

# Lentivirus Tat Proteins Specifically Associate with a Cellular Protein Kinase, TAK, That Hyperphosphorylates the Carboxyl-Terminal Domain of the Large Subunit of RNA Polymerase II: Candidate for a Tat Cofactor

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Received 24 August 1994/Accepted 6 December 1994

**Efficient replication of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) requires the virus transactivator proteins known as Tat. In order to understand the molecular mechanisms involved in Tat transactivation, it is essential to identify the cellular target(s) of the Tat activation domain. Using an in vitro kinase assay, we previously identified a cellular protein kinase activity, Tat-associated kinase (TAK), that specifically binds to the activation domains of Tat proteins. Here it is demonstrated that TAK fulfills the genetic criteria established for a Tat cofactor. TAK binds in vitro to the activation domains of the Tat proteins of HIV-1 and HIV-2 and the distantly related lentivirus equine infectious anemia virus but not to mutant Tat proteins that contain nonfunctional activation domains. In addition, it is shown that TAK is sensitive to dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole, a nucleoside analog that inhibits a limited number of kinases and is known to inhibit Tat transactivation in vivo and in vitro. We have further identified an in vitro substrate of TAK, the carboxyl-terminal domain of the large subunit of RNA polymerase II. Phosphorylation of the carboxyl-terminal domain has been proposed to trigger the transition from initiation to active elongation and also to influence later stages during elongation. Taken together, these results imply that TAK is a very promising candidate for a cellular factor that mediates Tat transactivation.**

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), the causative agents of AIDS, are closely related lentiviruses, a subfamily of retroviruses (73). Equine infectious anemia virus (EIAV) is a distantly related lentivirus that produces a slow, progressive disease in horses (11). HIV and EIAV encode a crucial gene, *tat*, whose protein product regulates virus gene expression and is required for efficient virus replication. Tat is unique among transcriptional activators in that its *cis*-regulatory target is an RNA element, TAR, that is found at the 5' ends of nascent virus transcripts. HIV TAR RNA is highly structured and appears to function simply as a binding site for Tat, since heterologous RNA elements can substitute for TAR when the appropriate RNA binding domain is fused to Tat (65, 67). While the sequence of EIAV TAR is sufficiently divergent from HIV TAR RNA that HIV and EIAV Tat (E-Tat) proteins do not cross-transactivate the heterologous promoter, the TAR element of EIAV is thought to function in an analogous manner to the HIV TAR element (10).

On the basis of sequence comparison, several regions of homology among the lentivirus Tat proteins have been defined (Fig. 1). The HIV Tat and E-Tat proteins contain a conserved core region and a basic region; the HIV Tat proteins share an additional conserved cysteine-rich region. Biochemical and genetic experiments have established that Tat proteins possess two functional domains, an RNA binding domain required for interaction with TAR RNA and the activation domain thought to function by recruiting a cellular factor to the virus promoter (reviewed in reference 34). For the HIV-1 Tat protein (Tat-1), the basic region is necessary and sufficient for TAR binding in

vitro, although amino-terminal sequences are also required for RNA binding in vivo (7, 13, 45, 63). The carboxyl-terminal region of E-Tat in addition to the basic region is required to form the RNA binding domain of E-Tat (20). Through protein fusion experiments, the activation domains of HIV Tat proteins have been shown to comprise the cysteine-rich and core regions of HIV Tat proteins, as well as nonconserved amino-terminal sequences (35, 60, 65, 68). The core region of E-Tat, which is functionally interchangeable with the core region from HIV, constitutes the activation domain of E-Tat, although its activity is influenced by sequences outside the conserved regions (20).

Although effects of Tat at the levels of both transcriptional initiation and elongation have been reported (24, 36, 41, 48, 68), the precise mechanism of action for the Tat proteins remains elusive. To understand the molecular mechanism of Tat, it is necessary to identify the cellular target(s) of the Tat activation domain. On the basis of genetic experiments, Tat-1, the HIV-2 Tat protein (Tat-2), and E-Tat have been predicted to interact with a common cellular factor (9, 46, 59). Therefore, the Tat cofactor is expected to bind specifically to the activation domains of all these lentivirus Tat proteins but to be unable to interact with activation domain mutants that fail to transactivate in vivo. Although several cellular proteins that can bind to Tat-1 in vitro have previously been reported (21, 32, 52), these proteins do not fulfill all of the criteria set for a Tat cofactor; since the specificity of binding between these proteins and Tat has not been demonstrated, their role(s) in Tat transactivation remains unclear. More recently, it has been shown that Tat-1 can interact directly and specifically with the TATA-binding protein (TBP) or the TFIID complex (TBP and associated factors) in vitro (37). It remains to be determined whether TBP specifically interacts with Tat-2 and E-Tat. Precisely how the interaction between TBP and Tat-1 results in increased transcriptional effects by Tat is not known.

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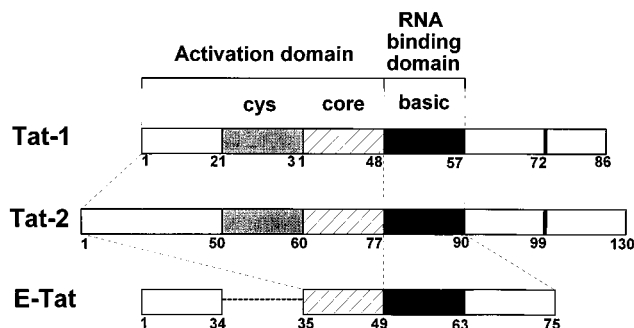


FIG. 1. Comparison of Tat-1, Tat-2, and E-Tat proteins. The regions of homology among Tat-1 (HXB2), Tat-2 (ROD), and E-Tat are shown. The regions that constitute the activation domain of each protein are indicated. The RNA binding domain is sufficient for TAR RNA binding *in vitro*, although amino-terminal sequences have been shown to be required for RNA binding of Tat-1 *in vivo*. The heavy lines after amino acid residues 72 of Tat-1 and 99 of Tat-2 represent the exon 1-exon 2 junctions of these proteins.

Recently, a specific interaction of Tat-1 and Tat-2 with a cellular protein kinase, Tat-associated kinase (TAK), was demonstrated (30). TAK phosphorylated Tat-2 (but not Tat-1) and an as yet unidentified 42-kDa protein. TAK associated with wild-type Tat-2 but not mutants of Tat-2 that contained deletions within the activation domain. It was shown that the activation domain of Tat-1 alone (although not full-length Tat-1) specifically interacted with TAK, since point mutations that abolished the function of the activation domain *in vivo* failed to interact with TAK *in vitro*.

In this study, we report that TAK binds specifically not only to Tat-2 but also to full-length Tat-1 as well as E-Tat. Thus, TAK fulfills the genetic criteria set for a Tat cofactor. We have further identified an *in vitro* substrate of TAK, the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II (RNAP II). Phosphorylation of the CTD has been proposed to trigger the transition from complex assembly to active elongation and to influence events during the elongation phase of transcription. Phosphorylation of the CTD by TAK suggests a relatively simple model for the mechanism of action of Tat. In addition, we present evidence that TAK activity is inhibited by dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), a nucleoside analog that has been shown to selectively inhibit Tat function *in vivo* and *in vitro*. Taken together, these results imply that TAK is an attractive candidate for a cellular cofactor that mediates Tat function.

## MATERIALS AND METHODS

**Preparation of bacterially expressed fusion proteins.** Plasmids used to express glutathione *S*-transferase (GST) fusions of wild-type and mutant Tat-2 (isolate ROD) proteins and GST-Tat-1 plasmids expressing isolate HXB2 86-, 72-, and 48-residue proteins have previously been described (30, 58). GST-Tat-1 plasmids that expressed isolate SF2 proteins were generously provided by R. Gaynor and colleagues. GST fusions of wild-type and  $\Delta$ 3C E-Tat proteins were kindly provided by D. Derse. The  $\Delta$ 3C mutant contains a deletion of the last three amino acid residues of the carboxyl terminus (8). The E-Tat core + T mutant contains an insertion of threonine between amino acid residues 36 and 37 within the activation domain. GST-E-Tat core + T fusions were constructed by PCR amplification by standard techniques, using the E-Tat core + T gene in the pRS vector (8). GST-Tat proteins were expressed and purified as described previously (30, 58). The GST-CTD expression plasmid (GCTD), obtained from B. Dynan and colleagues, was expressed and purified essentially as previously described (57).

**Preparation of HeLa cell nuclear extracts.** Nuclear extracts of HeLa cell suspension cultures were prepared by the method of Dignam et al. (22). Prior to use in *in vitro* binding and kinase assays, nuclear extracts were treated with 100 U of DNase per ml and 50  $\mu$ g of RNase per ml for 10 min at 37°C and precleared by successive incubations with glutathione-Sepharose beads (Pharmacia) and GST-loaded glutathione-Sepharose beads.

**CTD phosphorylation assay.** GST and GST-Tat fusion proteins (0.5  $\mu$ g) were bound to 10  $\mu$ l of glutathione-Sepharose beads (50% slurry) preequilibrated in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 5 mM dithiothreitol) by incubation for 15 min at 4°C with gentle rocking. Beads were pelleted and washed two times with EBC buffer that contained 0.075% sodium dodecyl sulfate (SDS) and once with EBC buffer. GST-Tat beads were subsequently incubated with precleared HeLa nuclear extracts for 60 min at 4°C with rocking. The resulting complexes were washed three times with EBC buffer that contained 0.03% SDS and once with Tat kinase buffer (TKB) (50 mM Tris [pH 7.4], 5 mM MnCl<sub>2</sub>, 5 mM dithiothreitol).

CTD kinase assays were performed by adding 50  $\mu$ l of a mix that contained TKB, 100 ng of GST-CTD, 2  $\mu$ M ATP, and 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci/mmol; New England Nuclear) to the bead complexes and incubating at room temperature for the indicated times. Complexes were pelleted briefly, boiled in Laemmli sample buffer, and resolved by electrophoresis on SDS-8% polyacrylamide gels.

## RESULTS

**Hyperphosphorylation of a CTD fusion protein by TAK.** Previously, we showed that Tat-2 and the activation domain alone of Tat-1 specifically associate with TAK (30). We proposed that TAK may mediate Tat function by phosphorylating a component of the transcription complex. Since phosphorylation of the CTD of the large subunit of RNAP II has been proposed to trigger the transition from complex assembly to active elongation and to influence transcriptional elongation, we considered the CTD to be a potential target of TAK phosphorylation. Therefore, we asked whether a kinase activity associated with Tat-2 could phosphorylate the CTD and give rise to the characteristic shift in electrophoretic mobility detected by SDS-polyacrylamide gel electrophoresis (PAGE).

Tat-2 expressed in bacteria as a fusion protein with GST was purified by selective adsorption to glutathione-Sepharose beads and then incubated with HeLa cell nuclear extract as described in Materials and Methods. A kinase assay was performed with a GST-CTD fusion protein (57) as the substrate and subsequently analyzed by SDS-PAGE (Fig. 2). As expected from our previous study (30), two phosphorylated forms of Tat-2, as well as a phosphorylated 42-kDa protein, were products of the reaction. The identity of the 42-kDa protein is not known, but the presence of this protein correlates with TAK activity; it may represent a subunit of the kinase that becomes autophosphorylated in the reaction. In addition to Tat-2 and the 42-kDa protein, two phosphorylated forms of the CTD were observed, a faster migrating form referred to as CTDA and a more slowly migrating form referred to as CTDO. The underphosphorylated CTDA form comigrated with bacterially expressed GST-CTD fusion protein (54). The two forms of the CTD displayed the expected migration rates for the GST-CTD fusion (see Fig. 3 to 6), as characterized by using the DNA-dependent protein kinase (DNA-PK) (57). For comparison, the migration of phosphorylated forms of the CTD labeled by purified cdk2/cyclin A kinase (28) is also shown in Fig. 2. The closely related cdk2/cyclin A kinase has previously been shown to phosphorylate the CTD with a resulting shift in mobility to the CTDO form (14). The cdk2/cyclin A kinase fully hyperphosphorylates the CTD (uppermost band) and also gives an incompletely hyperphosphorylated CTD form. Intermediate forms of the CTD have previously been observed with other CTD kinases (14, 56, 62a).

The shift in mobility of the CTD is characteristic of the change in migration seen with the full-length RNAP II subunit or the CTD alone expressed as a fusion with GST (6, 57). This suggests that the site of phosphorylation is within the CTD portion of the fusion protein rather than the GST portion. Furthermore, previous work has shown that TAK does not phosphorylate the GST portion of GST-Tat-2 protein (30), nor

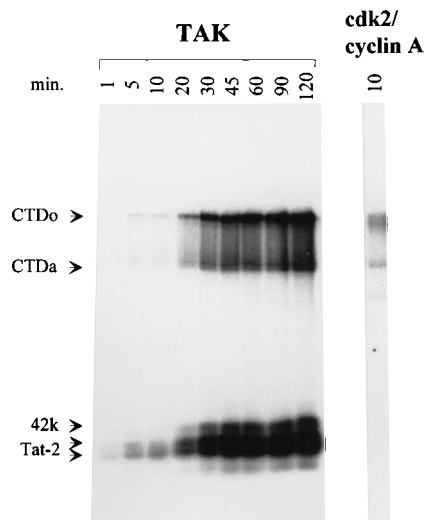


FIG. 2. TAK phosphorylates the CTD of the large subunit of RNAP II. Wild-type GST-Tat-2 bound to glutathione-Sepharose beads was incubated with HeLa cell nuclear extract as described in Materials and Methods. Kinase assays were performed by incubating washed bead complexes with 100 ng of GST-CTD-2  $\mu$ M ATP-10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP in TKB for the indicated times at room temperature. Lane cdk2/cyclin A, cdk2/cyclin A kinase expressed and purified from baculovirus (28) was incubated with 100 ng of GST-CTD-10  $\mu$ M ATP-10  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP in TKB for 10 min before the addition of Laemmli sample buffer. Products of the reaction were separated by electrophoresis on an SDS-8% polyacrylamide gel and visualized by autoradiography. The positions of the CTDa and CTDo forms, the two phosphorylated forms of Tat-2, and the 42-kDa protein discussed in the text are indicated by arrows. The phosphorylated product seen below the CTDa band in the cdk2/cyclin A lane is detected in the absence of GST-CTD and probably represents autophosphorylation of cyclin A.

does TAK phosphorylate GST expressed as a fusion with Tat-1 protein (see Fig. 4A).

Phosphorylation of both forms of the CTD by TAK was detected as soon as 1 min after the reaction was initiated, indicating a highly cooperative reaction (seen on longer exposures of the gel shown in Fig. 2). The level of phosphorylation increased with time (up to 120 min). GST alone did not bind a kinase activity that was able to hyperphosphorylate the CTD in our assay (see Fig. 3A, 4A, and 5), indicating that hyperphosphorylation of CTD was dependent on TAK activity.

Phosphorylation of the CTD has been detected on serine, threonine, and tyrosine residues (4, 6, 77). With Tat-2 as the substrate, TAK was previously shown to be a serine-threonine kinase (30). To determine the amino acid specificity of TAK for the CTD, phosphoamino acid analysis was performed on both forms of the CTD. Phosphorylation of CTDa and CTDo occurred almost entirely, if not exclusively, on serine residues (54).

**Hyperphosphorylation of the CTD is specific for a functional activation domain of Tat-2.** To investigate the specificity of hyperphosphorylation of the CTD, Tat-2 proteins that contained deletions in the activation domain were assayed for the ability to bind a kinase activity that was capable of shifting the mobility of the CTD. The  $\Delta$ 8/33 (deletion of residues 8 to 33) and  $\Delta$ 8/47 (deletion of residues 8 to 47) mutants displayed reduced transactivation activities in transient transfection assays performed in our laboratory (23, 60), although the equivalent of the  $\Delta$ 8/33 mutant was found to possess wild-type transactivation activity by others (4a). We previously showed that the  $\Delta$ 8/33 and  $\Delta$ 8/47 mutants bound TAK weakly, if at all (30). The mutants, 84 $\Delta$  and 90 $\Delta$ , which contained deletions at the carboxyl terminus of Tat-2 (truncations after residues 84 and 90, respectively) retained the ability to bind to TAK. The

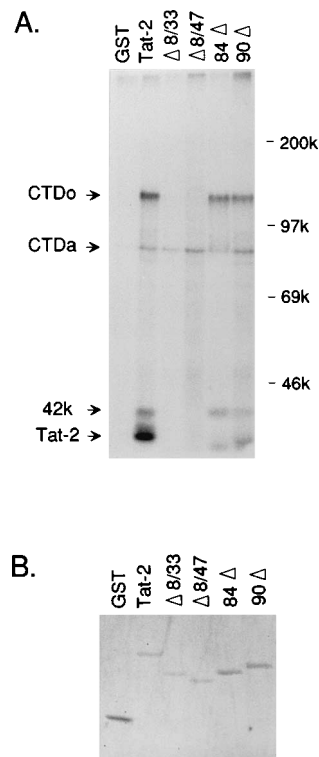


FIG. 3. Hyperphosphorylation of the CTD is specific for a functional activation domain of Tat-2. (A) Wild-type and mutant Tat-2 proteins fused to GST were bound to glutathione-Sepharose beads and incubated with HeLa cell nuclear extracts, and CTD kinase assays were performed as described in Materials and Methods. Kinase reaction mixtures were incubated for 45 min at room temperature, and products were separated by electrophoresis on SDS-8% polyacrylamide gels. Lane GST, the parental GST construct; lane Tat-2, wild-type Tat-2 fused to GST. GST-Tat-2 mutants  $\Delta$ 8/33 and  $\Delta$ 8/47 contain deletions within the activation domain (residues 8 to 33 and 8 to 47, respectively), and mutants 84 $\Delta$  and 90 $\Delta$  contain truncations at the C terminus (after residues 84 and 90, respectively) (30, 59). Arrows indicate the positions of the CTDa and CTDo forms, wild-type Tat-2, and the 42-kDa protein referred to in the text. (B) Equivalent amounts of the fusion proteins used in panel A were bound to glutathione-Sepharose beads, washed, and separated on an SDS-12% polyacrylamide gel. Fusion proteins were visualized by Coomassie blue staining.

results in Fig. 3A show that the kinase activity that bound to wild-type Tat-2 and the 84 $\Delta$  and 90 $\Delta$  mutants caused a shift in the mobility of CTD. The  $\Delta$ 8/33 and  $\Delta$ 8/47 complexes phosphorylated the CTDa form but did not give rise to the CTDo form. These mutants were present in kinase reactions at levels similar to that of wild-type Tat-2 protein as shown by Coomassie blue staining (Fig. 3B). Therefore, the ability to hyperphosphorylate the CTD in vitro correlates with Tat-2 proteins that contain functional activation domains. Phosphorylation of the faster migrating CTDa form in reactions with Tat-2 proteins that contain deletions in the activation domain is probably due to the activity of a kinase that binds to GST-Tat fusion proteins outside the activation domain (see Fig. 4A).

**Hyperphosphorylation of the CTD is specific for a functional activation domain of Tat-1.** In previous work, the interaction of TAK with full-length wild-type Tat-1 was difficult to detect probably in part because of the fact that Tat-1, unlike Tat-2, does not serve as a substrate of TAK. After the identification of the CTD as a substrate of TAK, the ability of full-length Tat-1 to interact with TAK was tested with the CTD as the substrate. The results in Fig. 4A show that wild-type full-length Tat-1 did interact with TAK, resulting in hyper-

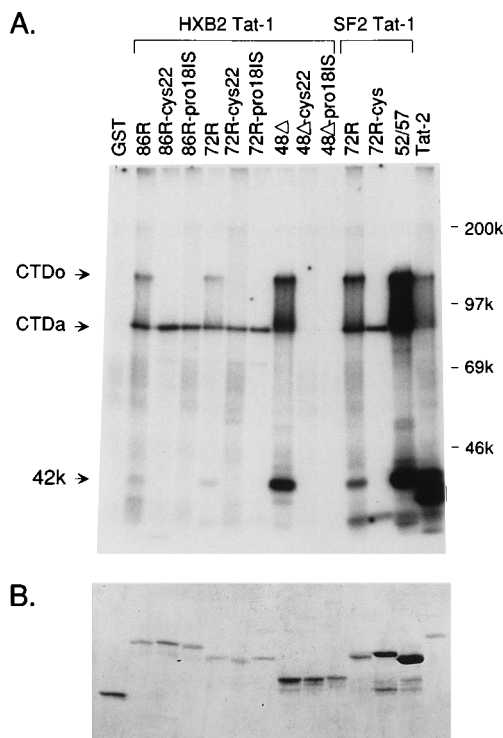


FIG. 4. Hyperphosphorylation of the CTD is specific for a functional activation domain of Tat-1. (A) Wild-type and mutant Tat-1 proteins fused to GST were assayed for CTD kinase activity as described in Materials and Methods. Kinase reaction mixtures were incubated for 60 min at room temperature. GST-Tat-1 proteins are derived from virus isolates HXB2 and SF2 as indicated. Lane GST, the parental GST construct; lanes 86R, 72R, and 48Δ, wild-type two-exon or one-exon Tat-1 (HXB2) or activation domain alone of Tat-1 (HXB2), respectively, fused to GST (30, 58). cys22 mutants contain a substitution of Cys for Gly at residue 22, and pro18IS mutants contain an insertion between residues 18 and 19. These mutations were constructed in 86R, 72R, and 48Δ backgrounds as fusions with GST (58). Lane SF2 Tat-1 72R, wild-type one-exon Tat-1 from isolate SF2 fused to GST. 72R-cys contains three substitutions of serine for cysteine in the activation domain, and 52/57 contains substitutions within the basic domain (50). Arrows indicate the positions of the CTDα and CTDβ forms and the 42-kDa protein referred to in the text. No phosphorylation of the CTDα form was seen with the mutants on longer exposure of the gel shown here or in repeated experiments with these mutants. (B) Portion of the reactions from panel A run on an SDS-12% polyacrylamide gel. Fusion proteins were visualized by Coomassie blue staining.

phosphorylation of the CTD in the kinase assay. By using the Tat-1 protein from virus isolate HXB2, phosphorylation of the CTDα form was seen with wild-type full-length 86R Tat-1 (two exons), 72R Tat-1 (one exon), and the activation domain of Tat-1 alone (48Δ). 72R possesses full transactivation activity in transient transfection assays (18, 66), while experiments with protein fusions of the amino-terminal 48 residues have established that this region constitutes the Tat-1 activation domain (35, 60, 65, 68). The hyperphosphorylated form of the CTD was not detected with two different mutants of Tat-1, cys22 (substitution of Gly for Cys at residue 22) and pro18IS (insertion of Glu and Phe between residues 18 and 19), in the 86R, 72R, or 48Δ background. These mutants have previously been shown to be nonfunctional in transactivation assays *in vivo* (61). It should be noted that the pro18IS mutant is predicted to retain wild-type structure on the basis of protease sensitivity studies (62), suggesting that the failure of the pro18IS mutant to bind TAK is not due simply to perturbation of protein structure.

Although phosphorylation of the faster migrating CTDβ form was evident for the cys22 and pro18IS mutants in 86R

and 72R backgrounds, this kinase activity is probably not relevant for Tat transactivation function since it binds outside the activation domain, as demonstrated by the failure of the 48Δ mutants to bind this activity. By using partially purified TAK preparations, it can be shown that the cys22 and pro18IS mutants in the 86R background fail to bind this nonspecific CTDα kinase activity (31).

The ability of Tat-1 to associate with TAK and result in hyperphosphorylation of the CTD was also tested with GST fusions of Tat-1 from virus isolate SF2. HXB2 and SF2 Tat-1 genes represent the most divergent Tat genes of HIV-1 isolates (51). As with HXB2 Tat-1, the slowly migrating CTDα form was seen with wild-type SF2 Tat-1 (72R) (Fig. 4A). 72R-cys, which contains three substitutions of serine for cysteine in the activation domain (obtained from R. Gaynor), failed to associate with a kinase that hyperphosphorylated the CTD. Hyperphosphorylation of the CTD was seen with 52/57, a potent transdominant mutant (50). This mutant contains substitutions of glycine and alanine in the basic domain but has an intact activation domain. The higher levels of CTD phosphorylation in the SF2 reactions most likely reflect higher levels of GST-Tat-1 fusion proteins in these reactions, as seen by Coomassie blue staining (Fig. 4B). Therefore, the results with Tat-1 obtained from two divergent isolates of HIV indicate that the interaction of Tat-1 with a kinase activity that hyperphosphorylates the CTD is specific for a functional activation domain of Tat-1.

In our previous study, the association of TAK with Tat-1 was detected with the activation domain alone (48Δ) but not with full-length Tat-1 (30). This study clearly shows that full-length Tat-1 does specifically associate with a cellular protein kinase, since reactions with wild-type Tat-1 proteins but not with mutant Tat-1 proteins display the shift in mobility of the CTD. Furthermore, low levels of the TAK-associated 42-kDa protein were detected with wild-type 86R and 72R Tat-1 proteins (Fig. 4A). The use of nuclear extracts, which contain higher TAK activity, rather than whole-cell extracts (used in our previous experiments) probably allowed the detection of the 42-kDa protein in these experiments. It is also possible that the interaction of TAK with Tat-1 is less stable than that with Tat-2 *in vitro* and therefore more difficult to detect.

**TAK specifically associates with E-Tat.** Since HIV Tat proteins and E-Tat are thought to function through a common cellular cofactor (9, 46), we asked whether E-Tat could interact with TAK. As shown in Fig. 5, wild-type E-Tat protein produced as a fusion with GST associated with a kinase activity that hyperphosphorylated the CTD. A mutant E-Tat protein, Δ3C, that contained a 3-amino-acid-residue deletion in the nonconserved C terminus of E-Tat also associated with the kinase activity that hyperphosphorylated the CTD. The Δ3C mutant protein is deficient in targeting the EIAV TAR RNA element and possesses <20% of the transactivation activity of wild-type E-Tat protein (8). However, a mutant, core + T, that contained an insertion within the conserved core region and retained only 10% of wild-type activity (19a) failed to interact with this kinase activity. This indicates that the interaction between TAK and E-Tat is specific for a functional activation domain of E-Tat.

In summary, TAK interacts specifically with Tat-1, Tat-2, and E-Tat. Thus, TAK meets the genetic criteria established for a cellular cofactor of Tat function. Interaction with TAK appears to be specific for lentivirus Tat proteins, since TAK did not associate with the transactivator protein Tax of human T-cell leukemia virus type 1, a member of a distinct class of complex retroviruses (31).

**TAK activity is sensitive to DRB.** DRB is a nucleoside an-

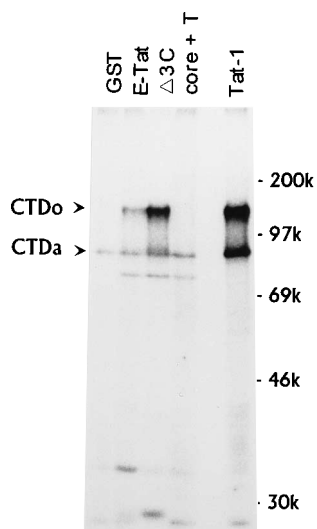


FIG. 5. Specific interaction between TAK and E-Tat. Wild-type and mutant E-Tat proteins fused to GST were assayed for CTD kinase activity as described in Materials and Methods. Kinase reaction mixtures were incubated for 60 min at room temperature. The  $\Delta 3C$  mutant contains a deletion of three amino acid residues in the nonconserved C-terminal region of E-Tat. The core + T mutant contains an insertion in the conserved core region (see Materials and Methods for further descriptions of these mutants). Equivalent amounts of GST fusion proteins were detected by Coomassie blue staining.

alog that selectively inhibits RNAP II transcription in vivo (25, 64) and in vitro (75). In vitro studies indicate that DRB inhibits RNAP II transcription at the level of elongation (12, 49). Interestingly, DRB has been demonstrated to preferentially inhibit Tat transactivation in vitro (48) and to inhibit Tat activity in *Xenopus* oocytes (5) and HeLa cells (72). DRB is thought to function by inhibiting the activity of a kinase(s) (76). A few protein kinases, including several kinases that phosphorylate the CTD, have been reported to be sensitive to this drug

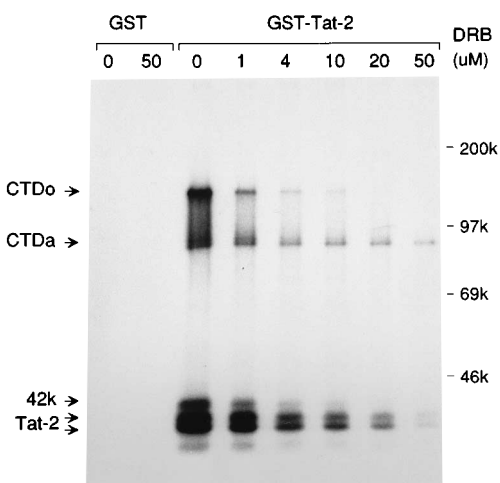


FIG. 6. TAK activity is sensitive to DRB. GST and wild-type GST-Tat-2 bound to glutathione-Sepharose beads were incubated with HeLa cell nuclear extracts, and kinase assays were performed as described in Materials and Methods except that the indicated concentration of DRB was included in each reaction mixture. Reaction mixtures were incubated for 45 min at room temperature. Arrows indicate the positions of the CTD $\alpha$  and CTD $\beta$  forms, the 42-kDa protein, and the two phosphorylated forms of Tat-2.

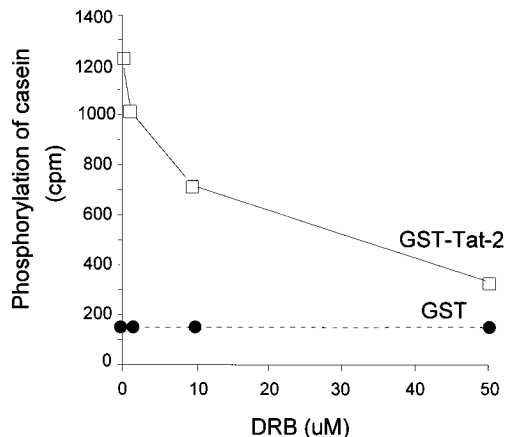


FIG. 7. Selective inhibition of CTD phosphorylation by DRB. GST and GST-Tat-2 bound to glutathione-Sepharose beads were incubated with HeLa cell nuclear extracts, and kinase assays were performed as previously described (30) except that 1 mg of casein per ml and the indicated concentration of DRB were included in each kinase reaction mixture. Kinase reaction mixtures were incubated for 30 min at room temperature, and products were separated by SDS-PAGE. Quantitation of phosphorylated casein in GST and GST-Tat-2 reactions, as indicated, was performed with a Betagen Betascope 603 scanner.

(see Discussion). Therefore, it was of interest to determine whether TAK activity was sensitive to DRB.

The ability of DRB to inhibit TAK activity was tested by examining the phosphorylation of Tat-2 and the CTD in the presence of different concentrations of DRB. Tat transactivation has been reported to be inhibited by 50% by a DRB concentration of 4  $\mu\text{M}$  in vitro (48) and of 6 to 18  $\mu\text{M}$  in vivo (5, 72). TAK activity was sensitive to DRB in this concentration range (Fig. 6). Quantitation of the gel shown in Fig. 6 with a Betagen Betascope 603 scanner revealed that the concentration of DRB required for 50% inhibition of TAK activity was 10  $\mu\text{M}$  with Tat-2 as the substrate and 2.5  $\mu\text{M}$  with the 42-kDa protein as the substrate. Phosphorylation of the CTD $\alpha$  form was inhibited by 50% at a DRB concentration of 2  $\mu\text{M}$ , while phosphorylation of the CTD $\beta$  form was inhibited by greater than 50% at a DRB concentration of 1  $\mu\text{M}$ .

To confirm that DRB was not causing general inhibition of kinase activity in our assay, the sensitivity of TAK to DRB was also tested with casein as the substrate. GST binds a kinase activity that is able to phosphorylate casein (Fig. 7). This nonspecific kinase activity was not inhibited by DRB concentrations of up to 50  $\mu\text{M}$ . Casein was phosphorylated to an approximately eightfold-higher level by the GST-Tat-2-associated kinase, and this activity was sensitive to DRB.

In conclusion, these results indicate that TAK is inhibited by DRB concentrations in the range that inhibits Tat transactivation in vitro and in vivo. This is consistent with a role for TAK in the DRB-sensitive step of Tat transactivation. Although further work is required to demonstrate that TAK is indeed a DRB-sensitive Tat cofactor, the correlation between the binding of the kinase activity that hyperphosphorylates the CTD in vitro with Tat proteins that are functional for Tat transactivation in vivo suggests that TAK represents a very promising candidate for a cellular factor involved in Tat transactivation.

## DISCUSSION

Transactivation of HIV gene expression by Tat is thought to be mediated by a cellular factor. Previously, we identified a candidate for such a Tat cofactor, a cellular protein kinase

activity, TAK, that specifically interacts with Tat-2 and the activation domain alone of Tat-1 (30). In this study, we demonstrated that not only the Tat-1 activation domain (residues 1 to 48) but also the two-exon (86R) and one-exon (72R) wild-type Tat-1 proteins interacted with TAK. The abilities of Tat-1 proteins to interact with TAK strongly correlated with their transactivation activities *in vivo*, i.e., Tat-1 proteins that contained point mutations that rendered them nonfunctional for transactivation *in vivo* failed to associate with this kinase *in vitro*. It was also shown that E-Tat specifically interacted with TAK. Thus, TAK interacts with Tat-1, Tat-2, and E-Tat precisely as predicted for a Tat cofactor by previous genetic experiments (9, 46). This study further identified a cellular substrate of TAK that has important implications for Tat function, the CTD of the large subunit of RNAP II. Finally, it was demonstrated that TAK activity is inhibited by DRB, a nucleoside analog that inhibits Tat transactivation *in vivo* and *in vitro*. These new results lend support to the proposal that TAK acts as a mediator of Tat function.

**Is TAK a novel protein kinase?** To date, a number of mammalian protein kinases that can phosphorylate the CTD *in vitro* have been identified; the relative roles of these kinases *in vivo*, however, have not yet been elucidated. Kinases that phosphorylate the CTD *in vitro* include the general transcription factor TFIIF (43), DNA-PK (57), and the cdc2 kinase (14). In preliminary experiments with a monoclonal antibody directed against the 62-kDa subunit of TFIIF (provided by D. Reinberg), we have been unable to detect TFIIF in complexes that contain TAK activity. TAK is unlikely to be DNA-PK because our assays have been performed in the absence of DNA, a condition under which DNA-PK has little or no activity. Furthermore, DNA-PK has been shown to be resistant to DRB (2). The cdc2 kinase displays sensitivity to DRB (14), but TAK does not appear to be related to cdc2 or cdk2, since unlike cdc2 or cdk2, TAK did not detectably phosphorylate histone H1 (29). Payne and Dahmus (56) and Stevens and Maupin (70) have described additional partially purified kinase activities that phosphorylate the CTD *in vitro* and are sensitive to DRB; like TAK, these kinases do not phosphorylate histone H1. Preliminary results indicate that the pair of CTD kinases characterized by Payne and Dahmus (56) do not associate with Tat-1 and Tat-2 with the specificity of TAK (31). Therefore, it appears that TAK may represent a novel protein kinase activity.

**Phosphorylation of the CTD.** While the experiments presented here do not address the functional significance of phosphorylation of the CTD by TAK *in vivo*, the ability of a kinase activity that associates with Tat activation domains to hyperphosphorylate the CTD of RNAP II *in vitro* is intriguing. The CTD contains an unusual, highly repetitive structure that consists of tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (17). Although the function of the CTD is not known, this domain is essential for cell viability (1, 3, 53). Two forms of RNAP II exist *in vivo*; they differ with respect to phosphorylation of the CTD and are involved at different points in the transcription cycle. The unphosphorylated IIa form preferentially enters the preinitiation complex, while the IIo form is associated with actively elongating complexes (15, 19). Roles for the phosphorylated CTD at various stages of the transcription cycle have been postulated. During the initiation phase of transcription, phosphorylation of the CTD has been proposed to disrupt the preinitiation complex and trigger the transition from transcription complex assembly to active elongation (19). It has been postulated that the phosphorylated CTD acts during transcriptional elongation to release RNAP II from paused or attenuated transcription complexes (69). The phosphorylated CTD has also been proposed to facilitate

transcriptional elongation by displacing nucleosomes ahead of the elongating complex (16). An additional role, the phosphorylated CTD as an attachment site for proteins involved in RNA processing, has been put forth (27). Given these different hypotheses and the number of kinases that phosphorylate the CTD *in vitro*, it seems likely that the CTD is regulated *in vivo* at different points in the transcription cycle by distinct kinase activities.

**Effects of DRB on transcriptional elongation and Tat transactivation.** The experiments presented here do not address the point in the transcription cycle at which phosphorylation of the CTD by TAK might occur. The sensitivity of TAK to DRB, however, suggests that TAK may function early during elongation. Recent studies that have investigated the sensitivity of transcription to DRB have concluded that DRB functions after the initiation of transcription but has no effect on elongation complexes that have transcribed more than 500 nucleotides (12, 39). Using extracts from *Drosophila* cells, Marshall and Price (49) have identified two classes of RNAP II complexes, abortive and productive. They have presented evidence that DRB functions by inhibiting a factor, P-TEF, that is required to convert abortive elongation complexes into complexes that are competent to transcribe full-length transcripts. Recently, it has been proposed that a mammalian P-TEF may be the DRB-sensitive mediator of Tat stimulation of transcriptional elongation (33). It is possible that TAK is this factor.

*In vitro* transcription experiments have demonstrated directly that Tat-1 stimulates elongation of the HIV-1 long terminal repeat through the formation of more-processive elongation complexes (38, 47). Two distinct classes of RNAP II complexes, a more-processive complex and a less-processive complex, have been reported to initiate from the HIV-1 promoter, and it has been proposed that Tat increases the relative proportion of highly processive elongation complexes (44, 48). This Tat-dependent increase in the abundance of more-processive complexes was preferentially inhibited by DRB. Consistent with the effects of DRB on TAK, Marshall and Price (49) reported that productive elongation complexes were inhibited by a DRB concentration of as low as 1  $\mu$ M, while abortive complexes were resistant to a DRB concentration of up to 50  $\mu$ M.

**Model of Tat transactivation.** Two models for the mechanism of action of Tat may be proposed on the basis of the work presented here and the published results of others (Fig. 8); these models are not mutually exclusive. In the absence of Tat, abortive elongation complexes are formed on the HIV promoter, resulting in the production of short, abortive transcripts (Fig. 8A). The role of Tat in both models proposed here is to recruit TAK to the TAR RNA element. TAK then phosphorylates the CTD of the large subunit of RNAP II, resulting in the formation of a more-processive elongation complex. The models differ with respect to the timing of the phosphorylation event. In the first model, TAK phosphorylates the CTD while the RNAP II preinitiation complex is still present on the promoter (Fig. 8B). This could increase the rate of transcriptional initiation as well as the processivity of elongation. In this model, the effects of Tat on initiation and elongation are coupled and the initiation complexes that form in the presence of Tat are processive for elongation. This model is consistent with the work of Yankulov et al., which suggested that the processivity of elongation complexes is determined during the initiation phase of transcription (74). In the second model, abortive elongation complexes initiate from the HIV long terminal repeat and become phosphorylated on the CTD by TAK after they transcribe through TAR RNA (Fig. 8C). This modification is proposed to convert abortive elongation com-

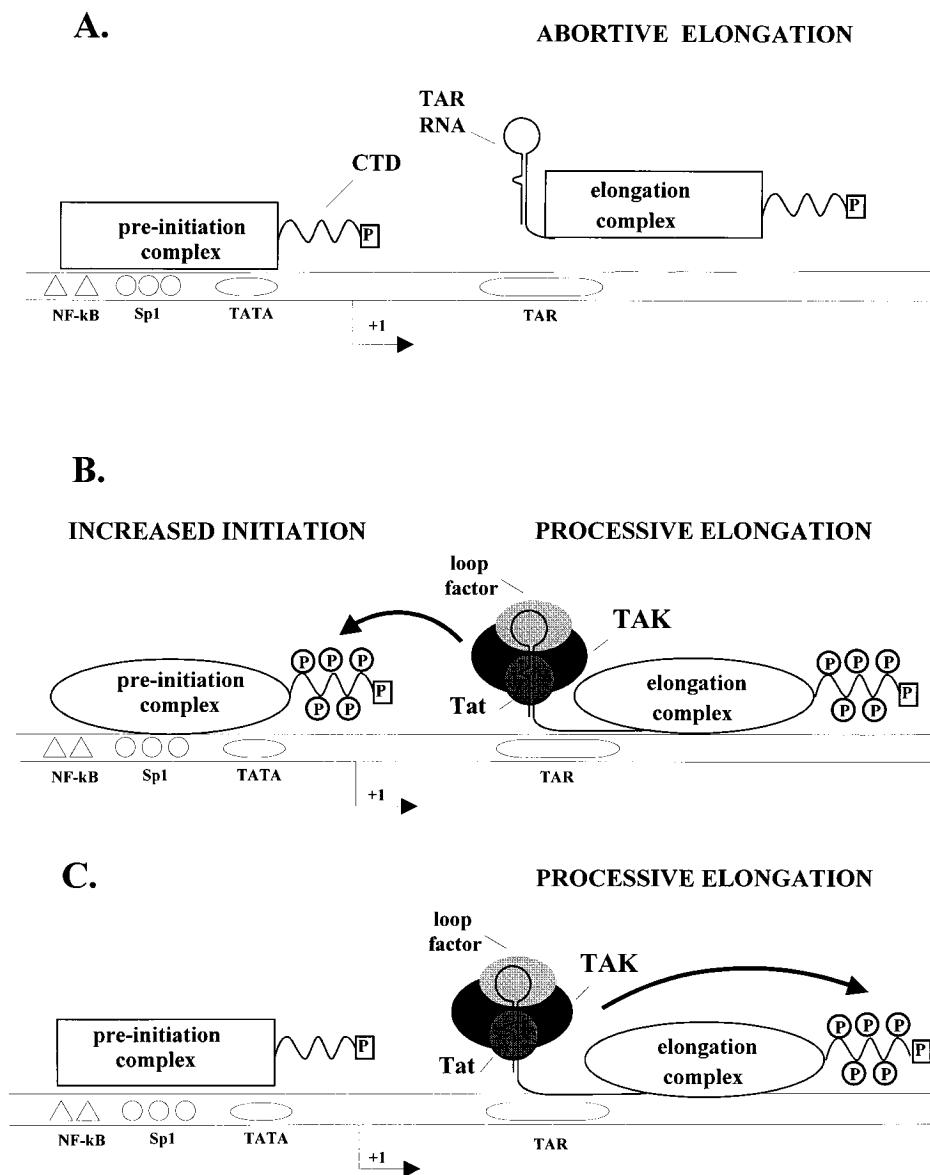


FIG. 8. Models of Tat transactivation. (A) In the absence of Tat, transcription complexes that initiate on the HIV long terminal repeat may be phosphorylated by a template-bound CTD kinase at a limited number of sites. This phosphorylation event may trigger the release of the preinitiation complex from the promoter to begin active elongation. Complexes formed in the absence of Tat are not highly processive for transcriptional elongation, and predominantly short, abortive transcripts are produced. (B) It is proposed that Tat recruits TAK to the TAR RNA element. Once bound to TAR RNA, TAK phosphorylates the CTD of the large subunit of RNAP II in the preinitiation complex. This may lead to an increase in transcriptional initiation as well as the formation of complexes that are highly processive for elongation. An increased number of transcripts and a higher proportion of full-length transcripts would be generated. (C) In this model, the transcription complexes that initiate from the HIV promoter are not highly processive for transcriptional elongation. Early during elongation of HIV RNA, it is proposed that the transcription complexes become modified by phosphorylation on the CTD by TAK, which has been recruited to TAR RNA by Tat. This modification results in the formation of highly processive transcription complexes, resulting in increased production of full-length transcripts. Depending on basal promoter activity, the mode of action of TAK depicted in panel B or panel C may predominate. These models do not imply the specific site(s) or number(s) of phosphorylation events on the CTD but simply suggest that an additional phosphorylation event occurs in the presence of TAK that increases the processivity of the elongation complex. The protein referred to as loop factor may represent an RNA binding domain of TAK or a distinct cellular protein. Square P, phosphorylation by a template-bound kinase; circle P, phosphorylation by TAK; rectangle, nonprocessive elongation complex; oval, highly processive elongation complex.

plexes to processive complexes, leading to an increase in the abundance of full-length transcripts. This model is consistent with TAK functioning analogously to P-TEF (see above). The latter model may provide an explanation for the proposed modification of transcription complexes that occurred during transcription through the TAR RNA region as suggested by the results of Graeble et al. (26). Consistent with this model, a

recent report has correlated hyperphosphorylation of the CTD with the passage of paused polymerase complexes to elongationally competent complexes on the *Drosophila* hsp70 gene (55).

The point at which phosphorylation of the CTD by TAK occurs could be influenced by the basal level of transcription. There have been several reports that the effects of Tat on elongation are predominant over initiation effects when there

is a high level of basal transcription (24, 40, 42). Under conditions of low basal promoter activity, phosphorylation of the CTD by TAK may occur primarily during the transcriptional initiation phase (Fig. 8B); under conditions of high basal transcription, phosphorylation of the CTD may occur predominantly after elongation has begun (Fig. 8C).

Recently, it was reported that Tat is capable of interacting directly and specifically with basal transcription factor TFIID (37). TFIID has also been shown to associate specifically with the nonphosphorylated form of the CTD (71). Therefore, it is conceivable that Tat, TAK, and TFIID may exist in a multi-subunit complex. Alternatively, and perhaps more likely, Tat may interact with TFIID and TAK independently at different points during the transcription process. One possibility is that the effects of Tat on transcriptional initiation might be exerted through TFIID, while the stimulation of elongation by Tat might be mediated by TAK. It is also possible that modification of another component of the transcription complex by TAK or a distinct cellular factor may be important for Tat transactivation.

Genetic experiments suggest that a cellular factor required for Tat transactivation binds to the loop region of TAR RNA. It is not presently known whether TAK is this cellular factor, since the experiments presented here were conducted in the absence of TAR RNA. In the models shown in Fig. 8, the protein referred to as loop factor could be an RNA binding domain of TAK or a distinct cellular factor. On the basis of a genetic analysis, Madore and Cullen (46) hypothesized that the interaction of Tat with a cellular factor was prerequisite for binding to TAR RNA. Tat clearly interacts with TAK in the absence of TAR RNA. It remains possible, however, that the interaction between Tat and TAK is stabilized by binding to TAR RNA, especially in the case of Tat-1. It will be of interest to determine whether TAK is capable of binding TAR RNA, either independently or in conjunction with Tat.

**Implications of CTD phosphorylation for transcriptional elongation.** The models depicted in Fig. 8 are consistent with the notion that RNAP II is regulated by multiple phosphorylation events on the CTD that occur in a precise temporal manner (19). Phosphorylation by a template-associated kinase may be required for the release of the initiation complex from the promoter, although as in the case of HIV long terminal repeats, these complexes may be abortive for elongation. An additional phosphorylation event (in this instance, by TAK) may be necessary for the formation of highly processive complexes. Although this second phosphorylation event could occur while the RNAP II complex is on the promoter (Fig. 8B) or at a subsequent point (Fig. 8C), the result would be the formation of more-processive complexes. It is further possible that the extent of phosphorylation correlates with the processivity of the elongation complex. Since the shift in mobility of the CTD by SDS-PAGE does not reflect the degree (number of phosphorylated sites) or specificity (serine and threonine versus tyrosine) of CTD phosphorylation, it is difficult to distinguish between different hyperphosphorylated forms of the CTD in elongating complexes. This model predicts, however, that highly processive elongation complexes are phosphorylated at additional sites relative to less-processive complexes. Because other strong transcriptional activators, such as the herpes simplex virus type 1 VP16 protein and the adenovirus E1A protein, appear to be able to stimulate elongation (74), hyperphosphorylation of the CTD may be a mechanism for increasing the processivity of elongation that is not unique to lentivirus Tat proteins.

#### ACKNOWLEDGMENTS

We are grateful to R. Gaynor and colleagues for SF2 GST-Tat-1 constructs, D. Derse for E-Tat constructs, and B. Dynan and S. Peterson for the GST-CTD construct. We thank W. Harper for generously providing the cdk2/cyclin A kinase. We also thank Moses Gold for construction of the GST-E-Tat core + T mutant, R. Javier and S. Marriott for comments on the manuscript, and D. Derse for helpful discussions.

This research was supported by NIH grant AI25308 (A.P.R.) and NIH postdoctoral fellowship AI08663 (C.H.H.). Some DNA sequencing of plasmid constructs was performed in the Nucleic Acid Core Laboratory (supported by NIH grant U01 AI30243).

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