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HIV-1 Tat transcriptional activity is regulated by acetylation

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The human immunodeficiency virus (HIV) trans-activator protein, Tat, stimulates transcription from the viral long-terminal repeats (LTR) through an RNA hairpin element, *trans*-activation responsive region (TAR). We and others have shown that *trans*-activator protein (Tat)-associated histone acetyltransferases (TAHs), p300 and p300/CBP-associating factor (PCAF), assist functionally in the activation of chromosomally integrated HIV-1 LTR. Here, we show that p300 and PCAF also directly acetylate Tat. We defined two sites of acetylation located in different functional domains of Tat. p300 acetylated Lys50 in the TAR RNA binding domain, while PCAF acetylated Lys28 in the activation domain of Tat. In support of a functional role for acetylation *in vivo*, histone deacetylase inhibitor (trichostatin A) synergized with Tat in transcriptional activation of the HIV-1 LTR. Synergism was TAR-dependent and required the intact presence of both Lys28 and Lys50. Mechanistically, acetylation at Lys28 by PCAF enhanced Tat binding to the Tat-associated kinase, CDK9/P-TEFb, while acetylation by p300 at Lys50 of Tat promoted the dissociation of Tat from TAR RNA that occurs during early transcription elongation. These data suggest that acetylation of Tat regulates two discrete and functionally critical steps in transcription, binding to an RNAP II CTD-kinase and release of Tat from TAR RNA.
Keywords: acetylation/HIV-1 Tat/p300/PCAF/transcriptional activity

Introduction

The human immunodeficiency virus type 1 (HIV-1) *trans*-activator protein, Tat, is absolutely required for productive replication (reviewed in Jeang and Gatignol, 1994). Tat is an atypical transcriptional activator that functions through binding, not to DNA, but to a short leader RNA, *trans*-activation responsive region (TAR) (Berkhout *et al*., 1989; Dingwall *et al*., 1989; reviewed in Jones and Peterlin, 1994). Several observations suggest that an as yet uncharacterized mechanism that regulates dissociation of Tat from TAR might be physiologically important. First, Tat protein is expressed at exceedingly low to virtually undetectable levels in virus-infected cells (Adams *et al*., 1994; Cannon *et al*., 1994; Peng *et al*., 1995). In contrast, the organization of the retroviral long-terminal repeats (LTRs) compels all viral transcripts to contain two bona fide copies of TAR. These TAR-containing RNAs are fully potent for Tat-binding and should competitively decoy the latter away from nascent promoter-associated TARs (Lisziewicz *et al*., 1993; Bohjanen *et al*., 1997; Corbeau and Wong-Staal, 1998). An estimate for productively infected cells is that TAR-containing transcripts exceed Tat by a stoichiometry of 100–1000 (K.-T.Jeang, unpublished observations). Hence, in order for a limiting amount of Tat to be functional in a setting of a large TAR RNA excess, a selective mechanism that either protects Tat association to or effects Tat dissociation from TAR should exist. Secondly, an increasing number of reports suggest that Tat does not exist bound statically to TAR RNA, but dissociates kinetically from TAR to bind either elongating RNA polymerase II, RNAP II (Keen *et al*., 1996, 1997; reviewed in Jones, 1997) or DNA-tethered promoter factors (Jeang *et al*., 1993; Cujec *et al*., 1997a; Garcia-Martinez *et al*., 1997a; Chun *et al*., 1998). In its unmodified form, the associative half-life of Tat for TAR RNA is 41 seconds *in vitro* (Wang *et al*., 1998); this duration is incompatible with the rapid kinetics required for Tat-directed transcription from the LTR (Jeang and Berkhout, 1992). Hence, deductively it seems likely that a post-translationally modified form of Tat that has a shorter half-life for TAR would exist intracellularly.

The optimal activity of Tat is further dictated by its association with two classes of cellular proteins, Tat-associated-kinases TAKs (Herrmann and Rice, 1993, 1995) [which include RNAPII C-terminal domain (CTD) kinases, TFIIH (Parada and Roeder, 1996; Cujec *et al*., 1997b; Garcia-Martinez *et al*., 1997b) and P-TEFb (Zhu *et al*., 1997; Garber *et al*., 1998; Wei *et al*., 1998; reviewed by Jones, 1997)] and Tat-associated histone acetyltransferases, TAHs [which include p300/CBP and p300/CBP-associating factor (PCAF) (Benkirane *et al*., 1998; Hottiger and Nabel, 1998; Marzio *et al*., 1998)]. TAKs effect processive transcription of RNAPII from the HIV-1 LTR promoter (Chun and Jeang, 1996; Okamoto *et al*., 1996; Parada and Roeder, 1996; Yang *et al*., 1996), while TAHs induce the activation of chromatinized HIV-1 LTRs (Benkirane *et al*., 1998; Marzio *et al*., 1998) presumably through acetylation of histones. Here, we show that TAHs also directly acetylate the Tat protein in two different

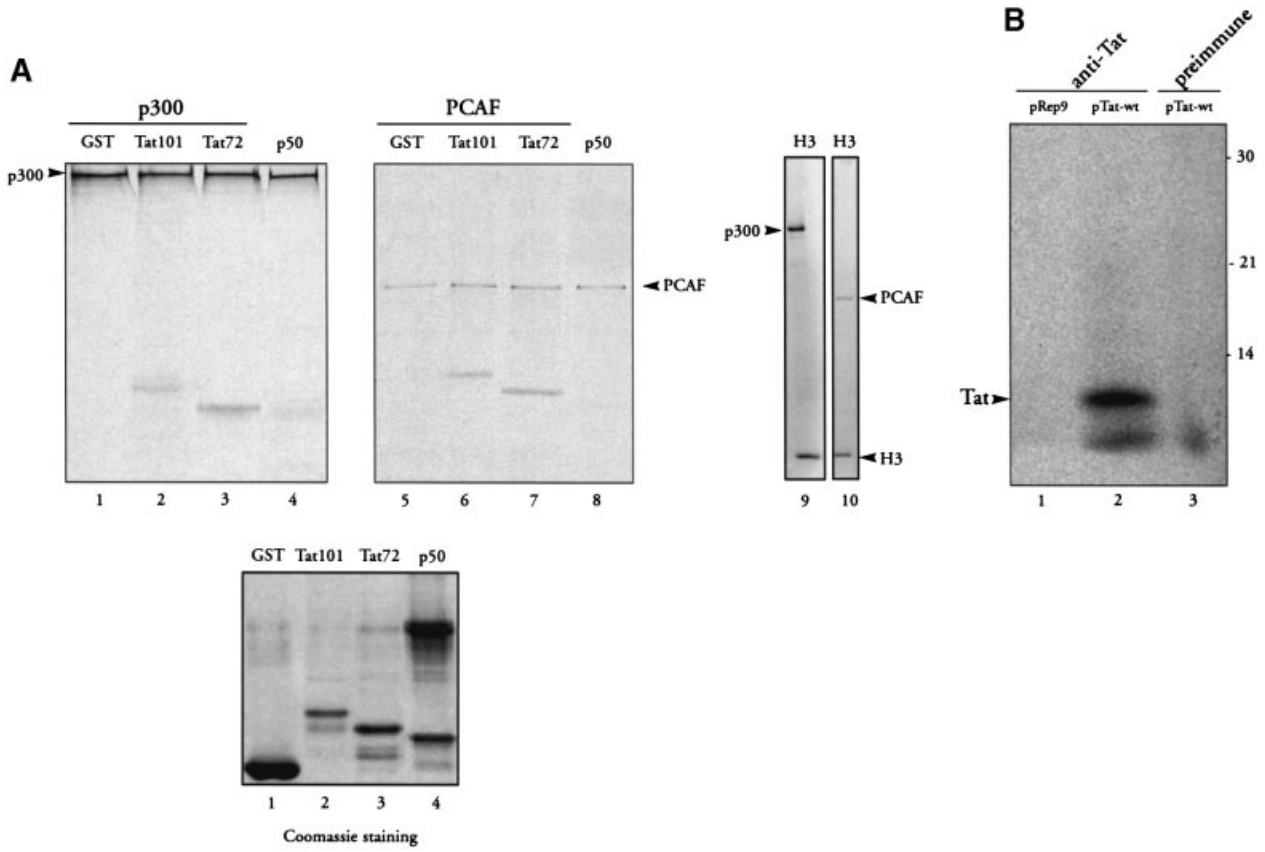


Fig. 1. HIV-1 Tat protein is acetylated *in vitro* and *in vivo*. (**A**) Tat protein is acetylated *in vitro* by p300 and PCAF. GST (lanes 1 and 5), GST–Tat101 (lanes 2 and 6), GST–Tat72 (lanes 3 and 7), GST–p50 (lanes 4 and 8) or histone H3 (lanes 9 and 10) were incubated in acetylation buffer with either recombinant p300 (lanes 1–4 and 9) or recombinant PCAF (lanes 5–8 and 10) for 1 h at 30°C. Reaction products were separated by 10% SDS–PAGE or by 15% SDS–PAGE for H3 samples and the gels were Coomassie Blue stained (bottom panel), dried and and visualized by autoradiography (top panel). (**B**) *In vivo* acetylation of Tat protein. HeLa cells were transfected with a control plasmid, pRep9, (lane 1) or with a Tat-expression plasmid, pTat-wt, (lanes 2 and 3). Extracts from transfected cells pulsed with [^{3}H]acetate were subjected to immunoprecipitation with Tat antiserum (lanes 1 and 2) or rabbit pre-immune serum (lane 3), and immunoprecipitated proteins were analyzed by 15% SDS–PAGE followed by autoradiography.

domains. Acetylation of Tat leads to two functional consequences. First, it promotes dissociation of Tat from TAR RNA; secondly, it modulates association of Tat to TAK. Thus, this novel post-translational modification governs two essential steps in HIV-1 transcription: binding of Tat to TAR and binding of Tat to TAK.

Results

Tat is acetylated in vivo and in vitro

Previously, we and others have shown that histone acetyltransferases, p300 and PCAF, are co-activators for the HIV-1 Tat protein (Benkirane *et al*., 1998; Hottiger and Nabel, 1998; Marzio *et al*., 1998). To test whether Tat is a substrate for p300 and/or PCAF, we performed *in vitro* acetylation assays using recombinant GST–Tat fusion protein together with recombinant p300 and PCAF. As shown in Figure 1A, both two exon (Tat101) and one exon (Tat72) forms of Tat were acetylated by p300 (lanes 2 and 3) and PCAF (lanes 6 and 7), respectively. No acetylation was observed when GST was supplied as substrate (lanes 1 and 5). GST–p50 (lanes 4 and 8) and histone H3 (lanes 9 and 10) were used as negative and positive controls, respectively. Coomassie Blue staining showed that equivalent amounts of protein were used in the acetylation reactions (Figure 1A, bottom panel). Using mass spectroscopy analysis, we estimated that ~15% of Tat is acetylated *in vitro* (data not shown). These findings demonstrate that Tat is acetylated *in vitro* by both p300 and PCAF.

To verify acetylation *in vivo*, HeLa cells transfected with either a Tat (one exon) expression plasmid (pTat-wt) or empty vector were biosynthetically labeled for 1 h with [^{3}H]sodium acetate. The cells were lysed and protein was immunoprecipitated using a Tat-specific antiserum. Figure 1B shows that intracellularly-expressed Tat was acetylated (lane 2). No signal was detected in control immunoprecipitates from either the same extract using pre-immune serum (lane 3) or from an anti-Tat immunoprecipitation of empty vector-transfected cells (lane 1). These results show that acetylation of Tat occurs *in vivo*.

Acetylation of the RNA-binding and activation domains of Tat by p300 and PCAF, respectively

As shown in Figure 1, p300 and PCAF can acetylate Tat independently *in vitro*. To determine whether the two reactions produce identical or different acetylations, we sought to determine the acceptor residues in Tat for p300 and PCAF. Thus, several synthetic peptides corresponding to amino acids 8–24 (P1), 23–43 (P2) and 39–54 (P3)

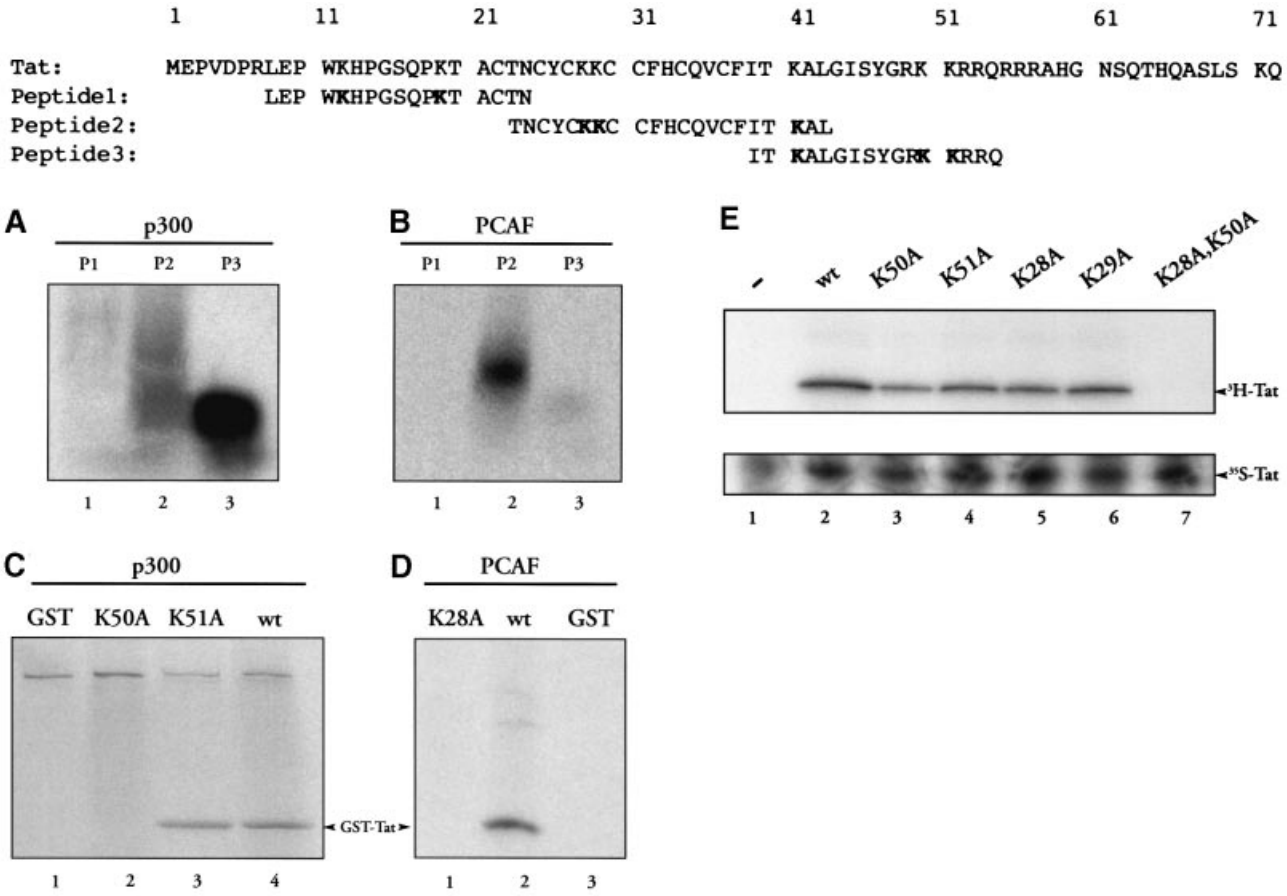


Fig. 2. Acetylation of Tat occurs at sites located in the RNA binding domain and activation domain. Top panel: the amino acid sequence of HIV-1 Tat (amino acids 1–72) is shown. Below that sequence are the sequences of peptides used in experiments shown in Figure 2A and B (peptide 1, 2 and 3). Bottom panel: p300 and PCAF differentially acetylate HIV-1 Tat protein. Synthetic peptides (5 μg) corresponding to amino acids 8–24 (P1), 23–43 (P2) and 39–54 (P3) of Tat were incubated with 250 ng of either p300 (**A**) or PCAF (**B**) and [^{14}C]acetyl-CoA for 1 h at 30°C. Reaction products were resolved in 16.5% Tris-tricine acrylamide gels followed by autoradiography. Lysine 50 and lysine 28 are substrates for p300 and PCAF respectively. (**C**) GST or GST–Tat72 containing either wild-type sequence (lane 4) or lysine 50 or 51 mutated to alanine (K50A, lane 2 and K51A, lane 3) were incubated with 100 ng of p300 for 1 h at 30°C. Reactions were analyzed on 10% SDS–PAGE followed by autoradiography. (**D**) GST or GST–Tat72 containing either wild-type sequence (wt, lane 2) or lysine 28 mutated to alanine (K28A, lane 1) were incubated in acetylation buffer with 100 ng of recombinant PCAF for 1 h at 30°C. Reaction products were separated by 10% SDS–PAGE and visualized by autoradiography. (**E**) *In vivo* acetylation of wild-type and mutant Tat proteins. HeLa cells were transfected with vector alone or with full-length Tat-expression plasmids having either wild-type sequence (wt) or containing single lysine to alanine mutations at residues 50 (K50A), 51 (K51A), 28 (K28A), 29 (K29A), or a double mutation at lysines 28 and 50 (K28A,K50A). Extracts from transfected cells pulsed with either [^{3}H]acetate or [^{35}S]cysteine + methionine were subjected to immunoprecipitation with Tat antiserum, and immunoprecipitated proteins were analyzed by 15% SDS–PAGE followed by autoradiography.

were tested as substrates for *in vitro* acetylation with either p300 or PCAF (Figure 2). Figure 2A shows that peptide P3 (lane 3) but not P1 or P2 (lanes 1 and 2) was acetylated by p300. In contrast, PCAF preferentially acetylated only P2 (Figure 2B, compare lane 2 with lanes 1 and 3). Coomassie Blue staining confirmed that equivalent amounts of peptide were used in the acetylation reactions (data not shown). Because P2 lies within the Tat-activation domain while P3 lies within the Tat-RNA-binding domain (reviewed in Jeang, 1996), these results suggest that p300 and PCAF mediate discrete post-translational modifications.

To resolve the acceptor residues further, we analyzed P3 and P2 sequences in detail. P3, spanning amino acids 39–54 of Tat, contains three potential acetylation sites at lysines 41, 50 and 51. Because K41 in P3 is also present in P2, which was not acetylated by p300 (Figure 2A, lane 2), this residue was excluded as a target for p300. To identify the p300 acceptor(s), GST–Tats with alanine substitutions at K50 or K51 were tested for *in vitro* acetylation. Consistent with results in Figure 1A, p300 acetylated the wild-type sequence (Figure 2C, lane 4). In contrast, a K50 to A50 change (K50A) completely abrogated p300-mediated acetylation when compared with that observed for wild-type GST–Tat or GST–TatK51A (compare lane 2 with lanes 3 and 4; Figure 2C).

We next examined PCAF acetylation of Tat peptide 2 (Figure 2B, lane 2). P2 contains three potential acceptors at K28, K29 and K41. K41 was excluded for the reason outlined above. To discriminate between K28 and K29, we constructed GST–Tats with alanine substitutions at K28 or K29 or at both residues. These GST–Tats were tested for *in vitro* acetylation. Figure 2D shows that PCAF-mediated acetylation was completely abrogated by the substitution K28 to A28 (K28A) when compared with that observed for wild-type GST–Tat (Figure 2D, compare lanes 1 and 2). Acetylation of K29A was comparable to the wild-type (data not shown). The p300 and PCAF results

taken together suggest that these two TAHs differentially modify the Tat protein in two discrete functional domains. Furthermore, comparison of Tat sequences from primate lentiviruses (HIV-1, HIV-2 and SIV) revealed absolute conservation of lysine residues 28 and 50. In contrast, lysines 29 and 51 are variable (Myers *et al*., 1995). The high conservation of lysines 28 and 50 suggests an important role for these residues in Tat function.

To confirm that acetylated lysine residues mapped *in vitro* were also acetylated *in vivo*, HeLa cells were transfected with full-length Tat-expression plasmids containing either wild-type sequence or single lysine to alanine mutations at residues 28, 29, 50 and 51, as well as a double mutation at lysines 28 and 50. Cells were pulse labeled with either [^{3}H]sodium acetate or [^{35}S]methionine + cysteine, lysed, and protein was immunoprecipitated using a Tat-specific antiserum (Figure 2E). *In vivo* acetylation was observed in cells transfected with wild-type Tat, Tat-K51A and Tat-K29A. A weaker signal was observed in cells transfected with Tat-K28A and Tat-K50A. No acetylation was observed in cells transfected with Tat-K28A,K50A. Metabolic labelling using [^{35}S]methionine + cysteine confirmed that the mutant Tat proteins were expressed to equivalent levels in transfected cells. Interestingly, complete abrogation of acetylation occurred only when both acetylation sites were mutated (Tat-K28A,K50A), suggesting that the acetylations by p300 and PCAF occur independently. This result also confirms that K28 and K50 are the sole acetylation targets in Tat.

Acetylation of Tat affects its transcriptional activity

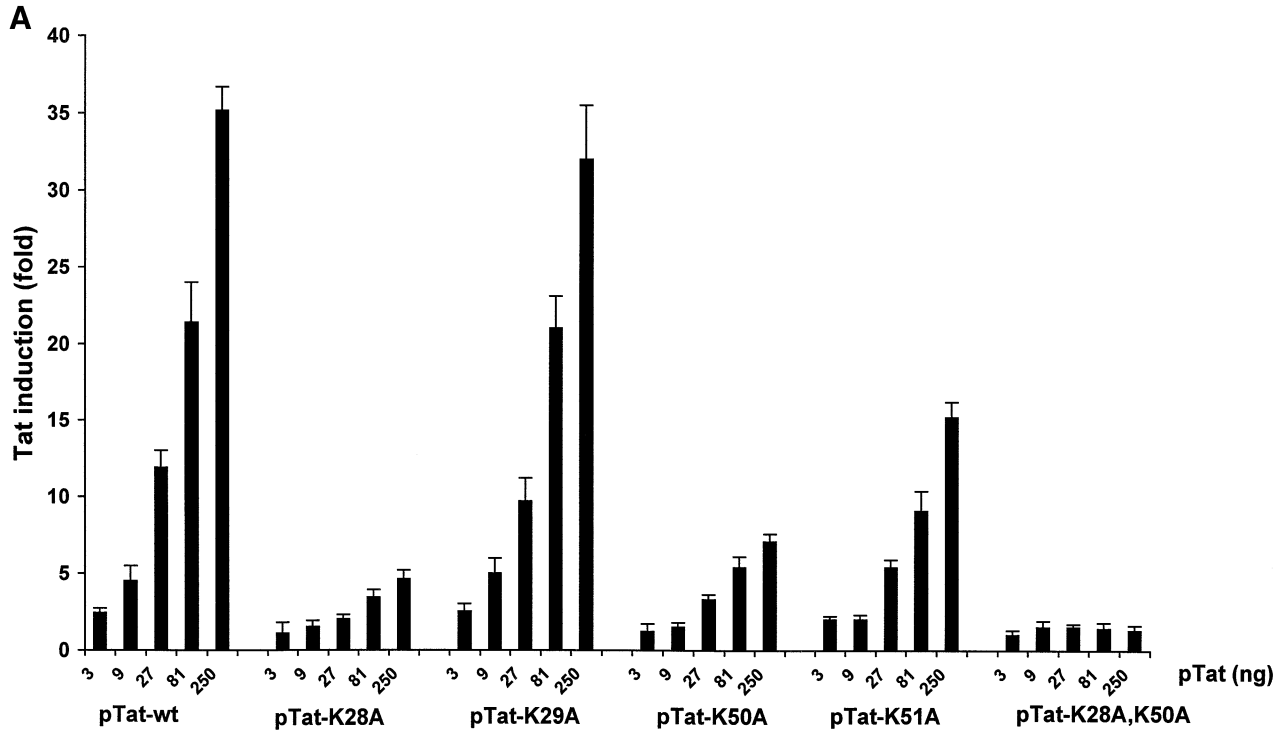
To address the *in vivo* significance of Tat acetylation, we compared the intracellular transcriptional activity of forms of Tat protein that can/cannot be modified by acetylation. Thus, the acetyl-acceptors K28 and K50, defined *in vitro* and *in vivo*, were mutated to alanine(s) either individually or in combination. As controls, full-length Tat proteins with changes in non-acceptor lysines, K29 to A29 and K51 to A51, were also created. Each of these cDNAs was positioned downstream of the RSV promoter for expression in eukaryotic cells. Immunofluoresence analysis showed that none of the mutations affected cellular localization and/or level of expression of Tat protein (data not shown). Hence, we assessed the effects of the lysine substitutions on Tat-mediated *trans*-activation. Co-transfections of SupT1 cells were performed using pLTRluc-wt reporter and increasing amounts of expression vector encoding either the wild-type or a mutant Tat protein and luciferase activity was measured in cell lysates. As shown in Figure 3A, depending on the amount of transfected plasmid, activation by wild-type Tat ranged from 2.4- to 35.2-fold. In comparison, activation by the K28A and K50A mutants ranged between 1.1- and 4.6-fold, and 1.2- and 7.1-fold, respectively. Thus, mutations at either K28A or K50A reduced Tat mediated *trans*-activation by ~2.2- to 7.7-fold and 2- to 5-fold, respectively. In contrast, mutation of K29 had no significant effect on Tat function (pTat-K29A; Figure 3A), while mutation of K51 affected by 2-fold Tat *trans*-activation (pTat-K51A; Figure 3A). Thus, it is unlikely that the effect of mutation from lysine to alanine is simply due to neutralization of charge, given that the same mutations at lysine 29 and 51 had no significant effect on Tat *trans*-activation. A profound decrease in intracellular Tat *trans*-activation was observed (27-fold) when K28 and K50 were simultaneously changed to alanines (pTat-K28A,K50A; Figure 3A). Overall, these findings correlate the integrity of Tat residues, which are acetylation acceptors *in vivo* (K28, K50) with intracellular *trans*-activation function.

Synergistic activation of HIV-1 promoter by Tat and deacetylase inhibitor, TSA

To assess whether Tat acetylation plays an important role in HIV-1 transcription, we used the chemical deacetylase inhibitor, TSA (Yoshida *et al*., 1990). We first determined that TSA inhibited Tat deacetylation *in vitro*. As shown in Figure 3B, incubation of *in vitro* acetylated Tat protein with HeLa extract leads to deacetylation of Tat protein (compare lane 3 with lane 1). In contrast, when acetylated Tat was incubated with HeLa extract in the presence of TSA, no deacetylation of Tat was observed. This result suggests that, as for histones (Yoshida *et al*., 1990; Van Lint *et al*., 1996a), TSA can inhibit the deacetylase activity present in HeLa extract that is responsible for Tat deacetylation.

If acetylation of Tat is relevant for transcriptional activity *in vivo*, then one predicts an enhancing effect from the deacetylase inhibitor, TSA. To examine this hypothesis, SupT1 cells were transiently co-transfected with pLTRluc-wt and increasing amounts (3–1300 ng) of wild-type Tat expression vector (pTat-wt; Table I). Cells were treated with TSA or mock-treated and assayed for luciferase activity. Results in Table I are presented as synergistic activation by Tat and TSA. Thus, in the absence of TSA, Tat *trans*-activation of the HIV-1 promoter occurred in a dose-dependent fashion ranging from 2.9- to 120-fold. Treatment of cells with TSA alone resulted in a 140-fold activation of transcription. Induction by TSA in the absence of Tat is likely to be explained by histone hyperacetylation (Van Lint *et al*., 1996a,b; Sheridan *et al*., 1997; Steger *et al*., 1998) and/or acetylation of non-histone proteins that bind to the HIV-1 promoter (Sheridan *et al*., 1997). Remarkably, when cells were exposed to increasing amounts of Tat and treated with TSA, a strong synergy was observed between the two activators, ranging from 14- to 126-fold (Table I). This synergism persisted even at saturating amounts of Tat protein (see 700, 1000 and 1300 ng of transfected Tat plasmid DNA), indicating that the observed effect was not the consequence of increased Tat expression due to activation of the RSV promoter by TSA.

Synergistic activation by Tat and TSA required an intact TAR element, since deletion of the 3-nt bulge of TAR abrogated this effect (Table I; pLTRluc-ΔTAR). This finding indicates that synergism was mediated by interactions at TAR RNA and not at the otherwise intact U3-promoter DNA sequences. We confirmed by RNase protection analysis, using an HIV-1 promoter-specific probe and a luciferase gene-specific probe, that the synergism between Tat and TSA occurred at the level of transcription (data not shown). Therefore, according to the definition of synergism described previously (Herschlag and Johnson, 1993; see Table I legend) the data presented in Table I shows that Tat and TSA act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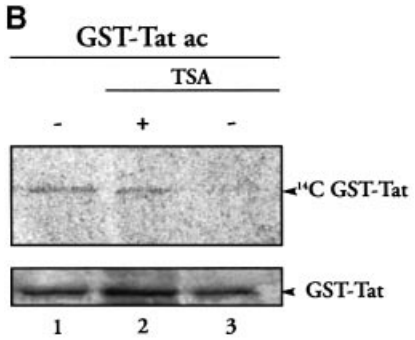
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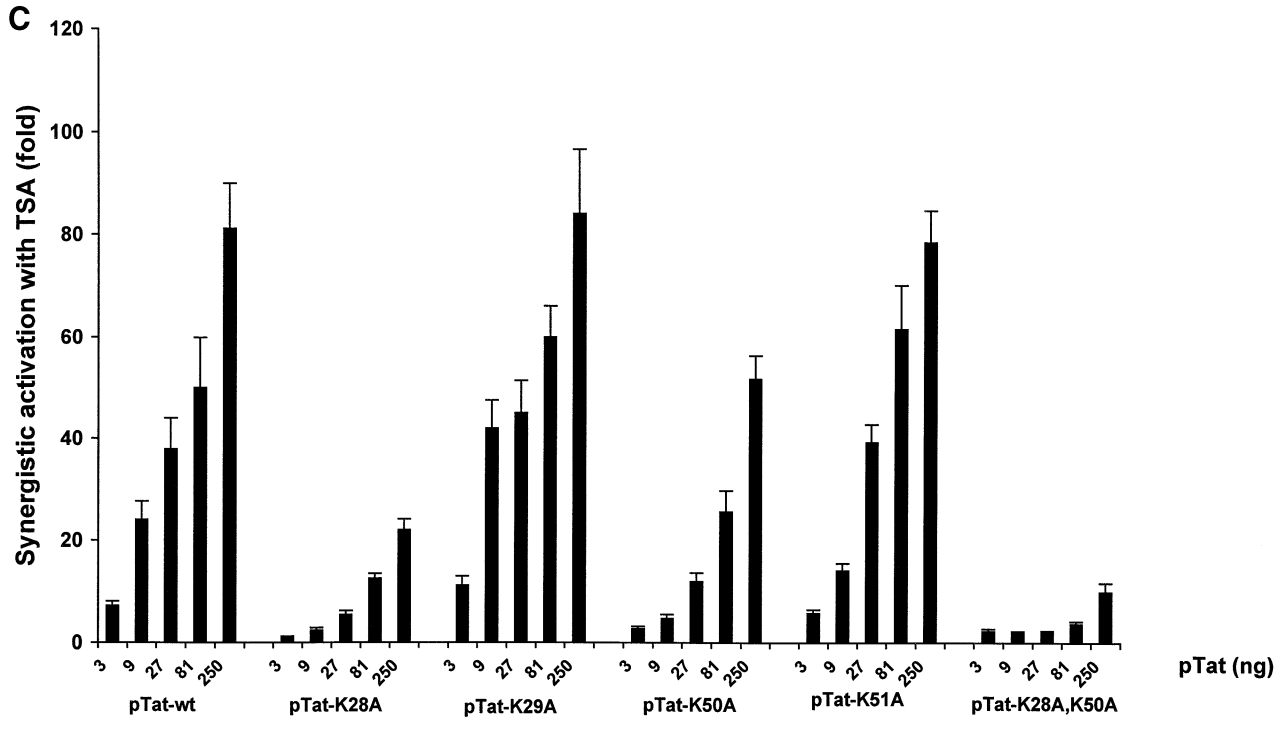
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pTat-K50A
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pTat-K28A,K50A

through the same pathway and are not functionally independent.

Tat/TSA synergism requires intact acetyl-acceptor residues

One explanation for the transcriptional synergism between Tat and TSA (Table I) is that the latter inhibits the deacetylation of the former, thereby influencing Tat function. If so, TSA should differentially affect the transcriptional activity of acetylation-competent versus acetylation-incompetent forms of Tat. To understand better the mechanism of transcriptional synergism between Tat and TSA, we repeated transfections in SupT1 cells using pLTRluc-wt with either the wild-type or mutant Tat expression vectors. Cells were treated with TSA or mock-treated, and assayed for luciferase activity. TSA strongly synergized with wild-type Tat (pTat-wt; Figure 3C) and with acetylation-competent Tat mutants (pTat-K29A and pTat-K51; Figure 3C) to levels of 7.2- to 81.1-fold, 11.2- to 84-fold and 5.8 to 78.4-fold, respectively. We note that, while K51 mutation resulted in a 2-fold reduction of Tat *trans*-activation (Figure 3A), no effect on synergism was observed. This example illustrates that a defect in Tat *trans*-activation is not correlated to a defect in Tat/TSA synergism. In contrast, TSA showed reduced activity with acetylation-incompetent forms of Tat that contained either individual or combined changes at lysines (Figure 3C). Indeed, we observed a 1- to 22-fold effect for pTat-K28A, which represents a ~8-fold decrease in synergy (Figure 3C); and 2.7- to 51.7-fold effect for pTat-K50A, which represents an ~4-fold decrease in synergy when compared with pTat-wt (Figure 3C). For the double-mutant (pTat-K28A, K50A; Figure 3C) we observed severely decreased effects from TSA (Figure 3C). Taken together, these results establish a correlation between loss of acetyl-acceptor lysines in Tat and reduced synergy with TSA. Therefore, this correlation is compatible with the functional synergy between Tat and TSA occurring as a consequence of acetylation at lysine residues in Tat.

Table I. Transcriptional synergism of Tat and histone deacetylase inhibitor, TSA, at the HIV-1 promoter

pTat-wt (ng)	R.L.U (–TSA)	R.L.U (+TSA)	Tat fold activation	TSA fold activation	Tat-TSA fold synergism
pLTRluc-wt					
–	0.128	17.87	1	140	
3	0.369	255.18	2.88		14
9	0.512	389.42	4		21
27	0.88	1101.28	6.8		59
90	1.55	1063.04	12.1		55
200	4.88	2096.55	38.1		92
400	5.14	2905.89	40.1		126
700	11.95	2847.16	93.3		96
1000	15.27	2877.05	119		87
1300	13.24	2379.71	103		77
pLTRluc-ΔTAR					
–	0.93	51.1	1	55	
3	0.75	25	0.8		0.5
9	0.52	36.7	0.55		0.7
27	0.65	54.1	0.7		1
90	0.57	194.1	0.6		3.8
200	1.67	280.2	1.8		5.3
400	1.45	246.4	1.55		4.7
700	2.02	319.7	2.17		6
1000	2.5	277.7	2.68		5.2
1300	1.95	291	2.09		5.5

SupT1 cells (6×10^6) were transiently co-transfected with 500 ng of either pLTRluc-wt or pLTRluc-ΔTAR and increasing amounts of an expression vector for Tat (0, 3, 9, 27, 90, 200, 400, 700, 1000 and 1300 ng of plasmid DNA). Cells were treated with TSA (450 nM) or mock treated. Transfections were normalized to 1800 ng of total vector DNA with pRep9 plasmid. Cells were harvested and luciferase activities were measured in cellular lysates and normalized with respect to protein concentration of the lysates (10 times more extract was used for the luciferase assays from samples transfected with pLTRluc-ΔTAR). Results are presented as relative light units (R.L.U) in absence or presence of TSA, Tat activation in absence of TSA (Tat fold activation), TSA activation in absence of Tat (TSA fold activation), and synergistic activation of Tat and TSA (Tat-TSA fold synergism). Synergistic activation of Tat and TSA was determined as previously described (Herschlag and Johnson, 1993) using the following formula: induction by (TSA+Tat)/induction by TSA-alone + induction by Tat-alone. A representative experiment of 10 repeated transfections is shown.

Acetylation of the RNA binding domain by p300 releases Tat from TAR RNA

p300 acetylates the K50 residue (Figure 2C) that lies in a portion of Tat involved in nuclear localization and RNA-binding. By immunofluorescence, we observed that acetylation of Tat on K50 does not affect its nuclear localization (data not shown). Thus, we analyzed the effect of Tat K50 acetylation on its interaction with TAR RNA. Tat proteins corresponding to residues 10–60 were chemically synthesized to contain either a non-acetylated lysine (Tat) or an acetylated lysine at residue 50 (Tat-K50ac). The affinities of the Tat and TatK50ac for ^{32}P-labeled TAR RNA in electrophoretic mobility shift assays were individually assessed. Thus, increasing concentrations of Tat were incubated with 10^4 c.p.m. of TAR RNA for 15 min at room temperature, and the complexes were analyzed by native polyacrylamide gel electrophoresis. Figure 4A shows that acetylated Tat bound TAR RNA with lower affinity than the non-acetylated form. Recently, it has been shown that cyclin T1 enhances the affinity and the specificity of Tat to TAR RNA (Wei *et al*., 1998). Thus, we analyzed the effect of Tat acetylation on Tat/cycT binding to TAR RNA (Figure 4B). TAR RNA was incubated either with various amounts of cyclin T in the

Fig. 3. Tat transcriptional activity is regulated by acetylation. (**A**) Mutation of acetyl-acceptor residues affects Tat-mediated transcription. SupT1 cells were transiently co-transfected with 500 ng of pLTRluc-wt and increasing amounts of an expression vector for either wt or mutated Tat (3, 9, 27, 81 or 250 ng of plasmid DNA). The results are presented as histograms indicating the induction by Tat (in fold) with respect to the activity of pLTRluc-wt in the absence of Tat, which was assigned a value of 1. A representative experiment of six repeated transfections is shown. (**B**) TSA inhibits Tat deacetylation *in vitro*. Acetylated GST–Tat (GST–Tat ac) was incubated overnight at 4°C with (lanes 2–3) or without (lane 1) HeLa extract in the presence (lane 2) or absence (lane 3) of TSA (500 nM). The reaction products were run on 10% SDS–PAGE and the gel was Coomassie Blue stained (bottom panel), dried and analyzed by autoradiography (top panel). (**C**) Mutation of Tat-lysine residues affects synergistic activation of the HIV-1 promoter by Tat and TSA. SupT1 cells were transiently co-transfected with 500 ng of pLTRluc-wt and increasing amounts of a vector expressing either wt or mutated Tat (3, 9, 27, 81 or 250 ng of plasmid DNA). Cells were treated with TSA (500 nM) or mock treated and luciferase activities were measured in cellular extracts. The results are presented as histograms indicating the synergistic activation of Tat with TSA (in fold). A representative experiment of six repeated transfections is shown.

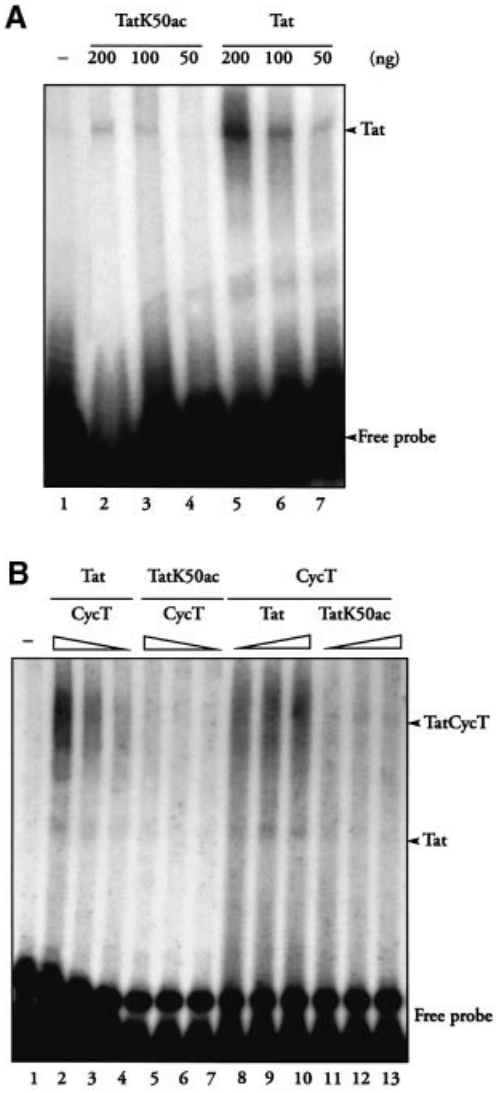

Fig. 4. Acetylation of Tat RNA binding domain decreases the affinity of the Tat/cyclin T complex for TAR RNA. (**A**) Analysis of the binding of acetylated Tat to TAR RNA. Various amounts of chemically synthesized Tat protein corresponding to amino acids 10–60 in non-acetylated form (Tat), or acetylated at lysine 50 (TatK50ac) were incubated with 10^4 c.p.m. of ^{32}P-labeled TAR RNA for 15 min at room temperature. Lane 1 corresponds to the probe alone. The reactions were resolved by 8% acrylamide gel electrophoresis and analyzed by autoradiography. (**B**) Analysis of the binding of acetylated Tat to TAR RNA in the presence of cyclin T. Various amounts of purified cyclin T (200 ng, lanes 2 and 5; 100 ng, lanes 3, 6 and 10–13; 50 ng, lanes 4 and 7) were incubated with either Tat (200 ng lane 10; 100 ng, lanes 2–4 and 9; 50 ng, lane 8) or TatK50ac (200 ng lane 13; 100 ng, lanes 5–7 and 12; 50 ng, lane 11) in the presence of ^{32}P-labeled TAR RNA for 15 min at room temperature. Reactions were resolved by 4% Tris–glycine acrylamide gel electrophoresis and the resulting complexes were analyzed by autoradiography. Lane 1 corresponds to cyclin T (200 ng) incubated with TAR RNA in the absence of Tat.

presence of Tat (lanes 2–4) or TatK50ac (lanes 5–7), or various amounts of Tat (lanes 8–10) or TatK50ac (lanes 11–13) in the presence of cyclin T. The resulting complexes were analyzed by gel mobility shift assay. As shown in Figure 4B, cyclin T bound to TAR RNA in a cooperative and dose dependent manner with Tat (lanes 2–4 and lanes 8–10) and did not bind TAR RNA in the absence of Tat (lane 1). Acetylated Tat (TatK50ac) bound with lower affinity to TAR, regardless of the concentration of either cyclin T (compare lanes 2–4 with lanes 5–7) or Tat (compare lanes 8–10 with lanes 11–13). Taken together, these results suggest that acetylation by p300 modulates the affinity of Tat/cyclin T for TAR RNA.

In vivo and *ex vivo* studies have demonstrated low to limiting levels of Tat expression in HIV-1 infected cells (Adams *et al*., 1994; Cannon *et al*., 1994; Peng *et al*., 1995). Based on the observation that levels of TAR RNAs greatly exceed Tat protein in infected cells (K.-T.Jeang, unpublished), the ability of the latter to dissociate from the former would 'recycle' functional protein and enhance the apparent concentration of this limiting activator in a manner advantageous for function. Indeed, consistent with this idea, Karn and colleagues have recently shown that Tat is released from TAR RNA during HIV-1 LTR-directed transcription (Keen *et al*., 1997). While the same authors have proposed an as yet uncharacterized co-factor function in the release of Tat from TAR, based on above findings, we wondered whether acetylation of Tat at lysine 50 by p300 might serve this mechanism. To test directly whether Tat pre-bound to TAR RNA could indeed be released by acetylation, a synthetic 5′-biotinylated TAR RNA was saturated first with maltose-binding protein (MBP)–Tat fusion protein. The RNA–protein complex was then immobilized onto streptavidin–agarose beads, washed extensively with buffer containing 100 mM KCl followed by three washes in acetylation buffer, then subjected to independent acetylations with either p300 or PCAF. The release of MBP–Tat bound to TAR RNA-biotin-strepavidin–agarose into the supernatant was then assessed by Western blotting using anti-Tat serum (Figure 5A). In parallel assays, we added [^{14}C]acetyl-CoA to the reactions and confirmed that Tat was comparably acetylated by either p300 or PCAF (Figure 5B). In addition, Coomassie Blue staining confirmed that equivalent amounts of GST–Tat were used in the acetylation reactions (data not shown). The effect of Tat acetylation on the release of MBP–Tat from TAR RNA into the reaction supernatant was assessed. Acetylation, *per se*, has very little effect on the release of Tat bound to TAR RNA, since very little Tat was found in the supernatant after acetylation by PCAF (Figure 5A, lanes 4 and 5). We reproducibly observed some release of Tat from TAR RNA in the presence of p300 but not PCAF (Figure 5A, compare lanes 3 and 5) suggesting that binding of p300 to Tat can alter the Tat–TAR interaction. In any case, specific acetylation of Tat by p300 accounted for a 2-fold increase of released Tat (Figure 5A, compare lanes 2 and 3).

Acetylation of the activation domain enhances Tat binding to Tat-associated kinase (TAK)

PCAF was found to acetylate K28, which lies within the cysteine-rich domain of Tat (Figure 2B). This domain has been shown to be involved in the interaction with cellular co-factor(s) that play a critical role in Tat-mediated *trans*-activation (reviewed in Jones and Peterlin, 1994). Therefore, we sought to determine how acetylation of K28 might affect the interaction of Tat with the Tat-associated RNAP II CTD-kinase (TAK; reviewed in Jones, 1997). To investigate this, wild-type GST–Tat fusion protein was acetylated or mock-acetylated *in vitro* with PCAF

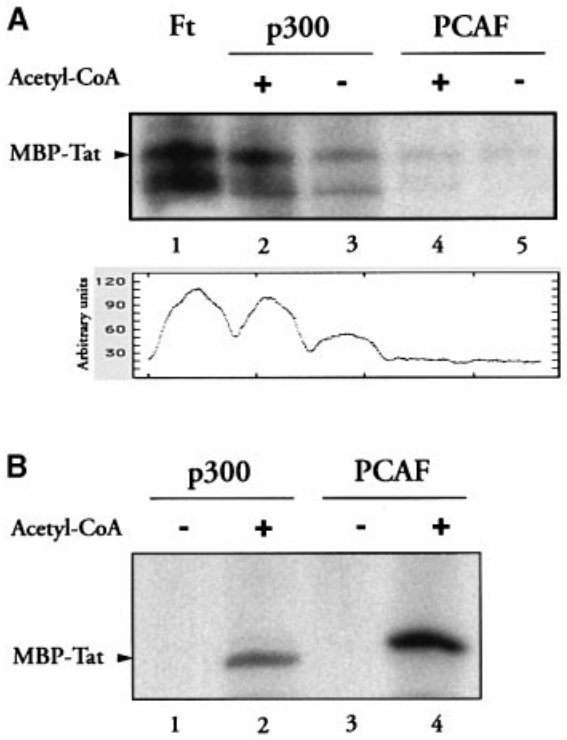

Fig. 5. Acetylation of Tat by p300 but not PCAF releases Tat from TAR RNA. (**A**) Top panel: synthetic biotinylated TAR RNA (2 μg) was incubated with MBP–Tat (5 μg) for 2 h at 4°C and the flow through (Ft) was collected. The complex was immobilized on protein A–streptavidin beads, washed 10 times in buffer containing 100 mM KCl and three times in acetylation buffer. Acetylation reactions were performed on the immobilized complex with 250 ng of either p300 or PCAF in the presence or absence of [^{14}C]acetyl-CoA as indicated. The reaction products were centrifuged and the supernatants were collected and analyzed by 10% SDS–PAGE. The presence of MBP–Tat protein in flow through (Ft) and supernatants was analyzed by Western blotting using Tat antiserum. Proteins were visualized by chemiluminescence. Bottom panel: quantification profile of MBP–Tat using NIH SXM image quantification program. *y*-axis is given as arbitrary units. (**B**) The amounts of ^{14}C-labeled Tat protein as a measure of relative acetylation efficiencies were analyzed by autoradiography.

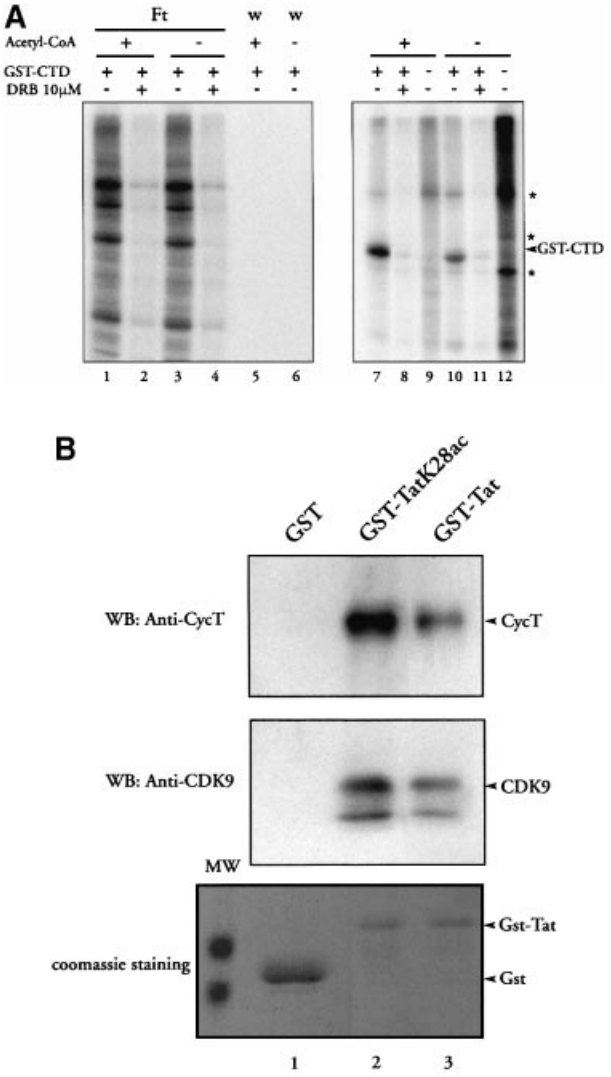

Fig. 6. Acetylation of Tat by PCAF regulates its binding to Tat-associated kinase. *In vitro* kinase assays for the phosphorylation of GST-CTD substrate using proteins pulled down from HeLa nuclear extract by either GST–Tat or PCAF acetylated GST–Tat as indicated. Flow-through (Ft), final wash (W) and elutions are shown. The kinase reactions were performed in the presence or absence of GST–CTD supplied as substrate and with or without an inhibitor, DRB, as indicated. GST–CTD is indicated by arrow; DRB-insensitive kinases are indicated by asterisks. (**B**) Acetylation of Tat by PCAF enhances its binding to CDK9/cyclinT. The experiment was performed as described for (A) except that after washes, the beads were resuspended in SDS–PAGE loading buffer and total amounts of CDK9 and cyclinT bound to the column were analyzed by Western blotting using either anti-cyclinT (top panel) or anti-CDK9 (middle panel). Bottom panel shows Coomassie Blue staining of samples run in parallel.

(Figure 6A). The acetylated and mock-acetylated products were then bound to gluthathione–Sepharose beads for 1 h at 4°C, followed by five washes with binding buffer. Bead-bound acetylated Tat and its non-acetylated counterpart were then separately equilibrated with HeLa nuclear extract for 2 h at 4°C. After equilibration, the two batches of beads were washed five times with binding-buffer (10 column volumes each time) and then eluted with KCl-salt buffer. Aliquots of bead flow-through, final wash fraction, and elution were assessed *in vitro* for CTD kinase activities (Figure 6A). Comparing the GST–CTD kinase activity eluted from acetylated versus non-acetylated GST–Tat, we show that acetylation by PCAF enhances the CTD-kinase activity associated with Tat (Figure 6A, compare lanes 7 and 10). Furthermore, the presence of a general kinase activity in eluates from non-acetylated Tat (Figure 6A, asterisks, compare lanes 9 and 12) suggests that acetylation of Tat by PCAF enhances the specificity for a CTD kinase. These results suggest that acetylation at K28 enhances Tat's binding and specificity for a CTD kinase.

CDK9/P-TEFb was recently identified as a CTD-kinase recruited by Tat to the HIV promoter (Mancebo *et al*., 1997; Yang *et al*., 1997; Zhu *et al*., 1997). We analyzed the effect of Tat acetylation at K28 on its binding to CDK9/cyclin T by Western blotting. As shown in Figure 6B, acetylation by PCAF enhanced the binding of Tat to CDK9/cycT1 by 3-fold (compare lane 2 with lane 3). Coomassie Blue staining of samples run in parallel showed that equal amounts of GST–Tat were used in the assay (Figure 6B, bottom panel). Thus, these results show that acetylation of Tat protein on K28 enhances the binding and the specificity of Tat to TAK.

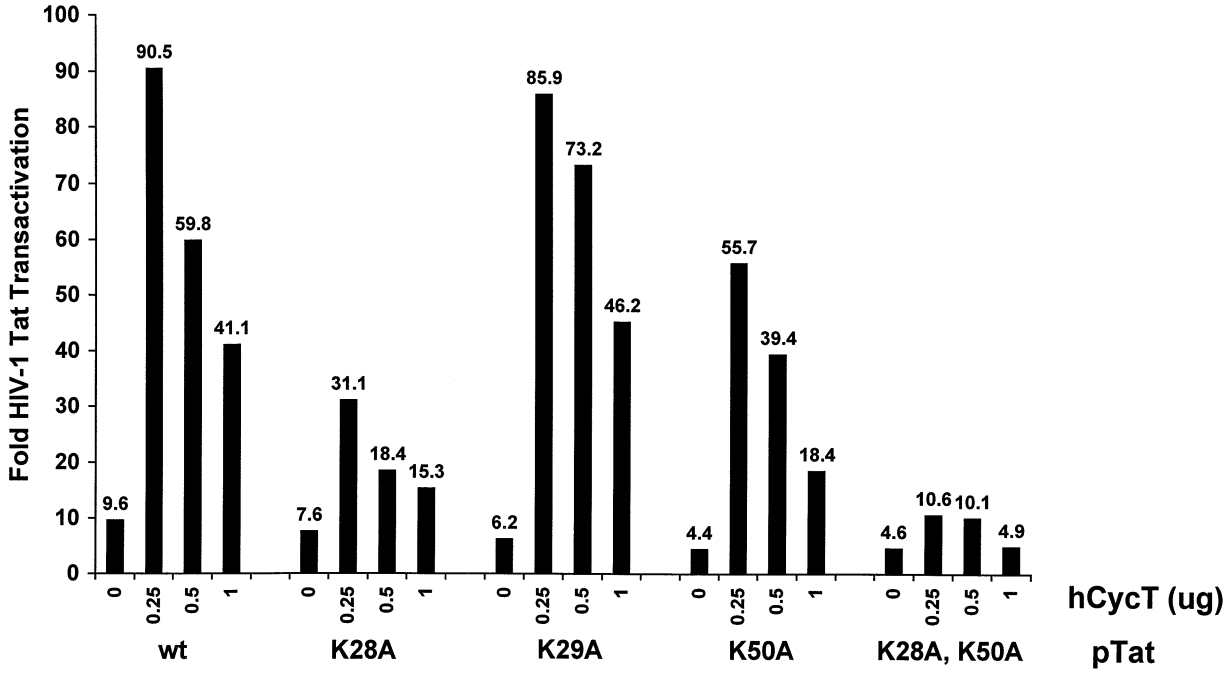

Fig. 7. Mutation of lysine 28 affects the ability of human cyclin T protein to support HIV-1 Tat trans-activation *in vivo*. NIH 3T3 cells were co-transfected with 1 μg of pLTRluc-wt, 200 ng of expression vector for either wt or mutated Tat, 100 ng of pRL-TK, and increasing concentrations of pCMVcyclT as indicated. The relative luciferase activity was calculated following normalization for Renilla luciferase activity expressed from the TK promoter present in the pRL-TK internal control plasmid. The fold HIV-1 Tat *trans*-activation was calculated relative to transfections in absence of Tat expression plasmids. A representative experiment of three repeated transfections is shown.

Enhancement of Tat trans-activation in NIH 3T3 cells by human cyclin T1 is affected by mutation of K28

Cyclin T1 is the predominant CDK9/P-TEFb-associated cyclin (Peng *et al*., 1998). Recently, it has been suggested that Tat recruits the CDK9/P-TEFb complex to the HIV-1 promoter through its interaction with cyclin T1 (Garber *et al*., 1998; Wei *et al*., 1998). Interestingly, human cyclin T1 (hCycT), but not murine cyclin T (mCycT), enhances Tat-mediated *trans*-activation of the HIV-1 LTR (Bieniasz *et al*., 1998; Garber *et al*., 1998). Thus, murine cells represent an interesting setting in which to confirm the role of K28 in the regulation of the Tat–TAK interaction. We surmised that enhancement of Tat *trans*-activation by hCycT would be impaired in the context of the HIV-1 Tat K28A mutation. To test this directly, NIH 3T3 cells were co-transfected with pLTRluc-wt reporter construct, and expression vectors encoding either wild-type or mutant Tat proteins, either in the absence or presence of increasing concentrations of a plasmid expressing full-length hCycT (Wei *et al*., 1998). Consistent with previous reports, *trans*-activation by wild-type Tat was enhanced ~10-fold by hCycT (Figure 7). This enhancement was unaffected by the K29A mutation. As expected, the K28A and K50A mutations did reduce overall *trans*-activation potential (~3-fold and ~2-fold) to within the range observed following transfection of SupT1 cells. However, the K50A mutation did not affect the relative ability of hCycT to enhance its *trans*-activation (up to 14-fold). In contrast, the K28A and the K28A,K50A mutants were both impaired for enhancement of Tat *trans*-activation by hCycT (4-fold and 2-fold enhancement, respectively). Since the interaction of Tat with hCycT/CDK-9/P-TEFb is crucial for activity in murine cells, these results support the biochemical evidence (Figure 6B) for a critical role of K28 in association to TAK.

Discussion

The coactivators p300/CBP and p300/CBP-associated cofactors (e.g. P/CAF, P/CIP-ACTR and SRC-1) contain an intrinsic histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogrysko *et al*., 1996; Yang *et al*., 1996; Chen *et al*., 1997; Spencer *et al*., 1997; Torchia *et al*., 1997). One current view postulates that recruitment of coactivators bearing HAT activity by promoter-bound transcription factors results in histone acetylation of nearby nucleosomes, thus enhancing access of the transcriptional machinery to DNA (Grunstein, 1997; Mizzen and Allis, 1998; Struhl, 1998). Conversely, some transcriptional repressors recruit histone deacetylases that inhibit transcription by deacetylating chromatin (Pazin and Kadonaga, 1997; Struhl, 1998). However, it is important to note that generalized increases in core histone acetylation do not necessarily induce widespread transcription. In some contexts, increased histone acetylation actually correlates with decreased transcription (reviewed in Pazin and Kadonaga, 1997), suggesting a complex relationship between acetyltransferase function and transcriptional activity.

The complexity of acetyltransferase function is further revealed in several recent studies that have shown that histones are not the sole functional substrates for HATs. Thus, CBP/p300 and PCAF can acetylate p53 (Gu and Roeder, 1997; Sakaguchi *et al*., 1998); PCAF, p300 and TAF250 can acetylate general transcription factors TFIIEβ and TFIIF (Imhof *et al*., 1997). GATA-1 (Boyes *et al*., 1998) and erythroid Krüppel-like factor (EKLF; Zhang and

Bieker, 1998) can both be acetylated by p300. Additionally, p300 can also activate *Xenopus* NF-Y through acetylation (Li *et al*., 1998), while acetylation by CBP represses the activity of T cell factor (TCF) (Waltzer and Bienz, 1998). Acetylation of HMGI(Y) K65 by CBP, but not PCAF, decreases its sequence-specific DNA-binding and results in destabilization and disassembly of the IFNβ gene enhanceosome. In contrast, HMGI(Y) K71 acetylation by PCAF seemingly does not influence high-affinity binding to DNA (Munshi *et al*., 1998). Considered together, these findings indicate that factor-acetylation (FAT; Gu and Roeder, 1997) can have divergent and context-dependent effects on DNA binding and function.

As yet, acetylation as a means for regulating RNA-binding factors has not been shown. Here, we report for the first time the functional acetylation of an RNA-binding transcription factor, HIV-1 Tat, by p300 and PCAF. p300 and PCAF have previously been shown to bind Tat and to potentiate transcriptional activation of the HIV-1 LTR (Benkirane *et al*., 1998; Hottiger and Nabel, 1998; Marzio *et al*., 1998), presumably through intracellular acetylation of histones. These intracellular findings of protein acetylation for HIV-1 transcription are consistent with two related observations. First, results shown here and elsewhere (Van Lint *et al*., 1996a,b) indicate a functional interaction of Tat with inhibitors of histone deacetylase, such as TSA and TPX. TSA and TPX are potent inducers of HIV-1 transcription in latently infected T-cell lines, and activation of the integrated HIV-1 promoter by these agents is accompanied by the loss or rearrangement of a positioned nucleosome (nuc-1) near the viral RNA start site. Secondly, *in vitro* studies examining cell-free transcription of the HIV promoter reconstituted into chromatin (Sheridan *et al*., 1997; Steger *et al*., 1998) also support a role for acetylation in LTR-directed transcription. Thus, TSA strongly induced HIV-1 transcription *in vitro* on nucleosomal DNA. This induction is correlated with an enhancer-dependent increase in acetylated histones (Sheridan *et al*., 1997) which resulted in greatly facilitated transcription reinitiation on chromatin *in vitro*. Furthermore, Steger *et al*. (1998) have demonstrated that *in vitro* HAT acetylation of either histone H3 or H4 stimulated HIV-1 transcription in a chromatin-specific fashion. These results agree fully with the recruitment of HAT co-activators by Tat as a critical step in HIV-1 proviral transcription.

In addition to the role of HAT activities on HIV-1 transcription, the current work demonstrates that FAT of Tat might be equally important. Indeed, the experimental findings suggest that synergism of TSA with Tat might emanate as much from an effect on FAT as from effects on HAT activities of p300 and PCAF. How might one then interpret the relevance of FAT for Tat activity? Clearly, a critical role for the Tat protein is to increase the abundance of processive transcripts that initiate from the HIV-1 LTR. Within this context, there are two critical essential interactions: (i) Tat must bind a nascent TAR RNA (Berkhout *et al*., 1989); and (ii) Tat must interact with cyclinT/CDK9/P-TEFb complex (reviewed in Jones, 1997). Our current findings indicate that FAT of Tat impacts both processes. Thus, PCAF acetylation at K28 regulates the binding and the specificity for the cyclinT/CDK9-kinase complex, and p300 acetylation at K50 within the RNA-binding domain of Tat influences its interaction with TAR RNA. Acetylation of K50 has been observed also by E.Verdin and colleagues (personal communication). Moreover, to our knowledge, Tat is the only known substrate for which there is a distinction between the acetylation activities of p300 and PCAF.

FAT activity of p300 and PCAF on Tat explains several biologically perplexing observations. A practical question exists as to why Tat is not rapidly squelched for activity in HIV-1 infected cells. The organization of the HIV-1 LTRs is such that every Tat-induced viral transcript contains two copies of TAR RNA, each fully competent to decoy Tat away from the promoter (Lisziewicz *et al*., 1993; Bohjanen *et al*., 1997; Corbeau and Wong-Staal, 1998). Rapid decoying/squelching of function does not occur, suggesting that the apparent concentration of Tat might be considerably higher than the very low amounts measured experimentally (Adams *et al*., 1994; Cannon *et al*., 1994; Peng *et al*., 1995). While it is possible that experimental determinations have underestimated hidden pools of Tat within cells, this would seem unlikely since both native (i.e. immunoprecipitation) and denaturing (i.e. Western blotting) techniques have consistently quantitated exceedingly low levels of Tat within cells. An attractive explanation that invokes FAT would be that the apparent concentration of Tat is increased if each molecule is (re)cycled between several TARs. This idea of facilitated release of Tat from TAR is, in fact, fully consistent with the observation from others (Keen *et al*., 1997) that although TAR is used to recruit Tat, this reaction is transient and continued Tat association to TAR is not required for the activated transcription complex. Thus, our demonstration of Tat acetylation by p300 at K50 provides a compatible mechanism for post-translational regulation of Tat-binding to TAR RNA.

Another unexpected finding from our study is that binding of Tat to TAK is regulated by TAHs. Tat has been shown to bind several different kinases including PKR, DNA-PK, TFIIH and CDK9/P-TEFb (McMillan *et al*., 1995; Chun *et al*., 1998; reviewed in Jones, 1997) and is a substrate for phosphorylation by PKR (McMillan *et al*., 1995; Brand *et al*., 1997). Reasonably, a means for dictating specificity of Tat association for kinases should exist. While additional mechanisms remain to be uncovered, a role for PCAF acetylation at K28 of Tat in regulating association with a 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)-sensitive CTD-kinase (CDK9/P-TEFb) is shown here. Probably, acetylation at K28 alters the protein conformation of Tat thereby enhancing its interaction with CDK9/P-TEFb. This is consistent with suggested conformational changes in p53 (Gu and Roeder, 1997) and GATA-1 (Boyes *et al*., 1998) occurring as a consequence of acetylation.

The independent acetylations of Tat by p300 and PCAF described in this report would need to be regulated in both a temporal and TAR-dependent manner. Because of the increasing number of known substrates for acetylation, it seems likely that acetyltransferase activities should be regulated. This regulation will act at two levels: induction of acetyltransferase activity and substrate specificity. To date, very little is known about the regulation of histone acetyltransferase activity. A clue to a possible mechanism of HAT regulation was provided by a recent report showing

that p300 HAT activity is regulated by phosphorylation (Ait-Si-Ali *et al*., 1998). It is possible, therefore, that the specific Tat acetylations by p300 and PCAF could be regulated by one or more of the kinase activities known to associate with Tat. Further work will be needed to understand the regulation of acetylation of Tat and, indeed, other substrates.

Our present study adds a new perspective to the current views on the molecular mechanisms governing Tat-mediated transcription. We implicate a FAT activity as being critically important for post-translational modifications that regulate the function of this RNA binding protein. While acetylation of DNA-binding transcription factors has accrued several important examples, the present demonstration for Tat represents the first invocation of this type of post-translational change for RNA-binding transcription factors. In addition, Tat is the first viral protein reported whose function is regulated by acetylation. Thus, in general, this suggests that acetylation of proteins is a larger common regulatory mechanism. More specifically, acetylation of Tat suggests a new route to block the activity of this viral factor selectively in HIV-infected cells.

Materials and methods

Plasmid constructs

To construct pLTRluc-wt, a 186 bp fragment containing the HIV-1 LAI 5′LTR (nt 345–531, numbering is according to the BRU provirus where nt +1 is the start of U3 in 5′LTR) was generated by PCR amplification of pLTR-CAT (Van Lint *et al*., 1994), digested with *Pst*I (site added in the 5′ primer) and *Hin*dIII (site added in the 3′ primer), and cloned into the *Pst*I–*Hin*dIII-restricted vector pGL2Basic (Promega). To construct pLTRluc-ΔTAR, pLTRluc-wt was used as a substrate for mutagenesis of the TAR region by the Transformer site-directed mutagenesis method (Clontech, Palo Alto, CA). The 3 nt bulge of TAR (nt 476–478) was deleted with the following mutagenic oligonucleotides: 5′-g^{nt494}agagctcccaggctctctggtctaacc-3′, 5′-cggtcgac**ggtacc**agacatgataag-3′. Mutated clones were fully resequenced after identification.

The plasmid pTat-wt (Ott *et al*., 1997) was used as a substrate for mutagenesis of the Tat open reading frame by the QuickChange Site-Directed Mutagenesis method (Stratagene). Four different mutations were generated with the following pairs of mutagenic oligonucleotide primers (mutations are highlighted in bold and underlined on the coding strand primer):

K28A: 5′- ccaattgctattgt**gc**aaagtgttgctttcattgcc-3′
K29A: 5′- ccaattgctattgtaaa**gc**gtgttgctttcattgcc-3′
K50A: 5′- ccttaggcatctcctatggcagg**gca**aagcggagacagcgacgaagacc-3′
K51A: 5′- ccttaggcatctcctatggcaggaag**gcg**cggagacagcgacgaagacc-3′

Mutated clones were fully resequenced after identification. The four mutated plasmids were designated as pTat-K28A, pTat-K29A, pTat-K50A and pTat-K51A, respectively. In addition, a construct containing combinations of the mutations described above were generated by site-directed mutagenesis (Stratagene). The resulting plasmid was designated pTat-K28A,K50A. To construct GST–Tat fusion proteins with an HA epitope in Tat C-terminus (added and underlined in 3′ primer), the Tat open reading frame from the wild-type (wt) and mutant Tat expression vectors described above was PCR-amplified using the following primers. *Bam*HI sites (highlighted in bold) were introduced into the PCR primers. Primer 5′:5′-cgc**ggatcc**ggagagccagtagatcctagactagagccctgg-3′; primer 3′:5′-gcg**ggatcc**taagcgtagtctgggacgtcgtatgggtagcctccttgctttgatagagaaacttgatgagtc-3′. *Bam*HI-restricted PCR fragments were cloned into the unique *Bam*HI site of pGEX-2T, placing the Tat protein in-frame with GST.

Cell culture and transfection

HeLa and NIH 3T3 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and transfected using calcium phosphate. Transfected NIH 3T3 cells were lysed and assayed for luciferase activity 48 h post-transfection. HIV-1 luciferase activity was normalized to pRL-TK (Promega), which encodes the Renilla luciferase from the TK promoter, as an internal control. SupT1 cells were cultured in RPMI 1640 GlutamaxI medium supplemented with 10% FBS (Gibco-BRL), and antibiotics and transfected using DEAE–dextran as previously described (Van Lint *et al*., 1994). At 20 h posttransfection, cells were treated or mock-treated with TSA (Sigma). At 42 h post-transfection, cells were lysed and assayed for luciferase activity (Promega). Luciferase activities derived from HIV-1LTR were normalized with respect to protein concentration using the Detergent-Compatible Protein Assay (Bio-Rad).

Fusion protein affinity chromatography

HIV-1 Tat wild-type and mutants (SF2 strain) were expressed as GST fusion proteins in BL21 (Pharmacia) as described (Benkirane *et al*., 1998). HeLa cell extracts (Cell Trends, Middletown) were prepared as described (Benkirane *et al*., 1998). Cellular extracts (100 μg) were incubated with 20 μl of the various protein-bound resins (immobilized at 0.2 μg/μl onto glutathione–Sepharose beads) for 2 h at 4°C. The resins were packed into columns, and the columns were washed with buffer B [20 mM HEPES–KOH pH 7.9, 20 mM KCl, 1 mM $MgCl_2$, 17% glycerol, 2 mM dithiothreitol (DTT)] containing 0.1 M KCl. Proteins were eluted with buffer B containing 1 M KCl. The eluates were desalted and concentrated using 10 000 mol. wt cutoff microconcentration tubes (Amicon) to a final volume of 100 μl in buffer B.

Protein acetylation and deacetylation assays

In vitro assays for protein acetylation were conducted using 1 μg of GST-fusion protein or 200 ng of H3 and 100 ng of either p300 or PCAF produced and purified from a baculovirus over-expression system (Ogryzko *et al*., 1996). Fifteen microliter reactions were incubated at 30°C for 1 h in buffer containing 50 mM Tris–HCl pH 8.0, 1 mM DTT, 0.1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM PMSF and 1 μCi/reaction ^{14}C-acetyl Coenzyme A (Amersham). Proteins were resolved by SDS–PAGE. Gels were fixed in 30% methanol and 10% acetic acid, washed with water, incubated for 2 h with 1 M salicylic acid, dried and exposed to X-ray film at –70°C.

Acetylation of chemically synthetized peptides (Synt:em, France) was performed as described above except that 5 μg of peptide and 250 ng of either p300 or PCAF were used per reaction. Peptides were resolved on 16.5% Tris-tricine gels (Bio-Rad). Gels were fixed, dried and exposed to film as described above.

For deacetylation assays, *in vitro* acetylated GST–Tat protein was incubated with HeLa extract overnight at 4°C in the absence or presence of 500 nM TSA. The GST–Tat protein was then purified by incubating the mixture with glutathione–Sepharose beads for 1 h at 4°C. The resin was washed extensively with buffer B containing 1 M KCl and equilibrated with buffer B. The beads were then resuspended in Laemmli buffer and resolved by 10% SDS–PAGE. Gels were fixed, dried and exposed to film as described above.

In vitro kinase assays

In vitro kinase reactions were performed in 20 μl final volume containing 50 mM Tris–HCl pH 7.5, 5 mM $MnCl_2$, and 5 mM DTT, 1 μCi of [γ-^{32}P]ATP. GST–CTD (200 ng) was added as substrate. Where indicated, the kinase reactions were performed in the presence or absence of 10 μM of DRB. Reactions were performed for 15 min at room temperature and terminated by the addition of SDS–PAGE sample buffer. Samples were resolved by 8% SDS–PAGE and visualized by autoradiography.

Western blot analysis

Column eluates were resolved by SDS–PAGE. Proteins were transferred to polyvinylidenefluoride (PVDF) membrane by semi-dry electroblotting (Millipore, Bedford) for 1 h at 400 mA. Membranes were incubated with the primary antibody overnight at 4°C, washed and incubated for 1 h with the appropriate secondary antibody (Tropix) for 1 h. Proteins were visualized by chemiluminescence (Tropix, Bedford) according to the manufacturer's protocol.

Electrophoretic mobility shift assay

TAR RNA was synthesized using T7 RNA polymerase from *Hin*dIII-digested pT7TAR by *in vitro* transcription reaction (Promega) containing [γ-^{32}P]UTP (Amersham). After treatment with RQ DNase, labeled TAR RNA was purified on a G-50 Sephadex column (Boehringer). Tat (10–60) peptides were chemically synthesized to contain either a non-acetylated lysine or acetylated lysine at residue 50. Peptides were purified to >95% homogeneity by mass spectral analysis purification procedures (Synt:em, France). Gel mobility shift reactions were performed as

previously described (Wei *et al*., 1998) except that the reactions were performed for 15 min at room temperature.

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