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# Host-cell positive transcription elongation factor b kinase activity is essential and limiting for HIV type 1 replication

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**ABSTRACT** **HIV-1 gene expression and viral replication require the viral transactivator protein Tat. The RNA polymerase II transcriptional elongation factor P-TEFb (cyclin-dependent kinase 9/cyclin T) is a cellular protein kinase that has recently been shown to be a key component of the Tat-transactivation process. For this report, we studied the requirement for P-TEFb in HIV-1 infection, and we now show that P-TEFb is both essential and limiting for HIV-1 replication. Attenuation of P-TEFb kinase activity either by expression of a dominant-negative cyclin-dependent kinase 9 transgene or through the use of small-molecule inhibitors suppresses HIV-1 gene expression and HIV-1 replication. Inhibition of HIV-1 replication is affected in a manner consistent with a direct and specific effect on P-TEFb and the known functional role of P-TEFb in Tat-activated transcription. Tat-activated expression of HIV-1 genes seems uniquely dependent on P-TEFb, as inhibition of P-TEFb activity and HIV-1 replication can be achieved without compromising cell viability or RNA polymerase II-dependent cellular gene transcription. Selective inhibition of the P-TEFb kinase may therefore provide a novel approach for developing chemotherapeutic agents against HIV-1.**

HIV-1 gene expression and replication require the viral transactivation factor Tat. Transcriptional activation by Tat is manifested on elongating transcription complexes where Tat alleviates an apparent block to RNA polymerase II (pol II) processivity at the HIV-1 long terminal repeat (LTR) promoter. In the absence of Tat, LTR transcripts terminate prematurely. Tat promotes the transition of abortive complexes to processive, elongation-competent complexes, thereby increasing the number of full-length transcripts elongated from the HIV-1 promoter.

The positive transcription elongation factor b (P-TEFb) was identified originally by virtue of its ability to stimulate RNA pol II transcriptional elongation *in vitro* (1, 2). The catalytic subunit of P-TEFb, PITALRE [now renamed cyclin-dependent kinase 9 (CDK9)], is a member of the family of CDKs (3). Recently, P-TEFb was shown to be required for Tat-dependent transcription (4–6). This conclusion is based both on *in vitro* biochemical experiments and transient transfection studies, including the observations that immunodepletion of P-TEFb from extracts competent to support Tat-activated transcription with anti-CDK9 antibodies abrogates Tat-dependent transcription *in vitro* (4, 5) and that transient overexpression of a catalytically inactive CDK9 mutant inhibits Tat-dependent reporter gene expression in intact cells (4, 6). In addition, cyclin T, the regulatory subunit of P-TEFb, binds directly to Tat; the association between Tat, cyclin T, and the HIV-1 TAR RNA element is proposed to facilitate the recruitment of Tat and P-TEFb to the HIV-1 LTR promoter (7). The observation that the interaction between the activation domain of Tat and the cyclin domain of cyclin T promotes cooperative binding to TAR and that this interaction seems to govern the species specificity of Tat further substantiates the importance of P-TEFb in Tat-activated transcription (7–9).

P-TEFb is one of several kinases that can hyperphosphorylate the pol II C-terminal domain (CTD; refs. 1, 5, 10, and 11). Maintenance of the hyperphosphorylated state of the CTD is required for processive, pol II transcriptional elongation (10–12). It is therefore believed that the requirement for P-TEFb in Tat-activation, specifically, and for the stimulation of pol II transcriptional elongation by P-TEFb, generally, is mediated by phosphorylation of the pol II CTD via the P-TEFb catalytic subunit CDK9. Because the CDK9 kinase activity of P-TEFb is required for all biological functions thus far ascribed to the P-TEFb complex, in this manuscript we refer to the P-TEFb kinase as CDK9.

Evidence that the kinase activity of CDK9 is essential for Tat-dependent transcription also derives from the study of kinase inhibitors identified in a random screen for inhibitors of Tat-activated transcription. Structurally discrete compounds initially identified as inhibitors of Tat-activated transcription were later shown to inhibit both CDK9 kinase activity and Tat activation with a high degree of correlation *in vitro* (4). The observation that inhibitors of CDK9 kinase activity can abolish Tat-dependent transcription from the HIV-1 LTR promoter (4) at drug concentrations that do not affect transcription from other pol II promoters suggests that Tat-dependent gene expression may be critically dependent on CDK9.

To test whether the CDK9 kinase can be exploited as a target to inhibit HIV-1 gene expression and HIV-1 replication selectively, we analyzed the effect of chemically or genetically interfering with CDK9 function in cells. The results of these studies substantiate the relevance of CDK9 to HIV-1 gene expression and HIV-1 infection and serve to define CDK9 as a potential target for antiretroviral drug development.

## MATERIALS AND METHODS

**Generation of Stable Cell Lines Expressing CDK9.** Parental cells (Tet-Off) expressing a Tet repressor/VP16 fusion were obtained from CLONTECH. Tet operator response constructs contained either wild-type or mutant (K48M, which contains a single amino acid substitution of methionine for an invariant lysine) FLAG-epitope-tagged CDK9 cDNAs. The parental cells were grown and transfected as described (4), except that after 36 h, complete selection RPMI medium 1640 containing

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Abbreviations: pol II, RNA polymerase II; LTR, long terminal repeat; P-TEFb, positive transcription elongation factor b; CTD, C-terminal domain; CDK, cyclin-dependent kinase; HTLV-1, human T lymphotrophic virus, type I; $CDK9_{mt}$, CDK9 mutant; $CDK9_{wt}$, CDK9 wild-type.

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360 μg/ml hygromycin, 100 μg/ml geneticin (G418), and 2 μg/ml tetracycline was substituted. Cells were plated under limiting dilutions, and resistant colonies were selected. Isolated colonies were expanded and then tested for expression of integrated FLAG–CDK9 by immunoprecipitation of CDK9 from [$^{35}$S]methionine-labeled extracts. Typically, 1–2 × $10^7$ cells were used per $^{35}$S-labeling experiment. Cells were washed three times with PBS, resuspended in 2 ml of serum-free medium, and incubated 1 h in 35-mm dishes; 200 μCi of [$^{35}$S]methionine was added to each well and incubated for 4 h at 37°C. Preparation of cell extracts and immunoprecipitation conditions were as described (4).

***In Vitro* Kinase Assays.** Lysates from 6–8 × $10^7$ cells were prepared and immunoprecipitated with CDK9 antibodies (Santa Cruz Biotechnology) as described (4). Kinase conditions were as described (5), except that a peptide containing four copies of the consensus CTD heptapeptide repeat was used as substrate at a concentration of 0.3 mM.

**Nuclear Run-On Transcription Assays.** Nuclei were prepared from infected cells by using a modification of the protocol described by Dignam *et al.* (13). Cells ($n = 2.5 \times 10^6$) were first washed with 1.0 ml of cold PBS. Cells were then pelleted, resuspended in 200 μl of cold buffer A (10 mM Hepes·KOH, pH 7.9/10 mM KCl/1.5 mM $MgCl_2$/0.5 mM DTT/0.2 mM PMSF), and allowed to swell on ice for 10 min. Cells were lysed by adding Nonidet P-40 to a final concentration of 0.3%, and the nuclei were collected by centrifugation and resuspended at $10^5$ nuclei per μl in nuclei buffer [50 mM Hepes·KOH, pH 7.9/5 mM $MgCl_2$/0.1 mM EDTA/20% (vol/vol) glycerol/5 mM mercaptoethanol] and stored at −80°C.

Nuclear run-on transcription was performed by using a modification of previously described methods (14, 15). Nuclei were transcribed in a reaction containing 25 mM Tris (pH 8.0), 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP, and 80 μCi of a [$^{32}$P]UTP (300 Ci/mmol) at 30°C for 30 min with gentle shaking. The RNA was treated with DNase and proteinase K for 30 min at 42°C. The RNA was then extracted with phenol chloroform, precipitated twice with isopropyl alcohol, resuspended in TES buffer (10 mM Tris, pH 7.4/10 mM EDTA/0.2% SDS), hydrolyzed in 0.2 M NaOH for 10 min in ice, and hybridized to cDNA probes immobilized in nitrocellulose filters. The cDNA probes used in these experiments mapped to TAR, *Gag*, *pol*, and *env* HIV genes and were 76, 489, 475, and 624 bp long, respectively. Filters were quantitated by PhosphoImager analyses, and results were normalized with respect to the uridine content of each probe.

## RESULTS

**CDK9 Inhibitors Block Acute and Chronic HIV-1 Replication.** Structurally discrete compounds previously shown to inhibit Tat-activated gene expression and CDK9 kinase activity selectively *in vitro* (4) were tested for the ability to block both acute and chronic HIV-1 replication in cells. As CDK9 is not required by the human T lymphotrophic virus, type I (HTLV-1) transactivator protein TAX (6), we evaluated the effect of CDK9 kinase inhibitors on chronic HTLV-1 replication as a control for general and/or nonspecific effects on pol II transcription. As shown in Table 1, three distinct CDK9 inhibitors were tested and all effectively blocked HIV-1 replication and viral spread in acutely infected H9 human T lymphoid cells. Inhibition of HIV-1 replication was attained at drug concentrations below those observed to cause cytotoxicity ($IC_{50}$s of 9.0, 2.5, and 1.0 μM vs. $EC_{50}$s of 38, 38, and 25 μM, respectively) and within the concentration range previously shown to inhibit Tat-activation and CDK9 kinase activity (4).

For each compound, doses observed to inhibit HIV-1 viral spread in the absence of cytotoxicity (10–20 μM) then were used to analyze the effect of CDK9 inhibitors on virus production from cells chronically infected with either HIV-1 or HTLV-1. As would be expected for inhibitors of HIV-1 gene expression, the CDK9 inhibitors attenuated the production of HIV-1 from persistently infected Molt-IIIB cells (Fig. 1*A*). At the concentrations used in this experiment, a >90% reduction in HIV-1 p24 viral antigen was observed and sustained over the course of a 3-day assay. In contrast, at these same concentrations, the CDK9 inhibitors had no effect on HTLV-1 p19 viral antigen production from chronically infected MT2 cells (Fig. 1*B*). The observation that Tat-dependent HIV-1 replication can be effectively blocked by CDK9 inhibitors in the absence of a discernable effect on TAX-dependent HTLV-1 production or gross cytotoxicity suggests a strict and selective requirement for CDK9 kinase activity in Tat-dependent HIV-1 gene expression.

Table 1. Inhibition of HIV-1 replication in acutely infected cells by CDK9 kinase inhibitors

| | $IC_{50}$, μM | | |
|---|---|---|---|
| Compound | CDK9 | Spread (H9) | Cytotoxicity (H9) |
| DRB | 3.0 | 9.0 | 38 |
| T172298 | 0.9 | 2.5 | 38 |
| T276339 | 4.0 | 1.0 | 25 |

The $IC_{50}$ of viral production in H9 human T lymphoid cells was determined as described (19). The cytotoxicity column shows the concentration of compound at which the first signs of cellular toxicity can be detected. Effects on cellular viability, growth, or morphology were scored as cytotoxic effects. The $IC_{50}$ values of ribofuranosyl benzymidazole (DRB), benzymidazole (T172298), and isoxazole (T276239) in a CDK9 *in vitro* kinase assay have been published (4) and are included to facilitate comparison of the relative potency of these compounds in both assays.

**Stable Expression of Catalytically Inactive CDK9 Does Not Affect Cell Viability and Has a Dominant-Negative Effect on P-TEFb Kinase and Tat-Dependent Transcription.** Although cytotoxicity was observed for each of the CDK9 inhibitors when the compounds were tested at higher concentrations (Table 1), the latter effects are likely due, at least in part, to a lack of absolute selectivity for these particular inhibitors (4) and interference with other cellular kinases. However, it is possible that the observed cytotoxicity might be a function of the level of CDK9 inhibition and that partial but not complete inhibition of CDK9 function can be tolerated by cells. To study the requirement for CDK9 kinase activity on HIV-1 replication and gene expression vs. cellular gene expression and viability more directly, we constructed Jurkat cell lines stably expressing either a catalytically inactive CDK9 transgene ($CDK9_{mt}$) or a wild-type CDK9 transgene control ($CDK9_{wt}$). The CDK9 mutant contains a single amino acid substitution of methionine for an invariant lysine residue (K48M) in subdomain two of the catalytic domain. Previously, we showed that transient overexpression of this catalytically inactive CDK9 mutant inhibits Tat-transactivated reporter gene expression (4).

Expression of both the wild-type and mutant CDK9 transgenes was placed under the inducible control of the tetracycline repressor (16). A FLAG-epitope tag was incorporated at the N terminus of both constructs to allow expression of the transgene proteins to be distinguished from endogenous CDK9 (Fig. 2*A* and *B*). Clones expressing the stably integrated CDK9 transgenes were identified by immunoprecipitation of $^{35}$S-labeled cell extracts with anti-CDK9 antibodies (Fig. 2*A*). As expected, anti-CDK9 antibodies immunoprecipitated the expressed CDK9 transgene and the endogenous CDK9 as well as cyclin T1 and a number of other polypeptides (Fig. 2*A*). Although the identity of the latter proteins is currently unknown, their apparent molecular masses suggest that they are related to CDK9-associated proteins that have been described

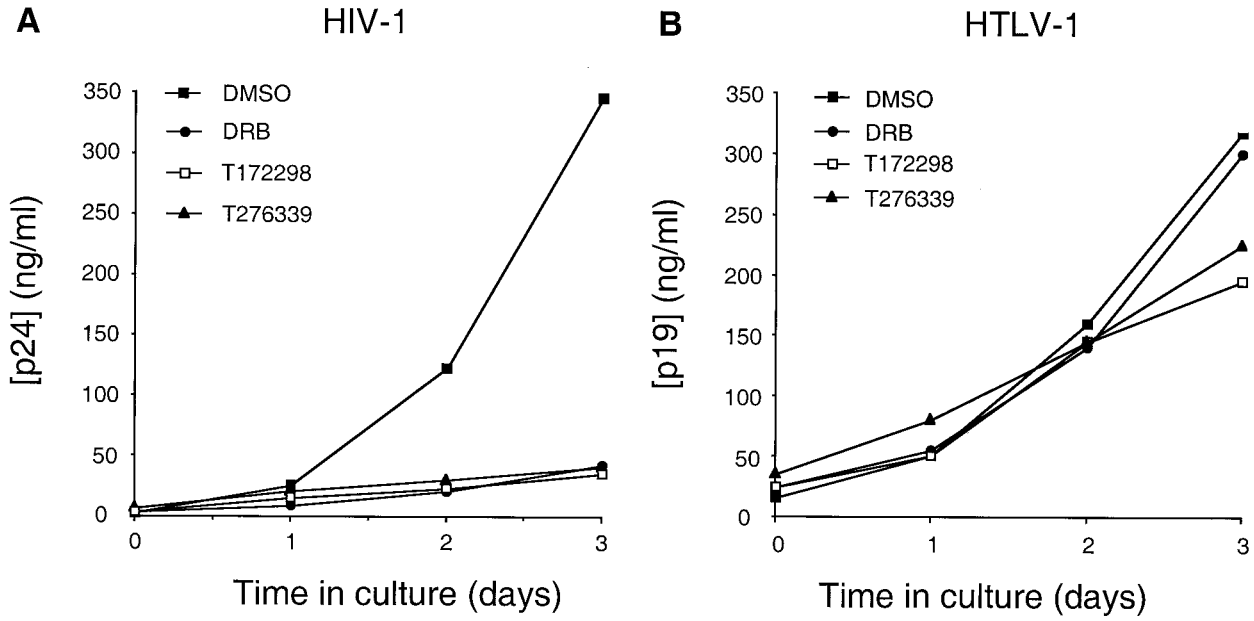


FIG. 1. (*A*) Effect of CDK9 kinase inhibitors on virus production from Molt-IIIB cells chronically infected with HIV-1. Molt-IIIB cells were washed three times in D-PBS (GIBCO/BRL), resuspended in RPMI medium 1640/20% (vol/vol) FBS (2 ml per well; GIBCO/BRL), and seeded in 24-well plates ($1 \times 10^5$ cells per well) in the presence of 0.2% DMSO, 20 $\mu$M DRB, 20 $\mu$M T172298, or 10 $\mu$M T276339. At 0, 24, 48, and 96 h after seeding, plates were centrifuged, and 100 $\mu$l of cell culture supernatant was removed from each well and stored at −70°C. Supernatants from Molt-IIIB cells were assayed for HIV-1 p24 viral antigen production by quantitative ELISA (Coulter) according to the manufacturer's instructions as described (19). (*B*) Effect of CDK9 kinase inhibitors on virus production from MT-2 cells chronically infected with HTLV-1. MT-2 cells were plated, and supernatants were prepared as described above. Cell culture supernatants were assayed for HTLV-I p19 viral antigen production by quantitative ELISA (Cellular Products) according to the manufacturer's instructions. (*A* and *B*) For both HIV-1 and HTLV-1, each data point reflects the mean viral antigen production from duplicate cultures.

(5, 17). Three clones expressing the wild-type CDK9 transgene and two clones expressing the kinase knockout CDK9 transgene were obtained. In each case, under noninduced conditions (i.e., in the presence of tetracycline), constitutive expression of $CDK9_{wt}$ and $CDK9_{mt}$ was observed (Fig. 2*B*). In the absence of induction, both the $CDK9_{wt}$ and $CDK9_{mt}$ FLAG transgenes were expressed in a 1:1 molar ratio with respect to endogenous CDK9 (Fig. 2*B*). The ratio of endogenous CDK9 to FLAG–CDK9 did not change significantly on induction (Fig. 2 *A* and *B*); therefore, the subsequent discussion focuses exclusively on results obtained under induced conditions unless otherwise noted.

As measured by the ability to hyperphosphorylate the CTD of pol II, ≈20% more CDK9-dependent kinase activity was recovered from immunoprecipitated extracts of the $CDK9_{wt}$ cells than from extracts of the parental Jurkat cells (Fig. 2*C*). In contrast, the amount of CDK9 kinase activity recovered from the $CDK9_{mt}$ cells was consistently ≈50% of that recovered from either the parental cells or $CDK9_{wt}$ cells (Fig. 2*C*). Because CDKs depend highly on their respective cyclin regulatory subunit(s), these data suggest that, at least in Jurkat cells, cyclin T may be limiting for CDK9 activity.

Although CDK9 kinase activity was modulated only modestly at the levels of $CDK9_{mt}$ transgene expression achieved in these cells, in transient transfection assays, Tat-activated reporter gene expression (LTR–luciferase) was substantially reduced. As shown in Fig. 2*D*, in the $CDK9_{mt}$ cells where CDK9 activity was reduced by 50%, Tat-dependent expression of luciferase was only 5% of that observed in either the parental cells or the $CDK9_{wt}$ cells (Fig. 2*D*). Although basal HIV LTR promoter transcription also was reduced slightly, the activation by Tat was reduced consistently by ≈10-fold in $CDK9_{mt}$ cells. Interestingly, expression of catalytically inactive CDK9 did not influence cell viability or pol II-dependent cellular gene expression. $CDK9_{wt}$ cells and $CDK9_{mt}$ cells had comparable growth kinetics both in the presence and absence of induction (Fig. 3*A*). Analyses of a human cDNA expression array containing 588 cellular genes (Atlas, CLONTECH) indicated that cellular pol II transcription generally was not affected by expression of either CDK9 transgene (not shown). These results are consistent with the normal phenotype of the cells and the lack of effect observed with CDK9 inhibitors on HTLV-1 replication as described above.

**Expression of a Dominant-Negative CDK9 Transgene Inhibits HIV-1 Replication.** The effective abrogation of Tat-dependent gene expression observed in $CDK9_{mt}$ cells suggests that CDK9 kinase activity is severely limiting for Tat-activated transcription. To investigate whether the diminished level of CDK9 kinase activity in the $CDK9_{mt}$ cells would be sufficient to impose a significant constraint on HIV-1 replication, we compared the ability of HIV-1 to replicate in the $CDK9_{wt}$, $CDK9_{mt}$, and parental Jurkat cells. In one set of experiments, a low multiplicity of infection was used and HIV-1 replication was monitored by p24 viral antigen production at days 1, 3, 5, 7, and 10 after infection (Fig. 3*B*). In a second set of studies, the multiplicity of infection was varied, and viral replication was assessed at days 3, 5, 7, and 10; representative data from day 10 are shown (Fig. 3*C*).

As expected for a productive, spreading viral infection, increasing p24 antigen production was observed on days 5, 7, and 10 in both the parental Jurkat and the $CDK9_{wt}$ cells (Fig. 3*B*). HIV-1 replication in the $CDK9_{wt}$ cells was slightly higher than the parental cell line; at each time point, the $CDK9_{wt}$ cells consistently produced 10–20% more p24 (Fig. 3*B*). In contrast, cells expressing the kinase knockout CDK9 transgene were severely impaired with respect to their ability to support HIV replication. In $CDK9_{mt}$ cells, p24 antigen production was reduced dramatically at each input level of virus, and viral replication remained minimal throughout the entire 10-day study, even at the highest multiplicity of infection (Fig. 3 *B* and *C*, respectively).

**Expression of a Dominant-Negative CDK9 Transgene Attenuates HIV-1 LTR Transcriptional Elongation.** To deter-

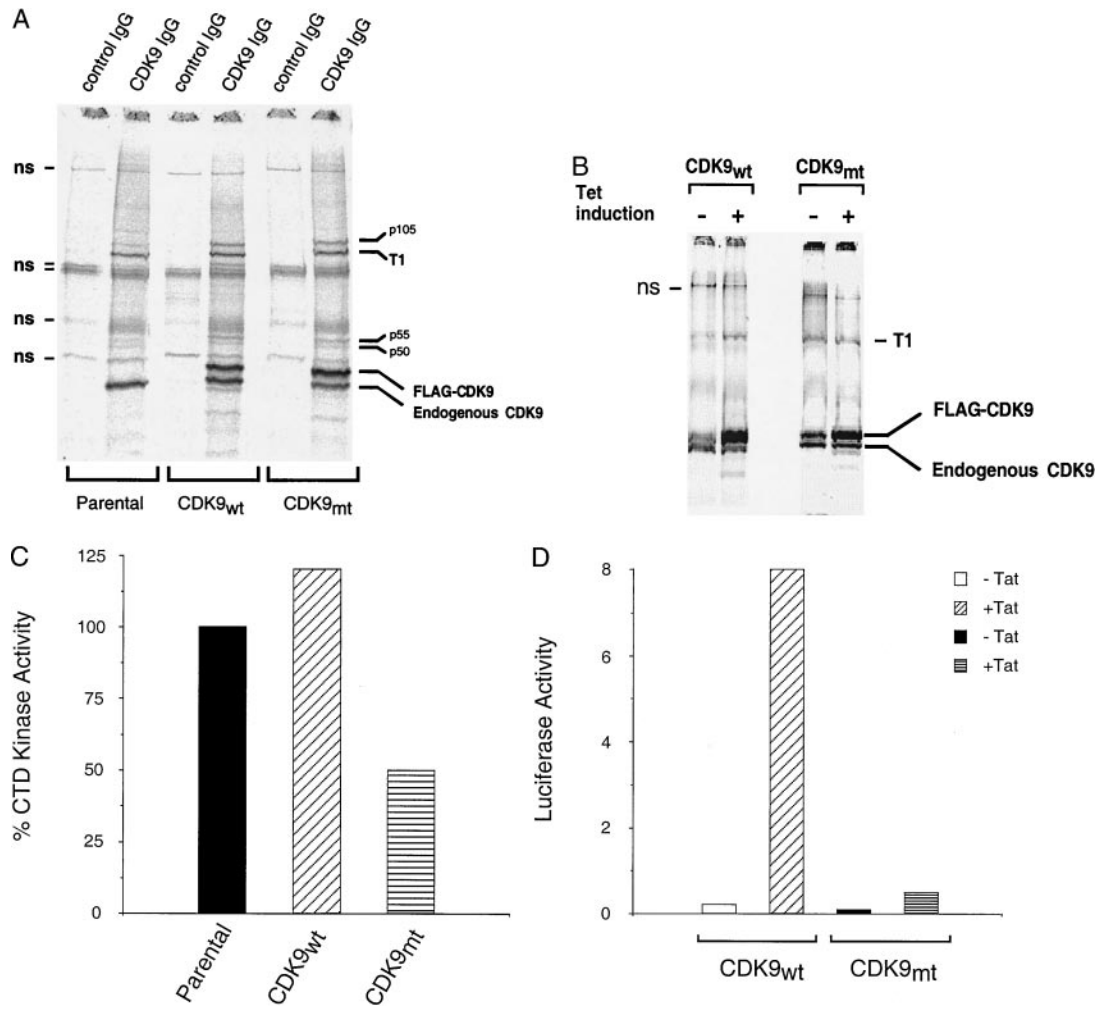


FIG. 2. (*A*) Expression of stably integrated FLAG–CDK9 in $CDK9_{wt}$ and $CDK9_{mt}$ Jurkat cell clones. Parental, $CDK9_{wt}$, and $CDK9_{mt}$ Jurkat cells were labeled with [$^{35}$S]methionine under conditions of induction (in the absence of tetracycline), and cell extracts were immunoprecipitated with anti-CDK9 or control antibodies as noted in the figure. Immunoprecipitates were analyzed by SDS/PAGE and visualized by autoradiography. As indicated, the stable FLAG–CDK9 transgene product migrates slightly higher than the endogenous CDK9. Cyclin T1 and polypeptides (of 105, 55, and 50 kDa) previously reported to coimmunoprecipitate specifically with anti-CDK9 antibodies (Santa Cruz Biotechnology) are indicated. Polypeptides immunoprecipitated by control IgG are also noted (ns). (*B*) Constitutive expression of FLAG–CDK9 in $CDK9_{wt}$ and $CDK9_{mt}$ cells. Cell extracts from [$^{35}$S]methionine-labeled $CDK9_{wt}$ and $CDK9_{mt}$ cells were immunoprecipitated with anti-CDK9 antibodies and analyzed as described above. Extracts were prepared from $CDK9_{wt}$ and $CDK9_{mt}$ cells both in the presence (+) and absence (−) of induction as indicated. (*C*) Extracts from $CDK9_{mt}$ cells have reduced levels of CDK9-dependent CTD kinase activity. Extracts from parental, $CDK9_{wt}$, and $CDK9_{mt}$ cells were immunoprecipitated with anti-CDK9 antibodies. Immunoprecipitates were washed and assayed for pol II CTD kinase activity as described (5) by using a CTD peptide with four heptapeptide repeats as substrate. Immunoprecipitations were normalized by both cell number and protein concentration, and the level of CTD kinase activity in the $CDK9_{wt}$ and $CDK9_{mt}$ cells is expressed as a percentage of the activity recovered from parental Jurkat cells. Nearly identical results were obtained in three independent experiments. (*D*) Tat-dependent transcription is impaired in $CDK9_{mt}$ cells. An HIV LTR–luciferase reporter was transfected either alone or together with a Tat expression vector into $CDK9_{wt}$ and $CDK9_{mt}$ cells. An average of four independent experiments performed in triplicate is presented. (Note: The experiments shown in *A* were performed with three times as many cell equivalents as those shown in *B*.)

mine whether a decrease in LTR-driven transcriptional elongation (i.e., a block of P-TEFb functional activity) was responsible for the block of HIV-1 replication seen in the $CDK9_{mt}$ cells, RNA from HIV-1-infected cells was analyzed by run-on transcription. Cells were infected at a high multiplicity and nuclei were harvested 48 h after infection. Four different probes were used to measure pol II density at various distances from the site of transcription initiation in the HIV-1 LTR. Each RNA signal was quantified and normalized with respect to the promoter-proximal TAR RNA sequence as described in *Materials and Methods*.

Expression of the kinase knockout CDK9 decreased HIV-1 transcription in a manner consistent with an effect on elongation. Promoter-distal HIV RNA transcripts were reduced by >6-fold, and the difference in pol II density between the $CDK9_{wt}$ and the $CDK9_{mt}$ cells increased proportionally with respect to the distance from the start site of transcription (Fig. 4). The finding that HIV-1 transcription is specifically attenuated at the level of elongation in cells expressing the mutant CDK9 is consistent with the observation that $CDK9_{mt}$ cells have lower amounts of functional P-TEFb (Fig. 2*C*) and, thus, cannot efficiently support Tat-activated pol II transcription (Fig. 2*D*) and HIV-1 replication (Fig. 3).

## DISCUSSION

In these studies, we have shown that HIV-1 replication and gene expression have a strict requirement for the CDK9 kinase and a dramatic sensitivity to reduced levels of CDK9 kinase activity. A seemingly modest 50% reduction in CDK9 kinase activity results in a 6- to 20-fold reduction in Tat-activated reporter gene expression and viral transcription (Figs. 2 and 4,

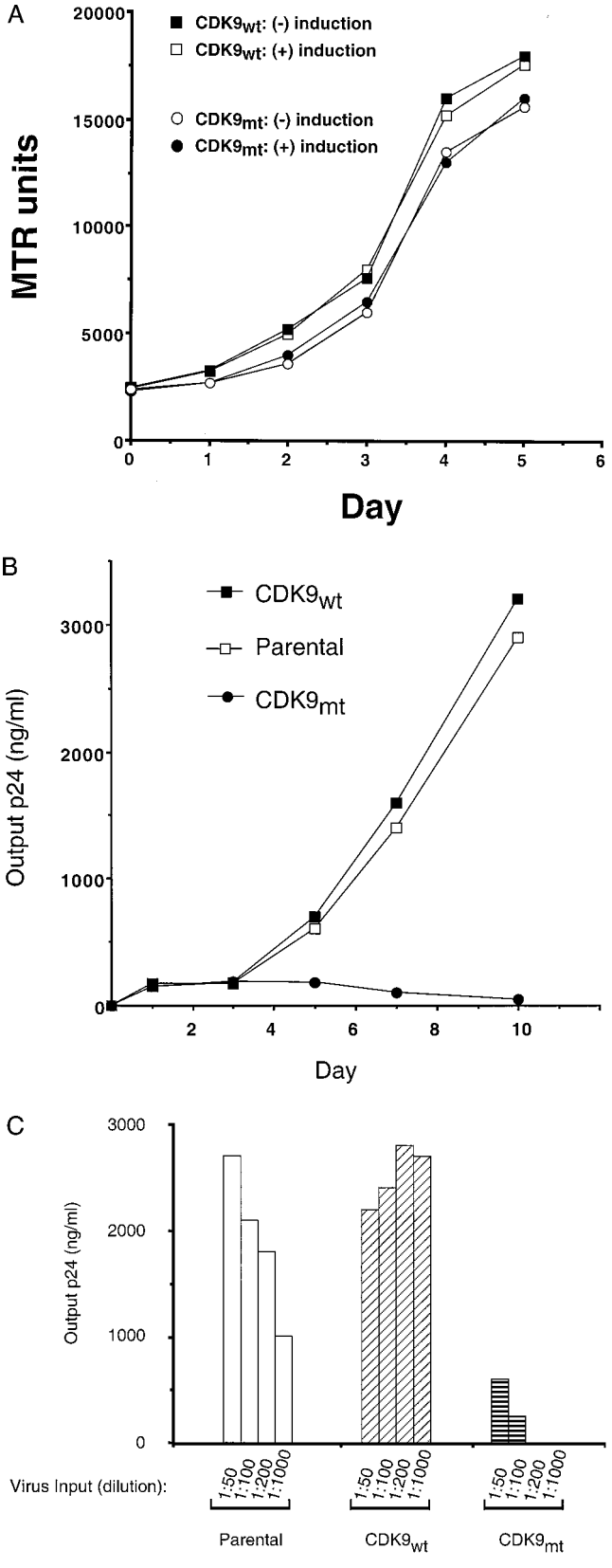


FIG. 3. (*A*) HIV-1 replication and replication of host cells in the presence of constitutive expression of $CDK9_{wt}$ and $CDK9_{mt}$ transgenes. The effect of transgene expression on cell viability was assessed by monitoring the growth kinetics of $CDK9_{wt}$ and $CDK9_{mt}$ cells in the presence and absence of induction (without and with tetracycline, respectively). Cells were plated in 15-cm plates at an initial density of $2 \times 10^4$ cells per ml in RPMI medium 1640 + 10% (vol/vol) FBS. Aliquots were taken daily for 5 days and stained with Alomar Blue in 96-well black luminometer plates. Each sample plate was incubated at 37°C for 3 h to allow incorporation of the stain, and then samples were read on a Cytofluor fluorescence multiwell plate reader (PerSeptive Biosystems, Framingham, MA) to measure the number of viable cells. (*B*) Parental Jurkat, $CDK9_{wt}$, and $CDK9_{mt}$ cells ($n = 1 \times 10^6$ per ml) were infected acutely with HIV-1 pHXB2d (Applied Biosystems, Columbia, MD). HIV-1 replication, quantitated at days 3, 5, 7, and 10 after infection by using 1:200 virus input dilution (125 ng/ml p24), is presented. Virus production was quantified by p24 antigen ELISA assay at days 3, 5, 7, and 10 after infection as described (14). (*C*) Infection was initiated by using virus at 1:50, 1:100, 1:200 and 1:1,000 (500, 250, 125, and 25 ng/ml p24, respectively). p24 antigen production at day 10 after infection for all virus dilutions is shown.

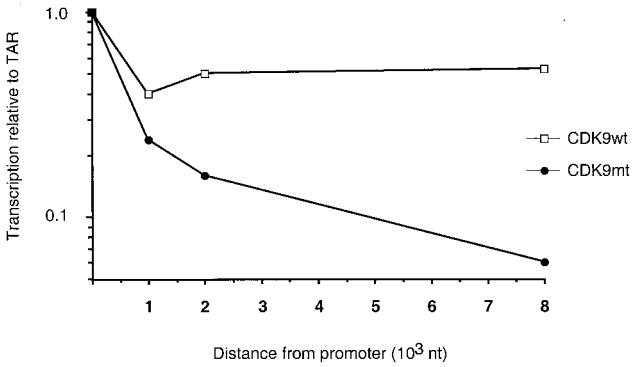


FIG. 4. HIV-1 transcriptional elongation is attenuated in $CDK9_{mt}$ cells. Nuclear run-on experiments were performed with nuclei isolated from $CDK9_{wt}$ and $CDK9_{mt}$ cells infected with a 1:50 dilution of input virus (500 ng/ml p24). Single-stranded DNA probes mapping to *tar*, *gag*, *pol*, and *env* were used to measure pol II density at various distances from the start site of transcription. The amount of viral RNA detected with each probe was normalized with respect to TAR. Numbers 1–8 in the horizontal axis correspond to nucleotides ($\times 10^3$) from the transcription start site. (Note: Similar results were obtained in experiments normalized by using an actin cDNA probe, and no difference in the expression-level actin was detected comparing each of the three cell lines.)

respectively) and a profound suppression of HIV-1 replication. Modulating the level of CDK9 kinase activity in cells either genetically or pharmacologically influences Tat-dependent gene expression in the absence of cytotoxicity and a general effect on pol II transcription, suggesting that CDK9 is limiting for HIV-1 gene expression.

We have shown that expression of a catalytically inactive CDK9 kinase transgene in cells at levels comparable to the level of the endogenous CDK9 exerts a dominant-negative effect on functional P-TEFb (Fig. 2*C*) and Tat transactivation (Fig. 2*D*), as well as HIV-1 gene expression and HIV-1 replication (Figs. 3 and 4). The dominant-negative phenotype observed for $CDK9_{mt}$ with respect to P-TEFb activity has several mechanistic implications for P-TEFb function, cellular gene expression, and Tat-activated transcription. The first implication is that the mutant version of CDK9 retains the ability to assemble with its cyclin T regulatory partner and that the assembly of nonfunctional complexes reduces the number of functional complexes. Second, the observation that the latter effect can be achieved at modest levels of CDK9 transgene expression suggests that cyclin T and its isoforms are limiting in Jurkat cells. Consistent with the above hypotheses, we find that cyclin T1 antibodies coimmunoprecipitate the mutant CDK9 and the endogenous CDK9 from extracts of $CDK9_{mt}$ cells (data not shown). The fact that $CDK9_{mt}$ cells are wild-type with respect to growth indicates that partial inhibition of P-TEFb complexes is well tolerated by most, if not all, transcription units of the host cell. In contrast, Tat activation, HIV-1 gene expression, and HIV-1 replication are markedly

inhibited under the same conditions. These data, together with the evidence that DRB and other CDK9 inhibitors block viral replication at concentrations that are not overtly cytotoxic (Table 1 and Fig. 1*A*), show that P-TEFb activity is limiting for Tat-activated gene expression and can be modulated in cells to inhibit effectively HIV-1 replication without compromising cellular transcription and viability.

The greater requirement of HIV-1 gene expression for CDK9, relative to cellular transcription, likely reflects the fact that the HIV-1 LTR promoter has evolved to produce nonprocessive transcription elongation complexes that are strictly rescued by the viral transactivator Tat. Although the specifics of the mechanism responsible for promoting nonprocessive complexes at the HIV-1 promoter are unclear, DNA elements in the LTR and/or the TAR-dependent pause of pol II (18) may facilitate the association of negative elongation factors (e.g., a CTD phosphatase). At the HIV-1 promoter, the specific recruitment of P-TEFb by the Tat–TAR complex (7–9) is essential for processive elongation and perhaps acts to counteract the action of negative effectors. It is possible that pol II CTD kinases other than P-TEFb can readily substitute for CDK9 in cellular but not HIV-1 transcription because of the highly specific viral mechanism, which has evolved to recruit P-TEFb to the LTR promoter and alleviate the elongation block.

Tat-activated HIV-1 gene expression, in general, and P-TEFb kinase activity, specifically, may represent particularly attractive targets for therapeutic intervention. In the context of a spreading viral infection, the effect of inhibiting Tat-activated gene expression is amplified through a series of cumulative effects, resulting in a dramatic effect on HIV-1 replication. Tat functions as part of a positive feedback loop for HIV-1 LTR transcription and full-length HIV transcripts are used not only as mRNA but also as new viral genomes. Thus, inhibition of Tat-dependent viral transcription simultaneously inhibits the synthesis of viral gene products (including Tat itself) and replication of the viral genome. The cumulative effect of inhibiting LTR-directed transcription therefore translates into a pronounced reduction in the final yield of virus, which is amplified subsequently over multiple rounds of infection.

Recent reports of HIV-1 strains that are resistant to both HIV-1 protease and reverse transcriptase inhibitors emphasize the need for novel therapies and targets that may not be vulnerable to the genetic flexibility of HIV-1. A potential strategy to bypass the ability of HIV-1 to adapt to traditional antiviral therapies is to develop agents that target a cellular factor essential for HIV-1 replication. Future studies aimed at analyzing the host requirement for CDK9 and P-TEFb and the viability of exploiting CDK9 as a therapeutic strategy will therefore be of great importance.

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