MOLECULAR AND CELLULAR BIOLOGY, Apr. 1991, p. 1883–1893
0270-7306/91/041883-11$02.00/0

Vol. 11, No. 4

An In Vitro Transcription Analysis of Early Responses of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat to Different Transcriptional Activators

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Received 24 August 1990/Accepted 9 January 1991

In this report we introduce a simple, fast, and reliable method to prepare whole cell or nuclear extracts from small numbers of cells. These extracts were used to study transcriptional activation of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) in vitro. Our results revealed that the time courses of activation of extracts derived from cells stimulated with the mitogenic lectin phytohemagglutinin (PHA) or with the tumor promoter phorbol 12-myristate 13-acetate (PMA) are different. PMA induces a rapid onset of increased in vitro transcription from the HIV-1 LTR, while PHA causes a slow and sustained response. The biochemical relevance of protein synthesis inhibition by cycloheximide treatment of cells was investigated. In these studies, PMA induction of a change in in vitro transcriptional activity is not dependent on protein synthesis. Cycloheximide alone is insufficient to induce activation. Oligonucleotide-mediated site-directed mutagenesis demonstrated that mutation of the TATA box in the LTR ablated initiation of both basal-level transcription and activation by extracts from cells stimulated with PMA. Surprisingly, mutation of both κB sites in the LTR reduced but did not eliminate the in vitro response to extracts prepared at early time points after PHA or PMA stimulation of Jurkat cells. The reduction was greater in extracts derived from cells treated with PMA. Deletion analysis of the HIV-1 LTR revealed at least one region (−464 to −252) capable of suppressing in vitro transcription in extracts from Jurkat cells stimulated by PMA. This result is consistent with early studies of the HIV-1 LTR in transient transfection assays. We therefore have been able to observe distinct regulatory events at early time points after cells are exposed to agents known to induce transcription of both the HIV-1 LTR reporter gene constructs and the HIV-1 provirus itself.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (15, 21, 29). The typical long latency before onset of symptoms may be related to the way in which the provirus is transcriptionally activated in response to intracellular signals. Among the major targets of HIV-1 are $CD4^+$ T lymphoblasts (29, 40, 41, 51). In in vitro tissue culture experiments using T lymphoblasts, production of the virus increases after the cells are exposed to mitogenic lectins and phorbol esters (27, 74). The stimulation is mediated through viral regulatory sequences localized largely in the long terminal repeat (LTR) (18, 36, 54, 61, 65, 68, 69). Certain transcriptional control sequences in the LTR have been genetically defined. A duplicated 10-bp sequence identified as an HIV-1 enhancer resides in the region −104 to −80 (relative to start site +1) of the LTR (36, 61, 68). Deletion analysis using transfection assays of the LTR uncovered an apparent negative regulatory region from −350 to −278 (61, 65). The specific sequence element(s) responsible for the effect within this region remains to be defined. Three activator protein 1 (AP-1) sites, however, are present in this region (19). Another control element in the HIV-1 LTR is the sequence motif Sp1 (61, 68). It is present as three tandem repeats (33). Both in vitro and in vivo studies showed that these repeats are important for HIV-1 LTR basal activity (22, 33, 55, 65). The TATA box in the HIV-1 LTR is required for basal and responsive transcriptional activity (55).

Previous studies by transfection experiments in T lymphoblasts showed that activity of the HIV-1 LTR increases dramatically following stimulation of cells with either phorbol 12-myristate 13-acetate (PMA) or phytohemagglutinin (PHA) or a combination of both (36, 54, 65, 67, 69). The AP-1 sequence element mediates transcriptional response to signals induced by the tumor promoter PMA has been found in many transcription control regions, including the HIV-1 LTR (9, 11, 20, 25, 32, 37, 42, 70, 71). Several constitutive and inducible cellular proteins that associate with this site have been identified. The molecular components and their functions in the PMA response pathway are not yet completely defined. However, we do know that protein kinase C is a crucial early mediator of response to PMA (56, 57). The mitogenic lectin PHA induces changes of cell surface proteins, but again the components of the signal transducing pathway(s) are in the early stages of characterization.

Though the HIV-1 LTR contains AP-1 binding sites that represent PMA response element sequences in several promoters, in vivo and in vitro deletion analysis demonstrated that inducibility of the HIV-1 LTR activity by PMA alone or in combination with PHA was largely dependent on the κB sites (14, 36, 65). Numerous cellular proteins that interact with the κB sites have been identified, and it has been demonstrated that the κB binding by several proteins is induced by activators of transcription (2, 4, 17, 18, 73). The ubiquitously expressed NF-κB belongs to this set of proteins. NF-κB was originally described as a tissue-specific factor that bound the κB site in the immunoglobulin κ light-chain gene enhancer (64). Subsequently, it was shown to bind to the duplicated 10-bp sequence of the HIV-1 enhancer κB core elements (54). Mutations of nucleotides within the κB binding site eliminate the binding of NF-κB and the association of several other proteins (18, 54, 73).

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These or similar mutations were also shown to abolish the increase in HIV-1 gene expression in PHA- and PMA-activated T lymphoblasts and other cell types (22, 54, 55, 65, 69, 73).

NF-κB has been purified as a heterodimer composed of p50 and p65 subunits (2). Recently, three groups (6, 24, 38) have cloned a gene that encodes a 105-kDa protein. A portion of that protein, of approximately 50 kDa, is related to *dorsal*, *rel*, and v-*rel*, is the p50 protein referred to as KBF1, and is the p50 component of NF-κB. KBF1 was identified to bind the enhancer site of class I major histocompatibility genes at a site that structurally resembles a κB site (5, 39). The p50 DNA-binding subunit of NF-κB is complexed in the cytoplasm with an inhibitory protein, I-κB (2, 3). It appears that I-κB and p50 associate only when p65 is present in the complex (4). Stimulation of cells with a variety of agents induces the release of NF-κB from I-κB and its translocation to the nucleus (2–4). I-κB is apparently phosphorylated in response to various stimuli and can then no longer associate with NF-κB (23).

The homology of p50 to the product of the c-*rel* proto-oncogene and to v-*rel* is interesting. c-*rel* is expressed primarily in murine B and T lymphocytes (7) and is transcriptionally responsive to various extracellular agents. The response does not require protein synthesis (8). c-*rel* has therefore been classified as an immediate-early gene like c-*fos*. v-*rel*, chimeric v-*rel*/c-*rel*, and the carboxy-terminal portion of c-*rel* have transcriptional activation activity (8, 26, 35). We (34) and others (38) have shown that v-Rel binds specifically to κB sites and have since shown that human c-*rel* encodes the κB-specific, inducible binding protein HIVEN86A (18, 45). v-Rel has been demonstrated to associate with several avian cellular proteins by immunoprecipitation analysis (52, 66). Whether cellular proteins identified to associate with human Rel (17a) modulate its activity remains to be determined.

The studies of activation of HIV-1 LTR expression thus far have focused primarily on a single, late time point (24 to 48 h) after PHA and PMA addition. Little attention has been directed to effects on HIV-1 LTR expression immediately following induction of changes in cellular growth. We were therefore interested in determining whether different pathways or components of the LTR are utilized in the earliest moments following stimulation of cells with PMA or PHA. Transient transfection or gel shift analyses are not sufficient for such studies. Therefore, we developed a microscale system for preparation of nuclear or whole cell extracts to permit study of the changes in transcriptional activity resulting from PHA or PMA stimulation. The microscale nuclear extract preparation is a modification of the methods of Dignam et al. (13) and Lee et al. (46). Using extracts from Jurkat cells ($CD4^+$ human T lymphoblasts), we were able to closely examine the changes in activity of the HIV-1 LTR induced by PMA or PHA. Consequently, we have elucidated different effects of the two agents on in vitro activation of HIV-1 LTR expression. We have also demonstrated that the κB sites are not sufficient to account for changes in transcriptional activation of the HIV-1 LTR induced by PMA or PHA.

MATERIALS AND METHODS

Preparation of DNA templates for in vitro transcription. DNA templates used for in vitro transcription analysis were prepared by polymerase chain reaction (PCR) (62) amplification of the HIV-1 LTR sequence. The fragment includes the sequence from −642 to +1 of the HIV-1 LTR, 80 bp of HIV-1 after the +1 start site, and 169 bp of chloramphenicol acetyltransferase (CAT) sequence (68; also see Fig. 2A). The sequence of the 5′ primer for the PCR reaction is CTCGAG ACCTAGAAAAACATGGAGCAAT. The sequence of the 3′ primer is GGTCTTTAAAAAGCCGTAATATCCAGC. The PCR reaction conditions are as follows: denaturation of the template at 95°C for 1 min, followed by a 1-min interval at 50°C to permit template and primers to anneal and then a 3-min incubation at 72°C for polymerase elongation of the primers. This cycle was repeated 25 times. An additional 15 min at 72°C at the end of 25 cycles was included to allow completion of the amplification. PCR products were electrophoresed on a 1% agarose gel. The correct-size product was excised and electroeluted from the gel. Gel-purified PCR product was either used directly after phenol-chloroform extraction or subjected to further purification through an ELUTIP (Schleicher & Schuell) according to the manufacturer's instructions. The PCR-amplified HIV-1 LTR was sequenced to check for mutations.

The reporter plasmids used as controls of the in vitro transcription, pE1B-CAT and pMLP-CAT, were gifts from M. Mathews (12); pβgl-HIV was a gift from N. Hernandez (60). pE1B-CAT and pMLF-CAT were linearized with *Eco*RI; pβgl-HIV was linearized with *Hin*dIII. About 1 μg of linearized plasmids was used in in vitro transcription assays.

DNA sequencing. The PCR-amplified (and therefore blunt-end) DNA templates were phosphorylated at the 5′ end by T4 polynucleotide kinase (10 U/μl; New England BioLabs) as described by Maniatis et al. (48). The templates were subcloned into the *Sma*I site of the PBSM13(+) vector (Promega). The recombinant plasmid was used to transform the competent bacteria, *Escherichia coli* JM109. Double-stranded DNA from positive clones was prepared and sequenced with the Sequenase kit (U.S. Biochemical Corp.). The sequencing reactions were analyzed on a 6% acrylamide–7.5 M urea gel.

Preparation of oligonucleotides for primer extension analysis. Oligonucleotides used for primer extension were 5′ end labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; ICN) according to the protocol of Maniatis et al. (48). The final reaction volume was 10 μl, which includes 3 pmol of primer, 1 μl of 10× kinase buffer (0.5 M Tris-Cl [pH 7.6], 0.1 M $MgCl_2$, 50 mM dithiothreitol [DTT], 1 mM spermidine, 1 mM EDTA), and 5 U of T4 polynucleotide kinase (10.35 U/μl; Stratagene). The reaction was carried out at 37°C for 30 min, and enzyme activity was destroyed by heating at 68°C for 10 to 15 min. The labeled primer was diluted with 90 μl of H_2O and stored at −20°C for future use. The sequence of the HIV-1 primer (+32 to +59) is 5′-GGGTTCCCTAGTTAGCCAGAGAGC TCCC-3′. The U2 primer sequence is 5′-CAGATACTACAC TTG-3′. It is complementary to a region in the first loop of the proposed secondary structure of U2 small nuclear RNA (snRNA) (50). The 3′ end of the primer is 27 bases from the 5′ end of U2 snRNA (1).

Microscale preparation of nuclear extract and whole cell extract. Jurkat cells were grown in RPMI medium plus 15% fetal calf serum. Cells were centrifuged from growth medium and resuspended in the same medium, and equal volumes were added to 60-mm culture dishes. Typically 8×10^7 cells (in 2 ml) were added to each dish. PMA or PHA was added according to the experimental design.

To prepare nuclear extracts, cells were collected and washed with phosphate-buffered saline (PBS) once and once with 200 μl of ice-cold buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5

mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) (13). Cells were lysed in 200 μl of buffer A by gently passing the cell suspension through a 28-gauge needle. This procedure is done with the tube containing the cells submerged in ice. The nuclei were collected by pelleting for 8 s in an Eppendorf microcentrifuge, and the supernatant was discarded. Crude nuclei were extracted with ice-cold buffer C (13) (20 mM HEPES [pH 7.9], 25% [vol/vol] glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), 60 μl for 100 μl of cell pellet, for at least 15 min on ice. An equal volume of buffer D^- (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT) was added, and the mixture was spun in an Eppendorf microcentrifuge for at least 10 min at 4°C. Supernatants were collected, and their volumes were measured. The protein concentration for each preparation was determined by using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

To prepare the whole cell extracts, cells were sequentially washed with PBS, 100 μl of ice-cold buffer A^+ (buffer A containing 20% glycerol), and finally 100 μl of ice-cold buffer A. Cells were lysed in 100 μl of buffer A as described for the nuclear extract. After centrifugation, the supernatant containing the cytoplasmic suspension was transferred to a new Eppendorf tube containing 20 μl of 100% glycerol and kept on ice. The pellet was extracted with buffer C on ice for 15 min. The cytoplasmic suspension and nuclear extract were combined and centrifuged for 15 min at 4°C. The supernatant was collected, and the volume was measured. The protein concentration of the preparation was determined. Typically, 8×10^7 cells yielded at least 500 μg of protein from a nuclear extract and approximately 2.5 mg of protein from a whole cell extract. When not assayed immediately, the nuclear and whole cell extracts were quickly frozen in liquid N_2 and stored at −70°C. Each preparation was adjusted to a final KCl concentration of 100 mM with buffer D^- prior to transcription assays. Extracts have been stored at −70°C for several months and remain active in in vitro transcription assays.

In vitro transcription and primer extension. In vitro transcription (28) reactions were performed according to Dignam et al. (13), with certain modifications. The final reaction volume was either 25 or 35 μl, which includes 1/10 volume of 10× Dignam buffer (250 mM HEPES [pH 7.9], 10 mM DTT, 100 mM KCl, 20% glycerol), 1/10 reaction volume of 80 mM $MgCl_2$, 1/10 volume of 6 mM nucleoside triphosphates (ATP, CTP, UTP, and GTP; pH 7), 50 to 150 ng of PCR-amplified DNA templates, and 1/2 reaction volume of nuclear extract or whole cell extract, fresh or frozen (frozen extract was thawed briefly at 30°C just prior to use). Poly(dI-dC) (Pharmacia) was added only to reactions containing whole cell extracts for binding of nonspecific cellular proteins, since much more nonspecific proteins reside in the whole cell extracts. Reactions were incubated at 30°C for 45 min and stopped by adding a solution containing 0.25% sodium dodecyl sulfate, 0.3 M sodium acetate (pH 5.2), and 0.5 mM EDTA plus 4 μl of proteinase K (10 mg/ml). The final volume did not exceed 200 μl. Protease digestion at 37°C for 45 min was followed by sequential extraction with an equal volume of phenol and then with an equal volume of phenol-chloroform. RNA was precipitated by adding 450 μl of 100% ethanol (15 to 30 min on ice). The RNA was centrifuged, and the pellet was washed and dried. The dried pellet was resuspended in 50 μl of H_2O–50 μl of 2× DNase I buffer (250 mM sodium acetate [pH 7], 20 mM $MgCl_2$, 10 mM DTT) plus 2 U of DNase I (1 U/μl; Promega) and 7.0 U of RNasin (28 to 40 U/μl; Promega). DNase I digestion proceeded at 37°C for 45 min, and the RNA was precipitated on ice after phenol-chloroform extraction. The RNA was collected by centrifugation, and the pellet was washed with 100% ethanol and dried.

For primer hybridization, the RNA pellet was resuspended in 10 μl of hybridization mix containing 2 μl of 5× hybridization buffer (400 mM KCl, 500 mM Tris-Cl [pH 8.3]), 1.5 μl of labeled HIV-1 primer, 4 μl of labeled U2 primer in an appropriate dilution (1:100 to 1:200), and 2.5 μl of H_2O. The hybridization mix was heated at 70°C for 2 min and then cooled to room temperature overnight or until the water temperature was cooled to less than 35°C. To extend the primed DNA, 10 μl of the elongation mixture was added to hybridized RNA-DNA. The primer extension mixture contained 0.4 μl of bovine serum albumin (1 mg/ml), 2 μl of 10× reverse transcriptase buffer (100 mM DTT, 60 mM $MgCl_2$), 2 μl of 5 mM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP, pH7), 2.5 μg of Dactinomycin (Boehringer Mannheim), and 10 U of reverse transcriptase (Life Science). The elongation reaction was incubated at 40°C for 1 h, stopped by addition of 2 μl of a fresh solution containing 0.1 μg of RNase A in 0.5 M EDTA, and further incubated at 37°C for 5 min. Then 80 μl of Tris-EDTA (TE) was added, and the mixture was extracted with an equal volume of phenol-chloroform. The extended products were precipitated on ice for 15 to 30 min. The pellet was collected by centrifugation, washed and dried. The pellet was resuspended in 6 μl of loading buffer (80% formamide, 0.5× Tris-borate, 0.5% bromphenol blue and xylene) and denatured at 80 to 90°C for 2 min prior to loading. Typically, 2.5 μl of each sample was loaded on a 6% acrylamide sequencing gel and electrophoresized at 1,200 V. The gel was dried and exposed to X-ray film (Kodak) with an intensifying screen at −70°C.

Site-directed mutagenesis. Site-directed mutagenesis was done according to Sambrook et al. (63). Plasmids that carry the PCR-amplified 900-bp fragment of the HIV-1 LTR were used to transform a *dut ung* F′ strain of *E. coli* (43, 44). A single ampicillin-resistant colony was inoculated into 1 ml of LB broth plus ampicillin (100 μg/ml) and incubated at 37°C for 6.5 h. While being shaken, 50 μl of the culture was transferred to 10 ml of LB broth plus ampicillin (100 μg/ml) with 1 μl of diluted phage VCS-M13 (stock titer, 1.8×10^{11}; 1:10 dilution; Stratagene). The culture was returned to 37°C for 1.5 h, and then kanamycin was added to a final concentration of 50 μg/ml. The culture remained at 37°C overnight with constant shaking.

To prepare single-strand phage DNA, the overnight culture was centrifuged and the pellet was discarded. For every 1.3 ml of supernatant, 200 μl of polyethylene glycol solution (20% polyethylene glycol, 2.5 M NaCl) was added and mixed. This mixture was kept at room temperature for 30 min and then centrifuged in an Eppendorf tube for 5 min at room temperature. The supernatant was discarded, and the pellet was resuspended in a total volume of 400 μl of TE. The suspension was extracted twice with an equal volume of phenol and once with phenol-chloroform. Phage DNA was precipitated with 100% ethanol and collected by centrifugation. The DNA pellet was washed, dried, and resuspended in TE solution. DNA concentration was estimated by comparison with a known amount of single-stranded phage DNA after separation in an agarose gel.

Approximately 0.3 pmol of single-stranded phage DNA prepared as described above was annealed to 3 pmol of a 5′-phosphorylated oligomer that carries a mutation in the

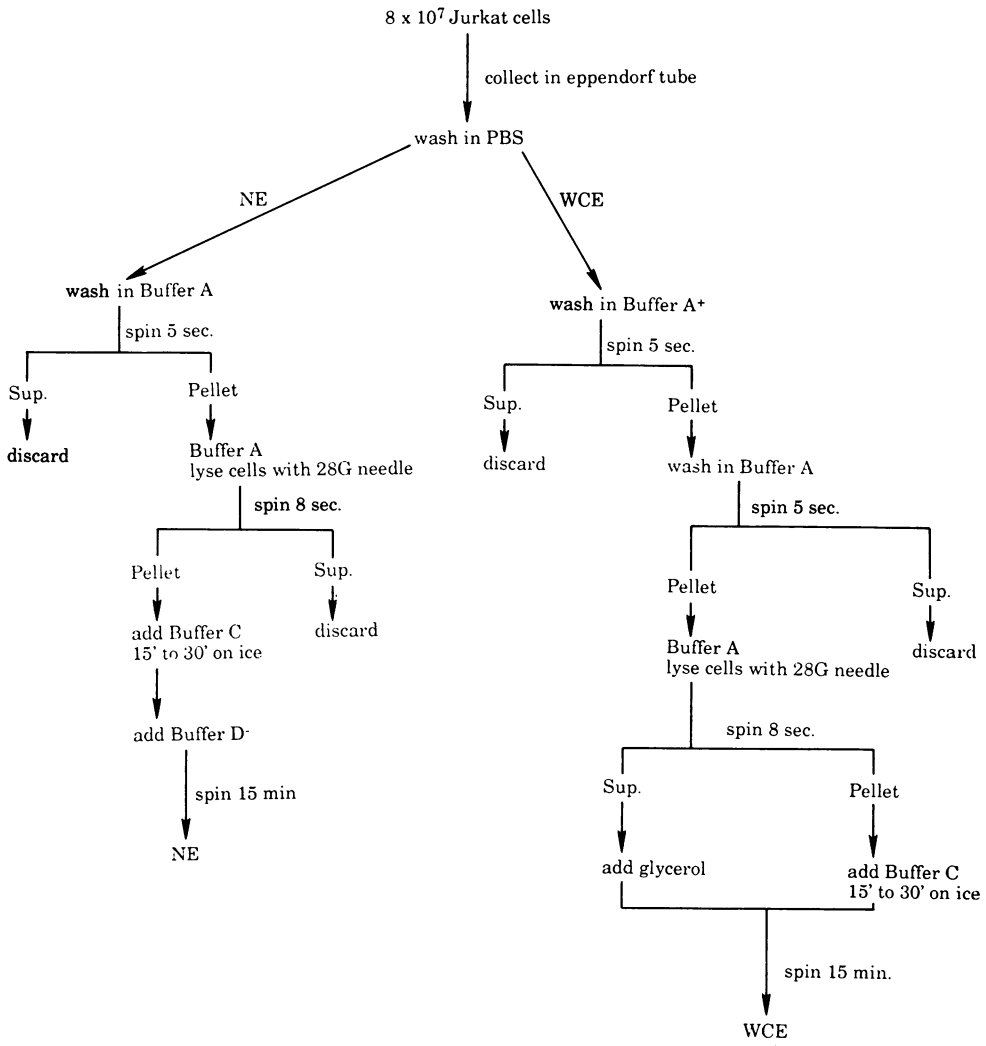

FIG. 1. Flow chart of microscale extract preparation. NE, Nuclear extract; WCE, whole cell extract; ', minutes; Sup., supernatant; G, gauge.

desired position in a 10-μl reaction volume of 20 mM Tris-Cl (pH 7.5)–10 mM $MgCl_2$–50 mM NaCl–1 mM DTT. The reaction mixture was kept at 65°C for 2 min and then slowly cooled to room temperature. The sequence of the mutant primer for the TATA box is 5'-AAGCAGCTGCGTCTCTGC AGCATCTGAGGGCTC-3'. The sequence of the mutant primer for the κB sites is 5'-CCCTGGAAAGTGAGCAGCG GAAAGTGAGTTGTAACAAGC-3'.

For elongation and ligation reactions, 10 μl of a mixture containing 20 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 500 μM deoxynucleoside triphosphate (dATP, dCTP, dGTP, and TTP), 1.5 mM ATP, 8 U of T4 ligase (8 U/μl; Boehringer Mannheim), and 1 U of T4 DNA polymerase (1 U/μl; New England BioLabs) was added to the above annealed reaction mixture. The elongation and ligation reaction mixture was kept at 37°C for 1.5 h. Half of the reaction product was used to transform JM109 or DH1 competent bacteria. Individual colonies were picked and sequenced as described above.

RESULTS

In vitro transcription using microscale nuclear extracts. Figure 1 is a flow chart that outlines the preparation of microscale nuclear and whole cell extracts. The application of this method to the study of temporal changes in in vitro transcriptional activity induced by either PHA or PMA is demonstrated in the following experiments.

The HIV-1 LTR DNA template used in the in vitro transcription experiments is a PCR-amplified 900-bp-long DNA fragment. It contains the HIV-1 LTR sequence starting at −642 and extending to the transcription start site at +1. It also contains 80 bp of coding sequence and 169 bp of CAT sequence (Fig. 2A). The use of PCR-amplified DNA fragments as templates eliminates any contribution of non-HIV-1 LTR sequences (contained in the plasmid vector) to regulation of in vitro transcription. The in vitro-transcribed HIV-1 CAT RNA product from each reaction is detected by the primer extension. The primer for the in vitro-synthesized RNA is shown in Fig. 2A; the expected product length from the primer extension reaction is 59 bases. An endogenous RNA, U2 snRNA, present in extracts, serves as a control for PHA or PMA effects on expression of endogenous genes and also for recovery of the products of the in vitro transcription reaction. Its in vitro-synthesized product, by primer extension, is 42 bases long.

PHA and PMA stimulations of transcription are different. Previously, several groups demonstrated increased produc-

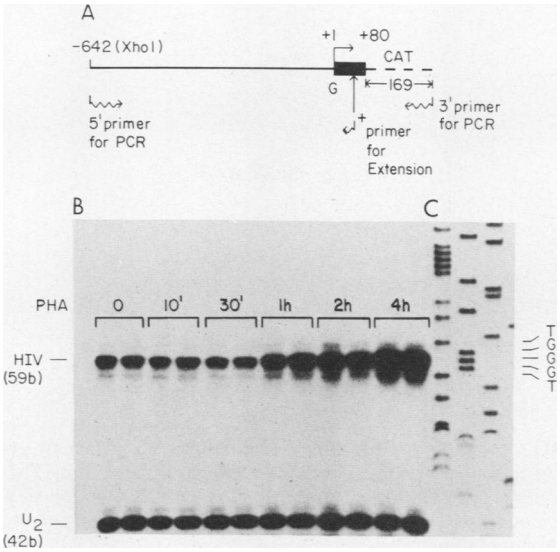

FIG. 2. Stimulation by nuclear extracts from PHA-stimulated Jurkat cells of transcription from the HIV-1 LTR. (A) Structure of the PCR-amplified HIV-1 template used for in vitro transcription. Symbols: ——, HIV-1 LTR sequence; ■, coding sequence of HIV-1 from +1 to +80; – – –, CAT sequence, 169 bp. 5′ and 3′ primers for PCR are primers used for producing the DNA template by PCR. The primer for extension is the HIV-1-specific primer used for primer extension analysis. The expected length of the extension product for HIV-1 is 59 bases. (B) Time course study of transcriptional activation of the HIV-1 LTR, using nuclear extracts from Jurkat cells treated with PHA. PHA was added to growth medium at a final concentration 1 μg/ml for the times indicated (′, minutes). Cells were harvested at the same time that nuclear extracts were prepared and assayed before the extract was frozen. Samples at each time point are duplicates. b, Bases. (C) HIV-1 LTR template sequence.

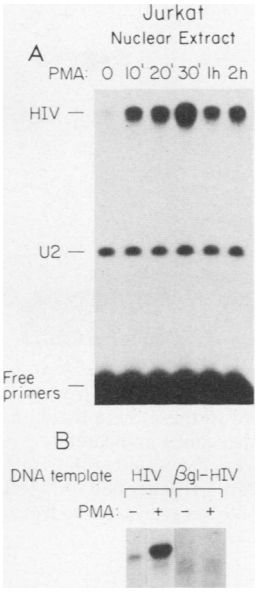

FIG. 3. (A) Time course study of transcriptional activation of the HIV-1 LTR, using nuclear extracts from Jurkat cells treated with PMA. PMA was added to the growth medium at a final concentration of 100 ng/ml for the times indicated (′, minutes). Cells from various time points were harvested simultaneously. (B) Evidence that β-globin gene promoter activity is not affected by PMA. A 100-ng amount of the HIV-1 LTR PCR template and 1 μg of linearized plasmid pβgl-HIV were used in in vitro transcription assays. The X-ray film for the HIV-1 LTR PCR template was exposed for approximately 36 h. The X-ray film for the pβgl-HIV template was exposed for 5 days.

tion of viral particles in HIV-1 infected, CD4$^+$ T lymphoblast cells upon stimulation of the infected cells with both PHA and PMA (27, 74). Increased CAT activity was also observed in HIV-1 LTR CAT plasmid-transfected HeLa cells when PHA and PMA were present in the growth medium for 24 h before assays of cellular extracts (36, 54, 65, 69). We were interested in whether addition of PHA or PMA to Jurkat cells would result in nuclear extracts that increased in vitro transcription directed by the HIV-1 LTR. Furthermore, we wanted to define the events resulting from stimulation by PHA or PMA at different intervals during the first 4 h of stimulation.

Jurkat cells were treated with either PHA or PMA for different intervals, and whole cell or nuclear extracts were prepared for in vitro transcription analysis. Each lot of PHA and PMA was tested to determine the dose that provided maximum induction (data not shown). Another variable that we were concerned with was the possible contribution of the 169 bp of CAT-coding sequence to regulation of in vitro transcription. A PCR-amplified template lacking the 169-bp CAT sequence was used in assays of nuclear extracts from PHA- or PMA-stimulated Jurkat cells. The results showed no difference between the HIV-1 LTR-plus-CAT and the HIV-1 LTR-without-CAT templates (data not shown). The template with CAT-coding sequences was used in all subsequent experiments.

The time course of activation of in vitro transcription of the HIV-1 LTR was examined during the initial 4 h of exposure to PHA. Induction of in vitro transcription from the HIV-1 LTR template was first detected at the 1-h time point and continued to increase during the subsequent 3 h (Fig. 2B). These in vitro HIV-1 LTR transcripts were correctly initiated, as seen by comparison with the sequence map of the PCR-amplified template (Fig. 2C). The level of endogenous U2 snRNA remained constant during this experiment. Additional experiments showed that stimulation of HIV-1 transcription in nuclear extracts of cells treated with PHA was typically detected between 30 min and 1 h. Maximum stimulation was between 4 and 6 h and declined slowly thereafter (data not shown). All correctly initiated transcription in vitro was ablated by addition of α-amanitin (3 μg/ml) to the reaction mixture (data not shown).

Figure 3A shows a time course of PMA stimulation of Jurkat cells and resulting effects on in vitro transcriptional activity. Jurkat cells were treated with PMA (100 ng/ml) for different times as indicated in Fig. 3A. Nuclear extracts from the cells were prepared for each time point and used for in vitro transcription. In contrast to PHA activation, stimulation of in vitro activity by PMA occurred abruptly upon addition of the tumor promoter. The maximum induction was detected between 20 and 30 min after exposure to PMA and decreased thereafter. U2 snRNA transcription did not vary under these conditions. The transcriptional activity of the rabbit β-globin gene promoter (Fig. 3B), the adenovirus E1B promoter, and the adenovirus major late promoter (data not shown) did not change when nuclear extracts from PMA-treated and untreated Jurkat cells were used. Therefore, the PMA induction of transcriptional activity in nuclear extracts derived from Jurkat cells is specific for the HIV-1 LTR.

We also studied whole cell extracts from Jurkat cells

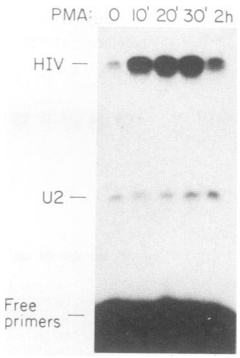

FIG. 4. Time course study of transcription activation of the HIV-1 LTR, using whole cell extracts from Jurkat cells treated with PMA. PMA was added to the growth medium at a final concentration 100 ng/ml for the times indicated (', minutes). Cells from different time points were harvested at the same time, and whole cell extracts were prepared as described in Methods and Materials. Whole cell extracts were assayed prior to freezing. Poly(dI-dC) (500 ng) was included in each reaction.

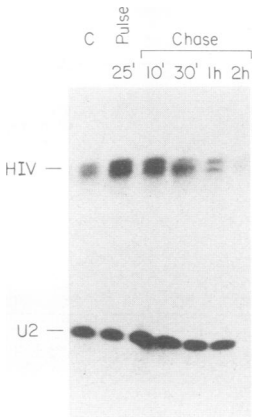

FIG. 5. Evidence that PMA stimulation is a transient process. Jurkat cells were treated with PMA (100 ng/ml) for 25 min. PMA was removed, and cells were grown in growth medium for the times indicated (', minutes). C, Control (nuclear extract from cells with no PMA stimulation).

treated with PHA or PMA for in vitro transcriptional activity. The preparation of whole cell extracts (Fig. 1) represents a slight modification of the microscale procedure for preparing nuclear extracts and is different from the traditional method (49). The overall PMA stimulation patterns when whole cell extracts (Fig. 4) are compared with nuclear extracts are similar, but whole cell extracts typically yield greater activity (Table 1). When whole cell extracts from PHA-stimulated cells were assayed, an inhibition of transcriptional activation was observed (data not shown). We do not know what causes this repressive effect but are investigating it further.

Several independent assays of whole cell or nuclear extracts were conducted to determine reproducibility of results. A quantitative comparison of each time point versus control for PHA or PMA stimulation from some of these experiments was calculated (Table 1). In each case, the protein concentration in the extract corresponding to each time point was quantified, and there was no significant difference among the values obtained. Equal amounts of extracts were used in each assay. In each case, the pattern of activation, i.e., PMA, rapid onset, versus PHA, slow and sustained, is always observed. However, the relative increase at each time point varies from experiment to experiment and probably reflects the subtle differences in the growth status of asynchronously dividing cells or the quality of preparation of different sets of extracts.

Further characterization of the PMA response. To further characterize the properties of the PMA response, a pulse-chase experiment was performed. Jurkat cells were treated with PMA for 25 min. PMA was removed, and cells were incubated in the medium without PMA for various times (Fig. 5). Nuclear extracts were prepared at each time point and used for in vitro transcription. Stimulation by PMA occurred at the 25-min time point and declined rapidly after removal of PMA (Fig. 5). In fact, in 1 h, activity returned to the level detected in extracts from unstimulated cells. Activation by PMA is therefore a transient process. Moreover, the activity at the 2-h time point was even lower than that of the control extract. This finding suggests a repressive effect on transcription and, with further study, may reveal how the cell quickly quenches the stimulating effect of PMA. U2 snRNA levels remained unchanged throughout the experiment.

Previous studies showed that in vitro transcription in HeLa nuclear extracts increased after PMA treatment, and this increase occurred in the absence of de novo protein synthesis (14). We treated Jurkat cells with cycloheximide alone or in combination with PMA. Cells were treated with cycloheximide for 4 h, with cycloheximide for 4 h plus PMA for the last 25 min, with PMA for 25 min, or with nothing added to the growth medium. Nuclear extracts from cells treated with cycloheximide alone did not yield increased in vitro transcription of the HIV-1 LTR template in comparison with the control (Fig. 6). PMA activation occurred as previously observed. Cycloheximide did not inhibit the activation

TABLE 1. Quantitation of transcriptional activation of the HIV-1 LTR from extracts of cells treated with either PHA or PMA[a]

Extracts	Relative activation									
	PMA					PHA				
	10 min	20 min	30 min	1 h	2 h	30 min	1 h	4 h	6 h	8 h
Nuclear	2.7	3.2	4.9	2.6	2.9	1.5	4.4	4.1	3.0	3.95
	1.6	2.9	2.7	2.3	1.9	1.9	1.8	2.2	2.9	2.1
Wholecell	3.45	5.45	4.1	5.0	3.5	ND	ND	ND	ND	ND
	4.9	5.8	3.5	—	2.6	ND	ND	ND	ND	ND

[a]Radioactive bands corresponding to the HIV-1 primer extension product were excised and counted in scintillation cocktail for 5 min. The counts per minute from each band was subtracted from the background counts and then normalized to the control (unstimulated) counts (assigned a value of 1). ND, Not determined; —, no data available.

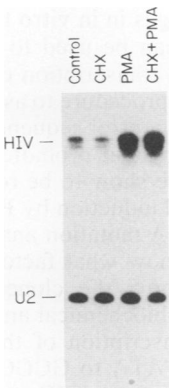

FIG. 6. Transcriptional activation of the HIV-1 LTR by PMA in the presence of a protein synthesis inhibitor. Nuclear extracts were prepared from untreated Jurkat cells (Control), Jurkat cells treated with cycloheximide (CHX; 10 μg/ml) for 4 h (CHX), Jurkat cells treated with PMA (100 ng/ml) for 25 min (PMA), and Jurkat cells treated with cycloheximide for 4 h and PMA for the final 25 minutes (CHX + PMA). Cells from different treatments were harvested at the same time.

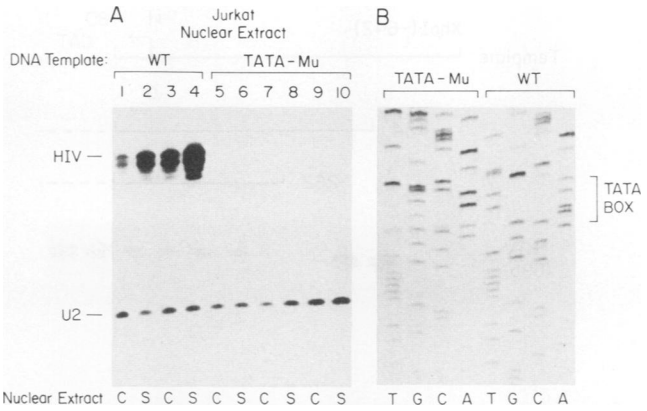

FIG. 7. Ablation by TATA box mutation of transcription of the HIV-1 LTR. (A) In vitro transcription with nuclear extract from Jurkat cells was performed with the WT HIV-1 LTR template (lanes 1 to 4) and with the HIV-1 LTR template mutated in the TATA box (TATA-Mu; lanes 5 to 10). The amount of DNA template used in lanes 1, 2, 5, and 6 was 40 ng; the amount in lanes 3, 4, 7, and 8 was 80 ng; the amount in lanes 9 and 10 was 120 ng. C, Nuclear extract from nonstimulated Jurkat cells; S, nuclear extract from Jurkat cells treated with PMA (100 ng/ml) for 25 min. (B) DNA sequences of TATA-Mu and WT templates.

effect of PMA; however, we have yet to observe a significant superinduction of in vitro transcriptional activity when the two agents are added to the same cells. Therefore, in Jurkat cells, the ability of PMA to induce an increase in in vitro-assayed transcriptional activity appears not to require de novo protein synthesis.

TATA mutations ablate transcription of the HIV-1 LTR. We were interested in the effects of various mutations on in vitro transcription of the HIV-1 LTR template. The first site that we studied was the TATA box because of its general importance to control of RNA polymerase II-catalyzed transcription. Previous studies have shown that mutations in the TATA box (TATA to GCGC) lead to a profound loss of both basal and induced HIV-1 LTR activity (55). In our study, we changed TATAA to GAGAC by site-directed mutagenesis (Fig. 7B). The rest of the LTR sequence was unchanged (Fig. 7B and data not shown). Wild-type (WT) HIV-1 LTR and mutant HIV-1 LTR (TATA-Mu) were compared for their transcriptional activities in unstimulated Jurkat cell nuclear extracts and nuclear extracts from Jurkat cells treated with PMA for 25 min (Fig. 7A). Consistent with previous experiments, transcription of the WT HIV-1 LTR template occurred in extracts from unstimulated cells (lanes 1 and 3) and increased significantly in extracts from cells stimulated by PMA (lanes 2 and 4). Lanes 1 and 2 contain approximately 40 ng of template, while lanes 3 and 4 contain approximately 80 ng. Irrespective of the amount of template added to the same extracts, the TATA-Mu template (lane 5 and 6, 40 ng; lane 7 and 8, 80 ng; lane 9 and 10, 120 ng) did not permit initiation of in vitro transcription. This finding demonstrates that initiation of transcription of the HIV-1 LTR template is dependent on the presence of an intact TATA box and that the effect of PMA on in vitro transcription of the HIV-1 LTR is dependent on the TATAA sequence.

Mutations in κB sites partially reduce transcriptional activation by PMA and PHA. It has been shown that deletion or site-directed mutation of the κB sites in the HIV-1 LTR (GGGACTTTCC to CTCACTTTCC or TCTACTTTCC) reduces DNA mobility shift and CAT activity in cells treated with PHA and PMA (2, 54, 55). In some of these studies, the effects were measured in cells expressing the HIV-1 regulatory protein, Tat (55). We used oligonucleotide-directed mutagenesis to change GGG in each of the two κB sites to CTC without otherwise altering the LTR (Fig. 8A). The mutant template (EN-Mu) was used at a concentration similar to that of the WT template in in vitro transcription assays of Jurkat cell nuclear extracts. In this experiment, extracts from PMA-stimulated Jurkat cells resulted in a 3-fold increase in transcription from the HIV-1 LTR template but only a 1.7-fold increase from the EN-Mu template (Fig. 8B). When PHA-stimulated nuclear extracts were assayed, the EN-Mu template gave a 2.5-fold induction, compared with a 3-fold induction with use of the WT HIV-1 LTR template. U2 snRNA levels remained the same. Additional experiments showed that reduction of PMA inducibility ranged from 20 to 40%, while reduction of PHA induc-

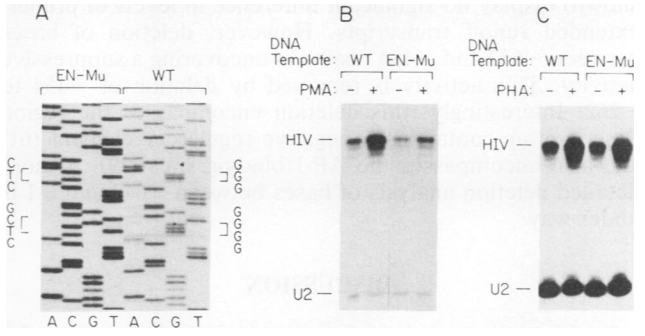

FIG. 8. Reduction by mutations in κB sites of transcriptional activation by PMA and PHA. (A) The DNA sequence of the HIV-1 LTR template was mutated at the two κB sites (EN-Mu) as indicated. (B and C) In vitro transcription was performed with the WT template (lanes 1 and 2) and with the EN-Mu template (lanes 3 and 4). Nuclear extracts were from Jurkat cells PMA stimulated for 25 min (+), (B), PHA stimulated for 4 h (+), (C), or nonstimulated (−). The amount of DNA template was 65 (B) or 100 (C) ng per reaction.

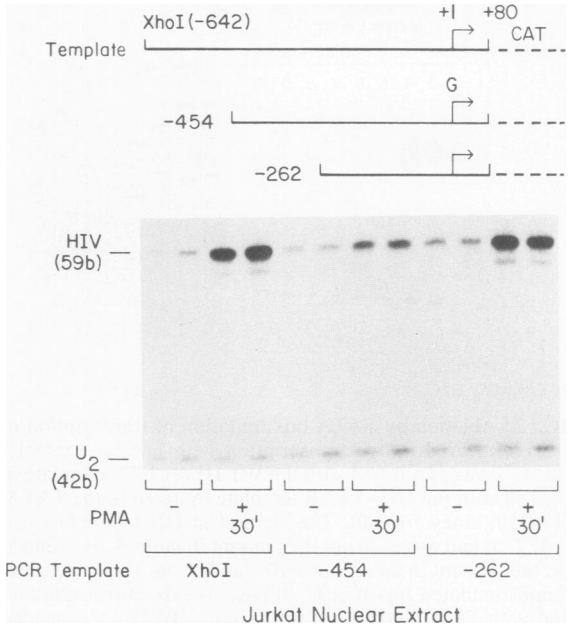

FIG. 9. Deletion analysis of the HIV-1 LTR. The schematic drawing illustrates the deletions and resultant templates. Jurkat cells stimulated or not for 30 min (30') with PMA were harvested, the nuclear extracts were divided into equal amounts, and duplicate assays of control versus stimulated cells were done for each template.

ibility was 10 to 20%. These data suggest that factors involved in PMA and PHA stimulation are quantitatively or qualitatively different. Moreover, other HIV-1 LTR elements, in addition to the κB sites, participate in the early response resultant from PMA or PHA stimulation of Jurkat cells.

Deletion analysis of the HIV-1 LTR. We analyzed the responses of three deletions of sequences in the HIV-1 LTR by using extracts from Jurkat cells stimulated for 30 min with PMA. Each assay is shown as a duplicate reaction, and the same batch of microscale nuclear extract was used for all assays. The full-length template (Fig. 9) and a template containing deletion to either −262 (Fig. 9) or −107 (data not shown) display no significant difference in levels of primer-extended runoff transcripts. However, deletion of bases between −642 and −454 results in uncovering a suppressive activity. This activity is removed by deletion of −454 to −262. Interestingly, this deletion encompasses the region described as containing a negative regulatory element (61) and also encompasses the AP-1 binding sites (19). A more detailed deletion analysis of bases between −642 and +1 is under way.

DISCUSSION

In this report we introduce a microscale procedure for preparation of cellular and nuclear extracts for in vitro transcription analysis. The microscale extract procedure enabled us to investigate biochemically the transcriptional activation of the HIV-1 LTR. Extracts from Jurkat cells were prepared for multiple time points during the early moments of stimulation with either PHA or PMA. All extracts were simultaneously assayed for in vitro transcription activity. Thus, this procedure provides the means to investigate rapid changes in in vitro transcriptional activity. In a similar way, it can be used to study effects of other biological reagents on transcription of the HIV-1 LTR. We expect the microscale procedure to assist in the investigation of other transcription control sequences in the LTR as well as in other viral and cellular promoters.

One element that we show to be required for both basal-level transcription and induction by PMA is the TATA box, as shown by our TATA mutation analysis. Although at this moment we do not know what factor(s) is involved at the HIV-1 LTR TATA box, the cloned TFIID (30, 31, 59) provides a source for biochemical analysis of its role in the TATA-dependent transcription of this promoter. Interestingly, the mutation, TATA to GCGC, in HIV-1 LTR-CAT plasmid-transfected Jurkat *tat*-III cells did not ablate the transcription (55). Biochemical analysis of microscale extracts from these cells and addition of Tat to various other microscale extracts should provide insight into the biochemical basis for these results.

Our results clearly demonstrate that in vitro activations of the HIV-1 LTR by PHA and PMA are different. Both reagents have been shown to induce increased production of viral particles in infected $CD4^+$ T lymphoblasts (27, 74). Therefore, while the endpoints may be the same, the pathways may be composed of different proteins and differential use of elements within the entire LTR. Although previous studies have shown NF-κB to be an important factor in PMA and PHA activation, our results indicate that other sequence elements and factors are required for activation. Mutation of the κB sites only partially reduced induction by either PMA or PHA. This finding contradicts those of Nabel and Baltimore (54), which credit this mutation with a dramatic reduction in the ability of the LTR to activate transcription in transient transfection assays of HIV-1 LTR-CAT constructs. The differences between our study and theirs may be due to the presence of Tat, a viral transactivator of HIV-1 (10, 53, 58, 61, 72), in their transfection system. The contribution of Tat can be determined in our assay system, and such studies are under way. However, recent in vivo studies by Leonard et al. (47) showed that deletions of κB sites did not interfere with viral replication in human T lymphocytes in a system in which Tat was present. Therefore, it is likely that some other regulatory element(s) in the HIV-1 LTR contributes to its replication and to its responsiveness to signals induced by PHA and PMA. Our studies suggest that it is essential to couple biochemical analysis of the transcriptional activity of the HIV-1 LTR with precise characterization of each protein that associates with the different control elements in the LTR.

The deletion analysis of the HIV-1 LTR presented in this report is limited, but it reveals a region that, when unmasked by removal of upstream sequences, is capable of suppressing an elevated level of in vitro transcript production in extracts of PMA-stimulated Jurkat cells. This finding suggests that in the full-length LTR various elements may be in competition, and the result observed is a higher order consequence of their respective interactions. This observation suggests a theoretical and methodological challenge because of a lack of definition of how many combinational possibilities exist and how the physical relationships (order of elements, distance between elements, etc.) of the units may affect potential interactions. Any regulatory element may have entirely different protein interactions when the element is tested individually or in combination with another unit. Simply deleting a single base at a time may not uncover the units, and site-directed mutations may unwittingly generate

a new binding site. An understanding of complex promoters will necessarily require genetic comparisons such as sequence analysis of multiple isolates of HIV-1 LTR to determine what regions of the LTR are evolutionarily conserved. Coupled with a direct examination of the proteins interacting with the regulatory unit, biochemical assays of the consequence of the interaction should lead to a more thorough understanding of how complex regulatory units function as the growth status of the cell changes.

Given the number of proteins that we (16–18) and others (2, 4, 73) have found associated with the κB site, it is likely that the role of the site is multifunctional. We have identified proteins that associate with the site in extracts from unstimulated and stimulated cells. These proteins vary quantitatively and qualitatively during stimulation. We now demonstrate biochemical differences in extracts from cells treated with the same agents that induce changes in protein-DNA interactions. We also demonstrate that biochemical analysis reveals effects of mutation of the κB sites in the HIV-1 LTR different from those observed when the LTR is assayed as a heterologous promoter of the bacterial CAT gene. Interestingly, our in vitro biochemical data correlate with assays of deletions of both κB elements from the HIV-1 LTR in assays of proviral replication. It is likely that the presence of κB sites and Sp1 sites and other known or yet-to-be-defined elements in multiple isolates of HIV-1 reflects the flexibility of the provirus to respond to changes in cell growth that may be dependent on or independent of the type of cell in which it resides.

ACKNOWLEDGMENTS

We acknowledge J. D. Watson for his support. We thank N. Hernandez, M. Laspia, J. Lee, and M. Massaro for comments on the manuscript; the CSHL oligonucleotide synthesis facility; Jim Duffy and Phil Renna for artwork and photography; and P. Smith and L. Korrol for preparation of the manuscript.

The work was sponsored by grants from the Mellam Family Foundation and by NIH NIAID grant AI27270. Y.L. is a Rita Allen postdoctoral fellow.

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