HIV-1 Tat Protein Interacts with Mammalian Capping Enzyme and Stimulates Capping of TAR RNA*

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HIV gene expression is subject to a transcriptional checkpoint, whereby negative transcription elongation factors induce an elongation block that is overcome by HIV Tat protein in conjunction with P-TEFb. P-TEFb is a cyclin-dependent kinase that catalyzes Tat-dependent phosphorylation of Ser-5 of the Pol II C-terminal domain (CTD). Ser-5 phosphorylation confers on the CTD the ability to recruit the mammalian mRNA capping enzyme (Mce1) and stimulates its guanylyltransferase activity. Here we show that Tat spearheads a second and novel pathway of capping enzyme recruitment and activation via a direct physical interaction between the C-terminal domain of Tat and Mce1. Tat stimulates the guanylyltransferase and triphosphatase activities of Mce1 and thereby enhances the otherwise low efficiency of cap formation on a TAR stem-loop RNA. Our findings suggest that multiple mechanisms exist for coupling transcription elongation and mRNA processing.

mRNA processing plays an important role in the expression of eukaryotic genes, and the earliest modification event is the formation of the 5′-terminal m7GpppN cap. Capping entails a series of three enzymatic reactions: (i) RNA triphosphatase removes the γ phosphate from the 5′-triphosphate end of the nascent mRNA to form a diphosphate terminus; (ii) RNA guanyltransferase transfers GMP from GTP to the diphosphate RNA terminus to form GpppRNA; and (iii) RNA (guanine-7) methyltransferase adds a methyl group to the N7 position of the cap guanine (1). RNA capping is essential for cell growth, transcription elongation and mRNA processing.

The CTD is unique to Pol II and consists of a tandemly repeated heptapeptide motif with the consensus sequence YSPTSPS that is differentially phosphorylated during the transcription cycle (22). Phosphorylation of the CTD correlates with the release of preinitiation complexes from the promoter and recruitment of the capping enzyme to the transcription elongation complex. In the budding yeast Saccharomyces cerevisiae, the guanylyltransferase (Ceg1) and methyltransferase (Abd1) bind directly to the phosphorylated CTD (15, 17). The mammalian capping enzyme, Mce1, a bifunctional 597-amino acid polypeptide with both RNA triphosphatase and guanylyltransferase activities, binds to the phosphorylated CTD but not to an unphosphorylated CTD (15, 16, 19, 21). Binding to CTD phosphorylated at Ser-5 of the YSPTSPS heptad stimulates the guanylyltransferase activity of Mce1 (21, 23). Although interaction between Pol II and capping enzymes offers an elegant explanation of the specific targeting of capping enzyme to nascent pre-mRNAs, it is conceivable that other factors are also involved in linking capping to transcription. For example, it was reported that hSpt5, the human homolog of yeast elongation factor Spt5, interacts directly with the mammalian capping enzyme and stimulates its guanylyltransferase activity (23). hSpt5 also plays a role in Tat transactivation of HIV-1 gene expression at the level of transcription elongation (24, 25).

HIV-1 Tat is a small RNA-binding protein required for efficient transcription of HIV genes. Tat binds specifically to a structured RNA element, TAR, located at the 5′-end of the nascent HIV transcript. Tat contains two important functional domains: an arginine-rich region that mediates the binding of Tat to TAR RNA and an activation domain that mediates interactions with cellular factors. Tat functions through TAR to control an early step in transcription elongation that is sensitive to protein kinase inhibitors and requires the Pol II CTD (26). Tat increases the processivity of RNA polymerase complexes that would otherwise prematurely terminate. This function of Tat is predicated on its ability to enhance the activity of a positive transcription elongation factor, P-TEFb (27).

Components of the P-TEFb complex required for its activity include a catalytic protein kinase subunit Cdk9 (previously known as PITALRE) (28, 29) and regulatory subunits cyclin T1, cyclin T2a, or cyclin T2b, which associate with Cdk9 and increase its kinase activity (29–31). Cyclin T1 interacts directly with the activation domain of Tat. When the proteins are bound to TAR RNA, Tat interacts with the bulge region, whereas cyclin T1 binds to the loop segment (31). Phosphorylation of the

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§ The abbreviations used are: Pol II, RNA polymerase II; HIV, human immunodeficiency virus; Mce1, mouse capping enzyme; CTD, C-terminal domain; HA, hemagglutinin; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
Pol II CTD by P-TEFb kinase is stimulated by Tat and leads to the formation of processive transcription elongation complexes. Because Tat transactivation and capping are both correlated with CTD phosphorylation at an early stage of transcription elongation, it is conceivable that Tat may interact physically or functionally with mammalian capping enzyme. Here, we show a direct association between Tat and Mce1 in vitro. We find that Tat stimulates mRNA capping in vitro by enhancing the triphosphatase and guanylyltransferase activities of Mce1. Moreover, Tat stimulates the capping of TAR mRNA, which is not guanylylated efficiently by Mce1, presumably because the 5′-terminus is encompassed within a stable RNA hairpin. We suggest a model whereby Tat stimulation of TAR mRNA capping contributes to the activation of HIV gene expression.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Wild-type and Mutant Tat—Recombinant wild-type HIV-1 Tat, hemagglutinin (HA)-tagged Tat, and Tat deletion mutants were expressed in Escherichia coli as glutathione S-transferase (GST) fusion proteins. These fusion proteins consisted of an N-terminal GST moiety followed by a thrombin cleavage site and variable C-terminal polypeptides segments comprising wild-type Tat(1–86), or without HA-tag or Tat(22–86) (a deletions of 4 amino acids 2 to 36 in the transduction domain); Tat48A (a deletion of amino acids 49–86 including the RNA binding domain). Recombinant fusion proteins were purified to apparent homogeneity from bacterial lysates by glutathione-Sepharose affinity chromatography. Briefly, lysates were mixed with a 1:ml slurry of glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were then washed four times with 20 ml of binding buffer A (50 mM NaCl, 2.6 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4) containing 1% Triton X-100, 1 mM EDTA, and 50 μg/ml phenylmethylsulfonyl fluoride. The immobilized GST-Tat beads were used in protein binding assays described below. Alternatively, the Tat proteins were recovered from glutathione-Sepharose beads by thrombin cleavage according to previously described procedures (47). The eluted Tat proteins were stored in buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, and 2.5 mM CaCl2 at −80 °C.

Recombinant Capping Enzymes—Full-length mouse capping enzyme Mce1, the N-terminal RNA triphosphatase domain Mce1-(1–210), and the C-terminal RNA guanylyltransferase domain Mce1-(211–597) were produced in bacteria as N-terminal His-tagged fusions and purified as described (19).

In Vitro Assay of the Binding of GST-Tat to Mammalian Capping Enzyme—Reaction mixtures containing 3–5 μg of the wild-type mutant (Δ22/66, 48A) GST-Tat proteins bound to glutathione-Sepharose beads and 1 μg of the wild-type or mutant Mce1 proteins in 300 μl of binding buffer (20 mM Tris-HCl, pH 7.9, 1% Triton X-100, 0.5% Nonidet P-40, 5 mM DTT, 0.2 mM ZnCl2, 0.1% bovine serum albumin) supplemented with protease inhibitor mixture (Amersham Pharmacia Biotech). The beads were washed six times with 1 ml of binding buffer A, and the bound proteins were then stripped from the beads by boiling in 25 μl of SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 12% glycerol, 4% SDS, 100 mM DTT, and 0.01% Comassie Blue G-250). Polypeptides on the gels were separated on 8% polyacrylamide gels. The gel contents were transferred to a polyvinylidene difluoride membrane, and the protein was detected by immunoblotting with a biotin-conjugated antibody against the HA tag. The blot was developed using a chemiluminescence kit.

Guanylyltransferase Assay—Guanylyltransferase activity of capping enzyme was assayed by the formation of the covalent enzyme-GMP intermediate. Reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 5 mM MgCl2, 1.25 μl [γ-32P]GTP, and capping enzyme and Tat proteins as specified were incubated for 15 min at 37 °C. The reaction was halted by addition of SDS to 1% final concentration. The reaction mixtures were analyzed by SDS-PAGE. Capping enzyme and Tat proteins as specified were visualized by autoradiography and quantified by scanning the gel with a phosphorimager.

RNA Triphosphatase Assay—RNA triphosphatase activity was assayed by liberation of P32 from [γ-32P]GTP-labeled poly(A) RNA synthesized by T7 RNA polymerase transcription. The template strand encoded the sequence for poly(A) RNA starting with G at position +1. In vitro transcription reactions were carried out as described previously (48) in the presence of [γ-32P]GTP. 5′-GTP-terminated 29-mer poly(A) was purified on a 15% polyacrylamide, 7 μm urea denaturing gel. RNA triphosphatase reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 10 μM [γ-32P]GTP-terminated poly(A), capping enzyme and Tat proteins as specified were incubated for 15 min at 37 °C. The reaction was halted by addition of 1 μl of 88% formic acid. Aliquots of the mixtures were applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.75 m potassium phosphate (pH 4.3). The release of P32 was quantified by scanning the TLC plate with a phosphorimager.

RNA Capping Assay—Triphosphate-terminated 17-mer RNA with no apparent secondary structure and TAR RNA containing a bulge and loop region (see Fig. 7A) were prepared by in vitro T7 polymerase transcription and then purified by electrophoresis through a 20% polyacrylamide, 7 μm urea gel as described previously (48). [32P]GMP was incorporated at internal positions in the RNAs by including [γ-32P]GTP in the transcription reactions. Capping reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM DTT, 50 μM GTP, 2.5 mM MgCl2, 10 pmol of RNA, 20 units of RNase inhibitor (Promega), and capping enzyme and Tat proteins as specified were incubated for 15 min at 37 °C. The reaction was quenched by adding 200 μl of stop solution (0.3 M Tris-HCl, pH 7.5, 0.3 mM sodium acetate, 0.5% SDS, 2 mM EDTA). The mixtures were extracted with phenol/chloroform/isooamyl alcohol (25: 24:1) and then with chloroform. RNAs were recovered by ethanol precipitation and then analyzed by electrophoresis through a 15% polyacrylamide gel containing 7 μm Tris-Borate-EDTA. Labeled RNA products were visualized by autoradiography. The internally labeled capped RNA product migrated more slowly than the uncapped substrate RNA. The extent of capping was quantitated by scanning the gel with a phosphorimager.

RESULTS

Tat Directly Interacts with Mammalian Capping Enzyme in Vitro—To test for interaction between Tat and mammalian capping enzyme, a purified GST-Tat fusion protein was linked to glutathione-Sepharose beads, and the beads were incubated with purified recombinant full-length Mce1. After washing the beads with buffer to remove unbound protein, the bound Mce1 was recovered from the beads by treatment with thrombin, which cleaved the GST-Tat fusion protein between the GST and Tat domains (Fig. 1A). Released Mce1 was detected in the supernatant fraction by immunoblotting with antiserum raised against the C-terminal guanylyltransferase domain (Fig. 1B, lanes 1, 2). Mce1 was not detected in the supernatant when the thrombin cleavage step was omitted (Fig. 1B, lane 3). Alanine mutations of the active site cysteine of the RNA triphosphatase domain of Mce1 (C126A) or the active site lysine of the guanylyltransferase domain of Mce1 (K294A) that abrogate the triphosphatase and guanylyltransferase activities, respectively, did not interfere with the binding of Mce1 to immobilized Tat (Fig. 1B, lanes 6 and 9). These results indicate that mammalian capping enzyme can interact directly with Tat in
vitro independent of the competence of Mce1 to catalyze phosphoryl or nucleotidyl transfer.

The C-terminal Segment of Tat Containing the RNA Binding Domain Suffices for Binding to Mammalian Capping Enzyme—Tat protein can be divided into two major functional domains (Fig. 2A). The transactivation domain (amino acids 1–48) is required for recruitment of cyclin T1 by Tat to the HIV-1 long terminal repeat (LTR) promoter (31). The C-terminal domain (amino acids 49–86) includes a basic region and is required for both RNA binding and nuclear localization of Tat (32). Two truncated versions of Tat, Tat\textsubscript{D2/36} and Tat\textsubscript{48D}, which are deleted in the transactivation domain and RNA binding domain, respectively, were expressed as GST fusion proteins and tested for binding to the guanylyltransferase domain of mammalian capping enzyme, Mce1–211–597. The guanylyltransferase bound to beads containing immobilized wild-type Tat and Tat\textsubscript{D2/36} (Fig. 2B, lanes 3 and 5), but not to Tat\textsubscript{48D} (Fig. 2B, lane 7). Similar results were obtained for binding of full-length Mce1 to the truncated Tat proteins (data not shown). We conclude that the Tat segment from amino acids 37–86 suffices for the binding of capping enzyme and that the transactivation domain per se does not interact with capping enzyme or its guanylyltransferase component.

Tat Binding Stimulates the Activity of Mammalian Guanylyltransferase—Are there functional consequences for the interaction of mammalian capping enzyme with Tat? To address this question, we tested the effects of full-length Tat and truncated Tat derivatives on the guanylyltransferase and triphosphatase activities of Mce1. The 597-amino acid mammalian capping enzyme consists of an N-terminal triphosphatase domain (amino acids 1–210) and a C-terminal guanylyltransferase domain (amino acids 211–597). The guanylyltransferase component of the enzyme catalyzes two sequential nucleotidyl transfer reactions involving a covalent enzyme-guanylate intermediate (33). In the first partial reaction, nucleophilic attack on the \(\alpha\)-phosphate of GTP by enzyme results in liberation of pyrophosphate and formation of a covalent adduct in which GMP is linked via a phosphoamide bond to the e-amino group of a Lys-294 (16, 19). The nucleotide is then transferred to the 5′-end of the RNA acceptor to form an inverted (5′)-(5′) triphosphate bridge structure, GpppN.
The extent of Mce1-[^32]P[GMP] complex formation during reaction with 1.25 μM GTP was proportional to input protein up to 0.15 μM Mce1 and leveled off as Mce1 was increased to 0.5 μM (Fig. 3A). In the linear range of Mce1-dependence, ~9% of the input protein molecules were labeled with [^32]P[GMP]. Enzyme-[^32]P[GMP] complex formation by 0.05 μM Mce1 was stimulated by Tat and TatΔ2/36, but Tat48Δ had no effect (Fig. 3B). Optimal stimulation (4–5-fold) was attained at a 2:1 molar ratio of Tat to Mce1 (Fig. 3C). The activity of the autonomous guanylyltransferase domain Mce1-(211–597) was similarly stimulated by Tat and TatΔ2/36, but Tat48Δ had no effect (Fig. 3B). Optimal stimulation (4–5-fold) was attained at a 2:1 molar ratio of Tat to Mce1 (Fig. 3C). The activity of the autonomous guanylyltransferase domain Mce1-(211–597) was similarly stimulated by Tat and TatΔ2/36, but not Tat48Δ (data not shown). Tat had no stimulatory effect on enzyme-GMP formation by purified recombinant yeast guanylyltransferase Ceg1 (data not shown). These results indicate that Tat interaction with Mce1 stimulates the guanylyltransferase activity of mammalian capping enzyme and the Tat domain from amino acids 37–86 suffices for this function.

Tat Stimulates the RNA Triphosphatase Activity of Mammalian Capping Enzyme—The RNA triphosphatase domain of mammalian capping enzyme displays extensive amino acid sequence similarity to protein tyrosine phosphatases and dual-specificity protein phosphatases. By analogy to the protein phosphatases, it is proposed that mammalian RNA triphosphatase executes a two-step phosphoryl transfer reaction involving a covalent enzyme-(cysteinyl-S)-phosphate intermediate (19, 34, 35). In the first partial reaction, nucleophilic attack by a cysteine thiolate (Cys-126 in Mce1) on the γ-phosphate of RNA results in release of diphosphate-terminated RNA and formation of a phosphoenzyme. The phosphate is then transferred from Cys-126 to water to release Pi. The phosphoenzyme intermediate has not yet been demonstrated directly. RNA γ-phosphate hydrolysis by 3 nM Mce1 was stimulated 6–8-fold by the inclusion of 6–18
nM Tat (Fig. 4B). Note that Tat by itself had no detectable RNA triphosphatase activity at the highest level of input Tat used in this experiment (data not shown). The remarkable finding was that Tat did not stimulate RNA γ-phosphate hydrolysis by 5 nM of the N-terminal RNA triphosphatase domain Mce1-(1–210), which catalyzed a similar level of basal RNA hydrolysis as 3 nM Mce1 (Fig. 4B).

To investigate whether Tat interacts directly with the RNA triphosphatase domain, we established an affinity chromatography assay using His-tagged capping enzyme domains immobilized on Ni$^{2+}$-agarose beads (Fig. 5A). The beads were incubated with HA-tagged Tat. After washing the beads with buffer to remove unbound protein, the bead-bound material was stripped from the beads with SDS, and the presence of HA-Tat in the SDS-eluate was detected by immunoblotting with antibody directed against the HA tag.

The results presented thus far demonstrate that Tat interacts with the isolated RNA triphosphatase and guanylyltransferase domains of mammalian capping enzyme. Whereas Tat-binding stimulates the guanylyltransferase activity of Mce1 and the C-terminal domain, Tat-stimulation of the RNA triphosphatase reaction appears to occur only in the context of the full-length Mce1.

**Tat Stimulates RNA Cap Formation by Mce1**—The enzymatic addition of an unlabeled cap guanylate to the 5′-end of an internally labeled RNA molecule results in a characteristic slowing of the electrophoretic mobility of the RNA, equivalent to about a 2-nucleotide increase in apparent chain length (36). The change in mobility upon addition of capping enzyme has been used to detect cap formation on RNAs as long as 78 nucleotides (37). Here we studied the complete capping reaction of Mce1 using a synthetic 17-mer triphosphate-terminated RNA substrate that was labeled internally with [32P]GMP (Fig. 6A). Incubation of 0.5 μM RNA substrate with Mce1 in the presence of 50 μM cold GTP and magnesium chloride resulted in transfer of GMP to the 5′-end, yielding a capped species that migrated more slowly than the input substrate RNA (Fig. 6B). Formation of the capped species was dependent on inclusion of GTP in the reaction mixture (not shown). The extent of capping was proportional to input Mce1 and saturated at 0.4 μM Mce1 with about 70% of the input RNA being capped (Fig. 6C). In the linear range of enzyme-dependence, 1 pmol of RNA was capped per pmol of the Mce1.

The level of capping catalyzed by 50 nM Mce1 (1 pmol of input Mce1) was increased 6-fold by Tat (Fig. 6D). The stimulation was Tat concentration-dependent and saturation was attained at a 4:1 molar ratio of Tat to Mce1. An identical capping stimulation profile was observed for the deletion mutant TatΔ2/36 containing an intact RNA binding domain. Tat48Δ
containing the activation domain had no salutary effect on RNA capping (Fig. 6D). The ability of Tat or its component domains to stimulate cap formation by Mce1 correlated perfectly with the capacity to bind to Mce1 (Fig. 3). Tat Enhances TAR RNA Capping—Tat functions through TAR RNA to control an early step in transcription elongation that depends on the Pol II CTD (26, 38). In light of our findings that Tat stimulates mammalian capping enzyme, we envisioned that Tat might enhance the capping of its target TAR mRNA, which might otherwise be inefficiently capped because the 5’-end is encompassed within a stable RNA duplex (39). To test this hypothesis, we prepared internally labeled Non-TAR RNA (17-mer) and TAR RNA (29-mer) substrates (Fig. 7A) and tested them for capping in the same reaction mixtures with limiting Mce1. Capping reactions containing 75 nM Mce1 and 250 nM each of the Non-TAR and TAR substrates were supplemented with increasing amounts of Tat protein and the labeled products were resolved by PAGE (Fig. 7B). In a competitive situation in the absence of Tat (Fig. 7B, lane 2), Mce1 favored the Non-TAR substrate (1 pmol of cap formed) over the TAR substrate (0.3 pmol of cap formed) (Fig. 7C). Tat enhanced capping of both Non-TAR and TAR RNA substrates at a 2:1 ratio of Tat to Mce1 (Fig. 7C). Yet, the relative Tat stimulation of capping of the TAR RNA (5-fold) was greater than that of the Non-TAR substrate (2-fold).

**DISCUSSION**

Selective targeting of caps to Pol II transcripts in vivo is achieved, at least in part, through direct physical interaction of the capping apparatus with the phosphorylated CTD of Pol II. In addition to recruiting capping enzyme to the Pol II elongation complex, the phosphorylated CTD stimulates the guanylyltransferase activity of the mammalian capping enzyme (21). This simple and appealing model for targeting and regulation of capping is belied by the underlying complexity of CTD phosphorylation (and dephosphorylation) and by mounting evidence that regulation of transcription elongation is a key facet of cotranscriptional mRNA processing. The data presented here illuminate a new pathway of capping enzyme recruitment and activation by the HIV Tat protein. Our findings contribute to an emerging picture of how elongation and processing are coupled, especially during HIV gene expression.

**FIG. 6.** Tat enhances RNA cap formation. *A,* sequence of the 17-mer substrate RNA used in capping reactions. *B,* capping reactions contained 10 pmol of internally labeled 17-mer RNA and Mce1 as specified. The radiolabeled reaction products were analyzed by PAGE. An autoradiogram of the gel is shown. The positions of capped and uncapped RNAs are indicated on the left. *C,* the extent of cap formation in (B) is plotted as a function of input enzyme. *D,* effect of Tat on RNA capping. In these reactions, we used 10 pmol of RNA, 1 pmol of Mce1, and increasing concentration of various Tat proteins. Quantitative analysis of Mce1 capping activity in the presence of various Tat sequences.
In mammalian cells, the timely acquisition of the cap may promote subsequent mRNA-specific processing steps (splicing and polyadenylation) and protect the nascent mRNA from exonucleolytic decay. A clear advantage would accrue from a mechanism whereby capping is restricted to Pol II complexes that are committed to productive elongation, insofar as the capping of short transcripts that are subsequently aborted and released would generate a population of non-coding capped RNAs that could compete with bona fide mRNAs in cap-dependent transactions. CTD hyperphosphorylation has often been correlated with the establishment of a stable elongation complex, but there is little information as to the exact nature of the phosphorylation array at any point in the transcription cycle and there is still uncertainty concerning the relative contributions of different CTD kinases (TFIIH and P-TEFb) to the establishment and remodeling of the CTD phosphorylation array. The timing during early elongation of the critical CTD phosphorylation steps that permit capping enzyme recruitment are not well defined and may even vary for different transcription units. Although it is clear that short nascent transcripts (on the order of 30 nucleotides) can be capped, such results reflect the action of the capping enzymes on arrested polymerase elongation complexes, where there is no kinetic competition between ongoing elongation and capping. The RNA size threshold for capping in this experimental setting simply reflects the steric constraints on capping of RNA chains held within the RNA binding pocket of the polymerase. The unimpeded rate of elongation by RNA polymerase (~20 nucleotides/s) is faster than estimates of the rates of the RNA triphosphatase and guanylyltransferase reactions (reviewed in ref. (40)), which sets up a situation in which RNA polymerase might "outrun" the capping enzyme. It is not known whether capping enzyme has a narrow or wide window for action on nascent 5' ends in vivo, i.e. whether capping enzyme would dissociate from the elongation complex after polymerase has proceeded a certain distance down the transcription unit.

Thus, there is advantage in imposing an elongation checkpoint to maximize the opportunity for the capping apparatus to bind the elongation complex. This scenario has been studied most thoroughly for HIV transcription, where the negative elongation factor DSIF induces an elongation block that is overcome by P-TEFb, a cyclin-dependent protein kinase that phosphorylates the CTD (27) (Fig. 8). One key function of the Tat-TAR RNA complex is to recruit P-TEFb to the nascent HIV mRNA through an interaction of the cyclin T1 subunit of P-TEFb with the activation domain of Tat. The CTD kinase function of P-TEFb is essential for Tat-TAR stimulation of HIV transcription, whereas the CTD kinase activity of TFIIH is apparently not critical for overriding the effects of DSIF (41, 42). Indeed, although TFIIH and P-TEFb are both associated with very early HIV elongation complexes halted at position +14, TFIIH dissociates by the time the polymerase moves to position +30 or +36 (prior to elaboration of the TAR site in the nascent RNA), whereas P-TEFb remains associated with the elongation complex at least up to position +79 when TAR is formed (43, 41). The presence of Tat triggers a new round of P-TEFb-catalyzed CTD phosphorylation on elongation complexes at position +79 (41).

An exciting connection between Tat, P-TEFb, and cap formation emerges from the recent report that Tat alters the phosphorylation site-specificity of P-TEFb in the context of the HIV transcription complex. P-TEFb phosphorylates Ser-2 of the CTD heptad in the absence of Tat, but it phosphorylates both Ser-2 and Ser-5 when Tat is present (41). CTD phosphorylation on either Ser-2 or Ser-5 suffices to bind mammalian capping enzyme, but CTD-stimulation of capping activity is specific for the Ser-5-PO4 CTD array (21). Thus, the Tat/TAR/P-TEFb complex helps craft a CTD array that both recruits and activates Mce1.

Here we have shown that Tat spearheads a second and novel pathway of capping enzyme recruitment and activation via a direct physical interaction between Tat and Mce1. Unlike the Tat-P-TEFb interaction, which requires the N-terminal transcriptional domain of Tat, the binding of Tat to capping enzyme is via the C-terminal domain that includes the TAR RNA binding site. The Tat-Mce1 interaction results in a significant stim-

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**FIG. 7.** Tat enhances capping of TAR RNA. A, sequence and secondary structure of TAR RNA (29-mer) used in the RNA cap formation assay. B, capping reaction mixtures (20 µl) contained 50 µM GTP, 75 nM Mce1, 250 nM Non-TAR and 250 nM TAR RNAs and increasing amounts of Tat (0, 1.5, 3, 6, and 9 pmol). The radiolabeled reaction products were analyzed by PAGE. An autoradiogram of the gel is shown. The positions of capped and uncapped TAR and Non-TAR RNAs are indicated by arrows. C, the extent of cap formation is plotted as a function of the Tat/Mce1 molar ratio for Non-TAR RNA (right y axis) and TAR RNA (left y axis).

**FIG. 8.** Emerging connections between CTD phosphorylation, capping, and transcription elongation. See text for details.
Stimulation of mRNA Capping by Tat

The formation of stable RNA secondary structures in which the 5’ end of the HIV transcript is encompassed within a duplex stem may limit access of the 5’-terminal active sites of the capping enzyme (39). Such a stem structure is formed by nascent HIV mRNA even prior to the synthesis of the TAR sequence and, although the secondary structure changes after TAR is formed, the 5’ end remains held within a duplex stem (45). We observed a structured TAR substrate was capped less effectively by purified Mce1 than by RNA with no apparent secondary structure. Although Tat stimulated the capping of both Non-TAR and TAR RNAs, the fold-stimulation of the structured TAR substrate was greater than that of the unstructured transcript. Thus regulation of capping by Tat would have the most impact where capping is inherently weak. Finally, a Tat-dependent enhancement of mRNA cap formation may account for the finding that Tat stimulates the translation of mRNAs synthesized from the HIV transcription unit (46).

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