Actinomycin D and α-amanitin are commonly used to inhibit transcription. Unexpectedly, however, the transcription of the human immunodeficiency virus (HIV-1) long terminal repeats (LTR) is shown to be activated at the level of elongation, in human and murine cells exposed to these drugs, whereas the Rous sarcoma virus LTR, the human cytomegalovirus immediate early gene (CMV), and the HSP70 promoters are repressed. Activation of the HIV LTR is independent of the NFκB and TAR sequences and coincides with an enhanced average phosphorylation of the C-terminal domain (CTD) from the largest subunit of RNA polymerase II. Both the HIV-1 LTR activation and the bulk CTD phosphorylation enhancement are prevented by several CTD kinase inhibitors, including 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. The efficacies of the various compounds to block CTD phosphorylation and transcription in vivo correlate with their capacities to inhibit the CDK9/PITALRE kinase in vitro. Hence, the positive transcription elongation factor, P-TEFb, is likely to contribute to the average CTD phosphorylation in vivo and to the activation of the HIV-1 LTR induced by actinomycin D.

α-Amanitin and actinomycin D are commonly used inhibitors of transcription. α-Amanitin binds to the largest subunits of RNA polymerase II (RNAP II)1, 2 and RNAP III (3), with RNAP II being the most sensitive. As a consequence, the incorporation of new ribonucleotides into the nascent RNA chains is blocked (4). Actinomycin D is generally thought to intercalate into DNA thereby preventing the progression of RNA polymerases, with RNAP I being the most sensitive (5, 6). In previous work, we have shown that the average phosphorylation of RNAP II C-terminal domain (CTD) increases in cells exposed to actinomycin D (7, 8). The activity of RNAP II is regulated by multisite phosphorylation on the CTD (9). The underphosphorylated CTD mediates multiple protein-protein interactions involved in the assembly of a preinitiation complex. The subsequent phosphorylation of the CTD occurs along the initiation of transcription and contributes to disrupt some of the interactions that lead to the assembly of the preinitiation complex on promoters. Phosphorylation of RNAP II at this step is required to elongate transcription and mediates the recruitment of various enzymatic complexes involved in processing of the primary transcript (10–12). In contrast, phosphorylation of the CTD prior to the formation of the preinitiation complex represses the expression of specific genes (13). Hence, the increase in average phosphorylation of the CTD promoted by actinomycin D raises the possibility that different genes may have different susceptibilities to this drug.

Several cyclin-dependent kinases (CDK) have been shown to phosphorylate the CTD and regulate transcription. CDK7, and its partner, cyclin H, are subunits of the general transcription factor, TFIH, a component of the preinitiation complex (14, 15); CDK8 and its partner cyclin C belong to the RNAP II holoenzyme (16, 17); CDK9/PITALRE, and its partners, cyclins T1 and T2, are subunits of the transcription elongation factor P-TEFb (18). 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is another widely used transcriptional inhibitor (19) that inhibits CDK7 (20) and CDK9/PITALRE (21). The average CTD phosphorylation is decreased in cells exposed to DRB (7), suggesting that these kinases might contribute to global CTD phosphorylation in vivo.

Involvement of the CDK7 and CDK9/PITALRE kinases in transcription is probably best documented for the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) promoter. Transcriptional activation of the HIV LTR at the level of elongation is a key step for the viral replication cycle and has been extensively analyzed (reviewed in Refs. 22 and 23). Like other retroviruses, the HIV can integrate in the cellular genome and remain silent for an indefinite period (24). In the latently infected cells, the basal transcription directed by the HIV LTR is inefficient as most of the transcription initiation events abort approximately 60–80 nucleotides (including the TAR RNA) downstream of the transcription initiation site (25). A great variety of stimulations switch the latent-infected cells to cells producing viral proteins including Tat and viral particles. The Tat protein binds the TAR RNA and activates the transcription directed by the LTR promoter (reviewed in Refs. 26 and 27). Tat binds to a number of components of the basal transcriptional machinery such as TATA-binding protein or the RNAP II holoenzyme (28, 29). Phosphorylation of the CTD assisted by the viral protein Tat is essential in establishing an efficient transcription of the entire viral genome (reviewed in Ref. 30). The Tat protein first facilitates the phosphorylation of the CTD by CDK7. In a second step, Tat recruits the CDK9/PITALRE CTD kinase (21, 31).
Therefor, to investigate whether an enhanced CTD phosphorylation influences the efficiency of actinomycin D and -aminatin on transcription of identified genes, we followed their effect on the expression of a reporter gene driven by the HIV-1LTR or by the human cytomegalovirus (CMV) immediate early promoters. Unexpectedly, both drugs were found to promote an enhanced reporter expression when the corresponding cDNA was placed under the control of the HIV-1LTR promoter. This stimulation is shown to be at the level of elongation of transcription and is suggested to be linked to an enhanced average CTD phosphorylation which may involve the CDK9/PTB/LRE CTD kinase.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The luciferase cDNA was placed under the control of the Rous sarcoma virus (RSV) LTR, pRSVLuc, (32) or the HIV-1 ARV-2 LTR between nucleotides -167 and +46, pHIVLucA41 (33). Plasmid pLTRTTLuc contained the HIV-1 ARV-2 LTR wild type sequences from -644 to +83, plasmid pLTRxA1Luc derived from pLTRTTLuc, and the NF1 site was deleted and replaced by a BstII linker (31). Plasmid pLTR476Luc contained the luciferase cDNA controlled by the HIV-1 ARV-2 LTR wild type sequences from -177 to +83; in the plasmid pLTR361Sp1Luc derived from pLTR476Luc, the three Sp1 sites (75 to -50) have been replaced by the sequence 5'-ATATCGGTCC GGTCTGATCTG CTGGCC. Plasmids pTRXLxL, pTRBLuc, and pLTRBIARLuc contained the luciferase cDNA controlled by the HIV-1 5'LTR between, respectively, nucleotides -644 to +83, -489 to +83, and -489 to +52 (35). Plasmid pHSPLuc contains the luciferase cDNA under the control of the human HSP70 promoter (36), and plasmids pCMVTat and pCMVLuc contain the human cytomegalovirus (CMV) immediate early promoter followed by the cDNAs coding for either the Tat protein or luciferase (35). To generate plasmid pCM/VLuc, the CMV sequences between -224 and -18 (relative to the transcription initiation site) were amplified by PCR from plasmid pHCMVLuc using the primers 5'-GCGATCTGG CAGTCTGATCTG CTGGCC and 5'-CACAAGCCTG ACTGATCTGAC CGCAC GACGCGGCTC TTTGGCGTCT TCCAT, was end-labeled. The amplified fragment was digested with XhoI and PouII and inserted into the unique XhoI and PouII sites of pHIVLucA41. To generate plasmid pH/MVLuc, the pCMVLuc sequences between -18 and +738 were amplified by PCR using the primers 5'-GATATCCG GACGTCGTCGT TCTTATGTT TTTGGCGTCT TCCAT, was end-labeled. The amplified fragment was digested with PouII and BstEI and inserted into the unique PouII and BstEI sites of pHI-IVLucA41. To generate pmTATALuc, the pHIVLucA41 sequences were amplified by PCR using the primers 5'-GCAAAGGACA GCGCTGTCGTC GAGTCGACGCT GACGCGGCTC TTTGGCGTCT TCCAT and 5'-AAGCTGATAT CCTCGAGGAC CCGCTGTCGTC GAGTCGACGCT GACGCGGCTC TTTGGCGTCT TCCAT that replaced the TATA box sequence by GACA. The amplified fragment with XhoI and PouII and inserted between the unique XhoI and PouII sites of pHIVLucA41. All these plasmids were controlled by sequencing the 3' ends of the promoter fragments.

**Cells and Transfection**—Murine Ltk−, NIH 3T3, and human HeLa cells (MRL2 strain) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. The cells were dissolved in 1× Laemmli buffer, and the samples were heated for 5 min at 95 °C before loading on sodium dodecyl sulfate-5% polyacrylamide gels. The RNAP II largest subunit was detected with the POL 3/3 antibody that recognizes an epitope located outside the CTD (43). This monoclonal antibody was visualized by the enhanced chemiluminescence method.

**RNA Extraction and Northern Blots**—Total cellular RNAs were isolated by the guanidinium thiocyanate method. 15 μg of total RNA were denatured 15 min at 65 °C in 6% formaldehyde, run on a 1.2% agarose, 6.3% formaldehyde gel. Equal loading of each lane was checked by ethidium bromide staining. The gels were blotted onto Hybond N nylon membrane (Amersham Pharmacia Biotech) that were UV cross-linked and prehybridized at 65 °C for 1 h in 0.5 M NaHPO4, 1% bovine serum albumin, 1 mM EDTA, and 7% SDS and hybridized overnight at 65 °C to radiolabeled probes in the prehybridization solution. The membranes were washed twice at room temperature in 2× NaCl/citrate buffer (SSC), 0.1% SDS for 1 h and twice at 42 °C in 0.2× SSC, 0.1% SDS for 1 h. The membranes were autoradiographed. Quantification was performed with a Fuji BAS-100 PhosphorImager System. Before reprobing, the membranes were boiled for 10 min in 0.1% SDS to strip off the hybridized probe. DNA probes for luciferase from pRSVLuc (32), cytoplasmic actin from pPAL41 (38), mouse 18S ribosomal RNA from pMISE2 (39), WAF1 (40), and HSC73 from pRC82 (41) were labeled by random priming.

**Primers Extension and DNA Sequencing**—The Alu5 luciferase antisense primer, 5'-CTCTTATGGTT TTGGCGTCT TCCAT, was end-labeled with T4 kinase. 20 μg of total RNA were denatured 10 min at 75 °C and annealed to the primer overnight at 42 °C in 20 μl 10 mM Pipes, pH 6.4, and 400 mM NaCl, overlaid with 20 μl of mineral oil. Nucleic acids were precipitated in ethanol and redissolved in 20 μl of 50 mM Tris-HCl, pH 8.2, 6 mM MgCl2, 10 mM dithiothreitol, 100 μM of each of the four dNTPs, 0.1 unit/μl RNasin (Promega), and 1 unit/μl SuperScript II (Life Technologies, Inc). Reverse transcription (Life Technologies, Inc) extension was performed at 42 °C for 60 min using the Alu5 luciferase primer. Nucleic acids were precipitated in ethanol, redissolved in formamide loading buffer, and run on a 10% denaturating polyacrylamide-urea gel. The dried gels were exposed for autoradiography. DNA sequences were obtained using the Alu5 primer, plasmid pHIVLucA41, and a T7 Sequencing kit (Amersham Pharmacia Biotech).

**Single-strand DNA Probes and Run-on Assays**—The primers GC-CCTCAGATGTCTCAGTATA and CGGCTCAGCTCCTAAGGATG were used to generate the 5' probes (177 base pairs from -42 to +136), whereas the primers CAGCTATCCGACCGC and ATTCGCT-CTTCTGATTACCG were used for the M probe (127 base pairs from +1111 to +1238). These primers were used to amplify DNA fragments by PCR (30 cycles using 0.1 mM dNTP, 0.2 μM primers, 10 μl of mineral oil. The single-strand antisense probes were amplified from these DNA fragments by asymmetric PCR (30 cycles) using only one of the primers.

Run-on assays were performed following established procedures (42). 2 × 106 nuclei were allowed to transcribe in vitro for 20 min at 30 °C in the presence of [α-32P]UTP, non-radioactive ATP, CTP, and GTP with or without -aminatin (0.1 μM), and the resulting RNAs were iso-

**RESULTS**

α-Aminatin and Actinomycin D Activate the HIV LTR in Stably Transfected Cells—To investigate the dose effect of α-aminatin on the expression of a reporter gene under the control of a defined promoter, firefly luciferase activity was followed in lysates from μH6L6 cells exposed to varying concentrations of the drug for 24 h. This clonal cell line was derived from murine Ltk− cells stably transfected with the pCMVLuc plasmid, in which the luciferase cDNA had been placed under the control of an HIV LTR. Unexpectedly, increasing amounts of luciferase activity were found in lysates from μH6L6 cells exposed during 24 h to α-aminatin up to 30 μg/ml (Fig. 1A). The highest stimulation (~28-fold) was achieved at 10 μg/ml, a rather elevated concentration. To extend these observations to other cell systems, we stably trans-
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Transcriptional Inhibitors Enhance HIV LTR-driven mRNA Accumulation—To establish that the increase in luciferase activity reflected an increase in the corresponding mRNA, total RNA was isolated from muHL6b cells incubated with actinomycin D. The luciferase activity in the lysates was strongly enhanced when the cells were exposed 24 h to actinomycin D after transfection (Fig. 2). The strongest effects were obtained around 0.1 μg/ml drug, and higher concentrations led to a drop in activation. The optimal drug concentration was lower than with the related huHL6 cells as the calcium phosphate treatment exacerbated an extensive cell death. The transient transfection assays allowed us to compare different promoters. Therefore, HeLa cells were transfected with plasmids associating the luciferase cDNA to other promoters such as the Rous sarcoma virus LTR (RSV), the cytomegalovirus early promoter (CMV), or the human HSP70 promoter (there was no need to stress the cells to observe a relatively high basal level of HSP70-driven luciferase expression). In the two latter cases, the luciferase activity in the lysates decreased with increasing concentrations of actinomycin D (Fig. 2). However, the Rous sarcoma virus LTR resisted inhibition up to 0.1 μg/ml actinomycin D.

Thus, moderate actinomycin D or α-amanitin concentrations enhance luciferase synthesis in murine and human cell lines stably transfected with a plasmid associating the HIV-1 LTR to the luciferase cDNA. But no stimulation was observed when actinomycin D (0.2 μg/ml) and α-amanitin (10 μg/ml) were added simultaneously (data not shown). This finding as well as the bell-shaped dose-response curves suggests two opposing effects: an activation and an inhibition. At high drug concentrations, the latter overcomes.

Activation by Actinomycin D Is a Characteristic of the HIV LTR in Transient Transfection Assays—The increased expression of the luciferase gene in stably transfected cells might relate to a positional effect in the region of plasmid DNA insertion. Indeed, Tat transactivation of the HIV-1 LTR has been reported to differ for integrated versus unintegrated vectors (44). Therefore, HeLa cells were transiently transfected with the pHIVLucA41 plasmid. The luciferase activity in the lysates was strongly enhanced when the cells were exposed 24 h to actinomycin D after transfection (Fig. 2). The strongest effects were obtained around 0.1 μg/ml drug, and higher concentrations led to a drop in activation. The optimal drug concentration was lower than with the related huHL6 cells as the calcium phosphate treatment exacerbated an extensive cell death. The transient transfection assays allowed us to compare different promoters. Therefore, HeLa cells were transfected with plasmids associating the luciferase cDNA to other promoters such as the Rous sarcoma virus LTR (RSV), the cytomegalovirus early promoter (CMV), or the human HSP70 promoter (there was no need to stress the cells to observe a relatively high basal level of HSP70-driven luciferase expression). In the two latter cases, the luciferase activity in the lysates decreased with increasing concentrations of actinomycin D (Fig. 2). However, the Rous sarcoma virus LTR resisted inhibition up to 0.1 μg/ml actinomycin D.

These results, which were also obtained upon transient transfection of NIH 3T3 cells (not shown), suggest that the effect of actinomycin D is a characteristic of the HIV LTR promoter.

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FIG. 1. Actinomycin D and α-amanitin activate HIV LTR-driven luciferase expression in stably transfected cell lines. Increase in luciferase activity in lysates from muHL6b (A) and huHL6 (C) cells that have been incubated with various concentrations of α-amanitin (A) and actinomycin D (B) for 24 h. C, time course variation of luciferase activity in muHL6b cells incubated with 0.2 μg/ml actinomycin D. An exponential curve fitting is drawn. The luciferase activity in control cells was taken as the unity.

FIG. 2. Actinomycin D activation is a characteristic of the HIV LTR in cell transient transfection assays. HeLa cells were transiently transfected with plasmids containing the luciferase gene under the control of different plasmids as follows: pHIVLucA41 (●), pCMVLuc (○), pRSVLuc (△), and pHS3PLuc (▲). Actinomycin D was added to the cells 24 h after transfection, and the cells were lysed 48 h after transfection. The luciferase activity in control cells was taken as the unity.
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Fig. 3. Northern blot analysis of RNAs isolated from cells treated with actinomycin D and α-amanitin. A, RNAs from muHL6b cells incubated 24 h with varying amounts of actinomycin D (left panel). RNA extracted at different time points from muHL6b cells incubated with 0.2 μg/ml actinomycin D (right panel). B, RNAs from huHL6 cells were incubated 24 h with varying amounts of actinomycin D. C, RNA from muHL6b cells or incubated with 10 μg/ml α-amanitin for 0, 16, and 20 h. The RNAs were blotted on nitrocellulose membranes after agarose gel electrophoresis. The membranes were dehybridized and rehybridized sequentially with radiolabeled probes. The position of the ribosomal RNAs detected by ethidium bromide staining is indicated.

The expected transcription initiation from the termination site on the pHIVLucA41 plasmid and likely corresponded to a full-length luciferase mRNA as the luciferase cDNA coding sequence spans over 1650 bases (32). In muHL6b cells treated for 24 h with actinomycin D, both RNA species markedly increased in a dose-dependent manner (Fig. 3A, left). The highest increase was observed with cells treated with 0.2 μg/ml actinomycin D. At higher concentrations (2 μg/ml), the signal corresponding to luciferase mRNA remained as in the controls and decreased at 20 μg/ml (not shown). The increase in luciferase mRNA was observable after 6 h of exposure to 0.2 μg/ml actinomycin D (Fig. 3A, right). When the muHL6b cells were exposed to α-amanitin, the 2.9 kilobase pairs of luciferase mRNA abundance also increased with time (Fig. 3B).

To evaluate the general transcriptional inhibition, the above-mentioned Northern blots were rehybridized with cellular genes probes. In RNAs prepared from muHL6b cells, the actin and the 70-kDa heat shock cognate (HSC73) probes each detected a single mRNA species with the expected sizes. When the cells were exposed to actinomycin D at concentrations above 0.02 μg/ml, both the actin and HSC73 mRNA abundance decreased indicating that these genes were inhibited (Fig. 3A). The decrease was more pronounced for HSC73 due to the shorter half-life of the corresponding mRNA in murine cells. Both signals were distinctly stronger with 0.2 μg/ml than with 2 μg/ml actinomycin D indicating that for this concentration, which was optimal for luciferase induction, the transcriptional inhibition was not complete. When the muHL6b cells were exposed to α-amanitin, the HSC73 mRNA levels also decreased (Fig. 3B).

When the RNAs were prepared from actinomycin-treated huHL6 cells, a strong increase in luciferase mRNA was also observed, but the minor species was less abundant than in muHL6b cells (Fig. 3C). Very low concentrations of actinomycin D (0.006 μg/ml) have been reported to promote an increase in p21WAF-1 mRNA in MRC5 human fibroblasts (45). However, no reliable changes in p21WAF-1 signals were found in huHL6 cells exposed to actinomycin D in the 0.02 to 0.1 μg/ml range. Primer extension using RNAs prepared from actinomycin D-treated huHL6 cells demonstrated that under these conditions, the luciferase mRNAs were correctly initiated at the usual +1 position (Fig. 4).

Thus, treatments that determine an enhanced luciferase mRNA accumulation lead to a near decrease in housekeeping gene mRNAs indicating a general transcriptional arrest. The increase in luciferase mRNA abundance was more pronounced with actinomycin D than with α-amanitin, as anticipated by luciferase activity determinations.

Increased Transcription Driven by the HIV LTR in Nuclei Isolated from Cells Treated with Actinomycin D—To establish that the accumulation of luciferase mRNA was due to an enhanced transcription of the corresponding gene, run-on assays were performed with nuclei prepared from huHL6 cells untreated or exposed to actinomycin D. The actinomycin D treatment determined a 13-fold increase in the luciferase gene signal (Fig. 5A). In contrast, the actin and HSC73 gene signals remained unaffected, and the ribosomal gene signal decreased more than 100 times as expected from the known high susceptibility of class I gene transcription (5). To demonstrate that the luciferase gene transcription in actinomycin D-treated cells was attributable to RNAP II transcription, the run-ons were also performed in the presence of α-amanitin in the assay (0.1 μg/ml). Addition of α-amanitin to nuclei prepared from control untreated cells did not affect the 18 S rRNA signal as expected.
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since RNA Pol I is insensitive to this compound; however, it completely wiped off the luciferase and the actin signals. The luciferase and actin signals as well as the remaining 18 S signal were also suppressed by addition of α-amanitin to nuclei from actinomycin D-treated cells. First, it should be emphasized that this finding does not conflict the data provided in Fig. 1A as in vitro α-amanitin poisoning of RNAPII II is likely to be fast and complete, whereas in vivo it is a slow, incomplete process controlled by the penetration of the drug (46, 47). Second, an involvement of RNAPII in ribosomal DNA transcription has been shown to occur in yeast cells that lack RNAPI activity (48).

To discriminate between an enhanced initiation of transcription and an enhanced elongation of transcription, the run-ons were repeated using short probes corresponding to either the 5′ end (5′-AS) or the middle (M-AS) of the luciferase gene. When RNAs were obtained from nuclei of untreated cells, 5′-AS probe hybridized much stronger than the M-AS probe (Fig. 5B). When RNAs were obtained from nuclei of actinomycin-treated cells, the 5′-AS probe decreased about 2-fold. But in contrast, the M-AS signal increased 18-fold. This result indicates that, in cells treated with actinomycin D, the transcription of the luciferase gene driven by the HIV-1 LTR is enhanced at the level of elongation.

**HIV LTR Activation by Actinomycin D Does Not Involve the TAR Sequence**—To dissect the promoter elements required for actinomycin stimulation, HeLa cells were transiently transfected with different plasmids containing fragments of the HIV LTR wild type or mutated and exposed or not to actinomycin D for 24 h before lysis. As the HIV LTR transactivation by the viral Tat protein has been extensively investigated, for standardization purposes, the same plasmids were cotransfected with a Tat expression vector in a parallel experiment. The luciferase synthesis driven by the CMV promoter (pCMVLuc) was inhibited by both actinomycin and Tat and was used as a reference (Table I, top part). In contrast, both actinomycin and Tat enhanced the luciferase synthesis driven by the HIV-1 LTR fragments of various lengths and isolates (plasmids pLTRWTLuc, pLTRXLuc, pLTRBLuc, pLTR476Luc, and pHIVLucA41) (Table I, top part). The actinomycin D treatment increased more than 10-fold the amount of luciferase, whereas Tat coexpression led to several 100-fold increases. In this set, the length of the LTR sequences upstream from the TATA box or trimming off the 3′ end nucleotides down to +46 minimally affected the Tat response and did not influence the actinomycin response. In contrast, deletion of the nucleotides downstream +32 (relative to the initiation site), in pLTRbTARLuc, resulted in a dramatic decrease in Tat inducibility (as expected since this deletion impaired the formation of the TAR RNA hairpin) and led to a 2-fold decrease in actinomycin D inducibility. Thus, the 14 nucleotides at the 3′ end of the TAR element (from +32 to +46) that are essential for the Tat response provided a limited contribution to the actinomycin response.

**DNA Sequences Upstream and Downstream from the TATA Box Confer HIV LTR Sensitivity to Actinomycin D**—To dissect further the DNA sequence elements involved in actinomycin D activation, we took advantage of a 10-base pair homology between the HIV and CMV TATA boxes in the core promoter elements (Fig. 6). The HI/MV promoter carried the HIV sequences upstream from the TATA box, and the TATA box fused to the CMV sequences downstream from it. The pHIV/MVLuc plasmid lacked the TAR DNA sequence and was not enhanced in a Tat cotransfection assay as expected (Table I, middle). However, its actinomycin inducibility was close to that of pLTRbTARLuc indicating that the HIV sequences between −19 and +32 were not involved.

The CM/IV chimeric promoter associated the CMV sequences upstream from the TATA box and the TATA box was fused to the HIV sequences downstream from it. The pCM/IVLuc contained the TATA sequence and was Tat-inducible although less efficiently than the parental HIV LTR (Table I, middle). The latter result confirmed a previous observation establishing that the TAR sequences remain functional within heterologous promoters (49). The pCM/IVLuc was actinomycin-inducible but with a lower efficiency than the parental pHIVLucA41 confirming that sequences downstream from the TATA box, overlapping the TAR element, contribute to the response.

**The NFκB Binding Sequences of the HIV LTR Are Not Required for Actinomycin D Stimulation**—Two NFκB-binding sequences, a cluster of three Sp1 elements, and the TATA box are regulatory elements that have been identified within the HIV LTR (22, 23). When the TATA box sequence (plasmid pHIVLucA41) was replaced by GACA (plasmid mTATA), the basal level of luciferase expression was around half that of its parental pHIVLucA41; the Tat stimulation, however, was abolished, and the actinomycin D stimulation was depressed (Table I, lower part). Disruption of the three Sp1 sites in plasmid pLTR361ASp1Luc minimally affected the basal level of expression compared with the corresponding wild type plasmid pLTR476Luc but led to a decreased actinomycin D inducibility, thereby suggesting the involvement of the Sp1-binding sites. The Tat stimulation dropped 10-fold with the Sp1-less plasmid. In plasmid pLTRA8BLuc, both the A and B NFκB sequences have been deleted from the corresponding wild type plasmid pLTRWTLuc. Luciferase synthesis was induced by actinomycin D to a similar extent in cells transfected by either plasmid. Involvement of NFκB in the actinomycin D-induced HIV-1 activation is therefore very limited.

In conclusion, the actinomycin stimulation of the HIV promoter requires the integrity of the HIV TATA box and Sp1 sites but is independent of the NFκB-binding sites.

**Activation of the HIV-LTR Is Sensitive to CDK9/PITALRE**

![Fig. 5. Run-on transcription from huHL6 nuclei and mapping of the transcription initiation site. A, nuclei were isolated from huHL6 cells that had been exposed or not to 0.1 μg/ml actinomycin D (AcD) during 18 h. The nuclei were allowed to transcribe in vitro in the presence of XTPs with (+ a-AM) or without (−) 0.1 μg/ml α-amanitin. In vitro transcribed RNAs were hybridized to linearized plasmids carrying the luciferase (LUC), actin (ACT), 70-kDa heat shock cognate protein (HSC), and ribosomal RNA (18S) sequences. DNA from plasmid pSP64 (SP6) was used as a negative control. B, nuclei were isolated from huHL6 cells that had been exposed (actinomycin D, AcD) or not (−) to 0.12 μg/ml actinomycin D during 8 h. In vitro transcribed RNAs were hybridized to single-strand antisense DNA probes. The 5′-AS probe extends from −42 to +136 relative to the transcription initiation site (30 transcribed uridines), whereas the M-AS probe extends from +1111 to +1238 (30 uridines), in the midst of the luciferase gene. The radioactivity hybridizing to each probe was quantified with a PhosphorImager.
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Table I

Molecular dissection of the DNA sequences involved in actinomycin D stimulation of transiently transfected plasmids

HeLa cells were transfected with the same amounts of luciferase expression vectors with or without plasmid pCMV Tat in 24-well plates. The DNA sequences between -29 and -19 in the HIV LTR and CMV promoters include the TATA box and are identical (Fig. 6). 24 hours after transfection, half of the wells with cells transfected with the luciferase expression vectors alone were exposed to actinomycin D (0.12 μg/ml). 48 hours after transfection, the cells were assayed for luciferase expression. The actinomycin D and Tat stimulations are relative to the untreated cells transfected with the vectors alone (taken as unity). The complete experiment was repeated three times independently, all plasmids were analyzed each time. The stimulations are given as the mean values obtained in the three experiments with groups of four wells. The luciferase activities provided in parentheses in relative light units (RLU) (mean of four wells) have all been obtained in the same experiment with untreated cells transfected with the expression vectors alone.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Actinomycin D stimulation</th>
<th>RLU</th>
<th>Tat stimulation</th>
<th>HIV sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMVLuc</td>
<td>0.45 ± 0.05</td>
<td>(443,000)</td>
<td>0.6 ± 0.1</td>
<td>Only CMV sequences (-522 to +97)</td>
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<tr>
<td>pLTR_{1} Luc</td>
<td>9.8 ± 1</td>
<td>(7,050)</td>
<td>292 ± 60</td>
<td>-644 to +83, ARV2 isolate</td>
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<tr>
<td>pLTR_{X} Luc</td>
<td>12.3 ± 1</td>
<td>(2,600)</td>
<td>644 ± 200</td>
<td>-644 to +83, LAI isolate</td>
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<tr>
<td>pLTR BLuc</td>
<td>11 ± 0.3</td>
<td>(3,100)</td>
<td>371 ± 50</td>
<td>-489 to +83, LAI isolate</td>
</tr>
<tr>
<td>pLTR{747} Luc</td>
<td>11.7 ± 0.7</td>
<td>(12,700)</td>
<td>248 ± 50</td>
<td>-177 to +83, ARV2 isolate</td>
</tr>
<tr>
<td>pHSV_{Luc} A1</td>
<td>12 ± 2</td>
<td>(8,060)</td>
<td>130 ± 30</td>
<td>-167 to +46, ARV2 isolate</td>
</tr>
<tr>
<td>pLTR{23TAR} Luc</td>
<td>4.3 ± 0.1</td>
<td>(24,600)</td>
<td>2 ± 0.5</td>
<td>-489 to +32, LAI isolate</td>
</tr>
<tr>
<td>pHi/MLuc</td>
<td>5.2 ± 2</td>
<td>(36,400)</td>
<td>0.5 ± 0.2</td>
<td>-167 to -19, fused to CMV downstream sequences (-18 to +97)</td>
</tr>
<tr>
<td>pCM/IVLuc</td>
<td>2.1 ± 0.5</td>
<td>(31,300)</td>
<td>22 ± 10</td>
<td>-18 ± 46, fused to CMV upstream sequences (-25 to -19)</td>
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<tr>
<td>pmTATAT Luc</td>
<td>2.9 ± 1</td>
<td>(3,800)</td>
<td>2 ± 1</td>
<td>-167 to +46, TATA box sequence replaced by GACA (pHSV_{Luc} A1)</td>
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<tr>
<td>pLTR{3}B Luc</td>
<td>8.7 ± 1</td>
<td>(2,700)</td>
<td>223 ± 0.1</td>
<td>-644 to +83, disruption of NFkB A and B sites in pLTRWT Luc</td>
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<tr>
<td>pLTR{361}Sp1Luc</td>
<td>2.2 ± 1</td>
<td>(5,000)</td>
<td>26 ± 0.1</td>
<td>-177 to +83, disruption of the Sp1 sites in pLTR{746} Luc</td>
</tr>
</tbody>
</table>

T163693 (not shown), had no significant effects when applied at concentrations below 20 μM.

Thus, the capacities to inhibit Tat and actinomycin D trans-activations are correlated. TRB, T276339, T172298, DBR, and H7 are strong inhibitors of CDK9/PITALRE, and out of this set, only T172298, DRB, and H7 inhibit CDK7 (37). Hence, CDK9/PITALRE is likely to be required for actinomycin stimulation as previously reported for the Tat-activated transcription of the HIV LTR (21, 31).

The Average CTD Phosphorylation Is Enhanced in Cells Exposed to Actinomycin D—CDK9/PITALRE phosphorylates the CTD of RNP II largest subunit (21), and actinomycin D enhances the average phosphorylation of the CTD in HeLa cells (7, 8). Indeed, the proportion in hyperphosphorylated (IIo) form of the CTD increased markedly in huHL6 cells exposed during 8 h to actinomycin D concentrations as low as 0.06 μg/ml (Fig. 8A). Identical effects were observed with murine cells (not shown).

To strengthen the link between CTD phosphorylation and HIV LTR activation, cells were treated with α-amanitin, and the lysates were analyzed by Western blot. In the muHL6 cells, both the intensities of the IIA and IIO forms decreased with time of treatment (Fig. 8B). Indeed, α-amanitin had been shown to promote the degradation of the largest subunit of RNP II in murine cells (47). Careful quantification of gel scans indicated that the IIA band intensity reproducibly decreased more rapidly than the IIO band, resulting in close to a 4-fold increase in the IIO/IIa ratio after 9 h of treatment. The increase in the IIO/IIa ratio was less than 2-fold in the HeLa-derivived huHL6 cells exposed to α-amanitin during the same period. This result strengthens the link between CTD phosphorylation imbalance and HIV LTR transcription as luciferase synthesis was much less inducible by α-amanitin in the huHL6 cells than in the muHL6 cells (Fig. 1C).

Thus, actinomycin D and α-amanitin treatment promoted an increase in the proportion of RNP II phosphorylated on its CTD in correlation with HIV LTR induction.

Inhibitors of CDK9/PITALRE Promote CTD Dephosphorylation in Mammalian Cells—DBR promotes a bulk dephosphorylation of the CTD (7). To identify which kinase was essential to the average CTD phosphorylation steady state in mammalian cells, the effect of the CDK9/PITALRE inhibitors was investigated in HeLa cells. Five of the eight inhibitors tested,
promoted average CTD dephosphorylation with treatment (Fig. 9A). TRB and T276339 were the most efficient (their effect was detectable above 6 μM) closely followed by DRB and H7, and T172298 was the least efficient. T172299, T163693, and T525636 had no detectable effects at 50 μM (not shown).

Although higher kinase inhibitor concentrations were required to observe average CTD dephosphorylation than to inhibit HIV LTR expression, both effects were correlated (Table II). The inhibition of Tat-activated HIV LTR expression is correlated with the capacity to inhibit CDK9/PITALRE in vitro (37), which suggests that this kinase provides an essential contribution to the average CTD phosphorylation in mammalian cells.

Addition of DRB prior to actinomycin D prevents the bulk CTD phosphorylation (8). Therefore, we evaluated the capacity of the various inhibitors of CDK9/PITALRE to prevent the CTD hyperphosphorylation induced by actinomycin D. In HeLa cells, the stronger inhibitors, TRB or T276339 at concentrations as low as 10 μM, were found to prevent the average CTD phosphorylation promoted by actinomycin D (Fig. 9B). DRB and H7 required higher concentrations (40 μM), and even higher concentrations of T172298 (60 μM) had to be used to observe any effect. The other compounds, T172299, T163693, and T525636 were inefficient up to 60 μM (not shown).

Thus, the capacity to inhibit the CTD phosphorylation promoted by actinomycin D correlates with the transcription inhibition efficiency. This correlation links CTD phosphorylation to the actinomycin-promoted HIV LTR stimulation and strongly suggests that CDK9/PITALRE is involved in both processes.

**DISCUSSION**

In this study we show that treatment of mammalian cells with actinomycin D and α-amanitin leads to a strong enhancement of transcription directed by HIV LTR promoters. This enhancement is observed both in stably transfected cells and during transient transfection assay. To observe a stimulation of RNAPII transcription in cells exposed to inhibitors of transcription is unexpected and entirely novel. This effect is a characteristic of the HIV LTR and is observed with moderate
The Accumulation of HIV-driven mRNAs Is Due to an Enhanced Elongation of Transcription—The accumulation of HIV-driven luciferase mRNAs in actinomycin D-treated cells is attributable to an enhanced transcription at the level of elongation since run-on transcription assays indicate a higher polymerase density in the midst of the luciferase gene in nuclei isolated from actinomycin D-treated cells, whereas the RNAPII density decreases at the 5′ end of the luciferase gene. There are several reports on class II mRNA accumulation in cells treated with inhibitors of transcription. For instance, treatment of cells with low concentrations of actinomycin D (0.007 μg/ml) trigger a p53-dependent accumulation of several mRNAs including p21WAF1, cyclin G1, and cyclin G2 mRNAs in human fibroblasts (45, 52). DNA-damaging agents and α-amanitin have independently been reported to block the degradation and therefore to promote the accumulation of the p53 protein, which is involved in p21WAF1 promoter activation (53, 54). Actinomycin D and α-amanitin have both been shown to promote an increase in tumor necrosis factor-α mRNA (55). Tumor necrosis factor-α activates the HIV-1 LTR through a p53-responsive element (56). However, the p53-mediated cellular responses to DNA damage are disrupted in HeLa cells (57), and no reliable changes in p21WAF1 mRNA levels are detected in actinomycin D-treated HeLa-derived huHL6 cells. Therefore, the accumulation of luciferase mRNAs described in the present work is unlikely to follow a p53-dependent mechanism. The mRNAs previously known to accumulate in actinomycin and amanitin-treated cells are unstable, and increasing their half-lives may lead to their accumulation. To our knowledge, a unique study reports that RNAPII transcription initiation rate increases on rRNA genes in cells exposed to concentrations of actinomycin D slightly lower than those used in the present study (58). Hence, this is the first report that elongation of RNAPII transcription can be stimulated in cells exposed to actinomycin D or α-amanitin.

Transcriptional Activation of the HIV LTR Does Not Involve the TAR nor the NFκB-binding Sequences—The transcriptional activation of the HIV LTR by actinomycin D is shown to be TAR-independent. The HIV LTR sequence between −19 and +32 overlapped the inducer of short transcripts element (25) and high affinity binding sites for LBP-1 (59). These factors are unlikely to be involved in the actinomycin response as the inducibility of the pH1/MVLuc chimera (HIV sequences −167 to −19) and pLTRΔTAR (HIV sequences −488 to +32) were similar (Table I). However, mutagenesis of either the TAR box or the Sp1-binding sites depresses the actinomycin mutagenesis-induced HIV LTR transcription and, as previously reported, the Tat-stimulated transcription as well (reviewed in Refs. 22 and 23).

Actinomycin D and topoisomerase inhibitors activate the NFκB factor (60), and many cases of NFκB-dependent activations of the HIV LTR have been reported. However, stimulation of the HIV LTR by Tat is NFκB-independent in the J-Han lymphoblastoid cells (61). Furthermore, an NFκB-independent activation of the HIV LTR has also been reported after treatment with phorbol ester (62, 63), UV irradiation (64), histone deacetylase inhibitors (65), protein kinase inhibitors (such as 2-aminopurine) (66), okadaic acid treatment (67), heat shock (68), and peroxovanadium compounds (69). Similarly, the NFκB-binding sequences are not required for actinomycin stimulation.

Involvement of the CDK9/PITALRE Kinase in HIV LTR Transcriptional Activation—The transcription from the HIV LTR has been extensively investigated. To summarize, in the absence of Tat, the HIV LTR directs the synthesis of a significant basal level of transcripts but most of them are short, transcription aborts after the TAR element. The TATA-binding protein would bind first to the promoter and recruit the RNAPII (70). Tat would interact with the TAR RNA hairpin and recruit histone acetylases on the LTR promoter (71–73). But an increased histone acetylation affects HIV LTR transcription only when integrated into the genome (65, 73); it is unlikely, therefore, to contribute to actinomycin D stimulation. More significant to our purpose, Tat mediates the recruitment of CDK7 (29) and CDK9/PITALRE (21, 31, 37) which phosphorylate the CTD and promote an efficient transcription elongation throughout the entire viral genome. Phosphorylation of the CTD by the CDK9/PITALRE of the positive transcription elongation factor, P-TEFb, might be the key step in switching the competence of RNAPII to proceed transcription beyond the TAR region. As the actinomycin-activated (this work) and the Tat-activated (37, 50) transcription are very sensitive to CDK9/PITALRE inhibitors in correlation with their inhibition efficiencies in vitro, this kinase is likely to contribute to both the Tat and the actinomycin D stimulation of the HIV LTR. In agreement with this hypothesis, actinomycin D (this work) and Tat both stimulate transcription at the elongation level.

The recruitment of P-TEFb by Tat involves a direct interaction between Tat and cyclin T1 but, in contrast to the human cyclin T1, the murine cyclin T1 is defective in binding to Tat (74, 75). Indeed, Tat transactivation of the HIV LTR is defective in rodent cells. However, actinomycin D is as efficient to stimulate the HIV LTR transcription in both human and murine cells. The actinomycin D treatment may bypass a requirement in Tat/cyclin T1 interaction to recruit P-TEFb.

Involvement of the CDK9/PITALRE Kinase in CTD Phosphorylation in Vivo—Here we report on a collection of compounds that promote CTD dephosphorylation in vivo with an efficiency correlating with their capacity to inhibit CDK9/PITALRE in vitro. Phosphorylation of the CTD is a dynamic process involving permanent phosphorylation and dephosphorylation reactions. Inhibiting CDK9/PITALRE would prevent CTD phosphorylation and result in the accumulation of dephosphorylated CTD. CTD dephosphorylation systematically required higher inhibitor concentrations than transcription impairment. However, it should be kept in mind that RNAPII exists under various structures, core enzyme and holoenzymes (76). As the holoenzymes are organized around the CTD, their reactivities toward CTD kinases and CTD phosphatases are expected to differ from one holoenzyme to the other. A specific
class of holoenzymes (with a characteristic kinase inhibitor sensitivity) is likely to be involved in the HIV LTR transcription. In contrast, the CTD dephosphorylation detected by Western blot averages the behavior of all classes of RNAP II molecules. Thus, the correlations between the in vitro and in vivo inhibitor effects strongly suggest that CDK9/PITALRE is an essential contributor to CTD phosphorylation in vivo.

Inactivation of KIN28, the yeast CDK7 homolog results in a rapid dephosphorylation of the CTD in yeast cells (77). However, the yeast CDK9/PITALRE homolog has not yet been identified and might also be the KIN28 gene product. In mammalian cells, both CDK7 and CDK9/PITALRE are likely to be essential to ensure CTD phosphorylation in vivo. Such a double requirement has been described for HIV promoter activation (30). Phosphorylation of the CTD is enhanced in cells exposed to actinomycin D and α-amanitin. Involvement of CDK9/PITALRE is suggested as this enhancement does not occur when its inhibitors are present.

An enhanced CTD kinase activity should promote an enhanced average CTD phosphorylation. Activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines is accompanied with an enhanced CDK9/PITALRE activity that is proposed to contribute to the high levels of HIV transcription (78). Alternatively, blocking CTD dephosphorylation that is proposed to contribute to the high levels of HIV transcription (78). Alternatively, blocking CTD dephosphorylation that is proposed to contribute to the high levels of HIV transcription (78).

Stress Activation of the HIV LTR—

Actinomycin D and the transcriptional initiation block caused by a “frozen” CTD prior to the formation of the preinitiation complex of transcription represses initiation of transcription on specific genes (13).

During severe stress, a block in CTD phosphate exchange due to CTD kinase and CTD phosphatase inactivations is likely to contribute to a general shut-off of transcription except for the heat shock genes (80–82). As the Tat protein remains bound to the elongating transcriptional complex (83) and to the RNAP II holoezyme (29), it is likely to “freeze” the RNAP II CT in a phosphorylated state in the vicinity of a transcriptionally active HIV LTR. Indeed, the Tat protein has evolved not to recruit CTD kinases (30) but also to inhibit a phosphatase that specifically dephosphorylates the CTD (84). The Tat protein represses several cellular genes at the transcriptional level (72), and it may be part of the HIV viral strategy to escape the transcriptional initiation block caused by a “frozen” CTD phosphorylation.

A General Inhibition of Transcription May Contribute to Stress Activation of the HIV LTR—The actinomycin D and the α-amanitin conditions that stimulate the HIV LTR are the same as those that promote the average CTD phosphorylation. Therefore, the bulk CTD phosphorylation promoted by these drugs may bypass the requirement in the viral protein Tat to recruit kinases to phosphorylate the CTD on the HIV LTR.

References:

Transcriptional Inhibitors Activate the HIV-1 Promoter
The Transcriptional Inhibitors, Actinomycin D and α-Amanitin, Activate the HIV-1 Promoter and Favor Phosphorylation of the RNA Polymerase II C-terminal Domain

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