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Increasing the Ratio of PP2A Core Enzyme to Holoenzyme Inhibits Tat-Stimulated

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We demonstrated previously that PP2A exists in many cell types as two abundant forms: (1) holoenzyme composed of two regulatory subunits, A and B, and a catalytic subunit C; and (2) core enzyme consisting of the A and C subunits. These two forms have different substrate specificities. Since published data suggested that HIV-1 transcription may be regulated by a cellular protein phosphatase, it was of interest to determine whether changing the ratio between PP2A core and holoenzyme affects HIV-1 gene expression. This question was addressed by expression in COS cells of an N-terminal mutant of the A subunit, AΔ5, which binds the C but not the B subunit. This resulted in an increase in the amount of core enzyme and a decrease in the amount of holoenzyme concomitant with the expected change in phosphatase activity. Tat-stimulated transcription from the HIV-1 LTR was inhibited 5-fold by mutant AΔ5, whereas mRNA synthesis directed by the actin promoter was not affected. Furthermore, virus production in COS, HeLa, and Jurkat T cells was inhibited 45-, 5-, and 3-fold, respectively, by mutant AΔ5. These results demonstrate that the balance between PP2A holoenzyme and core enzyme is important for HIV-1 gene expression and virus production. © 1997 Academic Press

INTRODUCTION

Transcription of the genome of human immunodeficiency virus-1 (HIV-1) is controlled by cellular factors that act on the 5′ long terminal repeat (LTR) of the integrated provirus (Jones and Peterlin, 1994). These factors include the basal transcription factor TBP, the proximal promoter-binding proteins NF-κB and SP1, and the distal enhancer-binding proteins LEF-1, Ets-1, and TFE-3 (Pazin *et al.*, 1996; Sheridan *et al.*, 1995). In addition, HIV-1 transcription is strongly activated by the viral factor Tat (Dayton *et al.*, 1986; Fisher *et al.*, 1986; Sodroski *et al.*, 1985a, 1985b), which binds to the RNA element TAR at the 5′ end of nascent transcripts (Gaynor, 1995; Rosen *et al.*, 1985). NF-κB is a dimeric protein held inactive in the cytoplasm by association with the inhibitor IκB (Grilli *et al.*, 1993). In response to extracellular signals, IκB becomes phosphorylated and subsequently degraded (Alkalay *et al.*, 1995; Brown *et al.*, 1995; DiDonato *et al.*, 1995; Finco *et al.*, 1994; Lin *et al.*, 1995; Miyamoto *et al.*, 1994; Palombella *et al.*, 1994; Thanos and Maniatis, 1995; Traenckner *et al.*, 1994). This liberates NF-κB which then migrates to the nucleus and binds to cognate enhancer sequences. It has been shown that IκB is phosphorylated *in vivo* in response to okadaic acid (OA), a tumor promoter and inhibitor of the serine/threonine-specific protein phosphatases type 1 (PP1) and type 2A (PP2A) (Cohen, 1989) as well as of the recently discovered enzymes PP3, PP4, and PP5 (Brewis *et al.*, 1993; Chen *et al.*, 1994; Honkanen *et al.*, 1991). The phosphorylated form of IκB is readily dephosphorylated *in vitro* by PP2A but not by PP1 or PP2B (Sun *et al.*, 1995). Therefore, OA may activate NF-κB by accelerating the phosphorylation of IκB and thereby stimulate HIV-1 LTR activity (Finco *et al.*, 1994; Lin *et al.*, 1995; Thevenin *et al.*, 1990). However, OA is also a strong inducer of the HIV-1 LTR in the absence of NF-κB (Vlach *et al.*, 1995). This effect is dependent on SP1 whose phosphorylation state increases during OA treatment. The effect of OA is enhanced in the presence of Tat. It has been suggested that increased phosphorylation of SP1, resulting from inhibition of PP2A by OA, promotes formation of active transcription complexes between SP1, Tat, NF-κB, and general transcription factors (Jeang *et al.*, 1993; Kashanchi *et al.*, 1994a, 1994b; Kerr *et al.*, 1993; Sune and Garcia-Blanco, 1995; Vlach *et al.*, 1995).

PP2A consists of three subunits, the 36-kDa catalytic C subunit and the 65-kDa regulatory A subunit, which together form the core enzyme, and the regulatory B subunit, which binds to the core enzyme yielding the holoenzyme (Mumby and Walter, 1993; Walter and Mumby, 1993). The A and C subunits both exist as two isoforms (α and β) and the B subunit as multiple forms, which are subdivided into three families, B, B′, and B″, unrelated to each other by primary sequence (Csortos *et al.*, 1996; Hendrix *et al.*, 1993; McCright and Virshup, 1995; Tanabe

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et al., 1996; Tehrani *et al.*, 1996). B subunits have important functions in regulating the substrate specificity (Agostinis *et al.*, 1992; Cegielska *et al.*, 1994; Ferrigno *et al.*, 1993; Kamibayashi *et al.*, 1991, 1994; Sola *et al.*, 1991) and possibly the subcellular localization of PP2A (McCright *et al.*, 1996; Sontag *et al.*, 1995; Tehrani *et al.*, 1996). In all cell lines examined, we found that the holoenzyme and the core enzyme are similarly abundant and that detection of the core enzyme was not an artifact of dissociation of the B subunit from the holoenzyme (Kremmer *et al.*, 1996). The A subunit polypeptide consists of 15 nonidentical repeats that form a rod-shaped molecule (Chen *et al.*, 1989; Hemmings *et al.*, 1990; Imaoka *et al.*, 1983; Walter *et al.*, 1989). The B subunit binds to repeats 1–10 and the C subunit to repeats 11–15 of the A subunit (Fig. 1) (Ruediger *et al.*, 1992, 1994). Binding of the C subunit to the A subunit occurs in the absence of the B subunit, whereas binding of the B subunit to the A subunit requires the presence of the C subunit (Kamibayashi *et al.*, 1991, 1992; Ruediger *et al.*, 1994).

While experiments with OA indicate that one or several phosphatases play an inhibitory role in HIV-1 transcription, they cannot distinguish which phosphatase is important. In this paper, we provide direct evidence that PP2A regulates HIV-1 gene expression. Transfection of cells with an N-terminal mutant of the A subunit, which associates with the C but not the B subunit, causes an increase in the ratio of PP2A core enzyme to holoenzyme and a corresponding change in phosphatase activity. Perturbing the ratio of the PP2A forms in this way strongly inhibits transcription from the HIV-1 LTR. Furthermore, a strong decrease in virus production was observed. Our results demonstrate that PP2A plays an important role in the life cycle of HIV-1.

MATERIALS AND METHODS

Expression plasmids

The A subunit constructs in pBluescript (Stratagene) have been described previously (Ruediger *et al.*, 1992) and were cloned as *Eco*RI–*Xba*I fragments into the eukaryotic expression vector pcDNA3 (Invitrogen), which utilizes the immediate early promoter/enhancer of the human cytomegalovirus (CMV). The β-galactosidase gene from pCMVβ (Clontech) was inserted into pcDNA3 as a *Not*I–*Not*I fragment and used as a control effector construct (pcDNA3-βGal). pCMVβ and pSV-βGal (Promega) were used as internal controls. Katherine Jones provided us with the reporter construct HIV-1 LTR luciferase (Sheridan *et al.*, 1995), and with expression plasmid pSV-Tat (previously termed TAT-1) utilizing the SV40 promoter/enhancer (Peterlin *et al.*, 1986). Flossie Wong-Staal provided us with expression plasmid pTat utilizing the HIV-1 LTR promoter/enhancer (Knight *et al.*, 1987). Jay Rappaport provided us with expression plasmid pHβA-Tat utilizing the human β-actin promoter/enhancer (Howcroft *et al.*, 1995). Michael Karin provided us with control reporter plasmid pActin-luciferase. The HIV-1 full-length genetic clone pNL4-3 from Malcolm Martin was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Adachi *et al.*, 1986). The pcDNA3, pSV-Tat, and pTat constructs contain the SV40 origin of replication permitting their replication in COS cells.

Cells and transfection

COS (Gluzman, 1981) and HeLa cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C in a humidified 10% CO_2 atmosphere. For transfections, 5×10^4 cells were plated per 3.5-cm well of 6-well plates. Transfections were carried out using LipofectAMINE from Gibco/BRL following the manufacturer's instructions. Jurkat T cells were maintained in RPMI 1640 with 10% fetal calf serum and transfected with DMRIE-C following Gibco/BRL's instructions. Transfection conditions were optimized for high expression of effector protein determined by Western blotting, and for high transfection efficiency determined by staining of fixed cells coexpressing β-galactosidase with X-gal (Gibco/BRL) (Sanes *et al.*, 1986). LipofectAMINE was used at 6 μl/ml, DNA at 1 μg/ml. Of the total DNA, 10–30% comprised reporter plasmid(s), and 70–90% was effector plasmid. In titration experiments, the total DNA amount was kept constant using vector DNA. Cells were transfected for 2–6 h, then the transfection solution was replaced by medium with 10% FCS until harvest.

Standardization of transfection

To eliminate the possibility that the DNA preparations for AΔ5 were more toxic than those for the A subunit or vector, all effector plasmids were prepared in parallel on silica gel columns (Qiagen). In addition, three independently prepared sets of plasmids yielded identical results. We also excluded the possibility that transfections with AΔ5 vectors were more toxic than those with A subunit vectors due to high expression levels for AΔ5. In fact, at identical DNA concentrations, expression levels for AΔ5 were always lower than those for A, but they had stronger effects on phosphatase activity (see Results). Furthermore, the cell numbers and the values for total cell protein after 48 h of transfection at high efficiency were identical for all effector plasmids. To control for transfection efficiency, the various transfection solutions contained equal amounts of β-galactosidase-producing reporter constructs, pCMVβ, pcDNA3-βGal, or pSV-βGal. By staining of fixed cells for β-galactosidase, equal transfection efficiencies for vector, A, or AΔ5 were found. At the same time, reduced color intensity was observed in AΔ5- compared to vector-transfected cells.

This observation was substantiated by the finding that β-galactosidase activity was reduced in AΔ5-transfected cells. Since AΔ5 inhibits the expression of β-galactosidase driven by the CMV or SV40 promoter/enhancer, it was not possible to normalize luciferase data against galactosidase activity. This finding indicates a broader, but not ubiquitous, involvement of PP2A in gene expression (see Results and Discussion).

FPLC

Transfections were carried out on 15-cm plates. After 48 h, cells were washed with TBS (10 mM Tris–HCl, pH 7.4, 150 mM NaCl) and harvested in 2 ml of buffer D (10% glycerol, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA) containing 1 mM dithiothreitol, 50 μM leupeptin (Sigma), 1 μg/ml aprotinin (Calbiochem), 0.2 mg/ml soybean trypsin inhibitor (Boehringer), and 1 mM Pefabloc (Boehringer). The cells were scraped off the plate and stored in liquid nitrogen. After thawing, they were homogenized by douncing and centrifuged at 4°C and 45,000 rpm in a SW 50.1 rotor for 25 min. The supernatants containing equal amounts of protein (2.5 mg) for vector-, A-, and AΔ5-transfected cells were fractionated on a Mono Q HR 5/5 column (Pharmacia LKB). Fractions were eluted with a salt gradient of 150 to 400 mM NaCl in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol. A total of 80 fractions, 0.5 ml each, was collected. Fractions 10 to 70 (0.1 ml each) were separated by SDS–PAGE and analyzed by Western blotting.

Western blotting

The ECL Western blotting detection system from Amersham was used with the following primary antibodies: rat monoclonal antibody anti-A subunit (6G3) (Kremmer *et al.,* 1996), rabbit anti-Bα subunit produced against the peptide KGAVDDDVAEADY coupled to BSA with bis-diazobenzidine as described (Walter, 1986), mouse monoclonal antibody anti-C subunit from Marc Mumby (Mumby *et al.,* 1985), and sheep anti-Tat (Biodesign). A variety of exposures of each Western blot was scanned using an Apple Macintosh Color One Scanner, and band intensities were analyzed with NIH Image software.

Phosphatase assay

Phosphorylase *a*, labeled with [^{33}P]ATP (1000 cpm/pmol), was used as a substrate in PP2A phosphatase activity assays as described (MacKintosh, 1993). A peptide, INGSPRTPRRGQNR, corresponding to amino acids 246–259 of the retinoblastoma protein was phosphorylated by cdk1/cyclin B under the conditions described by Agostinis *et al.* (1992) using [^{33}P]ATP (1000 cpm/pmol). The cdk1/cyclin B used in the reaction was expressed in Sf9 cells infected for 48 h with recombinant baculoviruses expressing human cyclin B and cdk1 at multiplicities of infection of 5 (Lin and Wang, 1992). The kinase/cyclin B complex was partially purified by immunoprecipitation using antibodies raised in rabbits against peptide YFNDLDNQIKKM that corresponds to the C-terminal sequence of cdk1 (Lee and Nurse, 1987). The stoichiometry of phosphorylation was 0.3 mol/mol, and the phosphopeptide was assayed at a final concentration of 5 μM.

Transfected cells were harvested at 48 h by washing with 20 mM Tris–HCl, pH 7.4, 150 mM NaCl followed by extracting for 4 min on ice with 0.5% Triton X-100, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 1 mM DDT, 1 mM Pefabloc, 50 μM leupeptin, 0.2 mg/ml soybean trypsin inhibitor, and 1 μg/ml aprotinin. The extracts were centrifuged at 14,000 rpm for 3 min at 4°C in a microfuge, and the supernatants were diluted for the phosphatase assay in 0.1% BSA, 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol. The phosphatase assay and extraction of released organic phosphate were performed as described (MacKintosh, 1993). PP2A activity was measured as the activity of a diluted extract, which could be inhibited with 1 nM okadaic acid, in the presence of 0.1 μM inhibitor-2.

Luciferase assay

Usually 48 h after transfection, cells were washed with PBS and extracted with 100 to 600 μl of either luciferase lysis buffer (1% Triton X-100, 100 mM Tris–acetate, pH 7.8, 10 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol), Triton X-100 buffer (1% Triton X-100, 50 mM Tris–HCl, pH 8, 150 mM NaCl, 3 mM $MgCl_2$), or buffer D (see above). The extracts (20 to 80 μl) were analyzed in 96-well plates using an Anthos Lucy 1 luminometer and WinLcom software. Each well was injected with 100 μl of luciferin solution (200 μM D-luciferin from Analytical Luminescence Laboratory, 2 mM ATP, 100 mM Tris–acetate, pH 7.8, 10 mM magnesium acetate, 1 mM EDTA). Light output was measured for 10 or 20 s without delay (De Wet *et al.,* 1987).

Galactosidase assay

Aliquots of 20 to 80 μl of the extracts analyzed for luciferase activity were mixed in 96-well plates with 200 μl of ONPG solution (0.6% *o*-nitrophenyl β-D-galactopyranoside, 100 mM sodium phosphate, pH 7.5, 0.85 mM $MgCl_2$, 0.85% 2-mercaptoethanol). The plates were incubated at 37°C for 20 to 120 min before the absorption was measured at 420 nm in an ELISA plate reader. Lysis buffer or extract of nontransfected cells, mixed with ONPG solution, was used to set the reader to zero (Rosenthal, 1987).

p24 assay

After transfection of cells on 6-well plates with pNL4-3 (2 to 20% of the total DNA) and A subunit constructs

(80 to 98%), the transfection solution was replaced with 1.3 ml medium containing 10% fetal calf serum. At different time points, 300 μl of medium were removed and mixed with 33 μl of 10× Abbott buffer containing Triton X-100. The 300 μl removed were substituted with 300 μl fresh medium. The samples were stored frozen and subjected, undiluted or diluted, to Abbott's HIVAG-1 enzyme immunoassay following the manufacturer's instructions. The p24 assays were performed by the virology CORE facility of the UCSD Center for AIDS Research.

Luciferase mRNA quantitation

Total RNA was isolated 48 h after transfection of cells grown on 5-cm plates. RNeasy Minipreps were performed following Qiagen's instructions. Up to 2 μg of total cellular RNA were translated *in vitro* using the TNT Coupled Wheat Germ Extract System from Promega. RNA polymerase and radioactive methionine were omitted. Promega's Complete Amino Acid Mixture was used. Reactions of 25 μl total volume, containing 12.5 μl wheat germ extract, were prepared directly in white 96-well plates. After 90 min at 30°C the plates were subjected to luciferase assays by injecting the stabilized substrate solution from Promega's Luciferase Assay System. Light output was measured for 30 s without delay. We found that (i) up to 2 μg of RNA in a 25-μl reaction result in a linear luciferase activity response, (ii) the luciferase activity is proportional to the luciferase mRNA amount within a given amount of total RNA, and (iii) cotranslation of AΔ5-mRNA contained in the total RNA as a result of the cotransfections does not affect the *in vitro* synthesis of luciferase (data not shown). This method is extremely sensitive permitting detection of luciferase activity, i.e., luciferase mRNA, within total RNA from 10^4 COS cells cotransfected with reporter constructs at 5 to 10% of total DNA. The method can detect as little as 0.1 pg of luciferase mRNA (Ruediger and Walter, manuscript submitted).

RESULTS

Experimental approach

The A subunit plays an important role in the regulation of PP2A by binding the B and C subunits and facilitating their interaction. B subunits can either stimulate or inhibit the activity of the core enzyme, depending on the substrate and the type of B subunit bound to the core enzyme (Agostinis *et al.*, 1992; Cegielska *et al.*, 1994; Ferrigno *et al.*, 1993; Kamibayashi *et al.*, 1991; Kamibayashi *et al.*, 1994; Sola *et al.*, 1991). We reasoned that N-terminal mutants of the A subunit, shown to bind the C but not the B subunit *in vitro* (Ruediger *et al.*, 1994), could be valuable tools for studying the function of PP2A *in vivo.* One expects that these mutants replace the wild-type A subunit in preexisting core and holoenzymes and compete with newly synthesized wild-type A subunit for newly synthesized C subunit, thereby causing an increase in the amount of core enzyme and a decrease in the amount of holoenzyme (Fig. 1). One also expects to find a change in phosphatase activity since the core and the holoenzymes differ in their substrate specificities. The holoenzyme is much more active than the core enzyme toward substrates phosphorylated by cyclin-dependent kinases (Agostinis *et al.*, 1992; Cegielska *et al.*, 1994; Ferrigno *et al.*, 1993; Kamibayashi *et al.*, 1994; Sola *et al.*, 1991), whereas the core enzyme is equally or more active than the holoenzyme toward most other substrates (Kamibayashi *et al.*, 1991). Thus, A subunit mutants should serve as a useful tool to determine whether PP2A is involved in specific cellular processes, and which form (holoenzyme or core enzyme) is involved.

Mutant AΔ5 does not bind the B subunit *in vivo* and increases the ratio of core enzyme to holoenzyme

We investigated whether mutant AΔ5 with a deletion of repeat 5 binds the C but not the B subunit *in vivo* as it does *in vitro* (Ruediger *et al.*, 1994). Extracts from AΔ5-

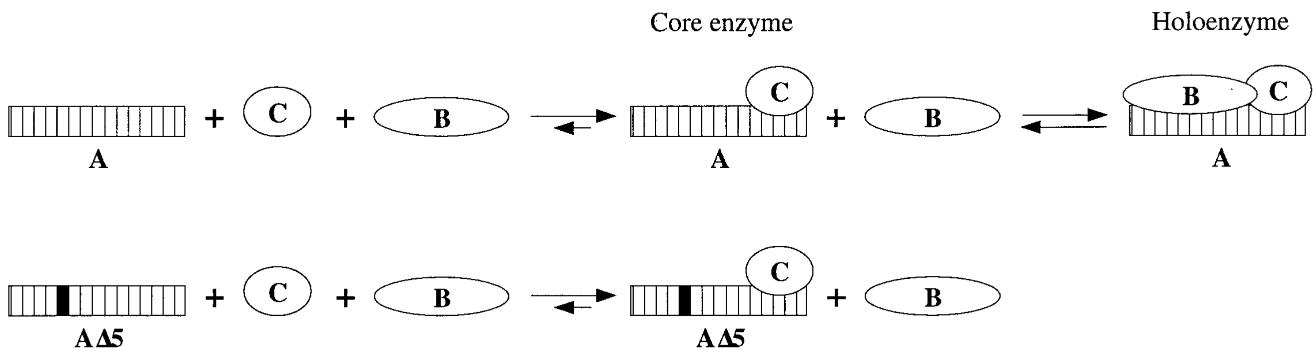

FIG. 1. Model of the action of N-terminal mutant AΔ5. In normal cells (upper row), the C subunit associates with the A subunit to form A-C core enzyme. The B subunit then binds to form the holoenzyme. In transfected cells (lower row), AΔ5 competes with wild-type A subunit for binding to the C subunit. AΔ5-C does not bind the B subunit.

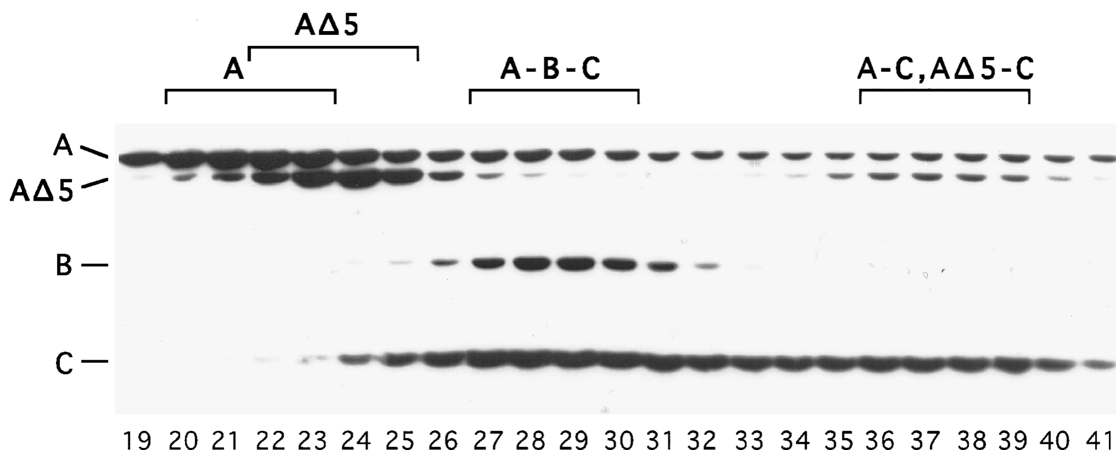

FIG. 2. Mutant AΔ5 associates with the catalytic C but not the regulatory B subunit of PP2A *in vivo*. COS cells were transfected with AΔ5 expression plasmid and extracted 48 h later with buffer D. The extract was fractionated on a Mono Q column by FPLC as described under Materials and Methods and analyzed by SDS–PAGE and Western blotting for A, Bα, and C. The fractions containing monomeric A and AΔ5, A-Bα-C holoenzyme, and A-C and AΔ5-C core enzyme are indicated. The experiment was performed twice in COS cells and several times in other cell lines with similar results.

transfected COS cells were fractionated on a Mono Q anion exchange column and individual fractions were analyzed for their content of A, AΔ5, Bα, and C. Figure 2 shows two peaks of AΔ5, one around fraction 24 corresponding to the free monomeric form and one around fraction 37 corresponding to AΔ5-C dimer and comigrating with endogenous core enzyme. Most importantly, there is no peak of AΔ5 in the region of endogenous holoenzyme (fraction 29). This demonstrates that AΔ5 is unable to associate with the B subunit *in vivo.*

By scanning the Western blots shown in Fig. 2, we determined that AΔ5-C constitutes 10% of all core and holoenzyme. This implies that 10% of all C subunit complexed with A and AΔ5 is bound to AΔ5. Since only 18% of the cells were transfected in this experiment (data not shown), AΔ5 bound to 55% of the C subunit in transfected cells. Consequently, the levels of core and holoenzyme in transfected cells dropped to 45% of untransfected cells. We assume that the levels dropped equally for both forms. Therefore, since untransfected COS cells contain $\frac{2}{3}$ holoenzyme and $\frac{1}{3}$ core enzyme, transfected cells contain approximately $\frac{1}{3}$ holoenzyme, $\frac{1}{6}$ core enzyme, and $\frac{1}{2}$ AΔ5-C. Thus, the concentration of holoenzyme dropped twofold and the concentration of core enzyme (A-C plus AΔ5-C) increased twofold in transfected cells.

The monomer region contained free AΔ5 and free A. The latter was presumably displaced from core and holoenzyme or was newly synthesized after transfection and unable to bind C subunit because of competition by excess AΔ5. Untransfected or control vector-transfected cells contained no free A subunit (data not shown). However, it is unclear why extracts from AΔ5-transfected cells contained so much free A subunit in comparison to the amount of AΔ5-C. If free A subunit is only generated through replacement or competition, then the molar amounts of free A and AΔ5-C should be equal. A small amount of the free A subunit in AΔ5-transfected cells may be derived from holoenzymes other than A-Bα-C. For example, it was reported that COS cells synthesize B′ mRNA and therefore may contain A-B′-C (Csortos *et al.,* 1996). We did not examine the Mono Q fractions for B′. The COS cells used here also contain a small amount of SV40 small T antigen that is bound to core enzyme, but we did not measure its concentration relative to total core and holoenzyme.

Expression of mutant AΔ5 reduces holoenzyme activity

To measure the decrease in holoenzyme activity and the increase in core enzyme activity, which are expected to result from expression of AΔ5, we used two substrates: phosphorylase *a* phosphorylated by phosphorylase kinase and Rb peptide phosphorylated by cdk1 kinase/cyclin B. Since the Δ5 mutation is distant from the C subunit binding region (repeats 11–15), it is reasonable to assume that core enzyme containing AΔ5 has the same activity and substrate specificity as core enzyme containing wild-type A subunit. Furthermore, since the holoenzyme is 100 times more active than the core enzyme in dephosphorylating the Rb peptide (Agostinis *et al.,* 1992), we expected that extracts from AΔ5-transfected cells would be less active than control extracts toward the Rb peptide. As shown in Table 1, AΔ5 transfection reduced the Rb peptide phosphatase activity to 71% in comparison to vector control. Taking into account a transfection efficiency of 40% in this experiment (data not shown), the activity toward the Rb peptide was reduced to 27% in transfected cells. This fourfold reduction

TABLE 1

Relative PP2A Activity in Cell Extracts and Transfected Cells

	Extract		Transfected cells	
	Phos *a*	Rb peptide	Phos *a*	Rb peptide
Empty vector	100	100	100	100
A subunit	98 ± 0.5	91 ± 2.5	95 ± 1.2	77 ± 6.2
AΔ5	111 ± 2.5	71 ± 3.0	128 ± 6.2	27 ± 7.5

Note. COS cells were transfected with empty vector or vector encoding wild-type A subunit or AΔ5, extracted after 48 h with Triton X-100 buffer, and assayed for PP2A phosphatase activity using phosphorylase *a* (Phos *a*) and an Rb peptide as substrates. Values are expressed as percentages of the PP2A activity in vector-transfected samples. Two transfections were assayed, one in duplicate and one in triplicate phosphatase assays, and the standard deviations are indicated. The experiment was repeated several times with similar results. The activity in transfected cells was calculated by multiplying the inhibition in the extract by 2.5 to compensate for the 40% transfection efficiency.

of holoenzyme activity suggests that the bulk of holoenzyme was converted to AΔ5-C. The cell fractionation showed a twofold reduction of holoenzyme (Fig. 2). Considering that two different methods were used, these values are in good agreement. The PP2A activity toward phosphorylase *a* was slightly stimulated by AΔ5, consistent with an increase in core enzyme, which is slightly more active toward this substrate than holoenzyme (Kamibayashi *et al.,* 1991). Expression of wild-type A subunit did not significantly change the PP2A activity toward phosphorylase *a* but reduced the Rb peptide activity to 77% in transfected cells. This effect may be caused by the large excess of A subunit (see below). In summary, expression of AΔ5 changed the specificity of PP2A in accordance with an increased ratio of core to holoenzyme.

A potential concern is whether the PP2A phosphatase activity measured in cell extracts truly reflects the activity in cells and to what extent an exchange of the wild-type A subunit in core and holoenzyme by AΔ5 takes place in the cell lysate. Although subunit exchange in extracts does occur (Ruediger *et al.,* 1992, 1994), this process is very slow, increasing continuously during a 24-h incubation and occurring faster at room temperature than on ice (unpublished). In order to minimize exchange, all phosphatase assays were carried out within 1 h after the preparation of extracts, which were kept on ice except for the actual assay period.

Inhibition of gene expression from the HIV-1 LTR by mutant AΔ5

Having established that mutant AΔ5 causes a marked change in the core enzyme to holoenzyme ratio and in the corresponding phosphatase activity, the possible involvement of this change in the control of HIV-1 LTR activity was investigated. COS cells were cotransfected with an HIV-1 LTR-luciferase reporter plasmid, mutant AΔ5 expression plasmid, and increasing amounts of Tat expression vector, pTat. Wild-type A subunit and empty vector were used as controls. As shown in Fig. 3, upper panel, grey bars, increasing amounts of Tat resulted in increased activity of the HIV-1 LTR up to a maximal stimulation of 50-fold in accordance with published reports (Dayton *et al.,* 1986; Fisher *et al.,* 1986; Gaynor, 1995; Jones and Peterlin, 1994; Sodroski *et al.,* 1985a, 1985b). Presenting the same data as a percentage of vector control, the lower panel of Fig. 3 shows the relative suppression of LTR activity by the A subunit and AΔ5. AΔ5 inhibited LTR activity 4-fold in the absence of Tat and at low concentration of Tat (0 to 0.001% pTat). This suggests that AΔ5 has an effect on general transcription factors or upstream enhancer-binding factors. As Tat levels in-

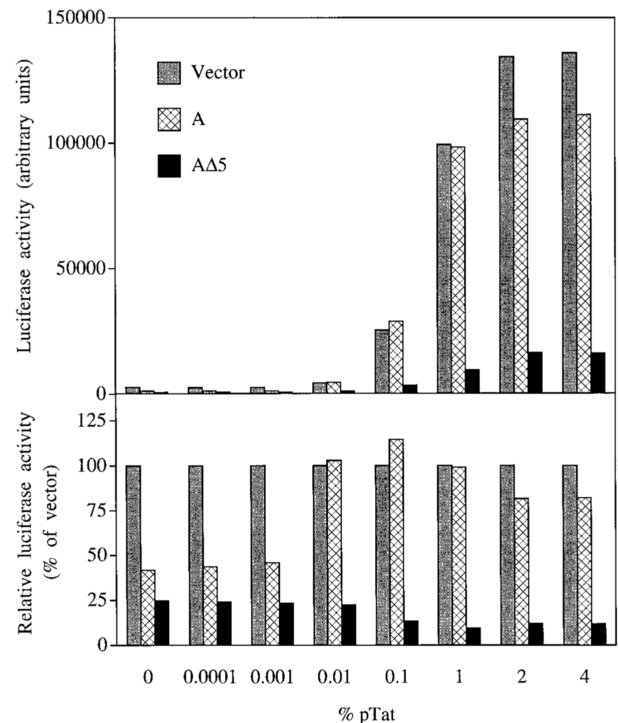

FIG. 3. Inhibition of Tat-enhanced HIV-1 LTR activity by mutant and wild-type A subunits of PP2A. COS cells were cotransfected with an HIV-1 LTR luciferase reporter construct (5% of total DNA), increasing amounts of a Tat expression vector (pTat, 0 to 4%), and with effector plasmids encoding the wild-type A subunit (A) or mutant AΔ5 (80%). Effector plasmid without insert (Vector) served as control. A β-galactosidase-expressing reporter (pSV-βGal, 15%) was also cotransfected to demonstrate equal transfection efficiencies of the various samples (see Materials and Methods). Cells were harvested 48 h after transfection. The upper panel shows absolute luciferase activities, while the lower panel shows relative luciferase activities (vector 100%) at each percentage of pTat. The lowest values, which resulted from transfections without Tat and with AΔ5, were 800 counts with a background of 200 counts. This experiment is representative of two; experiments without Tat and under fully Tat-stimulatory conditions were carried out numerous times with the same result.

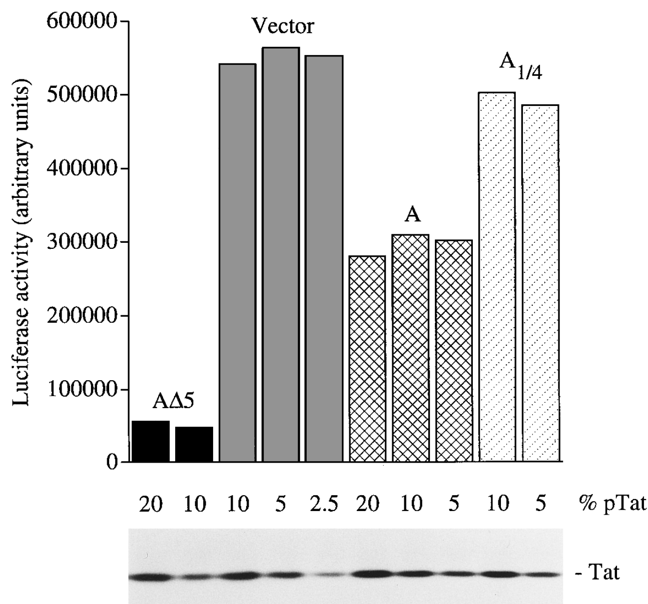

FIG. 4. Inhibition of Tat-enhanced HIV-1 LTR activity by mutant and wild-type A subunits is not due to inhibition of Tat expression. COS cells were cotransfected with an HIV-1 LTR luciferase reporter construct (1% of total DNA), increasing amounts of a Tat expression vector (pTat, 2.5 to 20%), and effector plasmids encoding the wild-type A subunit (A) or mutant AΔ5 (95%). Effector plasmid without insert (Vector) served as control. $A_{1/4}$ designates an effector plasmid mix containing 1/4 A subunit-encoding plasmid and 3/4 empty vector to adjust the amount of A subunit expressed to that of AΔ5. Cells were harvested 48 h after transfection with 150 μl of 1× Passive Lysis Buffer (Promega). An aliquot of 80 μl was mixed and boiled with 40 μl of 6× SDS–PAGE sample buffer. Gradient gels with 4 to 20% polyacrylamide (Novex) were used and Western blotted for Tat (lower panel). The upper panel shows the corresponding luciferase activities at various percentages of pTat. The experiment was repeated once showing the same result.

creased up to full stimulation of the HIV-1 LTR, the inhibitory potency of AΔ5 increased to 8-fold. This doubling of the inhibitory effect suggests that there might be a specific interference of AΔ5 with the transcriptional enhancer function of Tat (see also Fig. 5). The inhibition of Tat-dependent transcription by AΔ5 was not due to reduced expression of Tat, as demonstrated by Western blotting with Tat-specific antibodies. As shown in Fig. 4, cells transfected with AΔ5 and 10 or 20% pTat expression vector contained more Tat protein than cells transfected with vector and 2.5 or 5% pTat (lower panel). Nonetheless, the AΔ5-transfected cells suppressed LTR expression by a factor of 10 (upper panel).

Figure 3 also shows that a two- to threefold inhibition of LTR activity was caused by wild-type A subunit in the absence of Tat stimulation (0 to 0.001% pTat). This effect disappeared almost completely at increasing Tat levels (0.01 to 4% pTat). These experiments were carried out using equal amounts of expression vector for A subunit and AΔ5. However, under these conditions A subunit was expressed at four times higher levels than AΔ5 as demonstrated by Western blotting (data not shown). When corrected for equal levels of A subunit and AΔ5, the A subunit had essentially no effect (see $A_{1/4}$, Fig. 4, upper panel). To explain the lower expression of AΔ5, we assume that AΔ5, but not the A subunit, inhibited its own expression from the CMV promoter in pcDNA3, the expression vector for the A subunit constructs. This assumption is supported by the finding of reduced β-galactosidase production from CMV-driven reporter constructs due to AΔ5 coexpression (data not shown).

PP2A is an abundant protein in cells, each subunit accounting for approximately 0.1% of the total protein (Ruediger *et al.,* 1991). To determine the level of AΔ5 expression, transfections were carried out as described for Fig. 3 and the amounts of A subunit and AΔ5 were determined by Western blotting. Considering the transfection efficiency, there was a 40-fold excess of AΔ5 over endogenous A subunit and a 140-fold excess of exogenous over endogenous A subunit (data not shown). The AΔ5 overexpression resulted in the 2-fold increase in core enzyme and a corresponding decrease in holoenzyme, as shown in Fig. 2.

Mutant AΔ5 inhibits LTR- but not actin promoter-driven mRNA accumulation

To investigate whether AΔ5 inhibits HIV-1 LTR expression at the level of transcription, luciferase mRNA levels were determined. Since quantitating luciferase mRNA levels by Northern blotting or primer extension was not sensitive enough, we developed a new method involving isolation of total RNA from transfected cells, translation of the *in vivo*-produced RNA *in vitro* and measuring the activity of the *in vitro*-synthesized luciferase. As shown in Fig. 5, AΔ5 inhibited HIV-1 LTR-driven mRNA accumulation 2-fold in the absence and 5-fold in the presence of Tat. In contrast, it had no effect on actin promoter-driven mRNA levels. The increased effect of AΔ5 on LTR transcription in the presence of Tat provides direct support for the hypothesis mentioned earlier that AΔ5 interferes with the transcriptional enhancer function of Tat.

Inhibition of HIV-1 production

To investigate whether N-terminal A subunit mutants inhibit virus production, COS cells were cotransfected with the AΔ5 expression vector and pNL4-3, a full-length molecular clone of HIV-1. As shown in Fig. 6, left panel, p24 accumulation in the cell culture medium, a measure of virus yield, was inhibited 45-fold by AΔ5 in comparison to the vector control. The strongest inhibition was seen at 3 and 4 days after transfection. Wild-type A subunit inhibited HIV-1 production only 4-fold as compared to the 45-fold inhibition by AΔ5. The amounts of virus produced on day 1 were very low (see Legend to Fig. 6).

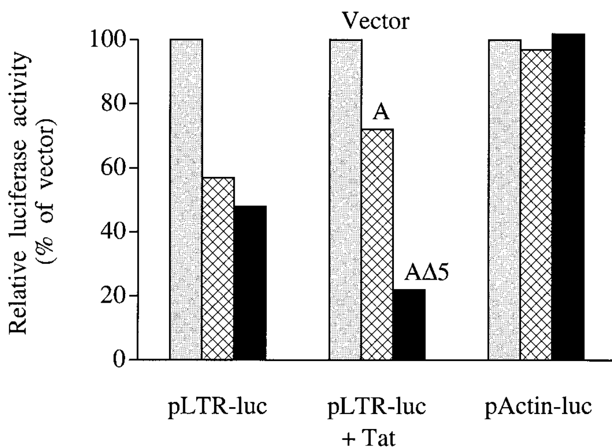

FIG. 5. Mutant AΔ5 inhibits Tat-enhanced HIV-1 LTR luciferase expression at the level of mRNA synthesis. COS cells were cotransfected with an HIV-1 LTR luciferase reporter construct (pLTR-luc, 8% of total DNA) in the absence and in the presence of a Tat expression vector (pHβA-Tat, 8%), or with an actin luciferase reporter construct (pActin-luc, 8%), and with effector plasmid encoding mutant AΔ5 (92 or 84%) or wild-type A subunit (1/4 A-encoding vector plus 3/4 empty vector). Effector plasmid without insert (empty vector) served as control. Cells were lysed with guanidinium isothiocyanate buffer, and RNA was isolated for *in vitro* translations followed by luciferase assays. This determines the extent of *in vivo* reporter gene inhibition up to the level of mRNA accumulation. Luciferase activities compared to vector transfections (100%) are shown. The values are the average of triplicates from two independent experiments. The absolute luciferase values from *in vitro* translations of AΔ5 samples were about 7,000 (pLTR-luc), 70,000 (pLTR-luc + Tat), and 200,000 (pActin-luc) with a background of 2,000 counts.

Therefore, the apparent 10-fold inhibition by the A subunit on day 1 is not meaningful. Since the same amounts of the A subunit and AΔ5 expression vectors were used for transfection, 4 times more A subunit than AΔ5 was produced (see above). Therefore, at equal expression levels the specific inhibition by the A subunit would be very low. To test whether other mutants with similar binding properties as AΔ5 also inhibit HIV-1 production, we used mutant AΔ1–4. This mutant has repeats 1 to 4 deleted and binds the C but not the B subunit *in vitro* (Ruediger *et al.,* 1994) and *in vivo* (data not shown). As shown in Fig. 6, second panel, AΔ1–4 inhibited HIV-1 production 9-fold and AΔ5 24-fold. As additional controls, we used mutants A8 and AΔ11–15. A8 with a deletion of amino acids 2 to 7 from the N terminus behaved like wild-type A subunit, which inhibited 3-fold. This result was expected because A8 and wild-type A subunit bind the B and C subunits equally well (Ruediger *et al.,* 1994). Mutant AΔ11–15, which has a deletion of repeats 11 to 15 and binds neither the C nor the B subunit *in vitro* (Ruediger *et al.,* 1994), also behaved like wild-type A subunit.

We also tested whether AΔ5 inhibits virus production in HeLa cells. As shown in Fig. 6, third panel, AΔ5 inhibited HIV-1 production by a factor of 5 compared to the vector control. Wild-type A subunit inhibited 2-fold. Presumably, this effect of the A subunit would be smaller if its amount was adjusted to that of AΔ5 (cf. Fig. 4). In Jurkat T cells we observed a 3-fold suppression of HIV-1 production due to AΔ5 expression (right panel). It should be noted that the expression levels of AΔ5 are much lower in HeLa and Jurkat T cells as compared to COS cells, since no episomal replication of the A subunit vectors occurs. This explains why effects of AΔ5 are smaller in HeLa and Jurkat T cells.

DISCUSSION

We have demonstrated that increasing the amount of PP2A core enzyme while decreasing the amount of holoenzyme inhibited Tat-dependent HIV-1 gene expression 5-fold at the level of mRNA synthesis. This perturbation of equilibrium between the two major forms of PP2A also inhibited HIV-1 virion production in COS, HeLa, and Jurkat T cells. Expression of N-terminal mutants of the A subunit altered the balance between holoenzyme and core enzyme toward core enzyme and changed phosphatase specificity correspondingly. We found that in AΔ5-transfected COS cells the activity for a holoenzyme-specific substrate (Rb peptide) was decreased approximately 4-fold, whereas that for a core enzyme-specific substrate (phosphorylase *a*) was slightly increased. In parallel, the amount of holoenzyme dropped 2-fold, whereas that of core enzyme (A-C plus AΔ5-C combined) increased 2-fold. The reason why the 2-fold increase in core enzyme produced only a slight increase in phosphorylase *a* phosphatase activity is that the core enzyme is only slightly more active toward this particular substrate than the holoenzyme and that the total amount of core enzyme plus holoenzyme remains constant. We assume that core enzyme and AΔ5-C have the same activity and substrate specificity, although this has not been directly proven. The quantitative effect of AΔ5 depends on the preexisting ratio of holoenzyme to core enzyme in cells. It is commonly believed that the holoenzyme is the predominant form, whereas the core enzyme is an artifact of dissociation and degradation of the B subunit occurring after cell lysis (Cohen, 1989; Shenolikar and Nairn, 1991; Zolnierowicz *et al.,* 1994). In this case AΔ5 would cause a many-fold increase in the amount of core enzyme (from zero to >50%). However, we have recently shown for a number of cell lines that the amounts of endogenous holoenzyme and core enzyme are similar (Kremmer *et al.,* 1996). Therefore, the increase in core enzyme due to AΔ5 expression is approximately 2-fold (from $\frac{1}{3}$ to $\frac{2}{3}$ in COS cells). The finding that a 40-fold overexpression of AΔ5 was required to obtain 2-fold effects on the levels of core and holoenzyme may be surprising. One reason for this observation could be that there was only a 24-h time span, from one day posttransfection until harvest at

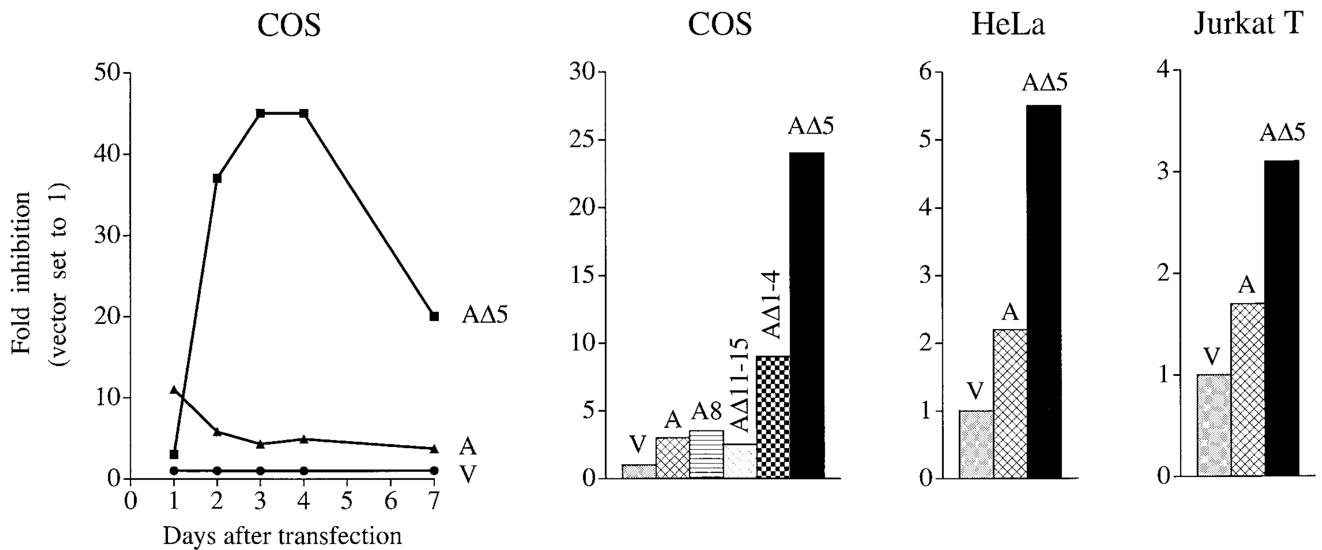

FIG. 6. Inhibition of HIV-1 production by mutant and wild-type A subunits. (Left panel) COS cells were cotransfected with a full-length, wild-type molecular clone of HIV-1, pNL4-3 (20% of total DNA transfected), and with effector plasmids encoding the wild-type A subunit (A) or mutant AΔ5. Effector plasmid without insert (Vector) served as control. Cell growth medium was sampled at different time points to determine the amount of p24 as a measure of HIV-1 released from the cells. The experiment was repeated four more times with a total of six independent sample sets yielding the same result. Inhibition by the A subunit ranged from 2- to 6-fold, and by AΔ5 from 10- to 45-fold. The amounts of p24 released for vector were 10 pg (day 1), 520 pg (day 2), 1300 pg (day 3), 4950 pg (day 4), 10200 pg (day 7). (Second panel) COS cells were transfected as described for the left panel, including additional mutants of the A subunit as indicated. The experiment was performed twice with the same result. The values shown are from the harvest at day 4; vector, 3200 pg of p24. (Third panel) HeLa cells were transfected as described for the left panel, except that pNL4-3 comprised 2% and the effector constructs 98% of the total DNA. The experiment was performed twice with the same result. The values shown are from the harvest at day 3; vector, 300 pg of p24. (Right panel) Jurkat T cells were transfected as described for the left panel and under Materials and Methods, except that pNL4-3 comprised 10% and the effector constructs 90% of the total DNA. At 24 h after transfection the cells were stimulated with PMA (Fluka) (60 ng/ml final), which was necessary to induce expression of the A subunit constructs. The experiment was performed three times with the same result. The values shown are from the harvest at day 2; vector, 240 pg of p24.

48 h, for replacement of endogenous A subunit by AΔ5. In this case one would predict that constant expression of AΔ5 in a permanent cell line would yield strong effects on the core to holoenzyme ratio at a significantly lower level of overexpression. The fact that a 2-fold change in the level of core and holoenzyme caused a 5-fold inhibition of transcription demonstrates that mutant AΔ5 is a powerful tool to study gene expression.

AΔ5 inhibited luciferase expression controlled by the HIV-1 LTR up to 88% and virus production up to 98%. Other A subunit mutants behaved as expected based on their B and C subunit binding properties observed *in vitro.* AΔ1–4 (deletion of repeats 1 to 4) had similar inhibitory effects as AΔ5, whereas AΔ11–15 (deletion of repeats 11 to 15), and mutant 8 (deletion of amino acids 2 to 7) behaved like the wild-type A subunit. These results confirm our hypothesis that the N-terminal mutants AΔ5 and AΔ1–4 are inhibitory because they bind the C subunit but not the B subunit (Ruediger *et al.,* 1994). It is highly unlikely that the inhibition by AΔ5 and AΔ1–4 occurred for reasons other than those proposed, e.g., some nonspecific effect on transcription. If nonspecific effects had occurred, they should be similar for all mutants and also affect transcription from the actin promoter. The small inhibition at extremely high levels of wild-type A subunit expression may be caused by some interaction with B subunit in the absence of C subunit. This would also result in an increase of core enzyme and a decrease of holoenzyme. The N-terminal mutants changed the concentration of both major forms of PP2A. Therefore, the question arises whether the inhibition of HIV-1 LTR activity and virus production resulted from the decrease in holoenzyme or the increase in core enzyme. Because of the observation that general phosphatase inhibition stimulates HIV-1 gene expression (Thevenin *et al.,* 1990; Vlach *et al.,* 1995) we favor the hypothesis that the increase in core enzyme rather than the decrease in holoenzyme was responsible for the observed inhibition.

The finding that the transcriptional inhibition of the HIV-1 LTR by AΔ5 increased from 2-fold in the absence to 5-fold in the presence of Tat suggests that AΔ5 interferes with the functional activity of Tat as a transcriptional enhancer. It is possible that AΔ5 expression leads to a change in the phosphorylation state of Tat or of the Tat-associated kinase, TAK. However, at present neither Tat nor TAK have been shown to be phosphorylated *in vivo* (Herrmann and Rice, 1993, 1995). Our study shows that alterations in the core to holoenzyme ratio affect HIV-1 gene expression. Whether such changes occur in the life cycle of HIV-1, e.g., during mitogenic activation of HIV-1

in resting T lymphocytes, is an interesting possibility that can be tested. It is important to note, however, that irrespective of whether or not alterations occur physiologically, our data argues that the given concentrations of both major forms of PP2A affect the degree of HIV-1 expression.

PP2A is involved in many cellular events, including cell growth and division (Lee, 1995), signal transduction (Mumby, 1995), and gene expression (Mumby and Walter, 1993; Schönthal, 1995). At present, little is known about its mechanism of action and how its activity is controlled. Most previous studies on the role of PP2A *in vivo* relied on the use of okadaic acid (OA), which, at low concentrations, was believed to specifically inhibit PP2A (Cohen, 1989). However, since other serine/threonine phosphatases have been discovered that are similarly sensitive to OA as PP2A (Brewis *et al.,* 1993; Chen *et al.,* 1994; Honkanen *et al.,* 1991), most conclusions regarding the role of PP2A are uncertain. The only PP2A-specific tool available until now was SV40 small T antigen, which binds with high affinity to the A subunit at the binding site for the B subunit. Small T antigen inhibits phosphatase activity toward several substrates. For example, it was demonstrated that it activates the MAP kinase signaling pathway by inhibiting the dephosphorylation of MAP kinase and MAP kinase kinase (Frost *et al.,* 1994; Sontag *et al.,* 1993). However, a serious drawback of small T antigen as a specific tool is the fact that it also has a transactivation function independent of its ability to bind and inhibit PP2A (Loeken, 1993; Porras *et al.,* 1996). It is difficult to distinguish between these functions since both could affect gene expression. In contrast to OA and small T antigen, the N-terminal mutants of the A subunit are specific tools to study the role of PP2A in all cellular events where its involvement is suspected.

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REFERENCES

Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. A. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59,** 284–291.

Agostinis, P., Derua, R., Sarno, S., Goris, J., and Merlevede, W. (1992). Specificity of the polycation-stimulated (type-2A) and ATP, Mg-dependent (type-1) protein phosphatases toward substrates phosphorylated by p34cdc2 kinase. *Eur. J. Biochem.* **205,** 241–248.

Alkalay, I., Yaron, A., Hatzubai, A., Orian, A., Ciechanover, A., and Ben-Neriah, Y. (1995). Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **92,** 10599–10603.

Brewis, N. D., Street, A. J., Prescott, A. R., and Cohen, P. T. W. (1993). PPX, a novel protein serine/threonine phosphatase localized to centrosomes. *EMBO J.* **12,** 987–996.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* **267,** 1485–1488.

Cegielska, A., Shaffer, S., Derua, R., Goris, J., and Virshup, D. M. (1994). Different oligomeric forms of protein phosphatase 2A activate and inhibit simian virus 40 DNA replication. *Mol. Cell. Biol.* **14,** 4616–4623.

Chen, M. X., McPartlin, A. E., Brown, L., Chen, Y. H., Barker, H. M., and Cohen, P. T. W. (1994). A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus. *EMBO J.* **13,** 4278–4290.

Chen, S.-C., Kramer, G., and Hardesty, B. (1989). Isolation and partial characterization of an M_r 60,000 subunit of a type 2A phosphatase from rabbit reticulocytes. *J. Biol. Chem.* **264,** 7267–7275.

Cohen, P. (1989). The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58,** 453–508.

Csortos, Csilla, Zohnierowicz, Stanislaw, Bako, Eva, Kurbin, Stephen, D., and DePaoli-Roach, Anna A. (1996). High complexity in the expression of the B′ subunit of protein phosphatase 2A. *J. Biol. Chem.* **271,** 2578–2588.

Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C., and Haseltine, W. A. (1986). The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell* **44,** 941–947.

De Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7,** 725–737.

DiDonato, J. A., Mercurio, F., and Karin, M. (1995). Phosphorylation of I kappa B alpha precedes but is not sufficient for its dissociation from NF-kappa B. *Mol. Cell Biol.* **15,** 1302–1311.

Ferrigno, P., Langan, T. A., and Cohen, P. (1993). Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases. *Mol. Biol. Cell* **4,** 669–677.

Finco, T. S., Beg, A. A., and Baldwin, A. S. J. (1994). Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors. *Proc. Natl. Acad. Sci. USA* **91,** 11884–11888.

Fisher, A. G., Feinberg, M. B., Josephs, S. F., Harper, M. E., Marselle, L. M., Reyes, G., Gonda, M. A., Aldovini, A., Debouk, C., and Gallo, R. C. (1986). The trans-activator gene of HTLV-III is essential for virus replication. *Nature* **320,** 367–371.

Frost, J. A., Alberts, A. S., Sontag, E., Guan, K., Mumby, M. C., and Feramisco, J. R. (1994). Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. *Mol. Cell. Biol.* **14,** 6244–6252.

Gaynor, R. B. (1995). Regulation of HIV-1 gene expression by the transactivator protein Tat. *Curr. Top. Microbiol. Immunol.* **193,** 51–77.

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23,** 175–182.

Grilli, M., Chiu, J. J., and Lenardo, M. J. (1993). NF-kappa B and Rel: Participants in a multiform transcriptional regulatory system. *Int. Rev. Cytol.* **143,** 1–62.

Hemmings, B. A., Adams-Pearson, C., Maurer, F., Muller, P., Goris, J., Merlevede, W., Hofsteenge, J., and Stone, S. R. (1990). a- and b-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* **29,** 3166–3173.

Hendrix, P., Mayer-Jaekel, R. E., Cron, P., Goris, J., Hofsteenge, J., Merlevede, W., and Hemmings, B. A. (1993). Structure and expression

of a 72-kDa regulatory subunit of protein phosphatase 2A. *J. Biol. Chem.* **268,** 15267–15276.

Herrmann, C. H., and Rice, A. P. (1993). Specific interaction of the human immunodeficiency virus Tat proteins with a cellular protein kinase. *Virology* **197,** 601–608.

Herrmann, C. H., and Rice, A. P. (1995). Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: Candidate for a Tat cofactor. *J. Virol.* **69,** 1612–1620.

Honkanen, R. E., Zwiller, J., Daily, S. L., Khatra, B. S., Dukelow, M., and Boynton, A. L. (1991). Identification, purification, and characterization of a novel serine/threonine protein phosphatase from bovine brain. *J. Biol. Chem.* **266,** 6614–6619.

Howcroft, T. K., Palmer, L. A., Brown, J., Rellahan, B., Kashanchi, F., Brady, J. N., and Singer, D. S. (1995). HIV Tat represses transcription through Sp1-like elements in the basal promoter. *Immunity* **3,** 127–138.

Imaoka, T., Imazu, M., Usui, H., Kinohara, N., and Takeda, M. (1983). Resolution and reassociation of three distinct components from pig heart phosphoprotein phosphatase. *J. Biol. Chem.* **258,** 1526–1535.

Jeang, K. T., Chun, R., Lin, N. H., Gatignol, A., Glabe, C. G., and Fan, H. (1993). In vitro and in vivo binding of human immunodeficiency virus type 1 Tat protein and Sp1 transcription factor. *J. Virol.* **67,** 6224–6233.

Jones, K. A., and Peterlin, B. M. (1994). Control of RNA initiation and elongation at the HIV-1 promoter. *Annu. Rev. Biochem.* **63,** 717–743.

Kamibayashi, C., Estes, R., Lickteig, R. L., Yang, S., Craft, C., and Mumby, M. (1994). Comparison of heterotrimeric protein phosphatase 2A containing different B subunits. *J. Biol. Chem.* **269,** 20139–20148.

Kamibayashi, C., Estes, R. C., Slaughter, C., and Mumby, M. C. (1991). Subunit interactions control protein phosphatase 2A: Effects of limited proteolysis, N-ethylmaleimide, and heparin on the B subunit. *J. Biol. Chem.* **266,** 13251–13260.

Kamibayashi, C., Lickteig, R. L., Estes, R., Walter, G., and Mumby, M. C. (1992). Expression of the A subunit of protein phosphatase 2A and characterization of its interactions with the catalytic and regulatory subunits. *J. Biol. Chem.* **267,** 21864–21872.

Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C. M., Roeder, R. G., and Brady, J. N. (1994a). Direct interaction of human TFIID with the HIV-1 transactivator tat. *Nature* **367,** 295–299.

Kashanchi, F., Shibata, R., Ross, E. K., Brady, J. N., and Martin, M. A. (1994b). Second-site long terminal repeat (LTR) revertants of replication-defective human immunodeficiency virus: Effects of revertant TATA box motifs on virus infectivity, LTR-directed expression, in vitro RNA synthesis, and binding of basal transcription factors TFIID and TFIIA. *J. Virol.* **68,** 3298–3307.

Kerr, L. D., Ransone, L. J., Wamsley, P., Schmitt, M. J., Boyer, T. G., Zhou, Q., Berk, A. J., and Verma, I. M. (1993). Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B. *Nature* **365,** 412–419.

Knight, D. M., Flomerfelt, F. A., and Ghrayeb, J. (1987). Expression of the art/trs protein of HIV and study of its role in viral envelope synthesis. *Science* **236,** 837–840.

Kremmer, E., Ohst, K., Kiefer, J., Brewis, N., and Walter, G. (1997). Separation of PP2A core enzyme and holoenzyme with monoclonal antibodies against the regulatory A subunit: Abundant expression of both forms in cells. *Mol. Cell. Biol.* **17,** 1692–1701.

Lee, M. G., and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2. Nature* **327,** 31–35.

Lee, T. H. (1995). The role of protein phosphatase type-2A in the *Xenopus* cell cycle: Initiation of the G2/M transition. *Sem. Cancer Biol.* **6,** 203–209.

Lin, B. T., and Wang, J. Y. (1992). Cell cycle regulation of retinoblastoma protein phosphorylation. *Ciba. Found. Symp.* **170,** 227–241.

Lin, Y. C., Brown, K., and Siebenlist, U. (1995). Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. *Proc. Natl. Acad. Sci. USA* **92,** 552–556.

Loeken, M. R. (1993). Multiple, distinct trans-activation functions are encoded by the simian virus 40 large T and small t antigens, only some of which require the 82-residue amino-terminal common domain. *J. Virol.* **67,** 7684–7689.

MacKintosh, C. (1993). *In* "Protein Phosphorylation" (D. G. Hardie, Ed.), pp. 197–230. Oxford Univ. Press, New York.

McCright, B., and Virshup, D. M. (1995). Identification of a new family of protein phosphatase 2A regulatory subunits. *J. Biol. Chem.* **270,** 26123–26128.

Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M., and Verma, I. M. (1994). Tumor necrosis factor alpha-induced phosphorylation of I kappa B alpha is a signal for its degradation but not dissociation from NF-kappa B. *Proc. Natl. Acad. Sci. USA* **91,** 12740–12744.

Mumby, M. (1995). Regulation by tumor antigens defines a role for PP2A in signal transduction. *Semin. Cancer Biol.* **6,** 229–237.

Mumby, M. C., Green, D. D., and Russell, K. R. (1985). Structural characterization of cardiac protein phosphatase with a monoclonal antibody: Evidence that the M_r = 38,000 phosphatase is the catalytic subunit of the native enzyme(s). *J. Biol. Chem.* **260,** 13763–13770.

Mumby, M. C., and Walter, G. (1993). Protein serine/threonine phosphatases: Structure, regulation, and functions in cell growth. *Physiol. Rev.* **73,** 673–699.

Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. *Cell* **78,** 773–785.

Pazin, M. J., Sheridan, P. L., Cannon, K., Cao, Z., Keck, J. G., Kadonaga, J. T., and Jones, K. A. (1996). NF-kappa B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. *Genes. Dev.* **10,** 37–49.

Peterlin, B. M., Luciw, P. A., Barr, P. J., and Walker, M. D. (1986). Elevated levels of mRNA can account for the trans-activation of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **83,** 9734–9738.

Porras, A., Bennett, J., Howe, A., Tokos, K., Bouck, N., Henglein, B., Sathyamangalam, S., Thimmapaya, B., and Rundell, K. (1996). A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays. *J. Virol.* **70,** 6902–6908.

Rosen, C. A., Sodroski, J. G., and Haseltine, W. A. (1985). Location of cis-acting regulatory sequences in the human T-cell leukemia virus type I long terminal repeat. *Proc. Natl. Acad. Sci. USA* **82,** 6502–6506.

Rosenthal, N. (1987). Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol.* **152,** 704–720.

Ruediger, R., Hentz, M., Fait, J., Mumby, M., and Walter, G. (1994). Molecular model of the A subunit of protein phosphatase 2A: Interaction with other subunits and tumor antigens. *J. Virol.* **68,** 123–129.

Ruediger, R., Roeckel, D., Fait, J., Bergqvist, A., Magnusson, G., and Walter, G. (1992). Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus. *Mol. Cell. Biol.* **12,** 4872–4882.

Ruediger, R., van Wart Hood, J. E., Mumby, M., and Walter, G. (1991). Constant expression and activity of protein phosphatase 2A in synchronized cells. *Mol. Cell. Biol.* **11,** 4282–4285.

Sanes, J. R., Rubenstein, J. L. R., and Nicolas, J.-F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* **5,** 3133–3142.

Schönthal, A. H. (1995). Regulation of gene expression by serine/threonine protein phosphatases. *Semin. Cancer Biol.* **6,** 239–248.

Shenolikar, S., and Nairn, A. C. (1991). Protein phosphatases: recent progress. *Adv. Second Messenger Phosphoprotein Res.* **23,** 1–121.

Sheridan, P. L., Sheline, C. T., Cannon, K., Voz, M. L., Pazin, M. J., Kadonaga, J. T., and Jones, K. A. (1995). Activation of the HIV-1 enhancer by the LEF-1 HMG protein on nucleosome-assembled DNA in vitro. *Genes. Dev.* **9,** 2090–2104.

Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F., and Haseltine, W. (1985a). Location of the trans-activating region on the genome of human T-cell lymphotropic virus type III. *Science* **229,** 74–77.

Sodroski, J., Rosen, C., Wong-Staal, F., Salahuddin, S. Z., Popovic, M., Arya, S., Gallo, R. C., and Haseltine, W. A. (1985b). Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat. *Science* **227,** 171–173.

Sola, M. M., Langan, T., and Cohen, P. (1991). p34cdc2 phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase $2A_1$. *Biochim. Biophys. Acta* **1094,** 211–216.

Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993). The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the Map kinase pathway and induces cell proliferation. *Cell* **75,** 887–897.

Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S., and Mumby, M. C. (1995). A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell Biol.* **128,** 1131–1144.

Sun, S-C., Maggirwar, S. B., and Harhaj, E. (1995). Activation of NF-kB by phosphatase inhibitors involves the phosphorylation of IkBa at phosphatase 2A-sensitive sites. *J. Biol. Chem.* **270,** 18347–18351.

Sune, C., and Garcia-Blanco, M. A. (1995). Sp1 transcription factor is required for in vitro basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. *J. Virol.* **69,** 6572–6576.

Tanabe, O., Nagase, T., Murakami, T., Nozaki, H., Usui, H., Nishito, Y., Hayashi, H., Kagamiyama, H., and Takeda, M. (1996). Molecular cloning of a 74-kDa regulatory subunit (B″ or delta) of human protein phosphatase 2A. *FEBS Lett.* **379,** 107–111.

Tehrani, M. A., Mumby, M. C., and Kamibayahi, C. (1996). Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. *J. Biol. Chem.* **271,** 5164–5170.

Thanos, D., and Maniatis, T. (1995). NF-kappa B: A lesson in family values. *Cell* **80,** 529–532.

Thevenin, C., Kim, S. J., Rieckmann, P., Fujiki, H., Norcross, M. A., Sporn, M. B., Fauci, A. S., and Kehrl, J. H. (1990). Induction of nuclear factor-kappa B and the human immunodeficiency virus long terminal repeat by okadaic acid, a specific inhibitor of phosphatases 1 and 2A. *New. Biol.* **2,** 793–800.

Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994). A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J.* **13,** 5433–5441.

Vlach, J., Garcia, A., Jacque, J. M., Rodriguez, M. S., Michelson, S., and Virelizier, J. L. (1995). Induction of Sp1 phosphorylation and NF-kappa B-independent HIV promoter domain activity in T lymphocytes stimulated by okadaic acid. *Virology* **208,** 753–761.

Walter, G. (1986). Production and use of antibodies against synthetic peptides. *J. Immunol. Methods* **88,** 149–161.

Walter, G., Ferre, F., Espiritu, O., and Carbone-Wiley, A. (1989). Molecular cloning and sequence of cDNA encoding polyoma medium tumor antigen-associated 61-kDa protein. *Proc. Natl. Acad. Sci. USA* **86,** 8669–8672.

Walter, G., and Mumby, M. (1993). Protein serine/threonine phosphatases and cell transformation. *Biochim. Biophys. Acta* **1155,** 207–226.

Zolnierowicz, S., Csortos, C., Bondor, J., Verin, A., Mumby, M. C., and DePaoli-Roach, A. A. (1994). Diversity in the regulatory B subunits of protein phosphatase 2A: Identification of a novel isoform highly expressed in brain. *Biochemistry* **33,** 11858–11867.