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The Human Immunodeficiency Virus Tat Proteins Specifically Associate with TAK In Vivo and Require the Carboxyl-Terminal Domain of RNA Polymerase II for Function

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Human immunodeficiency virus types 1 and 2 encode closely related proteins, Tat-1 and Tat-2, that stimulate viral transcription. Previously, we showed that the activation domains of these proteins specifically interact in vitro with a cellular protein kinase named TAK. In vitro, TAK phosphorylates the Tat-2 but not the Tat-1 protein, a 42-kDa polypeptide of unknown identity, and the carboxyl-terminal domain (CTD) of RNA polymerase II (RNAP II). We now show that the 42-kDa substrate of TAK cochromatographs with TAK activity, suggesting that this 42-kDa polypeptide is a subunit of TAK. We also show that the Tat proteins specifically associate with TAK in vivo, since wild-type Tat-1 and Tat-2 proteins expressed in mammalian cells, but not mutant Tat proteins containing a nonfunctional activation domain, can be coimmunoprecipitated with TAK. We also mapped the in vivo phosphorylation sites of Tat-2 to the carboxyl terminus of the protein, but analysis of proteins with mutations at these sites suggests that phosphorylation is not essential for Tat-2 transactivation function. We further investigated whether the CTD of RNAP II is required for Tat function in vivo. Using plasmid constructs that express an α-amanitin-resistant RNAP II subunit with a truncated or full-length CTD, we found that an intact CTD is required for Tat function. These observations strengthen the proposal that the mechanism of action of Tat involves the recruitment or activation of TAK, resulting in activated transcription through phosphorylation of the CTD.

The human immunodeficiency viruses, HIV-1 and HIV-2, encode closely related proteins known as Tat-1 and Tat-2, respectively, that act to stimulate transcription directed by the viral long terminal repeat (LTR) sequences. Tat function is essential for efficient viral replication in tissue culture and is likely to be necessary for the development of disease in infected individuals (1, 12, 17). Tat proteins activate transcription by binding directly to a *cis*-regulatory RNA genetic element, known as TAR RNA, formed at the 5′ end of all HIV RNAs (reviewed in references 10, 19, and 27). By binding to TAR RNA, the activation domain of Tat is able to interact with the RNAP II transcription complex to stimulate transcriptional elongation and, in some cases, transcription initiation (15, 28, 30, 33).

Although the mechanism whereby the Tat activation domain stimulates transcription has yet to be fully elucidated, we have proposed that this mechanism involves the recruitment or activation of a cellular protein kinase. This proposal has arisen from our observation that the activation domains of Tat-1, Tat-2, and the E-Tat protein of the distantly related equine infectious anemia virus bind in vitro to a cellular kinase termed TAK (Tat-associated kinase) present in HeLa cell nuclear extracts (25, 26). Mutations in the activation domains of these three Tat proteins that abolish transactivation function in vivo also abolish the ability to bind TAK in vitro. Because genetic experiments have suggested that the Tat-1, Tat-2, and E-Tat proteins function through a common cofactor (7, 32), the in vitro properties of TAK fulfill the genetic criteria established for such a cofactor.

TAK is a serine/threonine kinase that in vitro is able to phosphorylate a 42-kDa protein of unknown identity and Tat-2 but not Tat-1. Consistent with this specificity for Tat proteins, Tat-2 is phosphorylated in vivo but we and others have failed to detect phosphorylation of Tat-1 in vivo (13, 23, 25). Additionally, our studies have found that in vitro, TAK is able to hyperphosphorylate the carboxyl-terminal domain (CTD) of the largest subunit of RNAP II (26). The mammalian CTD consists of 52 repeats of the heptad consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Hyperphosphorylation of the CTD to the CTDo form is believed to regulate RNAP II; the CTDo form of RNAP II is present in actively elongating complexes, while the CTDa form (hypophosphorylated) is present in preinitiation complexes (8, 11, 52). On the basis of our previous work, we have proposed that the Tat activation domain may recruit or activate TAK, leading to hyperphosphorylation of the CTD and the resultant activation of transcriptional elongation and/or initiation. Our studies to date have suggested that TAK is not related to known CTDo kinases and may therefore be a novel cellular kinase (24, 26).

In this study, we show that the 42-kDa substrate of TAK copurifies with TAK activity through four chromatography columns, suggesting that it is a component of TAK which is autophosphorylated in kinase reactions. We also show that TAK can be specifically coimmunoprecipitated with Tat-1 and Tat-2 proteins transiently expressed in mammalian cells, indicating that Tat proteins associate with TAK in vivo. We also show that the in vivo phosphorylation site(s) of Tat-2 is at the carboxyl terminus, but analysis of proteins containing alanine substitutions for these phosphorylation sites suggests that phosphorylation of Tat-2 is not essential for its transactivation function. Additionally, using plasmid DNAs expressing α-amanitin-resistant RNAP II genes with a truncated or full-length CTD, we show that the CTD is required for Tat function in vivo. These results lend further support to the proposal that TAK is a cofactor recruited or activated by Tat, leading to activated transcription through phosphorylation of the CTD.

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MATERIALS AND METHODS

Cells and plasmids. Monolayer cultures of HeLa cells and COS cells were maintained in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Plasmids used for expression of activator proteins in mammalian cells were the GAL4 parental vector (pJDG [48]), GAL4-Sp1 (pJDG+SP1 [48]), the cytomegalovirus (CMV) parental vector (pCMV+30 [41]), CMV-Tat-1 (expresses the wild-type 86-residue two-exon HIV-1 Tat [41]), CMV-Tat-1/C22G (identical to CMV-Tat-1 except that cysteine 22 is mutated to glycine [41]), CMV-Tat-1/K41A (identical to CMV-Tat-1 except that lysine 41 is mutated to alanine [41]), CMV-Tat-2 (expresses the wild-type 99-residue first-exon HIV-2 Tat [41]), and CMV-Tat-2/C59A (also referred to as M59 [see Fig. 3], identical to CMV-Tat-2 except that cysteine 59 is mutated to alanine). Reporter plasmids used were a modified HIV-1 LTR-chloramphenicol acetyltransferase (CAT) containing six GAL4 DNA-binding sites and a deletion of the TAR element [G6(−83)HIVLTRΔTAR (49)], wild-type HIV-1 LTR-CAT (pUIIIR3 [46]), wild-type HIV-1 LTR-luciferase (2), and wild-type HIV-2 LTR-CAT (42). Plasmids used to express the α-amanitin-resistant large subunit of RNAP II (described in reference 20) were the CMV parental vector (pSTC), α-amanitin-resistant RNAP II containing the full-length (52-repeat) CTD (pHAWT), and α-amanitin-resistant RNAP II containing a truncated CTD with only 5 heptad repeats (pHAΔ5).

A PCR-based site-directed mutagenesis procedure (3) was used to construct plasmids expressing Tat-2 proteins with mutations in potential phosphorylation sites (see Fig. 3A). Tat-2 mutant genes were first inserted into the pGEM3Z (Promega) vector, and the entire coding region of each mutant was sequenced to verify their identities. Tat-2 genes were inserted into a variant of the pBC12/CMV vector (9) for expression in mammalian cells; the Tat-2 genes were also inserted into the pGEX2T vector (Pharmacia) for expression in *Escherichia coli* as fusions with glutathione-*S*-transferase (GST). GST-Tat fusion proteins were expressed in *E. coli* and purified as described previously (40).

Partial purification of TAK activity. TAK activity was partially purified from HeLa cell nuclear extracts as described previously (24). Briefly, nuclear extracts were dialyzed against buffer D (50 mM Tris-HCl [pH 8.0], 10% glycerol, 0.2 mM EDTA, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride)–50 mM KCl. The extracts were applied to a phosphocellulose P11 column (Whatman), and TAK activity was eluted with buffer D–0.5 M KCl. The eluate was dialyzed against buffer D–50 mM KCl and then applied to a Resource Q column (Pharmacia) in the same buffer; the peak of TAK activity eluted between 90 and 240 mM KCl. The eluate was adjusted to 150 mM KCl by dilution and applied to a heparin-agarose column (Bio-Rad) in buffer D–150 mM KCl; TAK activity eluted between 370 and 520 mM KCl. The eluate from the heparin-agarose column was estimated to represent an approximate 10-fold purification of TAK from the starting nuclear extract. Approximately 100 μg of the eluate from the heparin-agarose column was applied to a Superose 6 or Superose 12 gel filtration column (Pharmacia) that had been preequilibrated and run in buffer D–0.1 M KCl. The peaks of TAK activity from the Superose columns were estimated to represent an approximate 40- to 50-fold purification. At each stage of the purification, aliquots of fractions were diluted to ≤200 mM KCl and nuclease treated to reduce background in the kinase assay (the assay mixture was incubated at 37°C for 10 min with 100 U of DNase I per ml and 50 μg of RNase A per ml). Fractions were assayed for GST–Tat-2-associated CTD kinase activity as described below.

Kinase reactions. For immunoprecipitation (IP) kinase reactions, COS cells (6-cm culture dishes) were transfected with 1 μg of Tat expression plasmids by a DEAE-dextran procedure. At 48 h posttransfection, cell extracts were prepared by washing culture dishes with cold phosphate-buffered saline and cells were lysed by the addition of 0.6 ml of EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol) to each dish for 30 min on ice. Lysates were precleared with protein A-Sepharose beads (RepliGen) and then incubated at 4°C for 1 h with polyclonal antiserum directed against the HIV-1 or HIV-2 Tat protein (obtained from the AIDS Reference and Reagent Program). The lysates were then incubated with protein A-Sepharose beads for 1 h at 4°C. The immune complexes were collected by centrifugation in a microcentrifuge and washed three times with EBC buffer containing 0.03% sodium dodecyl sulfate (SDS). The immune complexes were then washed once with TKB/Mg buffer (50 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 5 mM dithiothreitol). Kinase assays were performed by adding 25 μl of a mix containing TKB/Mg buffer, 2.5 mM $MnCl_2$, 200 ng of GST-CTD (38), 5 μM ATP, and 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear) to the bead complexes and incubating the mixture at room temperature for 60 min. The complexes were pelleted briefly, boiled in Laemmli sample buffer, and resolved by electrophoresis on SDS-polyacrylamide gels. To verify that the wild-type and mutant Tat-1 proteins were present in immune complexes used for kinase reactions, a Tat-1 monoclonal antibody (5) was used in an enhanced chemiluminescence immunoblot assay to examine Tat-1 protein levels in immunoprecipitates. V8 phosphopeptide-mapping experiments were carried out as described previously (22); digestions were carried out with 0.05, 0.5, and 5 μg of V8 protease (Pierce Chemical Co.). Kinase assays were performed with GST-Tat fusion protein as described previously (24).

Transfections and transactivation assays. To evaluate expression levels, phosphorylation, and transactivation properties of Tat-2 proteins containing alanine substitutions for potential phosphorylation sites, COS cell cultures were transfected by a DEAE-dextran procedure. The indicated amount of expression plasmids for Tat proteins and 5 μg of the HIV-2 LTR-CAT reporter plasmid were cotransfected in 6-cm culture dishes, and CAT expression was measured at 48 h posttransfection by a standard assay as described previously (41). CAT assay results were quantified with a Betagen Betascope 603 scanner. As an internal reference to monitor transfection efficiencies, 5 μg of a β-galactosidase plasmid (pSVβ [31]) was included in transfections. To examine protein expression levels, cell cultures transfected with 1 μg of Tat-2 expression plasmids were labeled at 48 h posttransfection for 6 h in cysteine-deficient medium with 0.2 mCi of [^{35}S]cysteine (>600 Ci/mmol; NEN). To examine protein phosphorylation, cell cultures transfected with 1 μg of Tat-2 expression plasmids were labeled at 48 h posttransfection for 4 h in phosphate-free medium with 0.8 mCi of [^{32}P]orthophosphoric acid. After the labeling step, the cells lysates were prepared and IP reactions were performed as described previously (13).

To evaluate the requirement of the CTD for Tat or Sp1 function in vivo, HeLa cells were transfected in a double-transfection protocol by a liposome method as recommended by the manufacturer (LipofectAMINE reagent; Life Technologies, Inc.). Culture dishes (6 cm) were first transfected with 5 μg of plasmids expressing α-amanitin-resistant RNAP II genes plus plasmids expressing activator proteins (1 μg of Tat-1 plasmid, 2 μg of GAL4-Sp1 plasmid). At 24 h posttransfection, the cultures were retransfected with reporter plasmids (1 μg of HIV-1 LTR-CAT plasmid, 3 μg of plasmid containing GAL4 DNA-binding sites, 1 μg of HIV-1 LTR-luciferase plasmid) and the culture dishes were immediately treated with α-amanitin (final concentration in medium, 2.5 μg/ml; Boehringer-Mannheim). The α-amanitin treatment continued until the cell extracts were prepared at the indicated times. CAT enzyme levels were measured by standard procedures and were quantified with a Betagen Betascope 603 scanner. One CAT enzyme unit was arbitrarily defined as 1% conversion of chloramphenicol to its acetylated forms with 50% of the cell lysate obtained from a 6-cm culture dish. The amount of cell lysate was adjusted in CAT enzyme reactions such that conversion of chloramphenicol to its acetylated forms was less than 50%. Luciferase assays were performed with a commercial kit (Promega) and carried out as specified by the manufacturer. A Turner TD-20e luminometer was used to quantify luciferase assay results. To measure expression from the wild-type Tat-1 plasmid (1 μg) in transfections treated with α-amanitin, enhanced chemiluminescence immunoblots were performed by a standard procedure (22) with a Tat-1 monoclonal antibody (5).

RESULTS

The 42-kDa substrate of TAK is likely to be a TAK subunit. We have previously identified a cellular protein kinase activity, TAK, present in HeLa cell nuclear extracts that specifically binds in vitro to the activation domains of HIV Tat proteins (25, 26). In kinase reactions, TAK phosphorylates the HIV-2 Tat-2 protein but not the HIV-1 Tat-1 protein, a 42-kDa polypeptide whose identity is unknown, and recombinant CTD (25, 26). To further characterize TAK, we carried out a partial purification of TAK activity from HeLa cell nuclear extracts. Nuclear extracts were fractionated by sequential chromatography over phosphocellulose, Resource Q, and heparin-agarose columns. TAK activity was monitored during purification by incubating column fractions with a GST–Tat-2 protein attached to glutathione-Sepharose beads and performing kinase assays to measure the phosphorylation of Tat-2 and recombinant CTD. In these assays, GST–Tat-2 becomes phosphorylated on at least two sites, giving rise to two electrophoretic forms of the GST–Tat-2 phosphoprotein (25, 26). The recombinant GST-CTD substrate becomes hyperphosphorylated, resulting in the more slowly migrating GST-CTDo; the appearance of the CTDo form is indicative of the presence of a CTD kinase activity (11).

Fractionation of TAK over the final heparin-agarose column is shown in Fig. 1A. TAK activity eluted in column fractions 7 through 13, peaking in fraction 10. Of significance in these kinase reactions is the observation that the activity that phosphorylates the Tat-2 and the CTD substrates cochromatographs with kinase activity that phosphorylates the 42-kDa substrate. To verify that partially purified TAK binds specifically to a wild-type (wt) Tat activation domain, the peak of TAK activity from the heparin-agarose column was assayed with GST fusions of wt and transactivation-negative Tat-1 and Tat-2 proteins (Fig. 1B). The TAK preparation did contain a CTDo kinase activity that bound to the wt full-length Tat-2 and the wt Tat-1 activation domain (48Δ) fusion proteins but not to

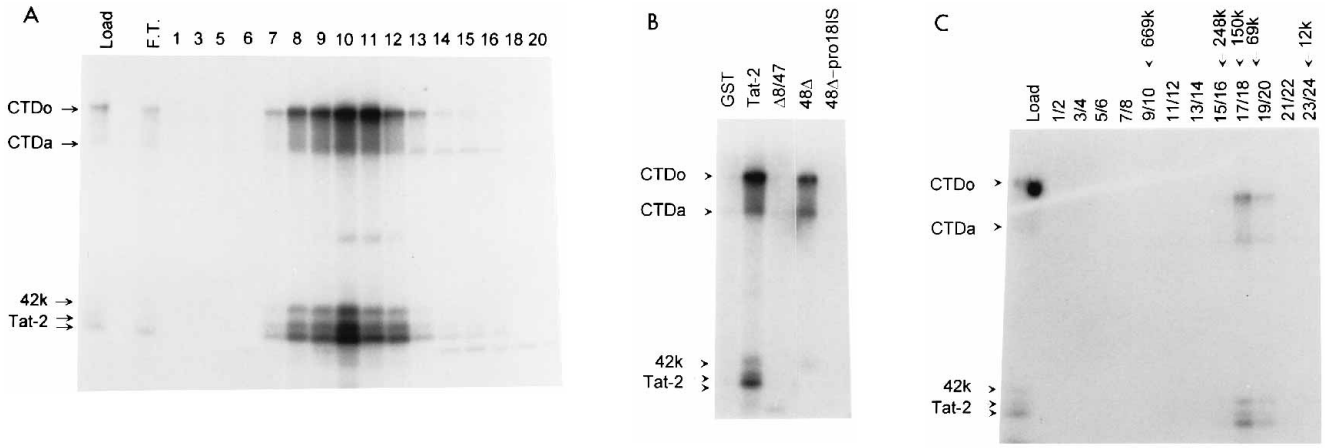


FIG. 1. Partial purification of TAK activity from HeLa cell nuclear extracts. (A) Fractions eluted from a heparin-agarose column were examined for TAK activity by using a wt GST–Tat-2 protein as described in Materials and Methods. The major products of the kinase assay are indicated on the left. The lane marked Load represents a kinase assay of a portion of the material loaded on the column, and the lane marked F.T. indicates the flowthrough of the column. (B) The peak of TAK activity from the heparin-agarose column was analyzed in the GST-Tat kinase assay with GST alone or GST fusions to wt Tat-2 (99R first exon), transactivation-negative Tat-2 mutant (Δ8/47, 99R with deletion of residues 8 through 47 in the activation domain), wt Tat-1 activation domain (48Δ), and transactivation-negative Tat-1 activation domain (48Δ-pro18IS, insertion of 2 amino acid residues after proline 18). The mutant Tat proteins possess nonfunctional activation domains in plasmid cotransfection transactivation assays (25, 26). (C) The peak of TAK activity from the heparin column was loaded on a Superose 6 gel filtration column, and the indicated pools of two column fractions were assayed for TAK activity as in panel A. The elution of molecular weight marker proteins from the column is indicated at the top. When the column was calibrated with the markers, individual fractions were analyzed. Relative to these markers, the peak of TAK activity was estimated to be 110 kDa.

the nonfunctional mutant Tat-2 (Δ8/47) and mutant Tat-1 activation domain (48Δ-pro18IS) fusions. As expected, partially purified TAK phosphorylated the wt Tat-2 fusion protein but not the wt Tat-1 activation domain fusion (Fig. 1B). Also as expected, the 42-kDa substrate was observed only in reactions with the wt Tat-2 protein and wt Tat-1 activation domain (the 42-kDa substrate is apparent on the original X-ray film in the reaction with wt Tat-1 activation domain). These results demonstrate that partially purified TAK binds specifically to the wt Tat-1 and Tat-2 activation domains.

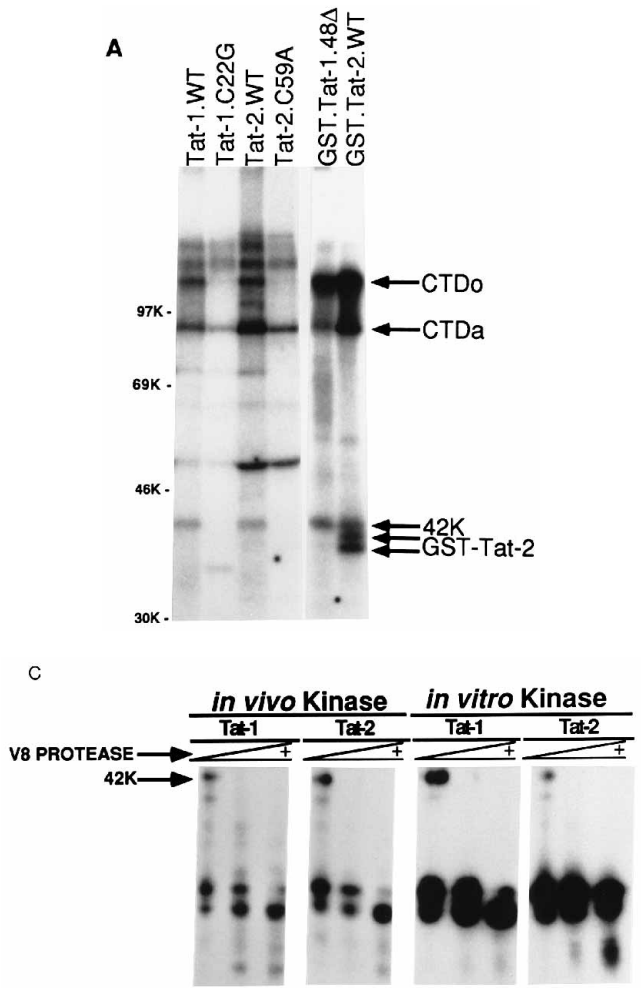
We note that with this partially purified TAK activity, the nonfunctional GST-Tat fusion proteins did not bind a kinase activity that phosphorylated the CTD to generate the CTDa or hypophosphorylated form. In kinase assays of nuclear extracts with these nonfunctional mutant Tat proteins, we previously observed a kinase activity that generated the CTDa (but not the CTDo) form in reactions (26). We believe that unfractionated nuclear extracts contain, in addition to TAK, a CTDa kinase activity that binds nonspecifically to Tat proteins outside the activation domain; this nonspecific activity is likely to have been separated from TAK during purification.

To estimate the native size of TAK, the TAK preparation after the heparin-agarose column was applied to a Superose 6 gel filtration column. Kinase assays of the column fractions indicated that the native size of TAK was approximately 110 kDa (Fig. 1B). Another TAK preparation was analyzed on a Superose 12 column, and TAK also eluted at the 110-kDa position (data not shown). Similar to the heparin-agarose column, the results with gel filtration showed that the activity that phosphorylates the Tat-2 and CTD substrates was not separable from the activity that phosphorylates the 42-kDa substrate. Experiments are presented below showing that the 42-kDa protein is phosphorylated in kinase reactions of Tat-TAK complexes coimmunoprecipitated from cells with anti-Tat antibodies (see below). Taken together, these results strongly suggest that the 42-kDa substrate is a TAK subunit that is autophosphorylated in kinase reactions. Because the native size of TAK is approximately 110 kDa, TAK is likely to be composed of more than one subunit.

HIV-1 and HIV-2 Tat proteins specifically associate with TAK in vivo. Our previous work showed that the in vitro properties of TAK fulfill the genetic criteria established for a Tat cofactor. However, if TAK is biologically relevant, it is crucial to demonstrate a specific interaction with Tat in vivo. To determine if Tat proteins associate with TAK or other cellular kinases in vivo, we looked for a kinase activity that could be coimmunoprecipitated with Tat proteins expressed transiently in mammalian cells. Plasmid DNAs expressing the wt Tat-1 and Tat-2 proteins were transfected into COS cells, cell lysates were prepared at 48 h posttransfection, immunoprecipitations were performed with a Tat-1 or Tat-2 antiserum, and kinase assays were performed (see Materials and Methods). As controls for specificity, mutant Tat proteins that contain single amino acid substitutions in the activation domains were also analyzed; the Tat-1 mutants C22G and K41A and the Tat-2 mutant C59A are inactive in vivo for transactivation of the HIV-1 and HIV-2 LTRs (29, 44, 47) (see Fig. 5). The products of the IP kinase reactions were analyzed on SDS-polyacrylamide gels (Fig. 2A and B). For comparison, in vitro kinase reactions with wt GST-Tat proteins bound to TAK from a HeLa nuclear extract were also analyzed (Fig. 2A).

TAK bound to GST–Tat-1(48Δ) or GST–Tat-2 was able to hyperphosphorylate the CTD, resulting in the more slowly migrating CTDo form; in these reactions, TAK also phosphorylated the 42-kDa substrate and the Tat-2 but not the Tat-1 fusion protein. The results of IP kinase reactions showed that the wt Tat-1 and Tat-2 proteins but not the nonfunctional mutant Tat proteins associated with a cellular kinase that was able to hyperphosphorylate recombinant CTD, resulting in the CTDo form (Fig. 2A). We note that immune complexes of the nonfunctional mutant Tat proteins did contain a kinase that phosphorylated the CTDa form. This nonspecific CTDa kinase may bind outside the activation domains, similar to a CTDa kinase that binds outside the activation domain of GST-Tat proteins and appears to separate from TAK activity during purification (discussed for Fig. 1C above).

To examine the phosphorylation state of Tat-1 and Tat-2 proteins in these types of reactions, IP kinase reactions were



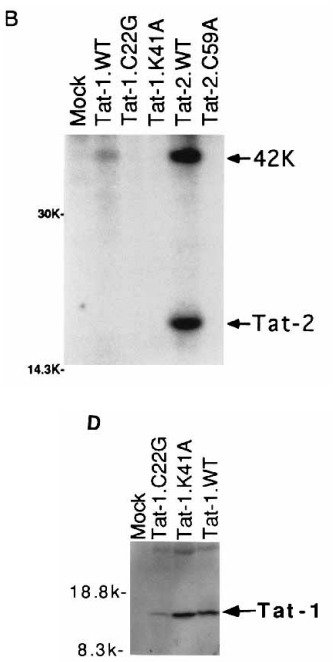


FIG. 2. TAK can be specifically coimmunoprecipitated with HIV-1 and HIV-2 Tat proteins. (A) Plasmids expressing wt Tat-1, Tat-1 mutant C22G, wt Tat-2, or Tat-2 mutant C59A as indicated were transfected into COS cells, and IPs were performed with a Tat-1 or Tat-2 antiserum. Kinase reactions with the immune complexes were carried out as described in Materials and Methods. The ^{32}P-labeled products of the reactions were analyzed on an SDS–9% polyacrylamide gel. The two lanes to the right of the IP kinase reactions contain the products of kinase reactions performed with a HeLa cell nuclear extract and purified GST–Tat-1 (48Δ, activation domain alone) and GST–Tat-2 (first exon). The positions of the recombinant CTD (CTDa and CTDo forms) and the 42-kDa phosphorylation products are indicated by arrows. (B) Plasmids expressing wt Tat-1, Tat-1 mutant C22G, Tat-1 mutant K41A, wt Tat-2, or Tat-2 mutant C59A proteins as indicated were transfected into COS cells, and kinase reactions of immunoprecipitates were performed as described for panel A. The lane marked Mock was mock transfected. The ^{32}P-labeled products of the kinase reaction were analyzed on an SDS–12% polyacrylamide gel. The 42-kDa and Tat-2 products are indicated by arrows. The Tat-2 and Tat-1 proteins analyzed in these studies migrate at the same position in SDS-polyacrylamide gels (39). (C) V8 phosphopeptide map of the 42-kDa substrate. The ^{32}P-labeled 42-kDa substrates of IP kinase reactions (denoted by in vivo kinase) with wt Tat-1 or Tat-2 protein and the 42-kDa substrates of GST–Tat-1 and GST–Tat-2 kinase reactions with a HeLa nuclear extract (denoted by in vitro kinase) were excised from an SDS-polyacrylamide gel and subjected to digestion with increasing amounts of V8 protease (0.05, 0.5, and 5.0 μg). The V8 digestion products were analyzed on an SDS–15% polyacrylamide gel. (D) COS cells were either mock transfected or transfected with the indicated Tat-1 expression plasmid, and 48 h later, IPs were performed with a Tat-1 polyclonal antiserum. The immunoprecipitates were loaded on an SDS–15% polyacrylamide gel, and Tat-1 levels were determined in an immunoblot with a Tat-1 monoclonal antiserum.

analyzed on a higher-percentage SDS-polyacrylamide gel (Fig. 2B). The 86-residue Tat-1 protein and the 99-residue Tat-2 proteins analyzed in this study comigrate in SDS-polyacrylamide gels (39). In agreement with our previous study (25), the Tat-2 protein was phosphorylated by a kinase activity bound to the wt Tat-2 protein whereas the mutant Tat-2 protein was not phosphorylated. Neither the wt nor mutant HIV-1 Tat protein was phosphorylated in IP kinase reactions. These data are consistent with GST-Tat kinase reactions and in vivo phosphorylation analysis, in which Tat-2 but not Tat-1 is phosphorylated (13, 23, 25). Although Tat-2 phosphorylated in vivo or in IP kinase reactions migrates as a single band in SDS-polyacrylamide gels, the GST-Tat kinase reactions generate two forms of GST–Tat-2 (25, 26) (Fig. 2A), suggesting that Tat-2 can be phosphorylated in the GST fusion at sites which are not phosphorylated in vivo at discernible levels.

We performed an immunoblot analysis to verify that the mutant Tat-1 proteins were present in immune complexes used in the IP kinase experiments. COS cells were transfected with plasmid vectors that expressed Tat-1 proteins, cell extracts were prepared at 48 h posttransfection, and IPs were carried out with the polyclonal Tat-1 antiserum used in the IP kinase assays. A Tat-1 monoclonal antiserum was then used in an immunoblot to determine the level of Tat-1 proteins in the polyclonal immunoprecipitates. The wt Tat-1 and K41A mutant were present at similar levels, while the C22G mutant appeared to be present at a lower level (Fig. 2D). However, the monoclonal antibody used in the immunoblot recognizes a Tat-1 epitope between amino acid residues 5 and 22 (5); the substitution of glycine for cysteine 22 in the C22G mutant may reduce recognition by the antibody, and the immunoblot may therefore underestimate the level of the C22G protein. IPs with the polyclonal Tat-1 antiserum from cells labeled metabolically with [^{35}S]cysteine showed that the C22G and wt proteins are synthesized at equivalent levels (43). Additionally, IPs with the Tat-2 polyclonal antiserum demonstrate that the C59A Tat-2 mutant used in the IP kinase reactions is synthesized at a level similar to wt Tat-2 (see Fig. 4 [in which C59A is referred to as M59]). We conclude that the mutant Tat proteins are present at levels similar to the wt proteins in immune complexes used in IP kinase reactions.

In IP kinase reactions with wt but not mutant Tat-1 and Tat-2 proteins, a 42-kDa protein that comigrated with the

42-kDa polypeptide labeled in the GST-Tat kinase reactions was phosphorylated (Fig. 2A and B). To determine if the 42-kDa proteins labeled in the two types of reactions are related, these 42-kDa proteins were excised from an SDS-polyacrylamide gel and digested with V8 protease (Fig. 2C). The results demonstrated that the 42-kDa polypeptides phosphorylated in GST-Tat and Tat coimmunoprecipitate reactions are very closely related and are likely to be identical polypeptides.

The results of these experiments demonstrate that a cellular kinase activity coimmunoprecipitating with the wt Tat proteins can phosphorylate the HIV-2 but not the HIV-1 Tat protein, the 42-kDa polypeptide, and recombinant CTD to generate the CTDo form. Because this substrate specificity is identical to that of TAK in GST-Tat kinase assays, we conclude that Tat proteins specifically associate with TAK in vivo. The 42-kDa polypeptide is clearly a HeLa cell protein, since its phosphorylation is observed in Tat-IP kinase reactions without the addition of recombinant GST-CTD (51). Moreover, the 42-kDa polypeptide is likely to be a component of TAK, since it was detected in both the IP kinase and GST-Tat kinase reactions and it appears to cochromatograph with TAK activity (Fig. 1).

Analysis of Tat-2 phosphorylation sites. Although TAK specifically associates with the activation domains of both Tat-1 and Tat-2, it appears capable of recognizing only Tat-2 as a substrate and phosphorylates it in vitro on serine and threonine residues (25). The serines and threonines in Tat-2 are clustered at the amino and carboxyl termini (Fig. 3A). Because the termini of the Tat-1 and Tat-2 proteins are not conserved (37), it is not necessarily surprising that Tat-1 is not a substrate of TAK. To map the phosphorylation sites of Tat-2 and assess whether their phosphorylation is functionally significant, we constructed Tat-2 genes containing alanine substitutions for clusters of serine and threonine residues which are conserved among different HIV-2 isolates (37). For these experiments, we used the 99-residue first exon of Tat-2, because this exon is essentially fully active in transactivation of HIV-1 and HIV-2 LTRs in plasmid DNA cotransfection assays (14, 16, 41). As diagrammed in Fig. 3A, mutant M3 contains substitutions at residues 3, 10, and 11; M22 contains substitutions at residues 22 and 23; and M85 contains substitutions at residues 85, 89, and 94. An additional mutant, M59 (alanine substitution for cysteine 59 [referred to as C59A in Fig. 2A and B]), was analyzed as a control, because the analogous mutation in the HIV-1 Tat protein at residue 30 is inactive for transactivation (29, 44, 47).

We used GST-Tat kinase assays to examine if these mutant Tat-2 proteins could bind TAK in vitro from HeLa cell nuclear extracts and if the proteins could serve as TAK substrates. Wild-type, M3, M22, and M85 GST fusion proteins possessed similar abilities to bind TAK from the HeLa cell nuclear extract, resulting in production of the CTDo product in the kinase reaction (Fig. 3B). In contrast, the GST-M59 protein appeared unable to bind TAK, and no CTDo product was observed. A longer exposure of the film in Fig. 3B showed that the 42-kDa substrate was phosphorylated with all GST-Tat-2 proteins except M59.

The phosphorylation patterns of GST-Tat fusions were similar for wt, M3, and M22 proteins, while phosphorylation of M59 and M85 proteins was greatly reduced. Because there is no CTDo product in the kinase reaction with M59, the low level of phosphorylation of M59 is probably the result of its inability to associate with TAK. However, the low level of phosphorylation of M85 cannot be explained by poor binding to TAK (see the CTDo product in Fig. 3B) but is probably due to the mutations at amino acid residues 85, 89, and/or 94 and suggests that these are the major phosphorylation sites by TAK in vitro.

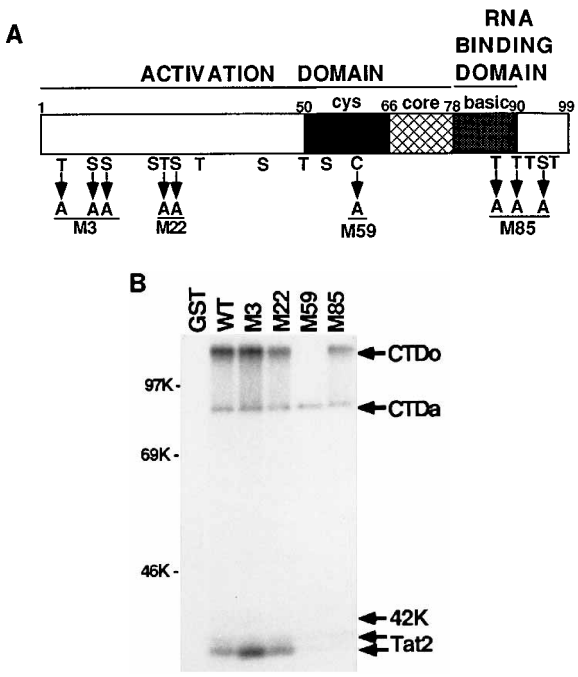


FIG. 3. Kinase reactions of GST-Tat-2 proteins containing alanine substitutions in potential phosphorylation sites. (A) Serine and threonine residues of the first coding exon of the HIV-2 Tat protein (ROD isolate) are shown. Tat-2 proteins analyzed in this study that contain alanine substitutions for the indicated residues are also shown. The indicated cysteine-rich region apparently binds metal ions with specific coordinations that may be necessary for proper protein conformation (18, 45); the core region has been identified by conservation of these sequences in the primate immunodeficiency viruses and equine infectious anemia virus (6); the basic region is the nuclear localization signal and also confers TAR RNA-binding properties (10, 19, 27). (B) The indicated GST-Tat fusion proteins were incubated with a HeLa cell nuclear extract and assayed for kinase activity as described in Materials and Methods. Products of the kinase reaction were analyzed on an SDS–9% polyacrylamide gel. The CTDa, CTDo, 42-kDa, and GST–Tat-2 products of reactions are indicated by arrows.

To examine the phosphorylation of mutant Tat-2 proteins in vivo, plasmid DNAs expressing wt and mutant Tat-2 proteins were transfected into COS cells. Cultures were then metabolically labeled with either [^{32}P]orthophosphoric acid or [^{35}S]cysteine, and IPs were performed. The results with [^{35}S]cysteine labeling indicated that the wt and four mutant Tat-2 proteins were expressed at similar levels (Fig. 4B). The results with ^{32}P labeling indicated that wild-type Tat-2 and M22 were phosphorylated at equivalent levels, whereas phosphorylation of M3 was reduced about 50% relative to the wt (Fig. 4A). This lower level of phosphorylation of M3 was observed in several independent experiments; the explanation for this observation is unclear. M59 was phosphorylated very weakly if at all in vivo. Because M59 does not bind TAK in vivo (Fig. 2A and B) or in vitro (Fig. 3B), a likely explanation for the lack of phosphorylation of M59 is the inability of this protein to associate with TAK in vivo. In contrast, M85 was not phosphorylated in vivo at a detectable level, although it is fully able to bind TAK in vitro (Fig. 3B). This suggests that the major phosphorylation site(s) in vivo for Tat-2 is at amino acid residue 85, 89, or 94 or combinations of these three sites. It is probable that the major site of phosphorylation is at residue 89, since a Tat-2 protein with alanine substituted for threonine 85 is phosphorylated in vivo at wt levels (13) and a GST-Tat-2 protein truncated at residue 90 is phosphorylated in vitro at its carboxyl terminus (25).

To examine the significance of phosphorylation of the Tat-2

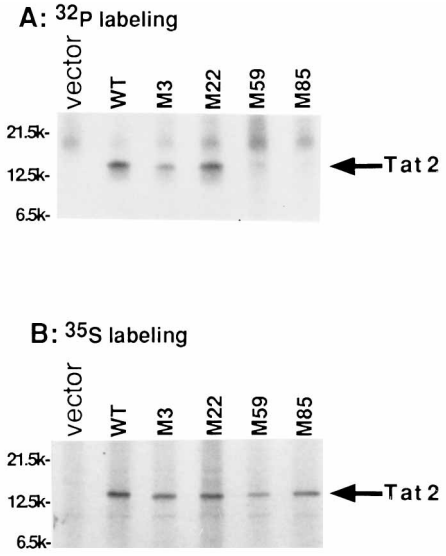


FIG. 4. Expression and phosphorylation of Tat-2 proteins containing alanine substitutions for potential phosphorylation sites. Plasmids expressing the indicated Tat-2 proteins were transfected into COS cells, cells were labeled with either [^{32}P]orthophosphoric acid (A) or [^{35}S]cysteine (B), and IPs were performed with a Tat-2 antiserum. Products of IPs were analyzed on SDS–15% polyacrylamide gels. Incorporation of ^{32}P into Tat-2 proteins was quantified with a Betagen Betascope 603 scanner.

protein on function, wt and phosphorylation site mutant proteins were tested in transactivation assays. Plasmids expressing Tat-2 proteins were transfected into COS cells with a plasmid containing the CAT reporter gene under control of the HIV-2 LTR, and CAT expression was measured at 48 h posttransfection (Fig. 5). Transactivation by wt, M3, M22, and M85 proteins was equivalent over a broad range of input plasmids, reaching maximum levels between 40- and 50-fold. M59 was unable to significantly transactivate the HIV-2 LTR, confirming the expected phenotype predicted from previous mutational analysis of the HIV-1 Tat protein (29, 44, 47). Transactivation assays were also performed in Jurkat cells, a T-lymphocyte cell line, and similar results were observed (data not shown). These results indicate that the amino acid substitutions in the M3, M22, and M85 proteins do not affect transactivation function in vivo. Because M85 is not phosphorylated in vivo at a detectable level, phosphorylation of the major site(s) in the carboxyl terminus of Tat-2 does not appear to be important for transactivation function in plasmid cotransfection assays. However, because the wild-type residues mutated in M85 are highly conserved in various HIV-2 isolates (37), it is possible that their phosphorylation is significant in natural infections. It is interesting that M59 is not phosphorylated in vivo or in vitro at significant levels, is unable to bind TAK in IP kinase or GST-Tat kinase assays, and is inactive in transactivation in vivo. These results suggest that the ability to bind TAK is important for the transactivation function of Tat.

RNA polymerase II CTD is required for Tat function in vivo. The observation that TAK efficiently hyperphosphorylates the CTD in vitro suggested a model in which TAK is recruited or activated by Tat, leading to phosphorylation of the CTD and to a resultant activation of transcription (26). To examine if the CTD is needed for Tat function, we used the system developed by Gerber et al. in which the requirement of the CTD for activated transcription of specific genes in vivo can be investigated (20). We modified the original protocol to allow facile measurement of reporter proteins rather than reporter RNAs. In this modification, a plasmid expressing an α-amanitin-resistant RNAP II gene and a plasmid expressing an activator protein were cotransfected into HeLa cell cultures by an efficient liposome transfection method. At 24 h posttransfection, cultures were retransfected with reporter plasmids and immediately treated with α-amanitin. In this manner, reporter plasmids should be efficiently transcribed only in cells that had previously been transfected and express an α-amanitin resis-

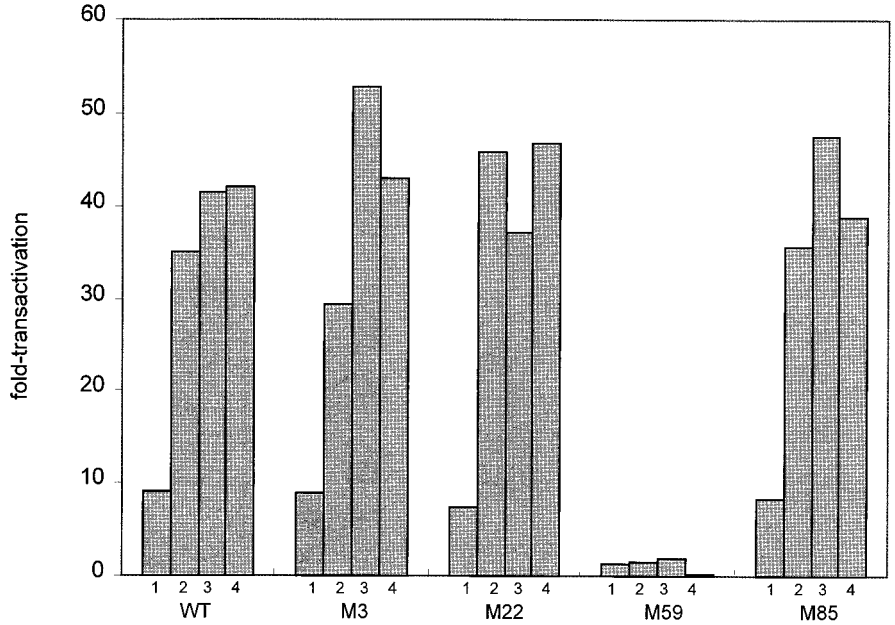


FIG. 5. Transactivation assays of Tat-2 proteins containing alanine substitutions in potential phosphorylation sites. The indicated amounts of plasmids expressing wt or mutant Tat-2 proteins (Fig. 3A) were cotransfected with an HIV-2 LTR-CAT reporter plasmid into COS cells. CAT expression was measured at 48 h posttransfection. The amount of CAT expression seen with vector plasmid (no Tat-2 gene) was used to calculate the fold transactivation by each Tat-2 expression plasmid. The values presented are the average of duplicate transfections and CAT assays. 1, 0.01 μg of activator plasmid; 2, 0.10 μg; 3, 1.0 μg; 4, 10.0 μg.

TABLE 1. Transactivation assays of Tat-1 activation of the HIV-1 LTR-luciferase reporter plasmid

α-Amanitin-resistant RNAP II present	Luciferase expression (fold activation) in[a]:				
	Tat-1	Expt 1[b]	Expt 2[c]	Expt 3[c]	Expt 4[c]
No (vector only)	Vector	4.88	3.03	4.21 (−)	1.02
	C22G	2.32 (0.5)	6.76 (2.2)	9.97 (2.4)	ND[d]
	wt	8.74 (1.8)	18.79 (6.2)	22.86 (5.4)	4.03 (4.0)
Yes (with truncated CTD)	Vector	6.09	6.51	5.23 (−)	3.0
	C22G	1.33 (0.2)	3.12 (0.5)	4.53 (0.9)	ND
	wt	8.97 (1.5)	31.77 (4.9)	11.83 (2.3)	2.26 (0.8)
Yes (with full-length CTD)	Vector	54.69	9.84	20.7 (−)	8.15
	C22G	28.84 (0.5)	3.98 (0.4)	311.5 (15.0)	ND
	wt	327.2 (6.0)	551.6 (56)	4,659 (225)	273.3 (33.5)

[a] Expressed in luciferase units. Fold activation expressed with respect to use of activator vector in the Tat-1 column.
[b] Harvested at 24 h posttransfection.
[c] Harvested at 48 h posttransfection.
[d] ND, not done.

tance gene. At 24 or 48 h after transfection of the reporter plasmids, cell extracts were prepared and expression of reporter proteins was measured. It is not feasible to use an internal reference plasmid in this protocol to normalize for transfection efficiencies, because most RNAP II promoters are likely to be affected by their own requirement for the CTD (20), and expression from the reference plasmid will be different with and without the CTD in the α-amanitin-resistant RNAP II complex. To deal with potential variations in transfection efficiencies in this study, we repeated each experiment multiple times. We obtained reproducible results with the transactivators tested, indicating that variations in transfection efficiencies are not a problem in this protocol.

We investigated whether transactivation of an HIV-1 LTR-luciferase reporter plasmid by HIV-1 Tat requires a full-length CTD (Table 1). Upon transfection of the control vector (no α-amanitin resistance gene), only low levels of luciferase expression were observed with activator vector, C22G Tat-1 mutant, or wt Tat-1. In four experiments with no transfected α-amanitin resistance gene, wt Tat-1 transactivation of the HIV-1 LTR-luciferase reporter ranged between 1.8- and 6.2-fold. This low level is therefore the background of Tat-1 transactivation in this method. Upon transfection of an α-amanitin resistance gene with a truncated CTD (only five heptad repeats), low levels of luciferase expression were observed for activator vector, C22G mutant, and wt Tat-1. In four experiments with the truncated CTD, wt Tat-1 transactivation ranged between 0.8- and 4.9-fold. Therefore, Tat-1 transactivation in the presence of the truncated CTD gene never exceed the background transactivation level. Upon transfection of an α-amanitin resistance gene with a full-length CTD, robust transactivation was observed with wt Tat-1. In four experiments, wt Tat-1 transactivation ranged between 6.0-fold (24 h after transfection of reporter) and 225-fold (48 h after transfection). We conclude that Tat-1 transactivation of the HIV-1 LTR-luciferase reporter plasmid requires the CTD in vivo.

We performed an immunoblot experiment to investigate expression levels of the Tat-1 protein under these conditions (Fig. 6). HeLa cell cultures were transfected with the wt Tat-1 expression plasmid and plasmids expressing α-amanitin resistance genes. At 24 h posttransfection, these cultures were treated with α-amanitin, and 48 h later, cell lysates were prepared. An immunoblot analysis showed that the level of Tat-1 protein was equivalent in cells transfected with the control vector (no α-amanitin resistance gene) and the α-amanitin resistance gene with truncated CTD, while the level of Tat-1 was slightly lower in cells transfected with the α-amanitin resistance gene with a full-length CTD. This result demonstrates that the lack of Tat-1 transactivation with the truncated CTD is not likely to be the result of low expression levels of Tat-1 protein.

To verify that the α-amanitin resistance gene with the truncated CTD was expressed and functional, we analyzed transcriptional activation by the cellular transcription factor Sp1, because Sp1 does not require the CTD for function (20). For these experiments, we used an activator plasmid that expresses a fusion of the Sp1 activation domain to the GAL4 DNA-binding domain (48). As a reporter plasmid, we used a plasmid based on the HIV-1 LTR; this plasmid, G6(−83)HIVLTR ΔTAR, contains 6 GAL4 DNA-binding sites positioned 5′ to HIV-1 sequences −83 to +82 (relative to the transcription start site) with the TAR RNA element deleted (49). The results of three experiments with these plasmids are presented in Table 2. Upon transfection of the control vector plasmid that does not express an α-amanitin resistance gene, a background level of Sp1 transactivation that ranged from 0.9- to 5.1-fold was observed. Upon transfection of a plasmid that expresses an α-amanitin resistance gene with a truncated CTD, Sp1 transactivation ranged between 10.3- and 17.5-fold, confirming that Sp1 function can occur in the absence of the CTD. Importantly, the total amount of CAT expression from the reporter with GAL4-Sp1 plus this α-amanitin resistance gene was higher than expression with GAL4-Sp1 and the control vector, indicating that the transfected α-amanitin resistance gene with

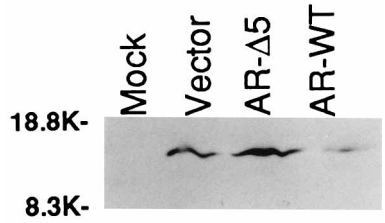


FIG. 6. Expression levels of Tat-1 protein in the presence of α-amanitin. HeLa cells were cotransfected with a plasmid expressing wt Tat-1 and plasmids expressing no α-amanitin resistance gene (vector), α-amanitin resistance gene with truncated CTD (AR-Δ5), or α-amanitin resistance gene with full-length CTD (AR-WT). At 24 h posttransfection, the cells were treated with α-amanitin, and 48 h later, whole-cell lysates were prepared. Expression levels of Tat were determined by immunoblotting. The lane labeled Mock represents a mock-transfected culture dish which was treated with α-amanitin.

TABLE 2. Transactivation assays of Sp1 activation of GAL4-CAT reporter plasmid and Tat-1 activation of HIV-1 LTR-CAT reporter plasmid

α-Amanitin-resistant RNAP II present	Sp1	CAT expression (fold activation) in[a]:			Tat-1	CAT expression (fold activation) in[a]:	
		Expt 1	Expt 2	Expt 3		Expt 1	Expt 2[b]
No (vector only)	GAL4	5.3	91	25.9	Vector	57	ND[c]
	GAL4-Sp1	27 (5.1)	80 (0.9)	66.2 (2.6)	wt	54 (0.9)	ND
Yes (with truncated CTD)	GAL4	7	18	9.1	Vector	87	154
	GAL4-Sp1	82 (11.7)	315 (17.5)	93.4 (10.3)	wt	79 (0.9)	401 (2.6)
Yes (with full-length CTD)	GAL4	37	30	14.7	Vector	1,125	285
	GAL4-Sp1	318 (8.6)	210 (7.0)	295 (20.1)	wt	4,530 (4.0)	>4,590 (>16)

[a] Expressed in CAT units. Fold activation expressed with respect to use of GAL4 in the Sp1 column and activator vector in the Tat-1 column.
[b] Harvested at 48 h posttransfection. In all other experiments, harvesting was done at 24 h posttransfection.
[c] ND, not done.

a truncated CTD was expressed and functional. Upon transfection of a plasmid that expresses an α-amanitin resistance gene with an intact CTD, Sp1 transactivation ranged between 7.0- and 20.1-fold.

To compare the use of luciferase or CAT as a reporter protein, we also examined Tat-1 transactivation of an HIV-1 LTR-CAT reporter plasmid. We note that the basal expression from the HIV-1 LTR-luciferase and HIV-1 LTR-CAT reporter plasmids was higher with the full-length CTD than with the truncated CTD (Tables 1 and 2). These data may reflect the requirement of the CTD for transcriptional activation through the two NF-κB sites in the reporter plasmids (at approximately −100 to −75 in the HIV-1 LTR). In the two experiments presented in Table 2, little or no Tat-1 transactivation of the CAT reporter was observed with the vector (no α-amanitin resistance gene) or with an α-amanitin resistance gene with a truncated CTD. In the two experiments with the α-amanitin resistance gene with the full-length CTD, significant Tat-1 transactivation was observed (4-fold [24 h after transfection of reporter] and >16-fold [48 h after transfection]). In summary, we conclude from these experiments with α-amanitin-resistant RNAP II complexes that Tat, unlike Sp1, requires an intact CTD for function in vivo.

DISCUSSION

Our previous studies identified a cellular serine/threonine protein kinase named TAK (Tat-associated kinase) that binds in vitro to the activation domains of the HIV-1, HIV-2, and equine infectious anemia virus Tat proteins (25, 26). In vitro, TAK phosphorylates the HIV-2 Tat protein (but not the HIV-1 Tat protein), a 42-kDa protein, and recombinant CTD to produce the hyperphosphorylated CTDo form. The data presented here demonstrate that a kinase activity with the same substrate specificity as TAK can be specifically coimmunoprecipitated with the HIV-1 and HIV-2 Tat proteins expressed in mammalian cells. Therefore, these observations indicate that Tat proteins associate with TAK in vivo.

We have also presented evidence here which strongly suggests that the 42-kDa polypeptide is a subunit of TAK that is autophosphorylated in kinase reactions. The 42-kDa substrate is phosphorylated by TAK in both coimmunoprecipitations and GST-kinase assays (Fig. 2). Furthermore, the kinase activity that phosphorylates the 42-kDa polypeptide cochromatographs with the activity that phosphorylates the CTD and Tat-2 (Fig. 1). Because gel filtration chromatography indicates that the native size of TAK is approximately 110 kDa (Fig. 1C), TAK is probably composed of more than one polypeptide.

Several lines of evidence suggest that TAK is relevant to Tat function. Analysis of numerous Tat proteins with loss-of-function mutations in the activation domain indicates that there is a precise correlation with in vivo function and the ability to bind TAK (25, 26). TAK is also sensitive to inhibition in vitro by DRB, a nucleoside analog that inhibits Tat function in vivo and in cell-free transcription reactions (4, 26, 34, 50). Furthermore, the biochemical activity of TAK and the observed effects of Tat on stimulation of transcriptional initiation and elongation can be readily reconciled with a model of transcriptional activation that involves phosphorylation of the CTD. A crucial prediction of this model is that Tat function should require an intact CTD. This prediction has been substantiated, because this study demonstrates that Tat function in vivo requires the CTD. In the present study, we did not address whether TAK directly phosphorylates the CTD in vivo, and it remains formally possible that the physiological target of TAK is another substrate whose function is somehow dependent upon an intact CTD. The definitive determination of the role of TAK in Tat transactivation will require the cloning of cDNAs for TAK subunits and subsequent genetic, biochemical, and immunological analyses.

Using immunological reagents, we have not detected a relationship between TAK and known CTD kinases, including the basal transcription factor TFIIH (24). It appears, therefore, that TAK may be a novel cellular protein kinase. It is conceivable that TAK may be related to the *Drosophila* transcription factor P-TEFb; P-TEFb is also sensitive to DRB, and it acts shortly after initiation to convert transcription complexes from an abortive to a productive elongation mode (35, 36). We have recently identified a cellular CTD kinase that binds in vitro to the activation domains of the adenovirus E1A protein and the herpes simplex virus VP16 protein but not to the activation domains of Tat proteins (24). Our analysis of this kinase indicates that it is distinct from TAK and is also unrelated to TFIIH. These observations suggest that strong viral transactivators may employ a general strategy for transcriptional activation that involves a direct interaction with CTD kinases.

Finally, a number of small molecules that selectively inhibit specific protein kinases both in vitro and in animals have been developed (21). If TAK does mediate Tat function, the past successes of pharmacology in developing kinase antagonists offer hope that therapeutically useful Tat inhibitors can be developed.

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