate. The pellet was resuspended in 90% formamide, heated at 85°C for 2 min, loaded on a 10% denaturing polyacrylamide gel containing 7 M urea, and run at 300 V. Full-length transcripts were excised from the gel, eluted, and repurified before use in the RNase protection gel mobility shift assay.

RNase protection gel mobility shift assay. A 5-μl amount of extract obtained by the method of Dignam et al. (9) (25 to 75 μg of protein in 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.8], 100 mM KCl, 0.5 mM dithiothreitol, 0.4 mM EDTA, and 20% glycerol) was incubated with 1 μl of 10 mM $MgCl_2$–1 μl of ^{32}P-TAR RNA (100 to 500 cpm)–3 μl of H_2O at room temperature for 10 to 15 min. RNase digestion was carried out with 2 μl of RNases A and T_1 (1 μg/μl and 5 U/μl, respectively) for 10 min. The RNase-protected TAR RNA-protein complex was then resolved on native 4% polyacrylamide gel (acrylamide-bisacrylamide, 80:1) at 100 V, essentially as described previously (29).

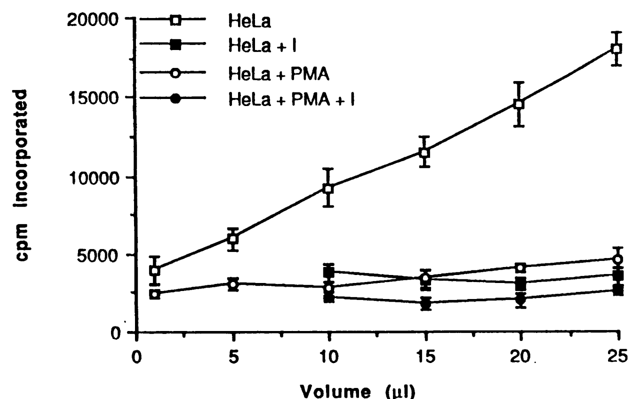

FIG. 1. PKC depletion. PKC levels of untreated and chronically PMA-treated HeLa cells were assayed with an in vitro phosphorylation assay specific for PKC as described in Materials and Methods. The cells were treated for 48 h in DMEM containing 10% fetal bovine serum and 100 ng of PMA per ml prior to harvesting. Following partial purification of PKC as described in Materials and Methods, increasing volumes of the extract were assayed for activity. Nonspecific phosphorylation levels were determined by incorporation of a competitive inhibitor (I) of PKC and omission of the lipids, as described in Materials and Methods. The means and standard deviations of three independent experiments are shown.

RESULTS

Effects of PKC depletion. HeLa cells were depleted of PKC by stimulation with PMA (100 ng/ml) for 48 h. Depletion of PKC was documented by an in vitro phosphorylation assay, as shown in Fig. 1. PKC-specific phosphorylation in PMA-treated cells was reduced to the level of nonspecific phosphorylation (compare HeLa + PMA with HeLa + I, Fig. 1). Therefore, the PMA treatment used has effectively depleted HeLa cell PKC activity.

The effects of PKC depletion on the host cells' ability to support Tat *trans* activation was determined by DNA-mediated transfection of HeLa cells. The indicator plasmid DNA used was HIV-1-CAT, in which the CAT reporter gene is under the control of the HIV-1 LTR. The control plasmid, CMV IE-CAT, has the CAT gene under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter (which lacks the TAR element). As shown in Table 1, the CAT response of the CMV IE-CAT plasmid was essentially the same in normal HeLa cells or HeLa cells cotransfected with pSVtat and in those that were depleted of PKC activity. Transfections with the HIV-1-CAT construct, on the other hand, showed over 62-fold inhibition of Tat-mediated *trans* activation in the PKC-depleted cells (Table 1). Thus, as reported previously (17), Tat-mediated *trans* activation is greatly reduced in PKC-depleted cells.

Phosphorylation dependence of TAR RNA-binding protein. Since the depletion of PKC activity did not affect the stability of Tat protein (17) (nor have we or others observed phosphorylation of HIV-1 Tat protein), we reasoned that the selective inhibition of Tat response may be mediated by the phosphorylation state of a specific host cell TCF (14, 27). Nuclear lysates (9) were prepared from normal and PKC-depleted HeLa and HeLa-Tat cells (cells stably transfected with HIV-1 *tat* under the control of the simian virus 40 early promoter; we have shown previously [14] that host protein binding to TAR RNA is not affected by the presence of Tat). The binding of TCF to the labeled in vitro-transcribed TAR RNA was assayed by RNase protection gel mobility shifts in nondenaturing conditions. The procedure (29) is a modification of one used to demonstrate sequence-specific binding of the HIV-1 Rev protein to the Rev-responsive element RNA (41). As shown in Fig. 2A, formation of the major TCF-TAR RNA complex is markedly inhibited in PKC-depleted lysates from both HeLa and HeLa-Tat cells. It is important to note that, compared with the total TAR RNA-protein complex (14), this sequence-specific RNase protection procedure yields a single ribonucleoprotein (RNP) complex, with p140 as the specific TAR RNA stem-binding protein (29).

To test the hypothesis that endogenous phosphatase activity might inactivate the TCF during the preparation of nuclear lysates from PKC-depleted cells, we used 10 mM microcystin-LR (a cyclic heptapeptide, which is a potent phosphatase inhibitor [22]) in the preparation of nuclear lysates. In both HeLa and HeLa-Tat cell lysates prepared with the phosphatase inhibitor, there was an insignificant change in TCF binding activity, suggesting that dephosphorylation during the preparation of the extract was not responsible for the reduced level of TCF binding to TAR RNA in

TABLE 1. Effect of TAR mutants on *trans* activation in normal and PKC-depleted HeLa cells[a]

CAT plasmids	PMA treatment[b]	CAT activity[c] (% conversion)		*trans* activation[d] (ratio)	Relative CAT activity[e]		Relative *trans* activation[f] (%)
		−pSVtat	+pSVtat		−pSVtat	+pSVtat	
CMV IE-CAT	−	0.97	4.11	4.24	1.00	1.00	100
	+	1.02	3.98	3.9	1.05	0.968	92
HIV-1 LTR-CAT	−	0.098	11.8	121	1.00	1.00	100
	+	0.120	0.232	1.94	1.23	0.02	1.6
TM37	−	0.225	3.45	15.3	1.00	1.00	100
	+	0.300	0.802	2.67	1.33	0.233	17

[a] CMV IE-CAT, wild-type HIV-1 LTR-CAT (containing nucleotides +1 to +57 of TAR sequences), or the TAR mutant TM37 was transfected into HeLa cells with or without pSVtat, an HIV-1 *tat* expression vector. Either 2 µg (CMV IE-CAT, HIV-1 LTR-CAT, or TM37) or and 0.5 µg (pSVtat) of plasmid was used per 5×10^5 cells. The data represent the averages of three experiments, with standard deviations of less than 20%.

[b] The cells used for transfection were either untreated or treated with PMA (100 ng/ml) for 24 h before and 48 h after transfection.

[c] Percent conversion of chloramphenicol to its acetylated versions.

[d] The ratio of chloramphenicol acetylation with pSVtat/without pSVtat.

[e] Relative CAT activity represents the CAT activity in PMA-treated cells versus that in untreated cells. Relative CAT activity of untreated cells corresponds to 1.00.

[f] Relative *trans* activation represents the *trans* activation for PMA-treated cells as a percentage of that for untreated cells.

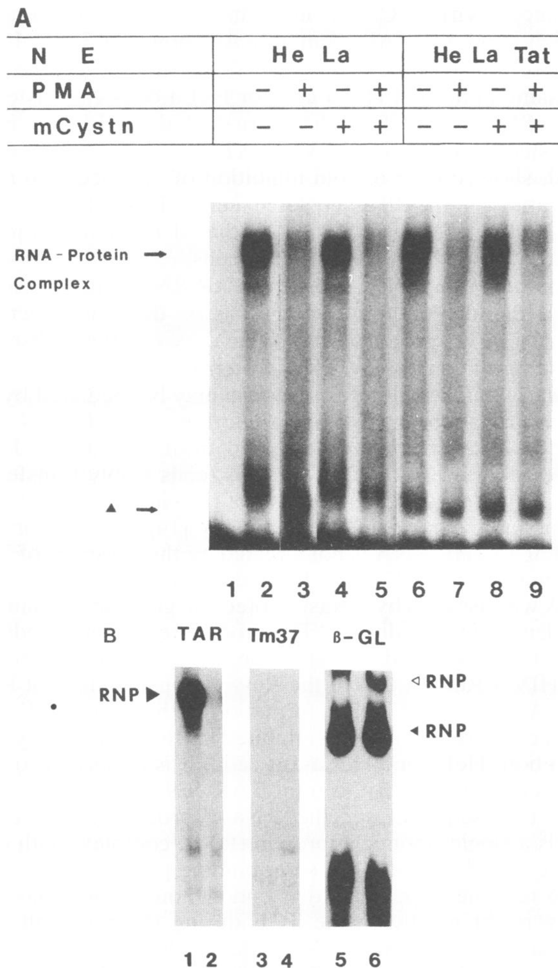

FIG. 2. (A) TAR RNA binding of nuclear factors. Nuclear extracts (NE) used were prepared from normal (lanes 2, 4, 6, and 8) or PMA-treated (lanes 3, 5, 7, and 9) HeLa and HeLa-Tat cells. Microcystin-LR (10 nM) was included as a phosphatase inhibitor. Lane 1 contains the ^{32}P-TAR RNA probe alone. The position of the specific RNA-protein complex is indicated by the arrow. The faster-migrating band, indicated by a triangle and an arrow, represents undigested TAR RNA probe and RNase complex. (B) Specificity of TAR RNA-nuclear protein interaction. Protein binding to the transcripts of wild-type TAR and its mutant was performed as described for panel A (except that in the case of the β-globin control [lanes 5 and 6], 2 mM $MgCl_2$ was used). Odd-numbered lanes, reactions with normal HeLa nuclear lysates; even-numbered lanes, reactions with PMA-treated cell nuclear lysates. The TAR RNA probes used were wild-type TAR (lanes 1 and 2) and TM37 (lanes 3 and 4), in which a base substitution (C-18→A) has been introduced in the TAR RNA stem region. Lanes 5 and 6 are globin-pre-mRNA RNP complex. The arrowhead indicates the specific RNP complexes formed.

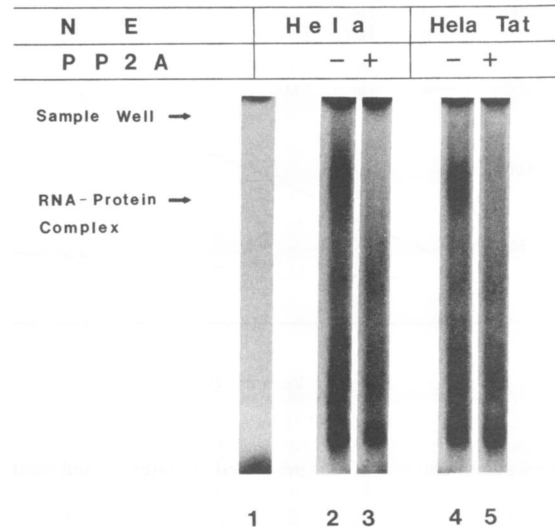

FIG. 3. Protein phosphatase 2A (PP2A) treatment in vitro blocks the binding of protein factor to TAR RNA. HeLa-Tat or HeLa cell nuclear extracts (NE) were treated with 2 U of PP2A (courtesy of D. Brautigan) at 30°C for 30 min prior to the addition of ^{32}P-TAR RNA (lanes 3 and 5). Lanes 2 and 4 are non-PP2A-treated lysates, and lane 1 is ^{32}P-TAR RNA probe alone. RNase protection gel mobility shift assays were performed as described in the legend to Fig. 2.

PKC-depleted cell lysates (Fig. 2A, compare lanes 2 and 3 with 4 and 5 and lanes 6 and 7 with 8 and 9).

Specificity of TAR RNA host protein binding. The experiments shown in Fig. 2B illustrate that the block in RNA-protein complex formation in PKC-depleted cell lysates is specific for the TCF-TAR RNA interaction. A truncated globin gene transcript (which retains 24 nucleotides of exon II and 30 nucleotides of exon III, the splice donor, the second intron, and the splice acceptor site; courtesy of Paula Grabowski, Brown University) formed similar RNP complexes in both normal and PKC-depleted HeLa cell lysates (Fig. 2B, lanes 5 and 6), suggesting that depletion of PKC does not lead to a nonspecific block in RNP formation.

Mutagenesis of the TAR RNA domain required for TCF binding suggests that it is distinct from previously reported TAR RNA-binding proteins in that it requires a TAR RNA base-paired stem region for binding (29). One such mutant TAR (TM37, in which a single base substitution, C-18→A, disrupts the C-18:G-42 base pair in the TAR stem region) was used to determine whether a specific mutation in the TAR RNA stem affects TCF binding and Tat-mediated *trans* activation. As shown in Fig. 2B, disruption of the C-18:G-42 base pair in TM37 leads to undetectable TAR RNA-protein complex formation (Fig. 2B, lanes 3 and 4).

The functional consequences of the TAR stem mutations were determined by the CAT assay, as shown in Table 1. The level of Tat-mediated *trans* activation seen in HeLa cells transfected with TM37 was about 12% of that seen with wild-type TAR. Thus, mutations which decrease the binding of TCF to TAR result in dramatically reduced levels of *trans* activation. The results of in vivo Tat-mediated *trans* activation suggest that the binding of TCF to TAR RNA in vitro correlates with Tat response in vivo. It should be noted, however, that the observed correlation of TCF-TAR RNA interaction and the dependence of Tat function on PKC levels do not demonstrate a direct effect of PKC on the host cell TCF.

Treatment of the nuclear lysates with the catalytic subunit of protein phosphatase 2A (courtesy of David Brautigan, Brown University) in vitro, prior to reaction with the ^{32}P-TAR RNA, abolished TAR-TCF complex formation (Fig. 3). Addition of the phosphatase inhibitor microcystin-LR, however, blocked the effect of protein phosphatase 2A on TCF-TAR complex formation (not shown), suggesting that the inhibition of complex formation is due to the protein

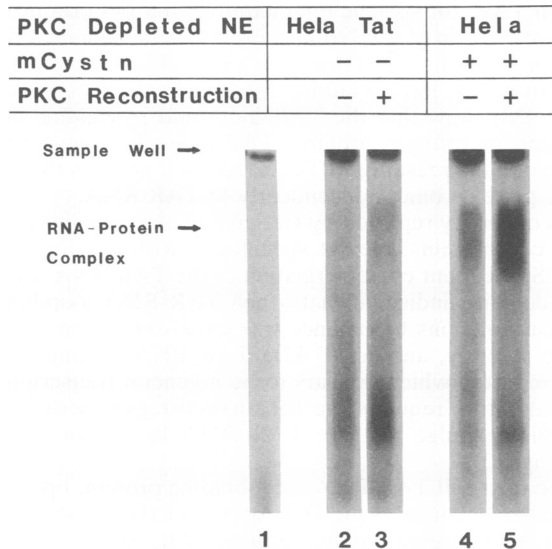

FIG. 4. Addition of purified PKC restores TCF binding to the TAR RNA. HeLa (lanes 4 and 5) and HeLa-Tat (lanes 2 and 3) nuclear lysates prepared from PKC-depleted cells were reconstituted (lanes 3 and 5) with 0.08 U of purified PKC (LIPIDEX) in the presence of 5 mM Mg^{2+}–0.4 mM Ca^{2+} at 30°C for 30 min before the addition of ^{32}P-TAR RNA. RNase protection gel mobility shift analysis of the TCF-TAR RNA complex was performed as described in the legend to Fig. 2A. Lane 1 represents TAR RNA probe alone.

phosphatase 2A treatment and not to any nonspecific RNase contamination.

Addition of PKC in vitro partially restores host protein binding. To test the hypothesis that the dephosphorylated state of TCF in PKC-depleted cell lysates might be the reason for its failure to bind to the TAR RNA, we added exogenous PKC preparations (6) (a mixture of PKC isoforms; LIPIDEX, Inc.) to the depleted nuclear extracts. As shown in Fig. 4, addition of purified PKC (0.08 U) partially restored TCF binding to TAR RNA in a nuclear lysate from PKC-depleted cells. Nuclear lysates prepared in the presence of microcystin-LR showed a more efficient restoration of the TCF-TAR binding upon reconstitution of PKC (Fig. 4, lanes 4 and 5), suggesting that phosphatase activity was present during the in vitro PKC reactions.

Artificial tethering of Tat to TAR confers PKC independence to *trans* activation. The observations above suggest that PKC, perhaps indirectly, regulates Tat-mediated *trans* activation via modification of cellular factors that either promote the functional interaction of Tat with TAR and/or are necessary for efficient *trans* activation. Several investigators have recently constructed chimeric Tat proteins with either DNA- or RNA-binding domains from heterologous proteins (2, 31, 35). Tat chimeric proteins *trans* activate the HIV-1 LTR when the TAR region is modified to contain the recognition site for the appropriate binding protein. By artificially tethering Tat to the TAR region, such systems might bypass the requirement for TCF-TAR binding. If the normal role of TCF is, first, to facilitate or stabilize a functional Tat-TAR interaction and, second, to mediate efficient *trans* activation, then the artificial constructs, by substituting only the binding component of the *trans* activation reaction, might be less efficient in *trans* activation than the wild-type system.

TABLE 2. Effect of PKC on Tat-mediated *trans* activation of wild-type and chimeric HIV-1 LTR-CAT constructs[a]

	Untreated	PKC - Depleted		PKC - Activated	
	Fold *Trans*-Activation	Fold *Trans*-Activation	Percent *Trans*-Activation	Fold *Trans*-Activation	Percent *Trans*-Activation
pHIVSCAT					
TAR CAT	1	1	100	1	100
Tat, CAT	88	9.2	10	230	261
Tat(1-67), CAT	69	7.5	11	183	265
Tat-CP, CAT	62	6.3	10	202	325
pHIVSRCAT					
R17 CAT	1	1	100	1	100
Tat, CAT	1	1	100	1	100
Tat(1-67), CAT	1	1	100	1	100
Tat-CP, CAT	15	16	107	13	87

[a] Wild-type pHIVSCAT (upper four lanes) or chimeric pHIVSRCAT (lower four lanes) plasmids (2 μg) were cotransfected into HeLa cells in the presence of the full-length pSVtat (Tat), first-exon pSVtat(1–67) [Tat(1–67)], or chimeric pSVtat(1–67)CP (Tat-CP) expression vector (1 μg). HeLa cells were depleted of PKC by exposure to 100 ng of PMA per ml for 24 h before and 48 h following transfection. For PKC activation, cells were treated with PMA (100 ng/ml) for 8 h before harvesting. Untreated HeLa cells were used as a control. CAT activity was assayed in the linear range of chloramphenicol conversion. Percent acetylation was normalized to the value for an internal transfection control (pXGH, mouse metallothionein promoter-driven hGH, as described in Materials and Methods). Data are expressed as relative CAT activity. Fold *trans* activation was determined as the ratio of relative CAT activity over the basal level of expression of the LTR-CAT vectors. Percent *trans* activation for each construct is expressed relative to that of untreated cells. The values used to calculate fold *trans* activation are the averages of three independent experiments, with a standard deviation of less than 20%.

The chimeric system developed by Selby and Peterlin (31) was used to test this hypothesis. The TAR region of an HIV-1 LTR-CAT reporter construct (pHIVSRCAT) was replaced by the operator region of bacteriophage R17, which is a stem-loop RNA target for the bacteriophage MS2 coat protein (7), and a chimeric Tat-coat protein (Tat-CP) was constructed by linking residues 1 to 67 of Tat to residues 2 to 129 of the MS2 coat protein [pSVtat(1–67)CP] (31).

Wild-type pHIVSCAT or chimeric pHIVSRCAT reporter plasmids (2 μg) were cotransfected into HeLa cells in the presence of full-length pSVtat, first-exon pSVtat(1–67), or chimeric pSVtat(1–67)CP HIV-1 Tat expression vectors (1 μg). HeLa cells were either depleted of PKC by prolonged exposure to PMA (100 ng/ml for 48 h) or PKC activated by short PMA treatment (100 ng of PMA per ml for 8 h). Untreated HeLa cells were used as *trans* activation controls for all constructs. Fold and percent *trans* activation relative to untreated cells were calculated for each construct and are shown in Table 2.

Basal expression from the wild-type LTR was increased

almost 2-fold by PKC depletion and 13-fold by PKC activation (data not shown). The chimeric LTR directed approximately twofold-lower levels of CAT expression in untreated HeLa cells and was less inducible by either depletion or stimulation of PKC activity than the wild-type LTR-CAT construct. These results indicate that sequences in the 5′ end of the mRNA leader are important for both basal transcription and PKC induction.

trans activation of wild-type pHIVSCAT by full-length Tat was 88-fold, while the first-exon pSVtat(1–67) stimulated expression somewhat less efficiently (69-fold), as expected (Table 2). The pSVtat(1–67)CP chimera was as efficient as pSVtat(1–67) in *trans*-activating the wild-type LTR, suggesting that the coat protein domain of the chimeric protein does not interfere with the *trans* activation function of Tat. Depletion of PKC resulted in a dramatic reduction in Tat-stimulated expression for all three constructs (Table 2); *trans* activation was decreased to about 10% of that detected in untreated cells. PKC activation, on the other hand, stimulated *trans* activation by either the wild-type, first-exon, or chimeric Tat construct two- to threefold, despite the high levels of PKC-induced basal expression (Table 2).

Neither full-length Tat nor pSVtat(1–67) was able to stimulate CAT expression from the chimeric pHIVSRCAT reporter, since the TAR region has been substituted with the R17 operator RNA stem-loop (Table 2). pSVtat(1–67)CP was able to *trans*-activate the LTR chimera 15-fold; thus, artificial tethering of Tat to the TAR region via the coat protein-R17 operator interaction partially restored its ability to *trans* activate the HIV-1 LTR. *trans* activation of pHIVSRCAT was only one-quarter as efficient as the *trans* activation of the wild-type pHIVSCAT by pSVtat(1–67)CP, indicating that other functions (of either TCF or other cellular factors interacting with TAR) may be necessary for efficient *trans* activation. The *trans* activation mediated by pSVtat(1–67) CP is, however, completely resistant to PKC depletion, since prolonged exposure to PMA did not affect the levels of pSVtat(1–67)CP-mediated *trans* activation (Table 2). In addition, the *trans* activation of the chimeric system is resistant to the PMA-induced enhancement of Tat activity observed with the wild-type LTR in PKC-activated cells. Thus, artificially tethering pSVtat(1–67)CP to the R17 operator region obviates the PKC requirement in Tat-mediated *trans* activation.

DISCUSSION

Based on a variety of studies which focused on the role of the TAR sequence in the regulation of HIV-1 *tat* gene response, it is clear that the TAR domain plays a crucial role in the functional interaction with Tat protein (3, 35). It is probable, however, that such an isolated Tat-TAR interaction is insufficient for optimal Tat response in vivo (10, 19, 30, 37). Since the structural integrity of TAR RNA is essential for Tat function (4) and the binding site of the cellular cofactor to TAR RNA substantially overlaps the TAR sequences required for Tat function (10, 14, 24, 29, 30, 33, 35, 37), we would argue that TAR RNA-TCF interaction forms an RNP (RNP complex) required for optimal Tat-mediated viral gene activation. It is interesting that the minimal Tat response domain of TAR (+17 to +43) was used as a probe to describe a large heteromeric complex containing a 185-kDa RNA-binding protein (33, 38), as well as a family of 110- to 70-kDa proteins (33). The sequence-specific RNase protection procedure that we used in these studies requires the TAR RNA lower stem region (present in full-length TAR) for specific interaction with a 140-kDa protein. The interaction of the 140-kDa protein is independent of the presence of the TAR loop or pyrimidine bulge primary structure (29). Recent studies by Sheline et al. (33) and Wu et al. (38) show that the TAR loop- and pyrimidine bulge-recognizing proteins promote TAR-dependent transcription in vitro in the presence of Tat, although it appears that these three proteins bind independently to TAR RNA.

As originally reported by Gatignol et al., a complex set of host cell proteins interact specifically with the TAR RNA (14). Subsequent characterization of the TAR RNA recognition domains indicated that a p68 TAR RNA loop-binding protein functions to enhance transcript elongation (24, 25). More recently, another 37-kDa TAR RNA-binding protein was reported which appears to be a general transcriptional activator that requires the base-paired region between the pyrimidine bulge and the TAR RNA loop region for its binding (13).

The host cell TAR RNA stem-binding protein, operationally described here as the TCF, appears to be a distinct TAR RNA stem region-binding protein (29), since a specific mutation that destabilizes the TAR structure, TM37, results in a marked depletion of the RNP complex. The block in TCF-TAR RNA binding in the TM37 mutant is functionally related to Tat response in vivo. As shown in Table 1, *trans* activation of the LTR TM37-CAT construct upon cotransfection of pSVtat into HeLa cells is eightfold less than with the wild-type TAR. Based on earlier work (2, 19, 31) and the results presented here, we suggest that activation of genes linked to the HIV LTR is regulated in response to functional Tat and that the specific binding of TCF to the TAR RNA plays a crucial role in stabilizing this process. Whether the 140-kDa TAR RNA stem-binding protein complements the functional interactions of the loop- and the pyrimidine bulge-binding proteins and Tat-TAR RNA interaction will require further studies with purified host cell factors. However, preliminary experiments suggest that the 140-kDa stem-binding factor complements the Tat-TAR RNA interaction, a result consistent with the studies described here, which indicate a stabilizing role of TCF in in vivo Tat response.

The strict dependence of Tat function on the orientation and the location of the TAR sequence has suggested that TAR serves primarily in a structural role as an RNA or, more accurately, an RNP target for Tat binding. Such a role for the TAR sequence in Tat-mediated *trans* activation has been addressed by using chimeric proteins in which the *trans* activation domain of Tat is fused to a heterologous RNA (Rev and MS2 coat protein) or DNA (c-*jun*) binding domain. These chimeric proteins can interact with appropriate RNAs (RRE and MS2 operator) or DNA (AP-1) targets substituted for the original TAR sequence (2, 31, 35). Such Tat fusion proteins were shown to permit transcriptional *trans* activation of a substituted HIV-1 LTR, albeit at a somewhat lower level than the normal system. We show here that *trans* activation by such an artificial construct in which the normal Tat-TAR interaction is bypassed is independent of PKC activity. This suggests that the PKC-sensitive step in *trans* activation involves the functional interaction of Tat with TAR RNA and strengthens the role for an essential cellular cofactor in this interaction. These experiments do not, however, argue for a direct role of PKC in the phosphorylation of TCF. Further in vitro studies will be required to demonstrate whether PKC has a direct role in the phosphorylation of the TCF.

The TCF we describe appears to be distinct from the other TAR RNA-binding proteins described thus far, both in its

recognition of the TAR RNA domain and in its PKC-dependent role in Tat activation. Neither the loop sequence nor the stem region between the pyrimidine bulge and the loop is essential for the binding of TCF (29). Interestingly, changes in the TAR stem domain have been reported to impair Tat-mediated *trans* activation. Mutations clustered in groups of three between positions 7 and 18 in TAR impair *trans* activation 3- to 4-fold, while mutations in positions 19 to 21, just before the pyrimidine bulge, cause a 20-fold decrease in *trans* activation (18).

The regulation of the functional interaction of TCF by phosphorylation reported here is similar to the response of a number of transcription factors (1, 16). In the case of Tat-mediated viral gene activation, the *cis*-acting target sequence is a unique RNP complex formed with TAR RNA and specific host cell protein (14, 27). Specific RNA-protein interactions have been demonstrated in mRNA splicing and translational regulation; however, the case described here represents a unique example of a specific RNP complex in transcriptional regulation which is responsive to cellular signal transduction pathways. These observations provide a new avenue to explore further the link between extracellular stimuli and transcriptional activation. Such studies may provide novel approaches to containing HIV-1 infection in the latent state.

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