JOURNAL OF VIROLOGY, Sept. 2001, p. 8524–8537 Vol. 75, No. 18
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.18.8524–8537.2001

Activation of Human Immunodeficiency Virus Transcription in T Cells Revisited: NF-κB p65 Stimulates Transcriptional Elongation

MICHELLE J. WEST, ANTHONY D. LOWE, AND JONATHAN KARN*

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, United Kingdom

Received 24 April 2001/Accepted 18 June 2001

Human immunodeficiency virus type 1 (HIV-1) is able to establish a persistent latent infection during which the integrated provirus remains transcriptionally silent. Viral transcription is stimulated by NF-κB, which is activated following the exposure of infected T cells to antigens or mitogens. Although it is commonly assumed that NF-κB stimulates transcriptional initiation alone, we have found using RNase protection assays that, in addition to stimulating initiation, it can also stimulate elongation from the HIV-1 long terminal repeat. When either Jurkat or CCRF/CEM cells were activated by the mitogens phorbol myristate acetate and phytohemagglutinin, elongation, as measured by the proportion of full-length transcripts, increased two- to fourfold, even in the absence of Tat. Transfection of T cells with plasmids carrying the different subunits of NF-κB demonstrated that the activation of transcriptional elongation is mediated specifically by the p65 subunit. It seems likely that initiation is activated because of NF-κB's ability to disrupt chromatin structures through the recruitment of histone acetyltransferases. To test whether p65 could stimulate elongation under conditions where it did not affect histone acetylation, cells were treated with the histone deacetylase inhibitor trichostatin A. Remarkably, addition of p65 to the trichostatin A-treated cell lines resulted in a dramatic increase in transcription elongation, reaching levels equivalent to those observed in the presence of Tat. We suggest that the activation of elongation by NF-κB p65 involves a distinct biochemical mechanism, probably the activation of carboxyl-terminal domain kinases at the promoter.

In addition to its normal lytic growth pathway, human immunodeficiency virus type 1 (HIV-1) is able to enter a latent state in which the integrated proviral genome remains transcriptionally silent for long periods. The ability to infect cells latently helps HIV to establish persistent infections despite strong humoral and cellular immune responses against the viral proteins. When T cells residing in lymphoid tissues are activated, the latent proviruses that they harbor can efficiently infect adjacent cells without the release of neutralizable virus particles through cell-to-cell transfer of virions (12, 30). A second consequence of latency during the development of HIV disease is that the latent proviruses create a large and stable reservoir of genetic variants from which strains carrying resistance to immune responses and therapeutic drugs can be selected (45).

Studies using viral long terminal repeats (LTRs) linked to reporter genes demonstrated that the transcription factor NF-κB plays a central role in the proviral activation pathway (28). In resting T cells and most established T-cell lines, NF-κB is sequestered in the cytoplasm by the inhibitor protein IκBα (for reviews, see references 1 and 2). Following exposure of T cells to antigen or treatment of the cells by mitogens such as phorbol myristate acetate (PMA) and phytohemagglutinin (PHA), NF-κB is unmasked due to the sequential phosphorylation, ubiquitination, and degradation of IκBα. Free NF-κB then translocates to the nucleus, where it can activate transcription from a wide variety of promoters, including the HIV LTR (11, 26, 28, 32). The HIV promoter contains two NF-κB binding sites located near the transcription start site. These sites are recognized by a variety of cellular proteins from the NF-κB/Rel family, including p52-p65 and p50-p65 heterodimers and p50 and p65 homodimers (4, 21). Binding to the NF-κB motifs in the HIV LTR is cooperative and can be enhanced by binding of SP-1 to adjacent sites in the promoter (21, 32).

Transcription from the HIV LTR during proviral activation is also regulated by the viral transactivator protein Tat. In the absence of Tat, initiation from the LTR is efficient, but transcription is impaired because the promoter recruits poorly processive polymerases that disengage from the DNA template prematurely (for reviews, see references 17 and 41). Activation of transcriptional elongation occurs following the recruitment of Tat to the transcription machinery by a specific interaction with an RNA regulatory element called TAR (18). After binding to TAR RNA, Tat stimulates a specific protein kinase complex called TAK (Tat-associated kinase) which contains a kinase subunit, CDK9, and its cyclin partner cyclin T1 (CycT1). CycT1 is able to bind directly to Tat and promote its binding to TAR RNA (44). The activated kinase is then able to hyperphosphorylate the large subunit of the RNA polymerase II (Pol II) carboxyl-terminal domain (14, 49).

When T cells carrying latent proviruses are activated, the low amounts of Tat that are produced initially create a powerful feedback mechanism that dramatically increases the overall transcription efficiency. This leads to a substantial rise in Tat levels and, after the expression of sufficient amounts of the

* Corresponding author. Mailing address: MRC Laboratory of Molecular Biology, Hills Rd., Cambridge CB2 2QH, United Kingdom. Phone: (44) 1223-402376. Fax: (44) 1223-412282. E-mail: karn@mrc-lmb.cam.ac.uk.

regulatory protein Rev, the eventual expression of the full complement of viral proteins.

Although the critical role played by NF-κB during the activation of HIV transcription is well established, the precise molecular mechanisms underlying its activity are still unknown. Most models of HIV activation assume that the increased levels of NF-κB exclusively stimulate initiation rates from the viral LTR. According to these models, Tat is produced initially simply because the promoter is "leaky" and a small fraction of the transcription complexes fortuitously synthesize full-length transcripts (24). However, it seems more likely that rather than relying on a stochastic process, specialized mechanisms exist that ensure early Tat production. An example of the type of mechanism that could be involved is activation of an enhancer element, since the addition of a strong cellular enhancer to the HIV LTR induces a high level of transcription even in the absence of Tat (46).

To test the hypothesis that HIV LTR carries an enhancer-like element that is utilized specifically during T-cell activation, we looked for evidence that NF-κB, or some other factor that is concomitantly upregulated during T-cell activation, can increase the elongation capacity of the polymerases recruited to the LTR in the absence of Tat. The results show that, unexpectedly, the p65 subunit of NF-κB is able to stimulate transcription elongation by a novel mechanism that operates in addition to its role in stimulating transcription initiation.

MATERIALS AND METHODS

Plasmids. The chloramphenicol acetyltransferase (CAT) reporter plasmid, D5-3-3, contains a wild-type HIV-1 LTR upstream of the CAT gene (3). Mutations in the NF-κB sites and surrounding sites were generated by site-directed mutagenesis using PCR. PCRs contained 10 ng of D5-3-3; 125 ng of each mutagenic primer; 200 μM dATP, dGTP, dCTP and dTTP; 1× *Pfu* polymerase buffer (Stratagene); and 2.5 U of *Pfu* polymerase (Stratagene) in a total volume of 50 μl. Reactions were carried out at 95°C for 30 s, 60°C for 1 min, and 68°C for 9 min for a total of 16 cycles. Template plasmid was digested with the methylation-sensitive enzyme *Dpn*I, and the mutated plasmid DNA containing staggered nicks was then transformed into endonuclease-minus *Escherichia coli* cells (ACE). The *Eco*RV (−340) to *Hin*dIII (+78) region of the new mutant κB and mutant spacer plasmids was sequenced to verify the mutation and then subcloned in place of the wild-type *Eco*RV to *Hin*dIII fragment of D5-3-3 to generate plasmids MJW-35 and MJW-36, respectively.

The luciferase reporter plasmid containing the wild-type LTR (LTR-FF) was generated by cloning the *Eco*RV-to-*Hin*dIII fragment of D5-3-3 into the *Sma*I and *Hin*dIII sites of pGL3 basic (Promega). The MJW-37 and MJW-38 luciferase reporter plasmids were generated by subcloning the *Eco*RV to *Hin*dIII fragments of MJW-35 (mutant κB) and MJW-36 (mutant spacer), respectively, into the *Sma*I and *Hin*dIII sites of pGL3-basic. MJW-39 [Δ(−340 to −80)] was derived by cloning the *Xho*I-to-*Hin*dIII LTR fragment of MTX-3 (38) into the *Xho*I and *Hin*dIII sites of pGL3-basic. MJW-40 [Δ(−340 to −104)] was generated in a similar manner by cloning the *Xho*I-to-*Hin*dIII LTR fragment of MTX-13 into pGL3-basic. RNase protection analysis of CAT reporter transcripts was carried out as described previously using the plasmids MTX-89 and MTX-147 to generate proximal and distal antisense probes, respectively (46).

Cell culture. The T-cell lines Jurkat and CCRF/CEM were maintained in RPMI 1640 medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml) supplemented with 10% fetal calf serum and were cultured at 37°C in 7% CO_2.

Transfections were carried out initially using Superfect (Qiagen) and later using the newer and more efficient transfection reagent Effectene (Qiagen). Cells were diluted 1 in 3 into fresh medium 24 h prior to transfection. Large-scale transfections for RNA analysis using Superfect were performed in 25-cm^3 flasks with 7.5 μg of HIV plasmid, containing a CAT or luciferase reporter gene and 0 to 200 ng of C63-4-1, which expresses Tat in *trans* from a Moloney murine leukemia virus LTR. To compensate for the variable amounts of C63-4-1 in the mixture, pUC12 DNA was also added. DNA samples were mixed in a total volume of 150 μl of medium containing no fetal calf serum or antibiotics and Superfect was then added at a ratio of 5 μl per μg of DNA. After a 10-min incubation at room temperature, 1 ml of normal growth medium was added to the transfection mixes, and the total volume then transferred to a 25-cm^3 flask containing 4 ml of cells resuspended at 1×10^6 to 1.25×10^6/ml in fresh media. The flasks were incubated at 37°C for 2 h, and 5 ml of normal growth medium was then added. For luciferase assays, small-scale transfections with Superfect were carried out in six-well dishes, and all quantities and volumes were reduced by approximately half. In addition, 1 μg of pRL-CMV (Promega) which contains the Renilla luciferase gene under the control of the cytomegalovirus (CMV) promoter was included as a transfection control.

Large-scale transfections carried out for RNA analysis using Effectene contained 1 or 2 μg of D5-3-3, 0 to 500 ng of C63-4-1, and pUC12 DNA to maintain constant DNA levels. DNA was mixed in a total volume of 300 μl of EC buffer (Qiagen), and 8 μl of Enhancer was added for every microgram of DNA present. Samples were then vortexed and incubated at room temperature for 5 min prior to the addition of Effectene (10 μl for every microgram of DNA). After vortexing the mixture, the samples were incubated for a further 5 min at room temperature, and 1 ml of normal growth medium was then added. The total mix was then added to a 25-cm^3 flask containing 4 ml of cells resuspended at 2.5×10^5/ml in fresh medium. The flasks were incubated at 37°C for 2 h, and 5 ml of normal growth medium was then added.

Jurkat and CCRF/CEM cells were activated 24 h after transfection by the addition of PHA (Sigma) to a final concentration of 10 μg/ml and PMA (Sigma) to a final concentration of 50 ng/ml.

Transfection mixtures for the RNase protection experiments utilizing the CDK9 mutant (D167N) contained 1 μg of D5-3-3, 250 ng of C63-4-1 and 0 or 0.5 μg of FLAG-D167N. Transfection mixtures for the luciferase assays contained 600 ng of LTR-FF, 0 or 50 ng of C63-4-1, and 1.5 or 3 μg of D167N. The FLAG-DNA content of all samples was maintained at a constant level by the addition of the vector DNA (CMV-FLAG).

Luciferase assays for the NF-κB overexpression experiments were carried out on cells transfected with 600 ng of LTR-FF, 0 or 40 ng of C63-4-1, and 150 to 600 ng of plasmids expressing the p65 or p52 subunits of NF-κB under the control of the Rous sarcoma virus (RSV) LTR (4). Large-scale experiments for RNA analysis were carried out in 25-cm^3 flasks using 1 μg of D5-3-3, 0 or 250 ng of C63-4-1 and 1.2 μg of pRSV-p65, or 600 ng of pRSV-p65 and 600 ng of pRSV-p52. Control samples contained 1.2 μg of pUC12 DNA. Cells were harvested after 48 h for analysis. Where indicated, cells were treated with trichostatin A (TSA; Sigma) at 24 h posttransfection and harvested after a further 24 h. Data from the luciferase assays were corrected for cell toxicity effects.

Toxicity assays were performed in parallel with luciferase assays by transferring 200 μl of transfected cell mix to a 96-well plate. These cells were treated in parallel with PMA and PHA on day 2, and cell proliferation was measured on day 3 by the addition of 40 μl of CellTiter 96 Aqueous One Solution Cell Proliferation Assay Reagent (Promega). Cells were then incubated at 37°C for 2 to 4 h, and the level of colored product was determined by the absorbance at 490 nm.

RNase protection assays. Total RNA was extracted from cells using Trireagent (Sigma) and then treated with 6 U of RNase-free DNase I (2 U/μl; HT Biotechnology Ltd.) for 30 min at 37°C in a total volume of 50 μl containing 1× NEB 2 buffer (New England Biolabs) and 80 U of RNasin (40 U/μl; Promega). RNA was then extracted with phenol-chloroform-isoamyl alcohol and precipitated in ethanol containing 0.5 M ammonium acetate. The DNase I treatment was repeated using 30 U of DNase I (10 U/μl; Boehringer Mannheim); and the RNA was resuspended in sterile water. Antisense probes were prepared exactly as described previously (46).

Each protection assay was performed on equal amounts of RNA (10 to 20 μg) and 20 to 40,000 cpm of probe as described previously (46). The digestion of single-stranded sequences was carried out using RNase T_1 (1,800 U/μl; Gibco-BRL) at a final concentration of 4.5 U/μl and RNase A at a final concentration of 5 ng/ml. Protected fragments were analyzed by electrophoresis through 6% polyacrylamide gels containing 6 M urea which were quantified directly by phosphorimagery or by laser densitometry of autoradiographs.

Luciferase assays. Cells transfected in six-well plates were harvested after 48 h, washed once in phosphate-buffered saline, and then lysed in 100 to 200 μl of 1× Passive Lysis Buffer (Promega) for 30 min at room temperature. Lysates (10 μl) were assayed using 50 μl of LAR II reagent and 50 μl of Stop and Glo reagent from the Dual Luciferase Assay System kit (Promega). Firefly luciferase and Renilla luciferase activity were measured sequentially using a microplate luminometer with an injection unit (Berthold detection systems).

Immunoblotting. Cell pellets were lysed directly in 1× GSB (50 mM Tris-HCl, pH 6.8; 4% sodium dodecyl sulfate; 10% 2-mercaptoethanol; 0.01% bromophe-

nol blue; 10% glycerol; 1 mM EDTA). Lysates were vortexed extensively and then heated to 90°C for 5 min prior to electrophoresis analysis using 10% NuPAGE Bis-Tris gels (Novex) and morpholinepropanesulfonic acid buffer. Proteins were transferred onto Protran nitrocellulose membrane (Schleicher & Schuell). CDK9 was detected using a rabbit polyclonal antibody (H-169 sc-8338; Santa Cruz Biotechnology, Inc.), and CycT1 was detected using a goat polyclonal antibody (C-20, sc-8128; Santa Cruz Biotechnology, Inc.). Complexes were then detected using a rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody and a rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (Dako), respectively, and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

The activation of basal transcription in mitogen-treated T cells is due to increased levels of both initiation and elongation. Transcription complexes that initiate from the HIV LTR are known to be poorly processive and are subsequently converted into a more processive form after the stimulation of CDK9 kinase activity by Tat. However, under certain circumstances it is possible to obtain significant increases in Tat-independent transcription elongation from the HIV LTR. For example, we have recently demonstrated that when a strong cellular enhancer is introduced into the HIV LTR it is able to promote the recruitment of elongation-competent transcription complexes (46).

Does a similar mechanism operate during the activation of latent proviruses? Transcription from the HIV-1 LTR has long been known to increase dramatically when NF-κB is activated in T cells that are stimulated by mitogenic agents that mimic the normal process of antigen-mediated T-cell activation in vivo (28) but, surprisingly, none of the published studies have examined whether NF-κB simply stimulates transcription initiation, or whether it also has more subtle effects on HIV transcription, including effects on elongation. We therefore decided to study HIV transcription in activated T cells using sensitive reporter assays and quantitative RNase protection assays.

To establish reliable cell culture conditions for these experiments, we first performed control experiments using luciferase reporter constructs transfected into Jurkat and CCRF/CEM T-cell lines. High levels of luciferase activity were only obtained following the treatment of the cells by the mitogens PMA and PHA (Fig. 1). Transcription from the viral LTR in the absence of Tat (basal transcription) was extremely low in unstimulated Jurkat cells (Fig. 1B) and CCRF/CEM cells (Fig. 1C) due to the combined restrictions on initiation and elongation. Tat was able to stimulate luciferase synthesis from the wild-type LTR by 7-fold in Jurkat cells and by 15-fold in CCRF/CEM cells; however, this still corresponded to comparatively low levels of transcription.

The increase in basal HIV transcription after mitogen stimulation of T cells is remarkably large and frequently exceeds the levels of transcription that can be achieved following the addition of Tat to unstimulated cells. For example, there was a mean 64-fold increase in basal transcription in Jurkat cells and a mean 19-fold increase in basal transcription in CCRF/CEM cells following PMA and PHA treatment. Addition of Tat activated transcription synergistically and led to extremely high levels of transcription as shown by the mean 664-fold increase in luciferase in the Jurkat cells and the mean 323-fold activation of luciferase in the CCRF/CEM cells. Furthermore, as shown in Fig. 2, mitogen-activated transcription resulted in luciferase levels that were greater than the levels obtained in Jurkat cells that had been transfected by Tat expression plasmid C63-4-1 over a wide concentration range (25 and 100 ng). At each Tat concentration, PMA and PHA acted synergistically with Tat to stimulate high levels of HIV transcription.

Although numerous studies have shown that basal transcription is activated in T cells, to our knowledge this is the first comparison of the levels of basal transcription in stimulated cells to Tat-activated transcription in unstimulated T cells. The fact that basal transcription is so dramatically reduced in unstimulated T cells suggests that there is a specific molecular mechanism that is used to repress HIV LTR transcription that is subsequently reversed following mitogen activation.

Additional control experiments, which are in agreement with earlier reports, showed that the efficient activation of transcription by PHA and PMA required functional NF-κB sites in the viral LTR (Fig. 1B and C). Deletion of all of the LTR sequences found upstream of the SP1 sites, including the NF-κB sites [Δ(−340 to −80)], also produced a promoter that was very poorly responsive to the mitogens.

Recent studies have identified a binding site for the NF-ATc protein that overlaps the NF-κB sites and includes the 4-bp spacer region separating these two sites (20). These results raised the possibility that NF-ATc could also contribute to HIV LTR transcriptional activation in T cells. In contrast to the results obtained when the κB sites were mutated, we found that LTRs carrying mutations of the spacer region that inactivate NF-ATc binding had wild-type activity (Fig. 1). These reporters showed the same levels of activation of basal transcription as the wild-type following stimulation by PMA and PHA. Furthermore, the extent of transactivation by Tat was unaffected by these mutations in either cell line (Fig. 1B and C). Potential NF-AT binding sites have also been identified in the region from nucleotides (nt) −253 to −213 upstream of the transcription start site. However, we found that, consistent with the results of other authors, the deletion of LTR upstream of the NF-κB sites [Δ(−340 to −104)] also had no measurable effect on the levels of basal and Tat-activated transcription in either the Jurkat or the CCRF/CEM cell line (22, 25). Thus, although NF-ATc might be able to substitute for NF-κB in certain circumstances, this protein does not play a central role in the activation of HIV transcription in established T-cell lines.

In order to compare initiation and elongation levels, transcripts produced after T-cell activation were next analyzed using RNase protection assays (Fig. 3). As described previously, hybridization to the promoter-proximal probe allows the detection of all transcripts that initiate at the LTR and provides an approximate measure of total transcription levels. Hybridization to the distal probe detects only those transcripts which have extended to residues nt +342 to +482 downstream from the transcription start site and therefore provides a measure of elongation (46). In order to compare the proportions of full-length transcripts to total initiations in different experiments, the data reported here were corrected for the specific activity of the probes. Following this correction, the level of full-length transcripts is expressed as a percentage of the amount of transcripts detected with the proximal probe to provide an approximation of the elongation efficiency. Since

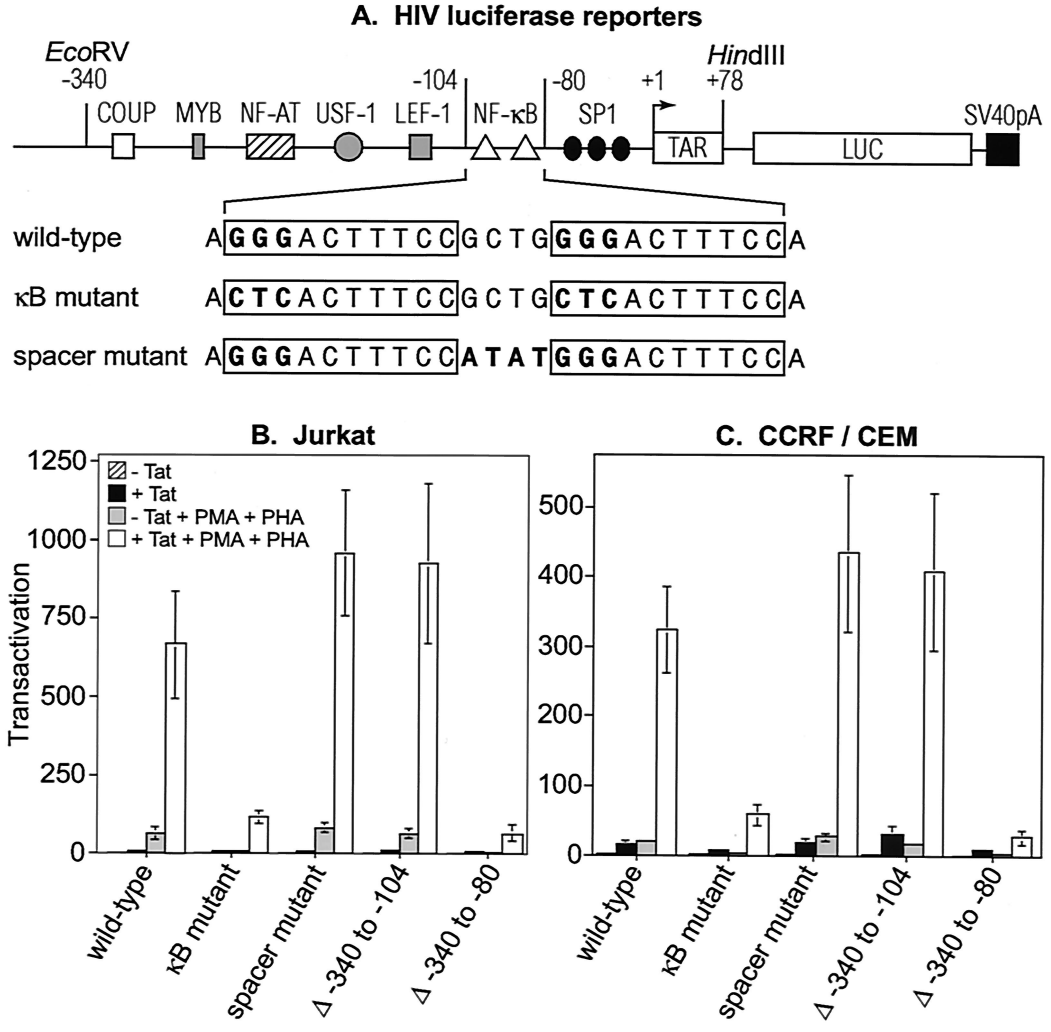

FIG. 1. The activation of the HIV-1 LTR after stimulation of T-cell lines by mitogens. (A) Luciferase reporter plasmids. The wild-type plasmid LTR-FF contains the *Eco*RV-to-*Hin*dIII fragment of the LTR (−340 to +78) cloned upstream of the firefly luciferase gene in pGL3 (Promega). The κB mutant plasmid (MJW-37) has a CTC substitution in the two NF-κB sites, and the mutant spacer plasmid (MJW-38) has a GCTG-to-ATAT change in the spacer region between these two sites. The Δ(−340 to −104) and Δ(−340 to −80) deletions were created by cloning the *Xho*I-to-*Hin*dIII (+78) fragments from plasmids containing the truncated LTRs (MTX-13 and MTX-3, respectively) into pGL3. (B) Jurkat cells. Cells were transfected with 4 μg of luciferase reporter in the absence or presence of 75 ng of the Tat-expressing plasmid C63-4-1 and 1 μg of the internal control plasmid, pRL-CMV. Cells were treated at 24 h posttransfection with PMA (50 ng/ml) and PHA (10 μg/ml) for a further 24 h. Data represent the mean ± the standard deviation of three independent experiments expressed as the fold transactivation over wild-type basal transcription. Results were also normalized to the expression of the Renilla luciferase gene expressed from cotransfected pRL-CMV. Since the CMV promoter was also activated by PMA and PHA, nonactivated and activated cells were normalized independently. (C) The activation of the HIV-1 LTR luciferase constructs in CCRF/CEM cells.

the data are expressed as the ratio of the signals obtained from the two probes, the experiment is internally controlled for variations in transfection efficiency and independent of variations in the recovery of RNA from the transfected cells.

In unstimulated Jurkat T cells, the level of distal transcripts in the absence of Tat is very low and represents only 1.9% of the total transcription (Fig. 3A). Addition of Tat (cells were cotransfected with 200 ng of the C63-4-1 plasmid) increased the levels of the distal transcripts to 18.4% of the total transcription but, as expected, Tat did not significantly affect the total amount of transcription detected by the proximal probe. These results are consistent with the well-established role of Tat in stimulating the processivity of a fraction of the polymerases that have initiated transcription.

After treatment of Jurkat cells with PMA and PHA there was a 4.3-fold increase in the level of transcripts that could be detected by the proximal probe. Thus, the total transcription levels from the LTR are markedly increased during activation of Jurkat cells by mitogens. Transcription in the mitogen-treated Jurkat cells can be further enhanced by Tat, and the proportion of long transcripts increased to between 17 and 25% of the total transcripts (Fig. 3A).

In CCRF/CEM cells, mitogen treatment also stimulated total transcription, but the effect was smaller than that seen in

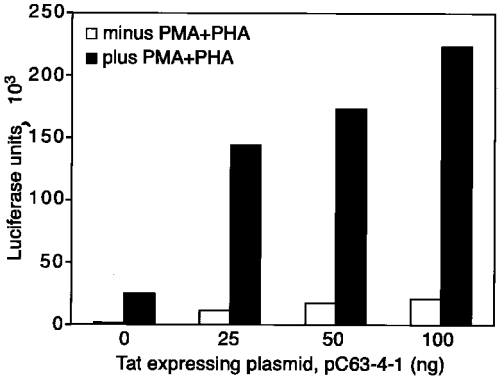

FIG. 2. Activation of transcription from the HIV-1 LTR in Jurkat cells stimulated by PMA and PHA in the presence or absence of Tat. Cells were transfected with 4 μg of luciferase reporter in the absence or presence of 25, 50, or 100 ng of the Tat-expressing plasmid C63-4-1 and 1 μg of the internal control plasmid, pRL-CMV. Cells were treated 24 h posttransfection with PMA (50 ng/ml) and PHA (10 μg/ml) for a further 24 h. The results were normalized to the expression of the Renilla luciferase gene expressed from cotransfected pRL-CMV.

the Jurkat cells. As shown in Fig. 3B, proximal transcript production increased only twofold following mitogen stimulation. Further activation of elongation by Tat was easily detected in the CCRF/CEM cells. After Tat addition, up to 26% of the transcripts are full length.

It is important to note that, although total transcription increased in both T-cell lines following stimulation by PHA and PMA, significant effects on elongation in the absence of Tat are also evident in these experiments. In the Jurkat cells, the proportion of full-length transcripts seen in the absence of Tat is increased by mitogen treatment from 1.9 to 7.2% of the total. A similar stimulation of elongation is also observed in mitogen-treated CCRF/CEM cells, where the proportion of Tat-independent full-length transcripts rises from 1.9 to 8.6% of the total transcripts. This disproportionate increase in elongation can be readily seen in the gel shown in Fig. 3B by comparing the intensities of the bands detected by the distal probe before and after treatment of the CCRF/CEM cells with PMA and PHA in the absence of Tat (compare Distal, 0 Tat, minus PMA + PHA to Distal, 0 Tat, plus PMA + PHA in Fig. 3).

Thus, the RNase protection experiments demonstrate that there are two components to the response of HIV LTRs to mitogen treatment in the absence of Tat. Although initiation levels increased 4-fold in the Jurkat cells and 2-fold in the CCRF/CEM cells after exposure to the mitogens, this increase in initiation does not fully correspond to the 16-fold increase in full-length transcripts seen in Jurkat cells or the 9-fold increase in full-length transcripts seen in CCRF/CEM cells.

NF-κB is required for the activation of basal transcription. To confirm that the increase in transcriptional elongation observed following mitogenic stimulation is mediated by NF-κB, additional RNase protection experiments were performed in Jurkat and CCRF/CEM cells transfected by constructs carrying mutations that inactivated both NF-κB sites in the LTR (Fig. 4).

In contrast to the wild-type promoter, the mutant LTRs did not show significant increases in transcription initiation or elongation in response to mitogen treatment. For example, in

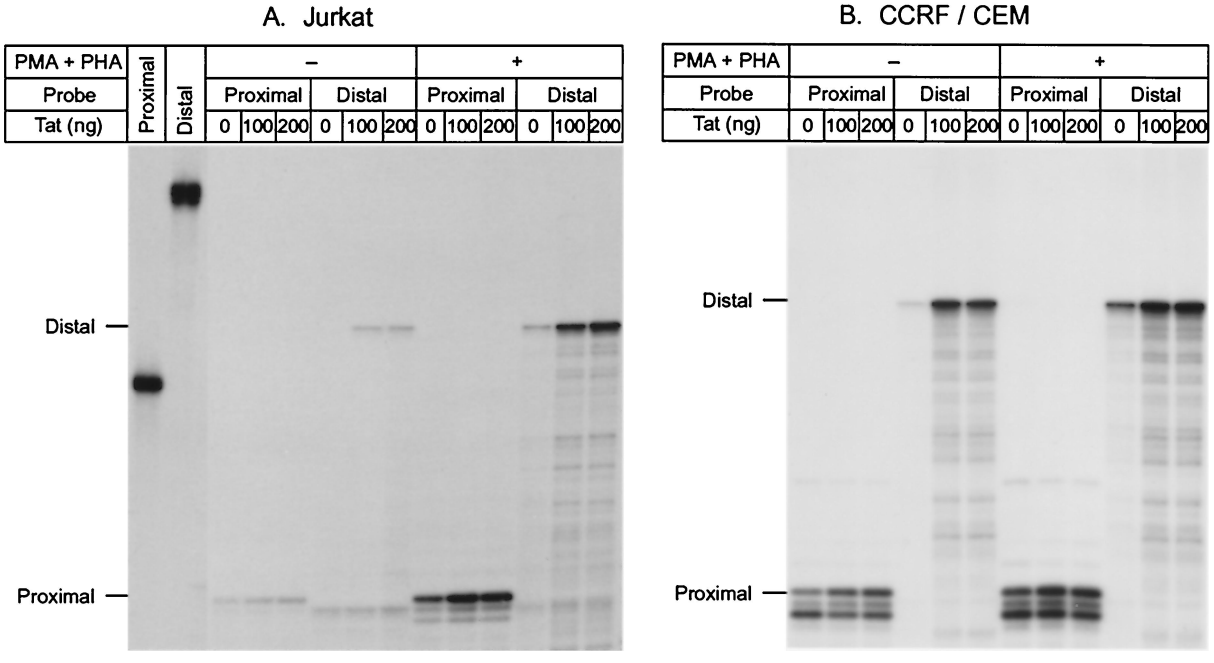

FIG. 3. RNase protection analysis of proximal and distal transcripts in activated T-cell lines. Jurkat cells (A) and CCRF/CEM cells (B) were transfected with 7.5 μg of a CAT reporter construct containing the wild-type LTR (D5-3-3) in the absence (−Tat) or presence (+Tat) of 100 or 200 ng of C63-4-1. Cells were treated with PMA and PHA as described in the legend to Fig. 1. Total RNA was then extracted and analyzed by hybridization to an antisense proximal (−10/+59, MTX-89) or distal (+342/+482, MTX-147) probe. The positions of the protected proximal product (59 nt) and protected distal product (140 nt) are indicated on the gel.

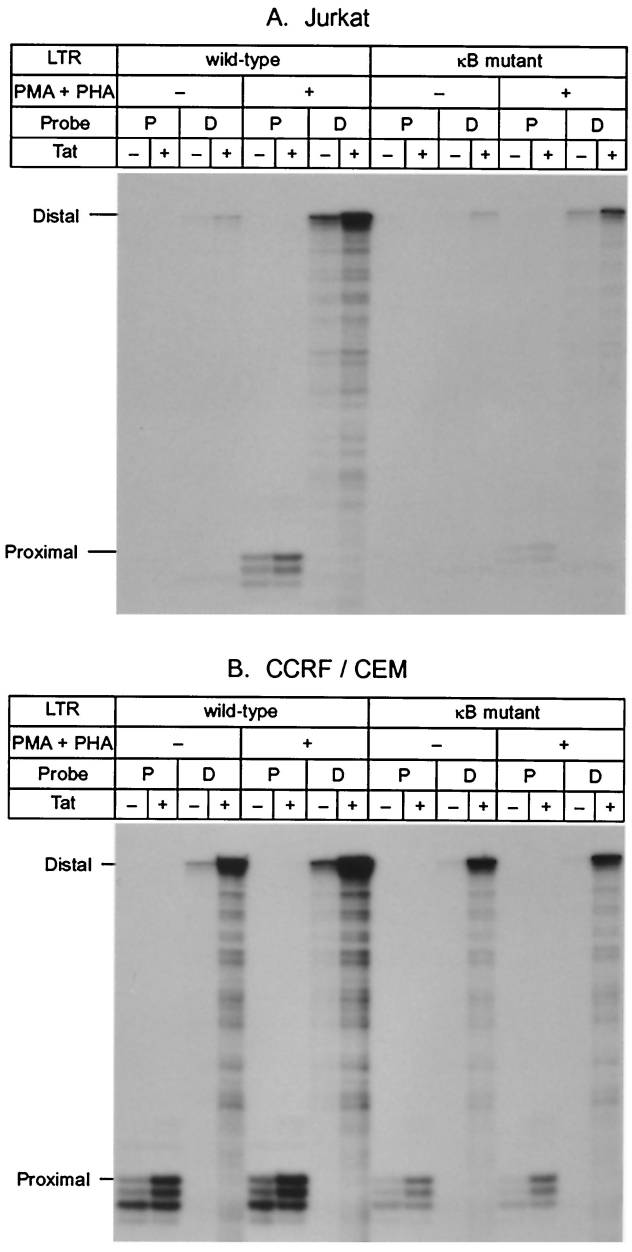

FIG. 4. The activation of transcription elongation by PMA and PHA requires functional NF-κB sites. (A) Jurkat cells were transfected with 1 μg of D5-3-3 (wild-type) or 1 μg of MJW-37 (mutant NF-κB sites) in the absence (−Tat) or presence (+Tat) of 250 ng of C63-4-1. Cells were activated with PMA and PHA, as indicated, and RNA levels were analyzed by RNA protection assays as described in the legend to Fig. 2. (B) CCRF/CEM cells were transfected with 2 μg of D5-3-3 or 2 μg of MJW-37 in the absence or presence of 500 ng of C63-4-1, and RNA was analyzed as in panel A. P, proximal probe; D, distal probe.

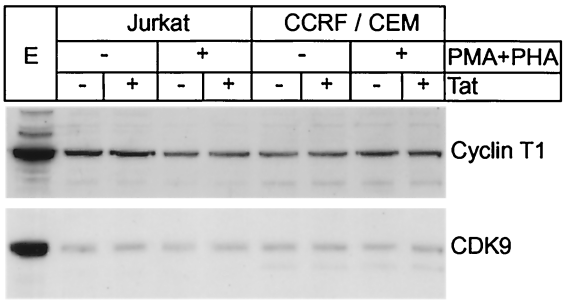

FIG. 5. Western blot analysis of levels of the CDK9 and CycT1 proteins in Jurkat and CCRF/CEM cells after stimulation by PMA and PHA. Whole-cell lysates were fractionated by electrophoresis through 10% NuPAGE Bis-Tris gels, and the Western blots were probed with anti-CDK9 or anti-cyclin T1 polyclonal antibodies.

the stimulated Jurkat cells (Fig. 4A), the NF-κB mutations reduced the levels of transcripts detected by the proximal probe to 24% of the levels of activated transcription from the wild-type reporter (Fig. 4A). The levels of full-length transcripts detected by the distal probe were reduced by a similar extent and were 31% of the wild-type levels in activated cells.

Because basal transcription is barely above the threshold of detection in this experiment, it was not possible to accurately measure whether the NF-κB mutations produced any changes in the elongation response in the Jurkat cells. We therefore performed similar experiments in the CCRF/CEM cells, where the basal transcription levels are substantially higher and the changes in initiation are less than twofold.

As shown in Fig. 4B, in CCRF/CEM cells the mutations in the NF-κB sites abolished both the increase in total transcription and the increase in elongation after treatment of cells with mitogens. The control experiments using the wild-type reporter gave similar results to those shown in Fig. 3. In this experiment, PMA and PHA increased the levels of wild-type transcripts detected by the proximal probe by 1.25-fold, a finding consistent with the small effects observed on initiation following mitogenic stimulation of CCRF/CEM cells. The addition of mitogens also resulted in the stimulation of elongation and the proportion of long transcripts detected in the absence of Tat rose from 6 to 12.2% (Fig. 4B).

Following activation of the CCRF/CEM cells, there was no detectable increase in elongation by reporters carrying the NF-κB mutations. The proportion of long transcripts remained at 5% following activation of cells carrying the mutant reporter (Fig. 4B). Significantly, Tat was able to stimulate transcriptional elongation even when the NF-κB mutations in the LTR were present. For example, addition of Tat in the presence of mitogens increased the proportion of long transcripts observed in the CCRF/CEM cells from 12.2 to 52% for the wild-type LTR, and from 5 to 45% in the experiment performed with the mutant LTR.

CDK9 and CycT1 levels remain constant in mitogen-stimulated T-cell lines. In addition to activating NF-κB, PHA and PMA could also stimulate the activity of elongation factors that contribute to HIV transcription. For example, the expression of both CDK9 and CycT1 increases when resting T cells are stimulated with a variety of mitogens or when promonocytic cells are induced to differentiate (7, 13, 48). This increase in TAK activity was shown to correlate with the increase in viral replication induced in a promonocytic cell line containing an integrated provirus (48). These observations led Price (36) to suggest that an increase in TAK activity is a primary mechanism used to regulate LTR transcription during T-cell activation.

We therefore measured CDK9 and CycT1 protein levels

after the treatment of both Jurkat and CCRF/CEM cells with PMA and PHA. As shown in Fig. 5, the levels of both proteins remained constant after mitogen activation or expression of Tat. In agreement with this result, Yang et al. (48) have reported that TAK enzymatic activity does not increase in Jurkat and CCRF/CEM cells that have been stimulated by PMA. Thus, the dramatic increase in HIV transcription in these cell lines does not appear to be due to upregulation of CDK9 or CycT1 expression and is most likely due to the activity of NF-κB itself.

NF-κB p65 selectively activates transcriptional elongation in T cells. In order to provide a direct demonstration that NF-κB, rather than another factor, was responsible for the increase in transcription elongation, a series of experiments were performed using plasmids expressing the individual subunits of NF-κB to stimulate HIV expression. The NF-κB/Rel family of transcription factors is made up of five proteins: p65 (Rel A), p50 (NF-κB-1), c-Rel, Rel B, and p52 (p49, NF-κB-2). The p50 and p52 subunits are synthesized as high-molecular-weight precursors (p105 and p100, respectively) that are proteolytically cleaved to generate their active forms. NF-κB always binds DNA as a dimer, and previous studies have shown that the p52-p65 heterodimer is the most effective activator of HIV-1 transcription in T cells (21). However, the binding of p50 and p52 homodimers to promoters has been observed in unstimulated T cells and macrophages. Similarly, activation of promoters by the p65 homodimer has been observed in mitogen-stimulated T cells (6).

To assess the roles played by the different forms of NF-κB in the activation of HIV transcriptional elongation, Jurkat and CCRF/CEM cells were transfected with different combinations of plasmids expressing the p65, p52, and p50 subunits of NF-κB in the presence of the HIV-1 LTR luciferase reporter. Consistent with the results of others (21), we observed that both p50 and p52 alone were poor activators of HIV-1 transcription in Jurkat cells (Fig. 6A). However, in contrast to Liu et al. (21), we observed that the p52-p65 and p50-p65 heterodimers were able to stimulate transcription from the LTR efficiently and to the same extent. Interestingly, these results also showed that p65 alone was able to activate basal transcription more efficiently than the p52-p65 or p50-p65 dimers (12.8-fold, compared to 6- and 5-fold, respectively). At the suboptimal concentrations of plasmids used in this experiment, the activation of transcription in the presence of Tat by p65 was lower than that observed for the heterodimers.

When titrations of p65 alone or p65 in combination with p52 were performed in Jurkat cells, we observed substantial increases in basal transcription (Fig. 6B). The activation of the HIV LTR by the p65 homodimer resulted in significantly higher levels of transcription than observed for the p52-p65 heterodimer throughout the titration range. For example, when the Jurkat cells were transfected with 600 ng of p65-expressing plasmid, basal transcription was stimulated 7.4-fold, whereas a combination of 300 ng of p65-expressing plasmid plus 300 ng of p52-expressing plasmid resulted in a stimulation of only 3-fold.

The activation of transcription by the NF-κB p65 homodimer was even more pronounced in the CCRF/CEM cells (Fig. 6C). Transfection of the CCRF/CEM cells using 600 ng of p65 produced a 29-fold increase in the basal transcription level, whereas there was only a 2.6-fold increase observed using the p52-p65 heterodimer (Fig. 6C). These results are consistent with our previous observations that the stimulation of basal transcription elongation following treatment of CCRF/CEM cells by PHA and PMA is greater than the stimulation seen in Jurkat cells.

The implication of the results obtained from the luciferase assays described above is that p65 selectively increases basal transcription in part by activating elongation. To test this hypothesis, RNase protection experiments were carried out on CCRF/CEM cells that were cotransfected with the HIV LTR CAT reporter and the p65- or p52-expressing plasmids (Fig. 7).

As shown in Fig. 7, p65 clearly stimulated elongation in CCRF/CEM cells. Although proximal transcript levels increased by 2.3-fold, there was a much greater increase in distal transcripts of up to 4.7-fold. Figure 7 also shows that the p52-p65 heterodimer was less effective at stimulating elongation than was p65 alone. Although the effect on initiation of p52-p65 was similar to that observed using p65 alone, distal transcript levels were stimulated only twofold. As described above, another measure of elongation is to calculate the proportion of long transcripts measured by the distal probe compared to the total transcripts measured by the proximal probe. In CCRF/CEM cells, p65 increased the levels of long transcripts from 7% in control cells to 14.2%. The p65-p52 heterodimer had a slightly smaller effect and increased the proportion of long transcripts to 10.7%. Tat was able to further stimulate transcription elongation. For example, in the presence of p65, Tat increased the proportion of long transcripts to 78%.

Thus, NF-κB can directly stimulate the processivity of transcription complexes recruited to the HIV LTR. The effects of NF-κB on transcriptional elongation appear to be primarily mediated by the p65 subunit of NF-κB, since a stronger effect is observed when the p65 homodimer is used to activate transcription than when the p65-p52 heterodimer acts as the activator.

NF-κB can stimulate HIV transcription under conditions where CDK9 activity is strongly inhibited. We next examined whether the stimulation of transcription observed in the mitogen-stimulated T-cell lines was dependent on the activity of the constitutively expressed CDK9 kinase. In order to inhibit the kinase activity selectively, cells were transfected with a catalytic mutant of CDK9 (D167N) alongside the appropriate reporter plasmids and then treated with PMA and PHA as in the previous experiments. The expression of D167N in *trans* inhibits strongly CDK9-dependent Tat-activated transcription in a wide variety of cell types (10, 23), but it has only minimal effects on CDK9-independent transcriptional elongation stimulated by a cellular enhancer (46).

Figure 8 shows the effect of the coexpression of D167N on wild-type LTR-luciferase reporter plasmid expression in Jurkat cells (Fig. 8A) and CCRF/CEM cells (Fig. 8B). Because CDK9 is essential for Tat-activated transcription, the expression of the CDK9 mutant strongly inhibited Tat-activated transcription in both cell lines. For example, in Jurkat cells (Fig. 8A), Tat-activated transcription in the presence of mitogen was reduced to 26 and 18% of control levels after cotransfection by 1.5 and 3 μg of the D167N plasmid. In CCRF/CEM cells (Fig. 8B) the level of Tat-activated transcription in the presence of

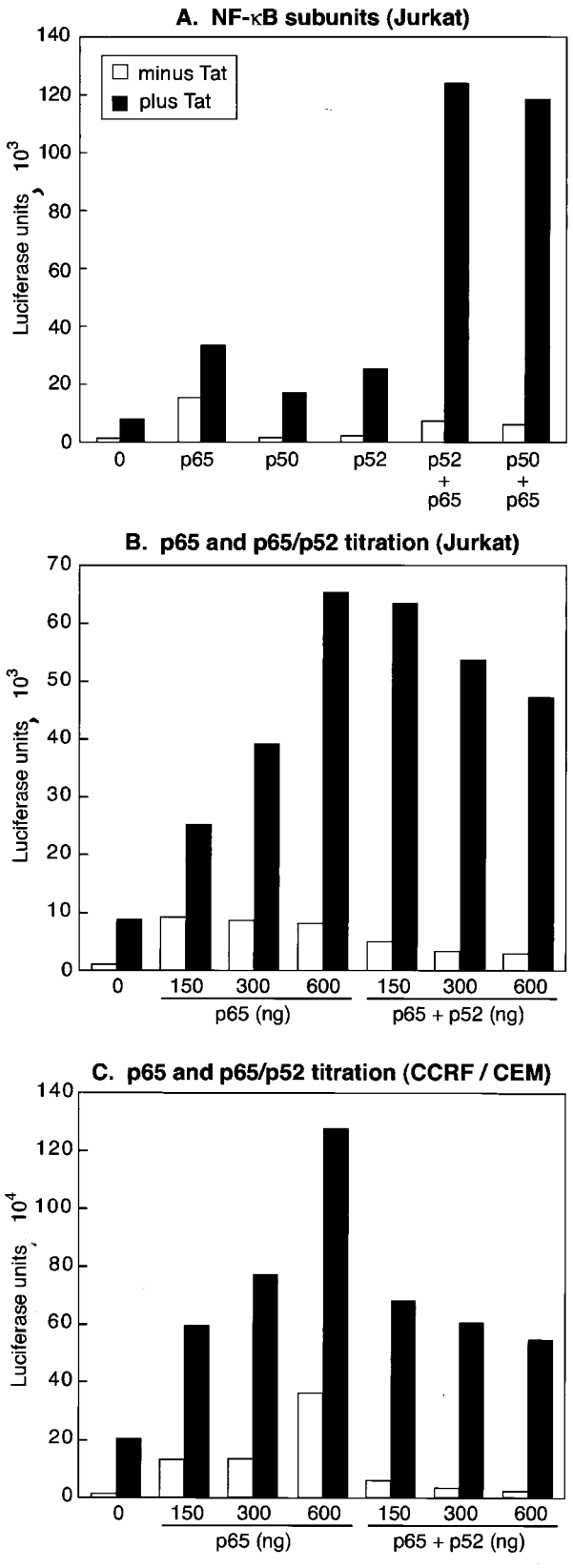

FIG. 6. NF-κB p65 activates transcription in the absence of Tat. (A) Jurkat cells were transfected with 600 ng of the wild-type LTR-FF (firefly luciferase) construct in the absence (minus Tat) or presence (plus Tat) of 40 ng of C63-4-1. Cells were cotransfected with 150 ng of RSV LTR constructs expressing the p65, p50, or p52 subunits of NF-κB, alone or in the indicated combinations. (B) Jurkat cells were transfected as in panel A but with increasing amounts of the p65-expressing plasmid, in the absence or presence of increasing amounts of the p52-expressing plasmid. For p65 and p52 coexpression, the indicated amounts of both plasmids were included in the transfection. (C) The effect of p65 and p52 expression on LTR-FF activity in CCRF/CEM cells.

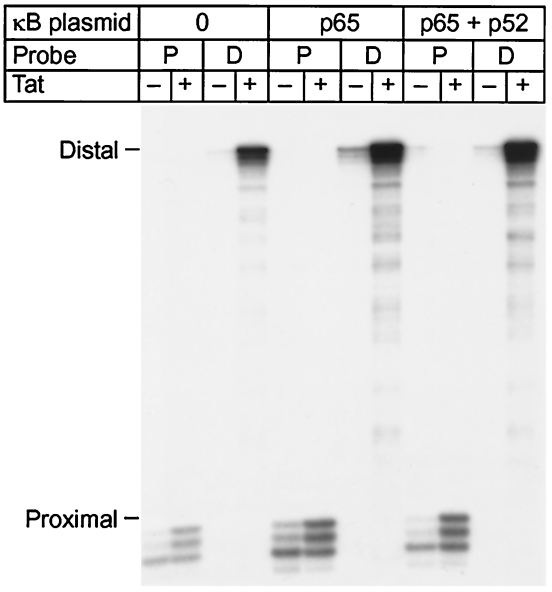

FIG. 7. NF-κB p65 stimulates transcription elongation. CCRF/CEM cells were transfected with 1 μg of the wild-type CAT reporter construct (D5-3-3) in the absence (−Tat) or presence (+Tat) of 250 ng of C63-4-1 and 1.2 μg of the p65-expressing plasmid, or with a combination of 0.6 μg each of the p65- and p52-expressing plasmids. Total RNA was extracted and analyzed with proximal and distal antisense probes as described in Fig. 2. P, proximal probe; D, distal probe.

mitogen was reduced to 36 and 16% of control levels in the presence of 1.5 and 3 μg of D167N, respectively.

In contrast, as shown in Fig. 8, basal transcription in mitogen-activated T cells (−Tat, +PMA, +PHA) is much less sensitive to the inhibition of CDK9 than Tat-activated transcription (+Tat, +PMA, +PHA). As a result, the net effect of adding D167N to cells treated with Tat and mitogens is to selectively inhibit the Tat-dependent component of transcription and reduce the overall levels of transcription to levels that are nearly identical to those seen in the absence of Tat. The insensitivity of mitogen-activated transcription to inhibition by D167N is further demonstrated by the observation that there is a large increase in luciferase activity after mitogen treatment of cells transfected by D167N. For example, there is a 5.4-fold increase in luciferase levels in Jurkat cells transfected by 1.5 μg of D167N and subsequently activated by PMA and PHA. Similarly, in Jurkat cells transfected with 3.0 μg of D167N and treated with PMA and PHA there was a 3.9-fold increase. These results therefore indicate that CDK9 is not a primary mediator of the response to mitogens.

RNase protection assays were also used to evaluate the role of CDK9 in the activation of HIV transcription initiation and

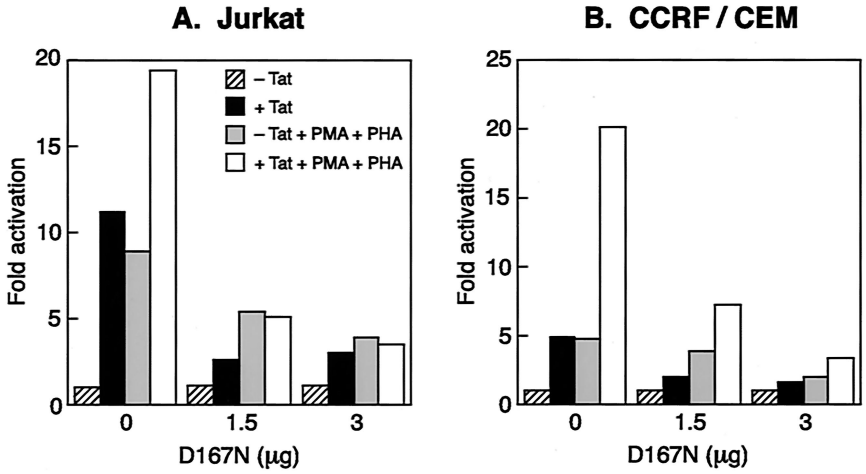

FIG. 8. Activation of HIV transcription by mitogens in T cells does not require high levels of CDK9 activity. (A) Jurkat cells were transfected with 600 ng of LTR-FF in the absence (−Tat) or presence (+Tat) of 50 ng of C63-4-1. Various amounts of a plasmid expressing a mutant form of CDK9 (FLAG-D167N) were included in the transfections where indicated. Vector DNA was used to keep the amount of FLAG plasmid constant in all samples. Cells were treated with PMA and PHA at 24 h posttransfection as in the legend to Fig. 1. Toxicity assays were performed in parallel, and the results were adjusted accordingly. (B) Effect of the expression of D167N in *trans* in CCRF/CEM cells.

elongation in the mitogen-activated T cells (Fig. 9). Since Jurkat cells show a much larger increase in proximal transcript levels after mitogen stimulation than CCRF/CEM cells, we used this cell line for the experiment. These experiments were performed using concentrations of D167N that fully inactivate Tat-dependent transcription but do not significantly inhibit basal transcription.

As shown in Fig. 9, the addition of PMA and PHA to control cells increased proximal transcript levels 4.5-fold. Under these conditions expression of D167N did not significantly inhibit total transcription, and proximal transcript levels remained at 82% of the levels detected in control cells. Addition of PHA and PMA to control cells resulted in a 10-fold increase in the level of transcripts detected by the distal probe. This large increase in full-length transcription was maintained in the presence of D167N. Thus, the level of distal transcripts remained at 78% of wild-type levels in the presence of 0.5 μg of the D167N-expressing plasmid. In contrast, at these levels of the D167N-expressing plasmid, Tat-dependent transcriptional elongation was nearly completely inhibited. In the absence of D167N Tat was able to increase the proportion of long transcripts from 19 to 36%. However, coexpression of D167N completely abolished the Tat activation effect, and the proportion of long transcripts increased from 11% to only 16%.

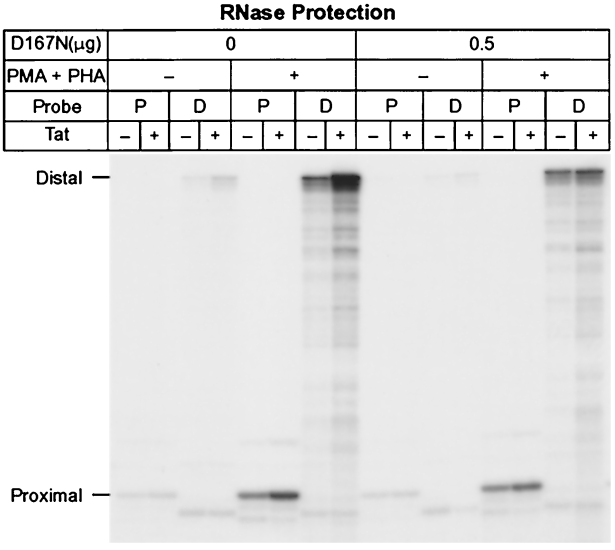

FIG. 9. Stimulation of initiation and elongation in mitogen-activated T cells does not require CDK9. Jurkat cells were transfected with 1 μg of the wild-type CAT reporter (D5-3-3) in the absence (−Tat) or presence (+Tat) of 250 ng of C63-4-1. A total of 0.5 μg of D167N was included in the transfections where indicated. Cells were treated with PMA and PHA as in the legend to Fig. 1, and total RNA was analyzed by hybridization to proximal (P) or distal (D) antisense probes.

Thus, NF-κB is able to stimulate both transcription initiation and elongation under conditions where CDK9 activity is strongly inhibited. In contrast, Tat-activated transcription shows a strict requirement for CDK9 and is thus mechanistically distinct. Although these results indicate that CDK9 is not stringently required for the NF-κB response, the partial inhibition of basal transcription in mitogen-treated T cells by high concentrations of D167N indicates that CDK9 probably plays a role in supporting transcription from the HIV LTR even in the absence of Tat.

NF-κB p65 stimulates elongation independently of histone acetylation. Activation of transcription from the HIV LTR by NF-κB is mediated, at least in part, by the recruitment of histone acetyltransferases to the promoter and the subsequent disruption of promoter-proximal nucleosomal structures (8, 27, 33, 39, 42). To determine whether the stimulation of transcriptional elongation mediated by NF-κB that we have observed can occur independently of histone acetylation, we performed experiments using the histone deacetylase inhibitor, trichostatin A (TSA). Exposure of T cells to TSA increases the acetylation of histones globally and, as a consequence, transcription from integrated HIV LTRs is strongly stimulated (42).

Jurkat and CCRF/CEM cells were transfected with the HIV

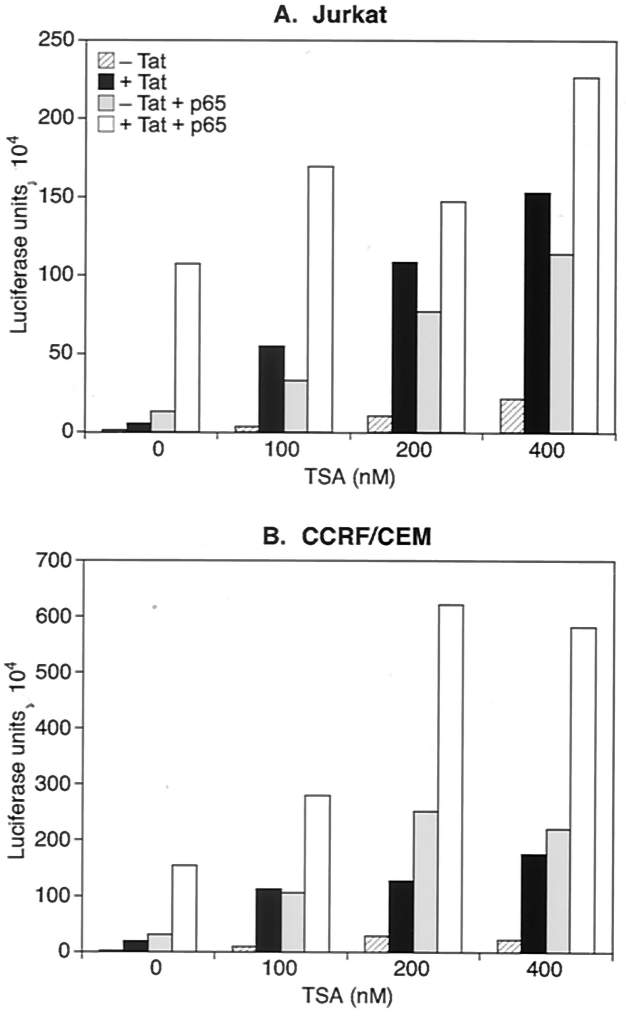

FIG. 10. Synergistic stimulation of transcription from the HIV LTR by treatment of cells with TSA and NF-κB p65. (A) Jurkat cells were transfected with 600 ng of LTR-FF in the absence (−Tat) or presence (+Tat) of 40 ng of C63-4-1 and in the absence (−p65) or presence (+p65) of 600 ng of the p65-expressing plasmid. Cells were treated with the indicated concentration of TSA at 24 h posttransfection and harvested for analysis after a further 24 h. Toxicity assays were performed in parallel, and the luciferase activities were corrected accordingly. (B) CCRF/CEM cells were transfected and analyzed as for panel A.

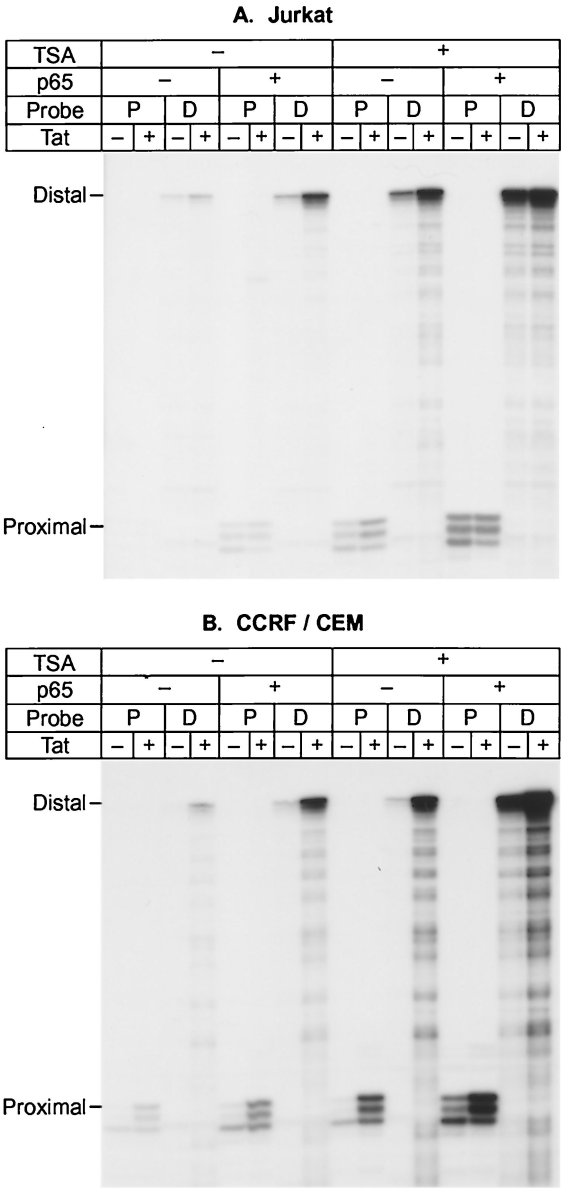

FIG. 11. p65 stimulates elongation from the HIV LTR in the presence of TSA. Jurkat cells (A) and CCRF/CEM cells (B) were transfected with 1 μg of D5-3-3 in the absence (−Tat) or presence (+Tat) of 250 ng of C63-4-1 and in the absence (−p65) or presence (+p65) of 1.2 μg of the p65-expressing plasmid. Cells were treated with 400 nM TSA at 24 h posttransfection and harvested after a further 24 h. RNA was extracted and analyzed as in Fig. 2. P, proximal probe; D, distal probe.

LTR luciferase reporter construct in the absence or presence of the NF-κB p65-expressing plasmid and treated with TSA 24 h posttransfection (Fig. 10). Although transcription from transiently transfected HIV LTR reporter constructs is not activated as strongly in response to TSA treatment as integrated proviruses since the plasmid DNA is not assembled fully into chromatin (42) large increases in HIV transcription can be observed following TSA treatment. For example, treatment of Jurkat cells with 400 nM TSA resulted in a 28-fold stimulation of basal transcription, and treatment of CCRF/CEM cells with 200 nM TSA resulted in a 14-fold stimulation of basal transcription (Fig. 10).

Significantly, we observed that p65 retained the ability to activate transcription even when cells were treated with TSA. In the experiment shown in Fig. 10 addition of p65 resulted in a further fivefold increase in luciferase activity in the TSA-treated Jurkat cells, and a further ninefold increase in luciferase activity in the TSA-treated CCRF/CEM cells. The results demonstrate clearly that p65 is able to stimulate transcription independently of histone acetylation.

To examine the effects of p65 on elongation in the presence of TSA in more detail, we performed RNase protection experiments (Fig. 11). When p65 was expressed in the presence of 400 nM TSA in Jurkat cells, the level of distal transcripts

increased 3.8-fold. Furthermore, the proportion of long transcripts increased from 32 to 57% of the total transcripts, demonstrating that p65 increased the elongation potential of the polymerases recruited to the chromatin-disrupted templates. Similar results were obtained when transcription was analyzed in CCRF/CEM cells. When these cells were treated with 400 nM TSA, the expression of p65 led to only a 4-fold increase in total transcription (proximal transcripts), whereas there was a >12-fold increase in distal transcript levels. Thus, expression of p65 increased the proportion of long transcripts from 12 to 46% of the total transcripts.

The remarkable increases in elongation that are mediated by p65 in the TSA-treated cells result in higher levels of distal transcripts than observed by addition of Tat alone. Because basal transcription is increased so dramatically, cotransfection of Tat and p65 into cells receiving TSA treatment results in only a minor further increase in transcription elongation. Thus, p65 is able to stimulate elongation strongly once chromatin structures have been disrupted by global histone acetylation.

DISCUSSION

Control of initiation and elongation during HIV proviral activation. Although initiation of transcription from the HIV LTR is generally efficient, only a small fraction of the transcription complexes which are recruited to the promoter are able to elongate efficiently in the absence of the viral regulatory protein Tat. Tat stimulates transcription elongation because it is able to activate a specific carboxyl-terminal domain (CTD) kinase, CDK9 (23, 44, 50), which phosphorylates RNA Pol II (14, 49), as well as the elongation factor SPT5 (15, 19, 35, 47). Efficient proviral activation therefore requires the early synthesis of Tat to initiate a positive feedback loop that results in a high level of viral mRNA synthesis.

How is the initiation of Tat synthesis following proviral activation achieved? Previous studies have shown that transcription from latent proviruses is restricted due to the absence of NF-κB (28, 32). It has been generally assumed that NF-κB stimulates initiation alone. If this were the case, then the initial synthesis of Tat could only arise if a subset of the transcription complexes initiating at the LTR are able to fortuitously transcribe the entire HIV genome. However, it seemed to us that rather than simply relying on random events, basal transcription from the HIV LTR includes some form of elongation control in order to guarantee that a threshold level of Tat synthesis is reached early during proviral activation.

The experiments described in this study provide strong evidence that both initiation and elongation are regulated when HIV transcription is activated in mitogen-stimulated T cells. Both processes are regulated by the p65 subunit of NF-κB, but the stimulation of initiation and elongation are achieved through distinct biochemical mechanisms.

Regulation of initiation by NF-κB. In agreement with previous reports that looked exclusively at the effects of NF-κB on overall transcription, the RNase protection assays described here show that transcription initiation is stimulated by NF-κB in the absence of Tat. Initiation increased 1.25- to 5.5-fold in CCRF/CEM cells and Jurkat cells that had been treated with mitogens or transfected with plasmids expressing NF-κB.

The results presented here show that the stimulation of transcription initiation by NF-κB does not require CDK9, and all of the transcription complexes initiating under these conditions are able to respond to Tat. The extent of the activation of initiation varies significantly between the Jurkat cells and CCRF/CEM cells, with the Jurkat cells showing the highest levels of induction. All of these properties make it likely that the effects of NF-κB on initiation are due to its ability to derepress chromatin structures (8, 42).

After integration into the host chromosome, the HIV-1 provirus is assembled into chromatin, with nucleosomes that are positioned at precise locations around the viral promoter (43). Nucleosome 0 (Nuc-0) and Nuc-1 are located at positions −415 to −255 and positions +10 to +155 with respect to the transcription start site (Fig. 10). Although the binding sites for SP1 and NF-κB remain accessible, Nuc-1 impedes transcription by occluding the initiation site.

There is strong evidence that Nuc-1 is disrupted after the activation of HIV-1 transcription by tumor necrosis factor alpha and PMA in transfected T cells (43) and latently infected cell lines (42). This modulation of chromatin structure appears to be mediated by recruitment of histone deacetylases to the HIV LTR by NF-κB. NF-κB is able to recruit multiple coactivators to promoters, including CBP (CREB-binding protein), p300, and members of the p160 family of coactivators (8, 27, 31, 33). Many of these coactivators possess histone acetyltransferase activity and are therefore capable of hyperacetylating histones at specific promoters. Consistent with this idea, the histone deacetylase inhibitor TSA has been shown to promote the hyperacetylation of histones and activate HIV-1 transcription (42).

Role of NF-κB subunits in the stimulation of transcriptional elongation. A second, more subtle, feature of the activation of the HIV LTR by NF-κB, which is documented here for the first time, is that it is also able to stimulate elongation. We have shown that the proportion of total transcripts extending to distal regions of the template was increased following activation, indicating that a proportion of the recruited polymerases had enhanced elongation properties. This demonstration that DNA elements present in the LTR can regulate elongation is consistent with our earlier observation that the insertion of a cellular enhancer into the LTR led to the recruitment of processive polymerases to this promoter and alleviated the requirement for Tat (46).

The stimulation of transcriptional elongation by NF-κB appears to be due to the p65 subunit. We have found that p65 homodimer is a more potent activator of transcriptional elongation than the p52-p65 heterodimer and that no effect on elongation is observed with the p52 homodimer. Similarly, it has been observed that the p50/Rel B heterodimer of NF-κB is able to stimulate transcriptional elongation through a pause site in intron I of the *c-myb* gene (40). Rel B is homologous to p65 and contains a transcriptional activation domain at its C terminus that is absent from p52. These observations suggest that the unique carboxyl-terminal activation domain of p65 is responsible for regulating elongation (5).

It is important to note that stimulation of transcription elongation by NF-κB involves a distinct biochemical mechanism. Disruption of chromatin structures by treatment of cells with TSA increases HIV transcription initiation and, to a lesser degree, elongation. Since Nuc-1, the major nucleosome dis-

rupted during HIV LTR transcriptional activation, is positioned downstream from the transcription start site, it is possible that histone acetylation itself could relieve a small block to transcriptional elongation imposed by the nucleosomal structure. However, dramatic further increases in transcriptional elongation are seen when p65 is added to TSA-treated cell lines. The effect of p65 under these conditions is as strong as the elongation enhancement mediated by Tat. Because basal transcription is increased so significantly, the addition of Tat under these conditions results in only a small further increase in elongation.

Role of TAK in proviral activation. Recent studies in T cells and monocytes have suggested that the induction of TAK might contribute to the escape from viral latency. TAK activity increases when resting T cells are stimulated with PHA and PMA as a result of an upregulation in CDK9 and CycT1 levels (7, 13). An increase in TAK activity is also observed when promonocytic cell lines are stimulated to differentiate into macrophages with PMA, although this appears to be mediated by an increase in CycT1 levels alone (13). This increase in TAK activity correlates with the increase in viral replication observed in a promonocytic cell line containing an integrated provirus (48). Our results show, however, that although we observed an enormous increase in LTR expression following T-cell activation, there is no concomitant increase in CDK9 or CycT1 protein levels in the cell lines that we have studied. Similarly, Yang et al. (48) reported that TAK activity does not increase in Jurkat and CEM cells after treatment by PMA.

It is likely that even in cells in which TAK is activated upon mitogenic stimulation, basal transcription levels are repressed due to chromatin assembly. Consistent with this hypothesis, we have found that the level of LTR transcription in the unstimulated promonocytic cell line U937 is below detectable levels and that stimulation of the LTR is associated with parallel increases in both NF-κB and CycT1 (data not shown).

Molecular mechanisms regulating HIV proviral induction. A model for the mechanism of proviral induction in T cells incorporating the ideas discussed above is shown in Fig. 12. In unstimulated T cells, transcription is repressed by the presence of extensive chromatin structure around the viral promoter. After exposure to mitogen or antigen, NF-κB translocates to the nucleus and binds to its recognition sites in the HIV LTR. NF-κB then directs the assembly of a multicomponent complex containing CBP, p300, p/CAF, SRC-1, and SRC-1-related proteins at the promoter. The acetylation of histones by this complex, possibly in combination with the activity of other chromatin remodeling factors, leads to the disruption of Nuc-1 and the stimulation of initiation (16).

Consistent with NF-κB's ability to depress chromatin, the increase in transcription initiation that we have observed in stimulated T cells is insensitive to the expression of a CDK9 mutant in *trans* that is able to efficiently inhibit CDK9-dependent Tat-activated transcription. Thus, although evidence from our own and other laboratories has shown that both CDK9 and CycT1 are present at the promoter (14, 34, 49), our results suggest that this kinase is not required for transcription initiation during T-cell activation.

It is also interesting in the context of this model to consider the different activation profiles we observed in Jurkat and CCRF/CEM cells. In Jurkat cells the effect of mitogenic stim-

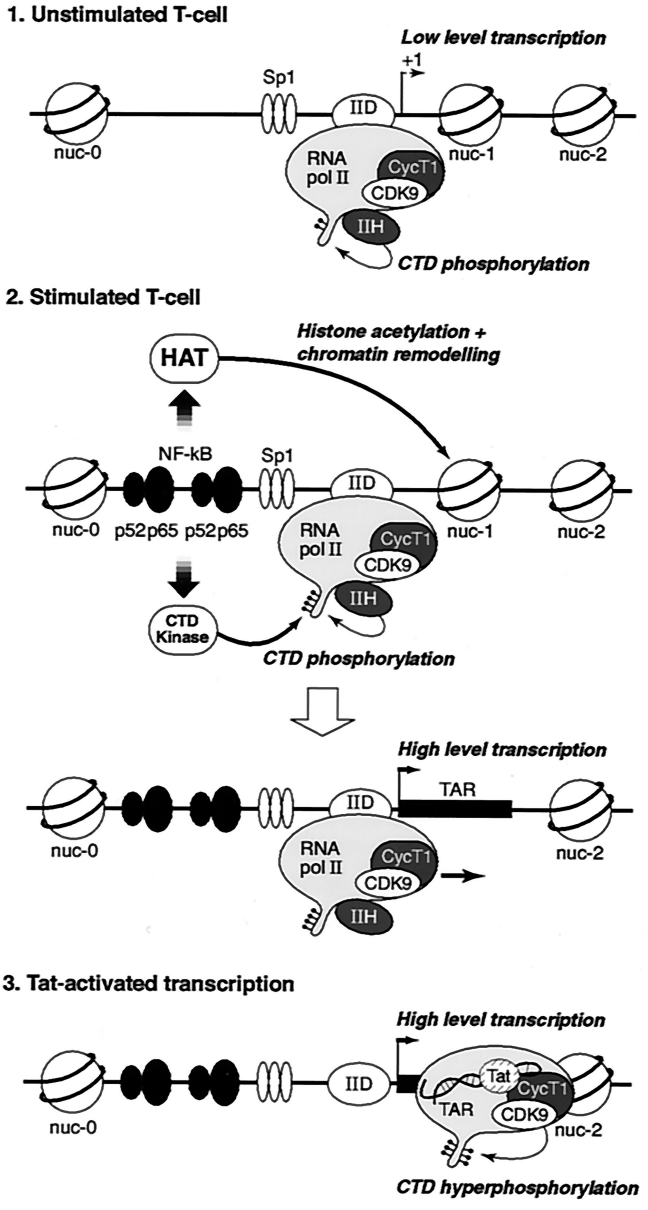

FIG. 12. Activation of HIV transcription during T-cell activation. (1) In unstimulated T-cells the integrated HIV-1 proviral DNA is assembled into chromatin, with nucleosomes positioned at defined positions along the DNA. The extensive chromatin structure around the HIV promoter helps to suppress transcription. (2) Following stimulation of T cells with antigen or mitogen, NF-κB translocates to the nucleus and binds to its recognition sites in the LTR. NF-κB associates with a coactivator complex containing histone acetyltransferases (CBP, p300, p/CAF, SRC-1, and SRC-1-related proteins). The histone acetyltransferase activity, together with additional chromatin remodeling factors, leads to the disruption of Nuc-1 and the stimulation of transcription initiation. The ability of NF-κB p65 to stimulate transcription elongation is probably due to the activation of a CTD kinase which catalyzes the phosphorylation of the CTD of RNA Pol II. (3) Tat produced during the early rounds of HIV transcription binds to the TAR RNA element present at the 5′ end of all viral transcripts and further stimulates transcription. Tat activation of transcription is due to the hyperphosphorylation of the CTD by CDK9.

ulation on initiation levels was always much greater than in the CCRF/CEM cells. Similarly, TSA stimulates elongation in the Jurkat cells to a greater extent than in CCRF/CEM cells. It is likely that these differences are the result of increased chromatin repression in the Jurkat cells resulting in lower levels of basal transcription. The chromatin derepression function of NF-κB would therefore lead to more pronounced increases in initiation from the promoter. In contrast, in CCRF/CEM cells there may be less chromatin structure around the promoter, and the effects of NF-κB on initiation are therefore less evident.

We have shown here that, in addition to stimulating initiation, NF-κB p65 is also able to stimulate elongation. This effect is particularly apparent when chromatin structures are disrupted by treatment of cells with TSA. The biochemical basis for this activity is not yet understood, but one attractive hypothesis is that the p65 subunit of NF-κB is able to stimulate CTD phosphorylation at the HIV promoter and that this, in turn, results in the stimulation of elongation. For example, Nissen and Yamamoto (29) have shown that NF-κB can stimulate the phosphorylation of serine 2 and serine 5 residues in the CTD of the Pol II complexes assembled at the promoters of the interleukin-8 and ICAM-1 genes. Repression of these genes by the glucocorticoid receptor correlates with its ability to bind to NF-κB and inhibit the phosphorylation of serine 2. Since the phosphorylation of the CTD of Pol II is a prerequisite for promoter clearance and efficient transcriptional elongation, it seems likely that the activation of transcriptional elongation by p65 is mediated by the phosphorylation of the CTD.

The three CTD kinases present in preinitiation complexes formed at the HIV promoter—CDK7, CDK8, and CDK9—can each be considered to be potential mediators of NF-κB activity. Our initial observations suggest that CDK9 may not be the primary mediator of responses to p65 since NF-κB-stimulated elongation is less sensitive than Tat-activated transcription to the expression of the CDK9 mutant in *trans*. The partial inhibition of mitogen-activated transcription that can be observed when cells are treated by high concentrations of D167N is possibly a reflection of the generalized contribution made by CDK9 to the maintenance of polymerase processivity rather than an indication that CDK9 is required specifically for the response to NF-κB. In agreement with these observations, we reported previously that the stimulation of transcriptional elongation by the immunoglobulin heavy-chain enhancer is also mediated by CDK9-independent mechanisms (46). Another candidate for the mediator role is the CTD kinase CDK8, which has substrate specificity that is distinct from CDK7 and CDK9 (37). CDK8 is present in HIV preinitiation complexes (data not shown) and in complexes formed with the viral activators E1a and VP16 (9). We are currently testing whether CDK8 or CDK9 can be activated by NF-κB.

In conclusion, the results described in this study provide new insights into the mechanisms involved in the stimulation of transcription during proviral activation. Studies using cell-free transcription systems should provide additional evidence that NF-κB can stimulate transcription elongation and permit the identification of additional components of the transcription machinery required for efficient proviral activation.

ACKNOWLEDGMENTS

We thank Martin Rowe for the gift of the NF-κB-expressing plasmids. We also acknowledge our colleagues in the LMB for their help and advice, and we especially thank Cyril Bourgeois, Catherine Isel, Young Kyeung Kim, and Marc Bailey.

REFERENCES

1. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
2. **Baldwin, A. S. J.** 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. **14:**649–681.
3. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 Tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO J. **9:**4145–4153.
4. **Duckett, C. S., N. D. Perkins, T. F. Kowalik, R. M. Schmid, E. S. Huang, A. S. J. Baldwin, and G. J. Nabel.** 1993. Dimerization of NF-κB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an IκB-alpha (MAD-3). Mol. Cell. Biol. **13:**1315–1322.
5. **Fujita, T., G. P. Nolan, S. Ghosh, and D. Baltimore.** 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. **6:**775–787.
6. **Ganchi, P. A., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1993. A novel NF-κB complex containing p65 homodimers: implications for transcriptional control at the level of subunit dimerization. Mol. Cell. Biol. **13:**7826–7835.
7. **Garriga, J., J. Peng, M. Parreno, D. H. Price, E. E. Henderson, and X. Grana.** 1998. Upregulation of cyclin T1/CDK9 complexes during T cell activation. Oncogene **17:**3093–3102.
8. **Gerritsen, M. E., A. J. Williams, A. S. Neish, S. Moore, Y. Shi, and T. Collins.** 1997. CREB-binding protein/p300 are transcriptional coactivators of p65. Proc. Natl. Acad. Sci. USA **94:**2927–2932.
9. **Gold, M. O., J.-P. Tassan, E. A. Nigg, A. P. Rice, and C. H. Herrmann.** 1996. Viral transactivators E1A and VP16 interact with a large complex that is associated with CTD kinase activity and contains CDK8. Nucleic Acids Res. **24:**3771–3777.
10. **Gold, M. O., X. Yang, C. H. Herrmann, and A. P. Rice.** 1998. PITALRE, the catalytic subunit of TAK, is required for human immunodeficiency virus Tat *trans*-activation *in vivo*. J. Virol. **72:**4448–4453.
11. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature **339:**70–73.
12. **Grossman, Z., M. B. Feinberg, and W. E. Paul.** 1998. Multiple modes of cellular activation and virus transmission in HIV infection: a role for chronically and latently infected cells in sustaining viral replication. Proc. Natl. Acad. Sci. USA **95:**6314–6319.
13. **Herrmann, C. H., R. G. Carroll, P. Wei, K. A. Jones, and A. P. Rice.** 1998. Tat-associated kinase, TAK, activity is regulated by distinct mechanisms in peripheral blood lymphocytes and promonocytic cell lines. J. Virol. **72:**9881–9888.
14. **Isel, C., and J. Karn.** 1999. Direct evidence that HIV-1 Tat activates the Tat-associated kinase (TAK) during transcriptional elongation. J. Mol. Biol. **290:**929–941.
15. **Ivanov, D., Y. T. Kwak, J. Guo, and R. B. Gaynor.** 2000. Domains in the SPT5 protein that modulate its transcriptional regulatory properties. Mol. Cell. Biol. **20:**2970–2983.
16. **Jones, K. A., and J. T. Kadonaga.** 2000. Exploring the transcription-chromatin interface. Genes Dev. **14:**1992–1996.
17. **Karn, J.** 1999. Tackling Tat. J. Mol. Biol. **293:**235–254.
18. **Keen, N. J., M. J. Churcher, and J. Karn.** 1997. Transfer of Tat and release of TAR RNA during the activation of the human immunodeficiency virus type-1 transcription elongation complex. EMBO J. **16:**5260–5272.
19. **Kim, J. B., and P. A. Sharp.** 2001. Positive transcription elongation factor b phosphorylates hSPT5 and RNA polymerase II carboxyl-terminal domain independently of cyclin-dependent kinase-activating kinase. J. Biol. Chem. **276:**12317–12323.
20. **Kinoshita, S., L. Su, M. Amano, L. A. Timmerman, H. Kaneshima, and G. P. Nolan.** 1997. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. Immunity **6:**235–244.
21. **Liu, J., N. D. Perkins, R. M. Schmid, and G. J. Nabel.** 1992. Specific NF-κB subunits act in concert with tat to stimulate human immunodeficiency virus type 1 transcription. J. Virol. **66:**3883–3887.
22. **Lu, Y., N. Touzjian, M. Stenzel, T. Dorfman, J. Sodroski, and W. A. Haseltine.** 1990. Identification of *cis*-acting repressive sequences within the negative regulatory element of human immunodeficiency virus type 1. J. Virol. **64:**5226–5229.
23. **Mancebo, H. S. Y., G. Lee, J. Flygare, J. Tomassini, P. Luu, Y. Zhu, J. Peng, C. Blau, D. Hazuda, D. Price, and O. Flores.** 1997. p-TEFb kinase is required for HIV Tat transcriptional activation *in vivo* and *in vitro*. Genes Dev. **11:**2633–2644.
24. **Marciniak, R. A., and P. A. Sharp.** 1991. HIV-1 Tat protein promotes

formation of more-processive elongation complexes. EMBO J. **10:**4189–4196.
25. **Markovitz, D. M., M. C. Hannibal, M. J. Smith, R. Cossman, and G. J. Nabel.** 1992. Activation of the human immunodeficiency virus type 1 enhancer is not dependent on NFAT-1. J. Virol. **66:**3961–3965.
26. **Moses, A. V., C. Ibanez, R. Gaynor, P. Ghazal, and J. A. Nelson.** 1994. Differential role of long terminal repeat control elements for the regulation of basal and tat-mediated transcription of the human immunodeficiency virus in stimulated and unstimulated primary human macrophages. J. Virol. **68:**298–307.
27. **Na, S.-Y., S.-K. Lee, S.-J. Han, H.-S. Choi, S.-Y. Im, and J. W. Lee.** 1998. Steriod receptor coactivator-1 interacts with the p50 subunit and coactivates nuclear factor κB-mediated transactivations. J. Biol. Chem. **273:**10831–10834.
28. **Nabel, G., and D. A. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature **326:**711–713.
29. **Nissen, R. M., and K. R. Yamamoto.** 2000. The glucocorticoid receptor inhibits NFkB by interferring with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. **14:**2314–2329.
30. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature **362:**355–358.
31. **Pazin, M. J., P. L. Sheridan, K. Cannon, Z. Cao, J. G. Keck, J. T. Kadonaga, and K. A. Jones.** 1996. NF-κB-mediated chromatin recongifuration and transcriptional activation of the HIV-1 enhancer *in vitro*. Genes Dev. **10:**37–49.
32. **Perkins, N. D., N. L. Edwards, C. S. Duckett, A. B. Agranoff, R. M. Schmid, and G. J. Nabel.** 1993. A cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer activation. EMBO J. **12:**3551–3558.
33. **Perkins, N. D., L. K. Felzien, J. C. Betts, K. Leung, D. H. Beach, and G. J. Nabel.** 1997. Regulation of NF-κB by cyclin-dependent kinases associated with the p300 coactivator. Science **275:**523–527.
34. **Ping, Y.-H., and T. M. Rana.** 1999. Tat-associated kinase (P-TEF-b): a component of transcription preinitiation and elongation complexes. J. Biol. Chem. **274:**7399–7404.
35. **Ping, Y. H., and T. M. Rana.** 2001. DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates pTEFb-mediated phosphorylation of RNA polymerase II and DSIF during transcription elongation. J. Biol. Chem. **276:**12951–12958.
36. **Price, D. H.** 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol. Cell. Biol. **20:**2629–2634.
37. **Ramanathan, Y., S. M. Rajpara, S. M. Reza, E. Lees, S. Shuman, M. B. Mathews, and T. Pe'ery.** 2001. Three RNA polymerase II CTD kinases display distinct substrate preferences. J. Biol. Chem. **276:**10913–10920.
38. **Rittner, K., M. J. Churcher, M. J. Gait, and J. Karn.** 1995. The human immunodeficiency virus long terminal repeat includes a specialised initiator element which is required for Tat-responsive transcription. J. Mol. Biol. **248:**562–580.
39. **Sheridan, P. L., T. P. Mayall, E. Verdin, and K. A. Jones.** 1997. Histone acetyltransferases regulate HIV-1 enhancer activity *in vitro*. Genes Dev. **11:**3327–3340.
40. **Suhasini, M., and R. B. Pilz.** 1999. Transcriptional elongation of c-*myb* is regulated by NF-κB (p50/RelB). Oncogene **18:**7360–7369.
41. **Taube, R., K. Fujinaga, J. Wimmer, M. Barboric, and B. M. Peterlin.** 1999. Tat transactivation: a model for the regulation of eukaryotic transcriptional elongation. Virology **264:**245–253.
42. **Van Lint, C., S. Emiliani, M. Ott, and E. Verdin.** 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. EMBO J. **15:**1112–1120.
43. **Verdin, E., P. J. Paras, and C. Van Lint.** 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. EMBO J. **12:**3249–3259.
44. **Wei, P., M. E. Garber, S.-M. Fang, W. H. Fischer, and K. A. Jones.** 1998. A novel cdk9-associated c-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop specific binding to TAR RNA. Cell **92:**451–462.
45. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, M. S. Saag, and G. M. Shaw.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117–122.
46. **West, M. J., and J. Karn.** 1999. Stimulation of Tat-associated kinase-independent transcriptional elongation from the human immunodeficiency virus type-1 long terminal repeat by a cellular enhancer. EMBO J. **18:**1378–1386.
47. **Wu-Baer, F., W. S. Lane, and R. B. Gaynor.** 1998. Role of the human homolog of the yeast transcription factor SPT5 in HIV-1 Tat-activation. J. Mol. Biol. **277:**179–197.
48. **Yang, X., M. O. Gold, D. N. Tang, D. E. Lewis, E. Aguilar-Cordova, A. P. Rice, and C. H. Herrmann.** 1997. TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. Proc. Natl. Acad. Sci. USA **94:**12331–12336.
49. **Zhou, M., M. A. Halanski, M. F. Radonovich, F. Kashanchi, J. Peng, D. H. Price, and J. N. Brady.** 2000. Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. Mol. Cell. Biol. **20:**5077–5086.
50. **Zhu, Y., T. Pe'ery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M. B. Mathews, and D. H. Price.** 1997. Transcription elongation factor p-TEFb is required for HIV-1 Tat trans-activation *in vitro*. Genes Dev. **11:**2622–2632.