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In Vivo Footprinting Analysis of Constitutive and Inducible Protein-DNA Interactions at the Long Terminal Repeat of Human Immunodeficiency Virus Type 1

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The regulation of the rate of transcription of human immunodeficiency virus type 1 is mainly exerted through the long terminal repeat (LTR) at the 5′ end of the provirus. A large number of *cis*-acting regulatory elements have been identified in the LTR by in vitro binding studies; the biological role of these sites within living infected cells, however, is still not clear. We have studied the interactions of nuclear proteins with the LTR in the U1 monocytic cell line by in vivo dimethylsulfate footprinting, using the ligation-mediated polymerase chain reaction technique. In this cell line, transcription of the virus, which is very low under basal conditions, is highly inducible by treatment with phorbol esters; therefore, this system is likely to represent a suitable cellular model to study viral latency. Independently of the level of viral transcription, major in vivo footprints appear at the two Sp1 sites adjacent to the enhancer, the downstream-positioned enhancer repeat, the NFAT binding site, and one of the purine-rich sites of the negative regulatory element. Upon transcriptional activation by phorbol myristate acetate, the only perturbation in the footprinting pattern is a dramatic increase in dimethylsulfate sensitivity of guanine at position −92 in the downstream enhancer repeat. This modification is correlated with the transient induction of two enhancer-binding activities, as determined by gel retardation assays. While the transcriptional rate is still increasing and the in vivo footprinting pattern is unchanged at up to 24 h postactivation, these enhancer-binding factors are considerably reduced at this time. Therefore, further levels of regulation have to be considered to explain the maintenance of the induced state.

The regulation of human immunodeficiency virus type 1 (HIV-1) gene expression plays a key role in triggering viral replication and, consequently, in disease development. Recent quantitative studies showed that HIV-1 is expressed throughout all stages of the disease (3, 18, 41, 45, 48); nevertheless, the levels of HIV-1 RNA expression, measured as the RNA/DNA ratio, directly correlate with disease stage (3, 18, 41), suggesting the involvement of viral transcriptional activation in disease progression.

The regulation of transcription of HIV-1 is modulated by the synergic action of the viral protein Tat and of cellular proteins with *cis*-acting viral regulatory elements in the long terminal repeat (LTR) at the 5′ end of the provirus. In the early phase of the infectious cycle, before the production of Tat, the regulation of transcription relies completely on nuclear factors of the host cell. On the basis of in vitro binding studies, analysis of deletion mutants viability, and transient transfection experiments, the LTR appears as a mosaic of binding sites for nuclear proteins (24). From the functional point of view, it can be divided into three main regions: the basal promoter region, encompassing the transcription start site, which exerts a positive basal effect on transcription; the enhancer region, which increases the effect of the basal region; and the negative regulatory element (NRE), extending upstream of the enhancer site, whose overall function is to down-regulate transcription (see Fig. 1 for a schematic representation of these genetic domains). A description of these binding sites and the cognate proteins is presented in references 24 and 26. In particular, the enhancer region mediates the transcriptional inducibility of the provirus in response to a variety of stimuli which trigger cellular activation and proliferation (12, 15, 55). This genetic element is composed of two repeats (κB sites [Fig. 1]) which are the targets of the inducible transcription factor NF-κB, a heterodimer composed of two subunits (p50 and p65) (30), that resides in the cytoplasm when complexed with the inhibitor IκB (2). Activating agents, such as phorbol esters and tumor necrosis factor alpha, promote phosphorylation and dissociation of IκB, subsequent migration of NF-κB into the nucleus, and consequent transcriptional activation. This initial model to explain viral inducibility through the enhancer element is probably an oversimplification, since, in addition to NF-κB, several other cellular and viral proteins have been reported to interact specifically with the NF-κB recognition motif. These include p50 and p65 homodimers, other Rel family members such as v-Rel and c-Rel (7) and p49 (52), and other apparently unrelated factors such as H2TF1 (6), EBP-1 (14), PRDII-BF1 (5), and TC-IIB (37). The physiological role of each of these factors still needs to be clarified.

At present, the overall outcome of the studies on the interactions of cellular proteins with the HIV-1 LTR led to the identification of more than 20 nuclear proteins able to bind to the LTR in vitro (24). In vitro binding studies, however, are limited in several respects. They may detect protein binding to sites that are unavailable in native chromatin, and they may fail to reveal sites which bind to proteins that are present in a low concentration in nuclear extracts or are displaced by more abundant proteins with overlapping specificities. Furthermore, in vitro analysis is insensitive to chromatin structure and to epigenetic modifications of DNA. For example, the activation

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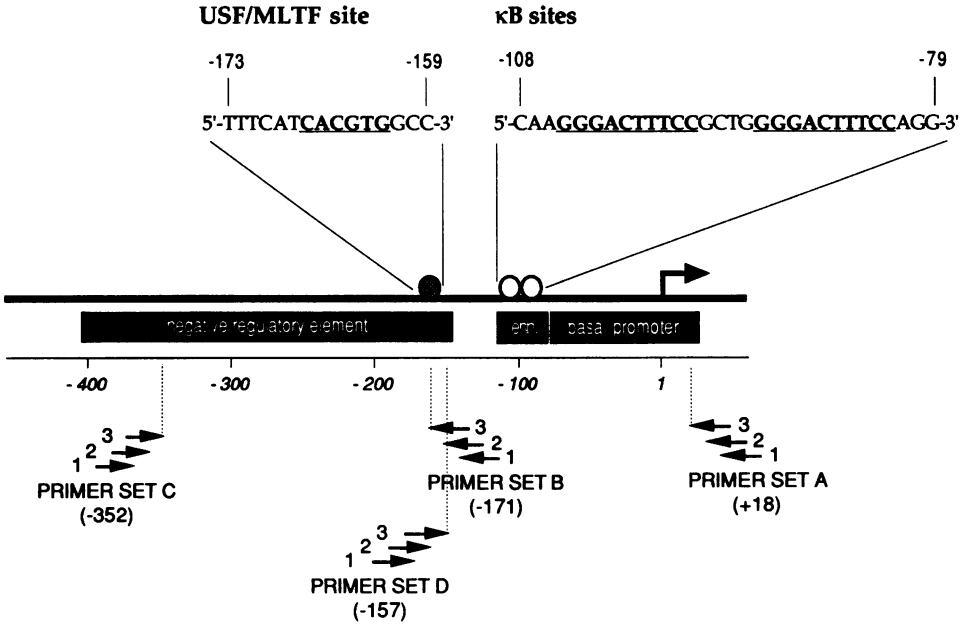


FIG. 1. Schematic representation of the HIV-1 LTR. The LTR is represented by a heavy horizontal line; the arrow on the right side indicates the start site of transcription (nucleotide +1 on the scale below). The sequences of the enhancer region (empty circles on the right side) and of the USF/MLTF binding sites (gray circle on the left side) are shown on top, with the core sequences indicated by boldface and underlining. The LTR is schematically divided into three functional regions (basal promoter, enhancer (enh.), and NRE), as described previously (26). Of the two repeats of the enhancer region, the one closer to the basal promoter is referred to as downstream positioned, while the one closer to the NRE is referred to as upstream positioned. The localization of the four primer sets used for the in vivo footprinting experiments is shown on the bottom. The sequences of these oligonucleotides have already been reported (17).

of the glucocorticoid-inducible rat tyrosine aminotransferase gene is dependent on a ubiquitous factor which binds to its cognate site only in cells actively transcribing the gene, as shown by in vivo dimethylsulfate (DMS) footprinting (8). Moreover, in vivo footprinting studies on the immunoglobulin heavy chain enhancer have revealed specific protein-DNA interactions which cannot be recreated in vitro (25). Finally, monitoring of changes in protein occupancy of specific targets within living cells has shown that activation of transcription may occur without any alteration at the level of protein-DNA interactions (33, 44, 46).

For these reasons, the determination of the protein-binding sites actually occupied in living cells represents a valuable tool in clarifying the regulation process. To this end, we have recently applied to the HIV-1 LTR an in vivo DMS footprinting method based on ligation-mediated polymerase chain reaction (PCR) (42). This method allows one to compare the methylation pattern of DMS-treated, naked DNA with the corresponding pattern obtained from DNA extracted from DMS-treated cells; any differences between the two patterns is likely to be due to protein-DNA interactions occurring in living cells. By this method, we have studied the contact points of nuclear factors in the H9/HIV-1_{IIIB} cell line supporting a highly productive viral infection (17). In this cell line, proteins appear to occupy the TATA box, the Sp1 sites, the two repeats of the enhancer region and, in the NRE, the USF/MLTF and NFAT sites, as well as two purine-rich sites from nucleotides −260 to −275 and −205 to −216, respectively.

In order to investigate the molecular mechanisms underlying viral latency and reactivation and to determine which are the regions of the LTR involved in this regulation, we have now extended this type of analysis to other HIV-1-infected cell lines suitable for induction and repression by chemical and biochemical agents. In this paper, we present data obtained with the U1 cell line, a derivative of the U937 monocyte/macrophage cell line, which harbors the viral genome and produces only low levels of viral progeny under basal conditions (20, 21). Induction by cytokines or phorbol esters causes a dramatic increase in viral mRNA expression and the production of larger amounts of virus (32, 49); therefore, this cells line represents a valuable model for the study of the maintenance of viral latency and the mechanisms responsible for viral reactivation (50).

MATERIALS AND METHODS

Cell lines and treatments. The human cell lines U937 (a promonocytic cell line) (57), U1 (an HIV-1-infected subclone of U937) (21) (kindly donated by G. Poli), and Jurkat (a lymphoblastoid cell line) were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml in a 5% CO_2 environment.

Cells growing at a density of 10^6/ml were stimulated by the addition of phorbol-12-myristate-13-acetate (PMA) (Sigma, St. Louis, Mo.) at a 10^{-7} M final concentration and were maintained until harvesting.

For each in vivo footprinting experiment, freshly prepared DMS at a 0.1% (vol/vol) final concentration was added to approximately 2.5×10^6 exponentially growing U1 cells treated with PMA as indicated above. After 5 min at room temperature, the cells were harvested, the nuclei were isolated by lysis with 0.1% Nonidet P-40, and DNA was extracted according to standard procedures (51). Control reactions with naked DNA treated in vitro with DMS were performed as described previously (17). G+A and C+T reactions were done

according to the sequencing protocol of Maxam and Gilbert (38).

Ligation-mediated PCR. Primer sets and conditions for ligation-mediated PCR (see Fig. 1 for a schematic localization of the primers on the HIV-1 LTR) were the same as those already described (17), with the following modifications. Ligation-mediated PCR was performed with *Thermococcus litoralis* DNA polymerase (Vent; New England Biolabs) according to the protocol of Garrity and Wold (23). To overcome problems due to high G+C content, the nucleotide analog 7-deaza-2′-GTP was included in the primer extension reactions and PCRs (39). First-strand DNA synthesis was performed by using a thermal cycle of 5 min at 95°C, 30 min at 60°C, and 10 min at 76°C, except for primer set D, for which the intermediate extension step was at 55°C. PCR was performed by using 18 cycles of 1 min at 95°C, 2 min at 67°C, and 3 min at 76°C. One-fifth of the PCR products was labeled by nine cycles of 1 min at 95°C, 3 min at 69°C, and 5 min at 76°C. The samples were then extracted twice with phenol and precipitated with ethanol. After resuspension in loading dye (42), one-sixth of the pellet was resolved on a 6% polyacrylamide sequencing gel. The gel was then dried and exposed for periods of from 3 h to overnight without an intensifying screen.

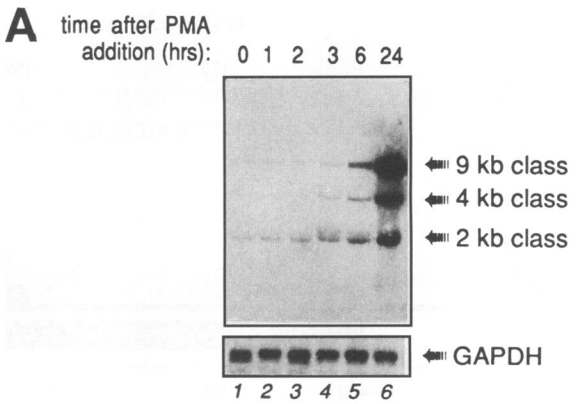
At least three experiments were performed with each primer set to ensure reproducibility of the DMS methylation patterns obtained.

RNA preparation and blot analysis. Total cellular RNA was harvested by the guanidinium isothiocianate method (13). Samples of 10 μg each were electrophoresed through a 1.0% formaldehyde-agarose gel, transferred to Hybond-N nylon filters (Amersham Corp.), prehybridized for 1 h in a solution containing 0.2 M $NaPO_4$, 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 250 μg of *Escherichia coli* RNA carrier per ml, and hybridized to a ^{32}P-labeled DNA probe in a solution with the same composition as the prehybridization solution at 60°C overnight. The probe was obtained by labeling a PCR-amplified fragment encompassing the HIV-1 LTR by the random-priming technique, according to standard procedures (51). The primers used for the amplification reaction were primer 2 set A and primer 2 set C (17) (Fig. 1). PCR was performed by using 25 cycles of 45 s at 95°C, 1 min at 65°C, and 2 min at 72°C. Following hybridization, blots were washed twice with 40 mM phosphate buffer (pH 7.2)–1% SDS at 65°C and then exposed overnight at −70°C with an intensifying screen.

p24 antigen analysis. Cell suspensions were cleared by low-speed centrifugation, and supernatants were frozen for subsequent p24 antigen determination. Culture supernatants were diluted appropriately before p24 quantitation and processed for reactivity by using a commercially available antigen capture kit (Abbott, Chicago, Ill.).

Gel retardation assays. Nuclear extracts from Jurkat, U1, and U937 cells were prepared according to the microscale preparation protocol described by Li et al. (35). Protein concentrations were determined by the Bradford assay with a commercial protein assay reagent (Bio-Rad, Richmond, Calif.). Stimulation of cultures was obtained by treatment with PMA (10^{-7} M final concentration) for the following time intervals: 5 h for Jurkat cells; 1, 3, 6, 12, and 24 h for U1 cells; and 1, 3, 6, and 24 h for U937 cells.

Oligonucleotides for gel retardation assays were synthesized by the Oligonucleotide Synthesis Service, International Centre for Genetic Engineering and Biotechnology, on an Applied Biosystems 380B synthesizer, using phosphoramidite chemistry. Oligonucleotides corresponding to one strand were end labeled with [γ-^{32}P]ATP and T4 kinase and annealed to the complementary oligonucleotide prior to use.



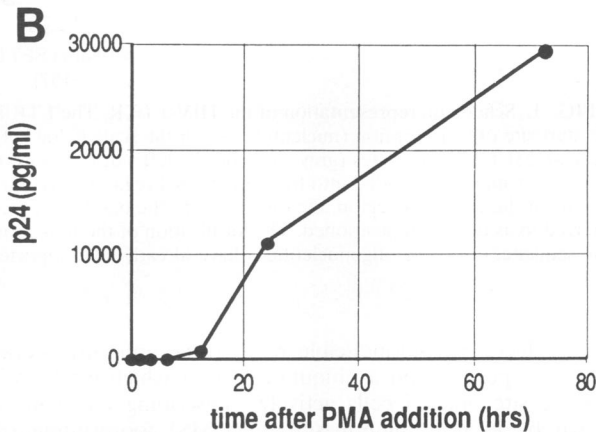


FIG. 2. Activation of HIV-1 expression in U1 cells by PMA. (A) Kinetics of HIV-1 RNA induction in U1 cells by PMA. Total RNA extracted from U1 cells at different times after PMA addition, as indicated on top of each lane, was extracted, resolved by gel electrophoresis, blotted, and hybridized to a DNA probe common to the 3′ end of all the HIV-1 transcripts. The apparent size of each transcript class, indicated on the right, was estimated on the basis of the position of rRNAs before blotting. In order to ascertain that the same amount of RNA was loaded in each lane, the same filter was subsequently hybridized with a probe specific for the ubiquitously and constitutively expressed human GAPDH gene (lower part). (B) Accumulation of p24 antigen in the supernatant of PMA-stimulated U1 cells. The amount of p24 antigen in the cell culture supernatants was determined by an antigen capture assay at different times after PMA addition.

Conditions for binding reactions with the κB-oligonucleotide probe (5′-GAGTGGGGACTTTCCAGGCTC-3′) were adapted from published procedures (47). Seven micrograms of nuclear extract was incubated with 10,000 cpm of probe in 20 μl (final volume) of 10 mM Tris-HCl (pH 7.5)–55 mM NaCl–20 mM KCl–1 mM dithiothreitol–1 mM EDTA–5% glycerol–2% Ficoll 400–0.2% Nonidet P-40–0.1 μg of denatured salmon sperm DNA–1 μg of poly[d(I-C)] · poly[d(I-C)]. After 30 min of incubation at room temperature, protein-DNA complexes were resolved on 5% native polyacrylamide gels containing 20 mM Tris base and 0.2 M glycine at room temperature.

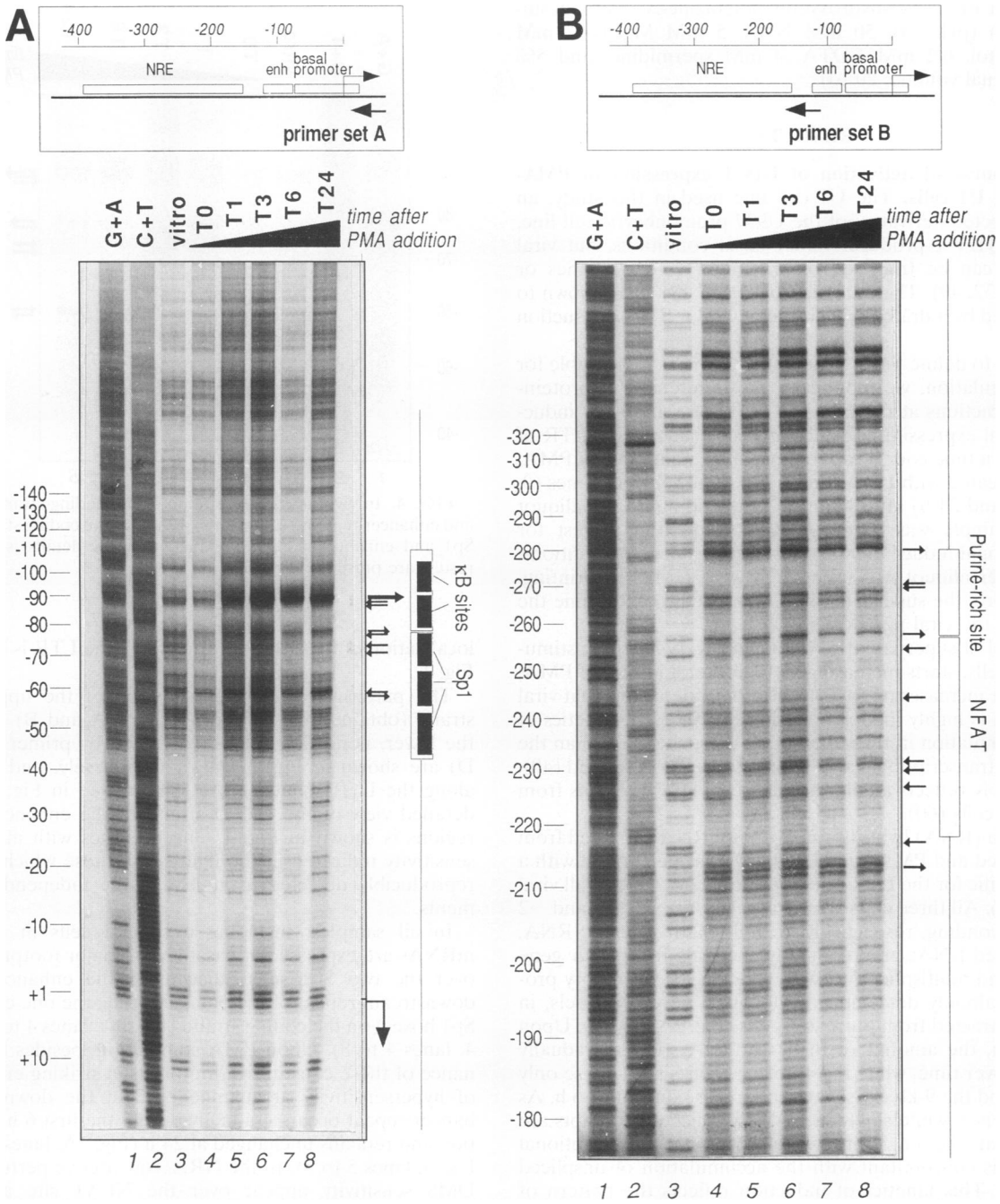


FIG. 3. In vivo DMS footprinting of the coding strand of the HIV-1 LTR at different times after PMA stimulation. The schematic localization of the primer sets utilized is indicated on the drawing at the top of each panel. The locations of the bands, with respect to the transcription start site, are indicated on the left of the autoradiograms. Lanes in both panels: 1 and 2, G+A and C+T sequencing controls, respectively; 3, naked DNA control (i.e., in vitro DMS-treated DNA of U1 cells); 4 to 8, in vivo DMS-treated samples of U1 cells stimulated with PMA for 0, 1, 3, 6, and 24 h, respectively. Protected guanines are shown by arrows pointing leftward on the right of the autoradiograms; hypersensitive guanines are indicated by arrows pointing rightward. In order to allow easy location of the purines with altered sensitivity on the LTR sequence, on the right of the autoradiograms the regions protected by in vitro footprinting experiments reported in the literature are indicated by open boxes; black boxes represent the locations of the consensus sequences for the factor binding sites. (A) In vivo footprinting using primer set A. The arrow pointing downward on the right of the autoradiogram represent the transcription start site. (B) In vivo footprinting using primer set B.

Competition experiments were carried out by mixing 100- or 200-fold molar excesses of cold oligonucleotide with the probe before incubation with nuclear extracts. Mild denaturation of extracts with formamide and sodium deoxycholate was performed as described previously (47).

The conditions for binding reactions with the USF/MLTF oligonucleotide probe (5′-CCGGTCGCATCACGTGACGA AGAG-3′) were the same as previously described (26). Five micrograms of nuclear extracts was incubated with 10^4 cpm of probe in a buffer containing 3 μg of poly[d(I-C)] · poly[d(I-C)],

20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM EDTA, 4 mM spermidine, and 5% glycerol (final volume, 20 μl).

RESULTS

Time course of activation of HIV-1 expression in PMA-stimulated U1 cells. The U1 cell line used in this study, an HIV-1-infected derivative of the U937 promonocytic cell line, is a poor HIV-1 producer under basal conditions, but viral activation can be triggered by treatment with cytokines or PMA (20, 32, 49). The induction of HIV-1 has been shown to be preceded by a dramatic increase in viral mRNA production (40, 50).

In order to define the molecular mechanisms responsible for this up-regulation, we monitored the differences in protein-DNA interactions at the HIV-1 LTR before and after induction of viral expression. To this end, we analyzed the LTR of U1 cells in a time course experiment of stimulation with PMA. U1 cells treated with PMA were harvested at various times (0, 1, 2, 3, 6, and 24 h) after addition of the stimulus. An aliquot of each sample was used for RNA extraction to test for transcriptional inducibility under our experimental conditions, and another aliquot was used for in vivo DMS footprinting. Furthermore, the supernatants were utilized to determine the amount of the viral p24 protein.

The level of supernatant p24 antigen is very low in unstimulated U1 cells, starts increasing at 12 h after addition of PMA, and further increases up to 72 h (Fig. 2B), confirming that viral production is highly inducible in this cell line. The kinetics of virus accumulation in the supernatant is much slower than the kinetics of transcriptional activation, as already described (40), and probably reflects a delayed release of HIV-1 virions from monocytic cells (60).

Northern (RNA) hybridization of total RNA extracted from unstimulated and PMA-stimulated cells was performed with a probe specific for the LTR (and therefore contained in all viral transcripts). All three classes of viral mRNAs (~9, ~4, and ~2 kb, corresponding, respectively, to full-length genomic RNA, singly spliced RNAs predominantly encoding for the *env* gene product, and multispliced RNAs encoding for regulatory proteins) are already detectable, although at very low levels, in samples extracted from untreated cells (Fig. 2A, lane 1). Upon stimulation, the amount of the 2-kb RNA species gradually increases over time, while the 4-kb class starts to increase only after 3 h and the 9-kb class starts to increase only after 6 h. As a consequence, while singly or multiply spliced transcripts are prevalent at the beginning, the process of transcriptional activation is concomitant with the accumulation of unspliced transcripts. This kinetics of induction reflects the pattern of transcription of acutely infected cells (16) and is similar to the one already described for the U1 cell line (40, 50). In our experiments, however, low levels of unspliced viral RNAs are already present before stimulation, and the kinetics of induction upon PMA addition is faster.

Constitutive and inducible protein-DNA interactions at the HIV-1 LTR examined by in vivo footprinting. In order to identify the DNA sites of the LTR actually occupied in living U1 cells and to determine the ones involved in the activation of transcription, we analyzed the U1 cells by in vivo DMS footprinting by using the ligation-mediated PCR technique (42) at different time intervals after PMA addition. Ligation-mediated PCR was performed by using four sets of primers able to identify both the coding and noncoding strands of the U3 region of the HIV-1 LTR as already described (17). The localization of these sets of primers on the LTR is indicated in Fig. 1.

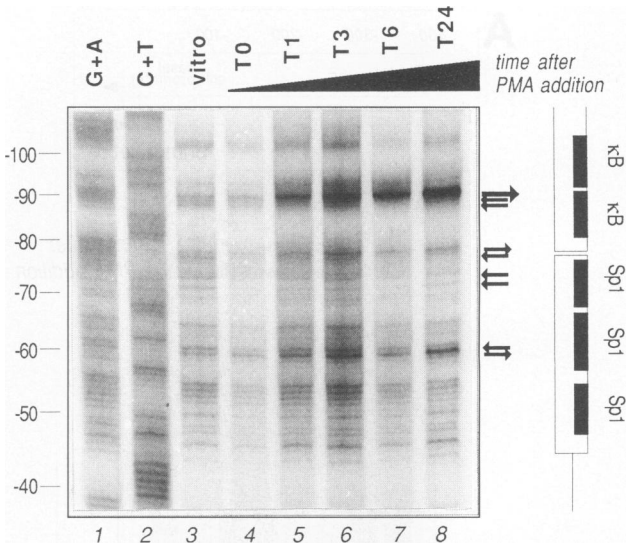


FIG. 4. In vivo DMS footprinting of the coding strand of the Sp1 and enhancer regions. A shorter exposure of the coding strand over the Sp1 and enhancer regions enlarged to show details is shown. The results are presented as in Fig. 3.

The patterns of DMS methylation of the upper, coding strand (obtained by using primer sets A and B) and that of the lower, noncoding strand (obtained by primer sets C and D) are shown in Fig. 3 and 5, respectively, and the results along the LTR sequence are summarized in Fig. 6. A more detailed view of the coding strand of the enhancer and Sp1 regions is shown in Fig. 4. The guanines with altered DMS sensitivity indicated in the figures are those which have been reproducibly detected in at least three independent experiments.

In all samples, including untreated cells in which viral mRNAs are expressed at basal levels, major footprints appear over the two Sp1 sites adjacent to the enhancer and the downstream repeat of the enhancer (i.e., the one closer to the Sp1 boxes) on the coding strand (Fig. 3A, lanes 4 to 8, and Fig. 4, lanes 4 to 8). Upon PMA stimulation, besides the maintenance of these constitutive footprints, a striking enhancement of hypersensitivity at guanine −92 at the downstream enhancer repeat occurs gradually during the first 6 h of stimulation and remains unchanged at 24 h (Fig. 3A, lanes 5 to 8, and Fig. 4, lanes 5 to 8). In the NRE, constitutive perturbations in DMS sensitivity appear over the NFAT site and over a purine-rich site which we found occupied also in infected H9 cells (17) (Fig. 3B, lane 4). The latter site has sequence similarity with a regulatory domain of the interleukin-2 promoter (22) and appears to be the target for different cellular proteins in vitro (17a, 27, 34, 61). The overall pattern of DMS sensitivity of the NRE remains unchanged after stimulation with PMA (Fig. 3B, lanes 5 to 8).

In the noncoding strand of the LTR there are no significant alterations in the sensitivity to DMS in the in vivo treated samples as compared to the in vitro controls, neither in the basal promoter and enhancer regions (Fig. 5A) nor in the NRE (Fig. 5B). In accordance with the results with the H9/HIV-1_{IIIB} system (17), the noncoding strand of the LTR appears to be less informative, as far as protein-DNA interactions are concerned, in U1 cells also. Upon PMA induction, the DMS

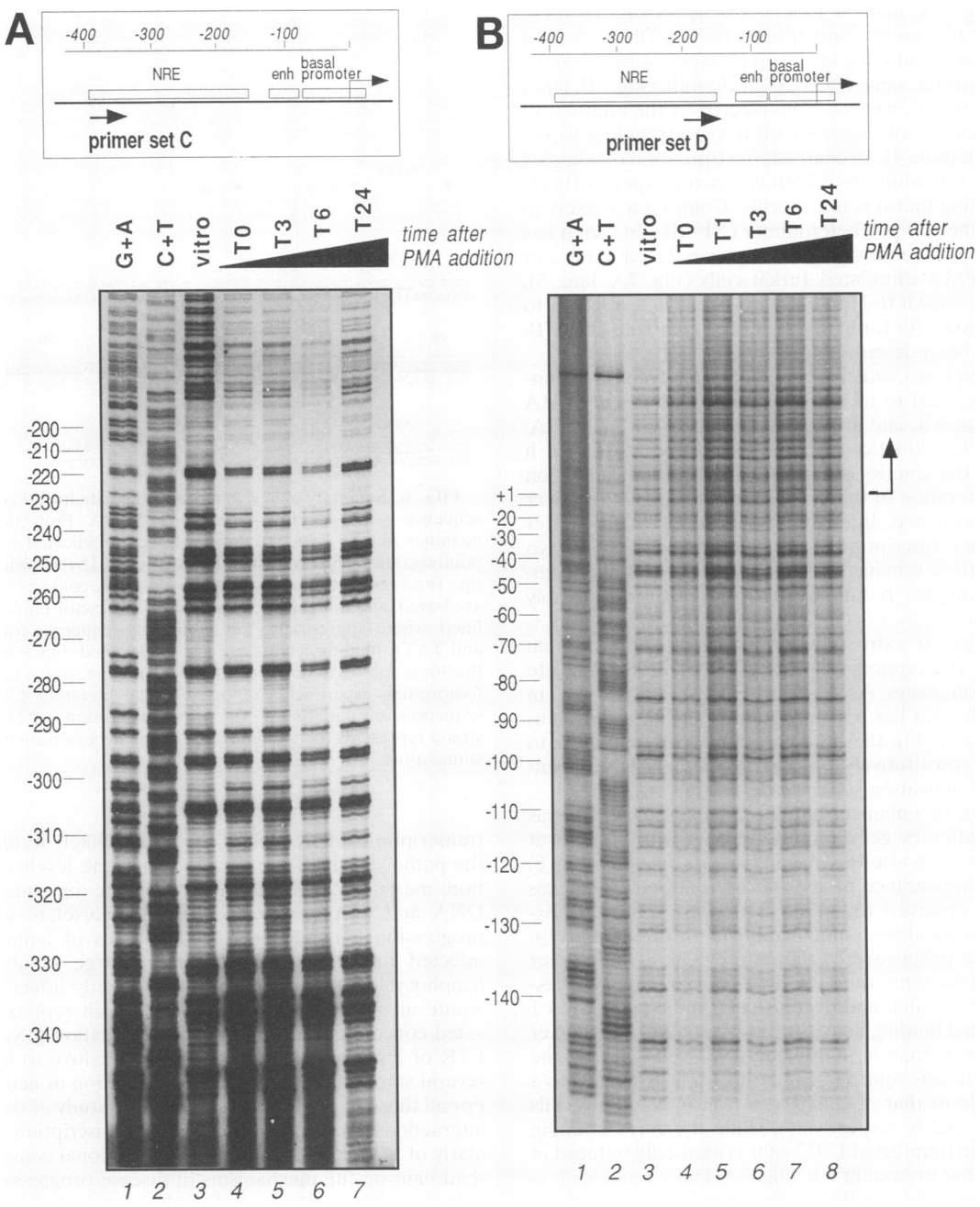


FIG. 5. In vivo DMS footprinting of the noncoding strand of the HIV-1 LTR after PMA stimulation. The results are presented as in Fig. 3. (A) In vivo footprinting using primer set C. (B) In vivo footprinting using primer set D. The arrow pointing upward on the right of the autoradiogram represents the transcription start site.

methylation pattern remains unchanged (Fig. 5A, lanes 5 to 7, and B, lanes 5 to 8).

These results are summarized in Figure 6 along the sequence of the LTR.

Kinetics of induction of enhancer-binding proteins in U937 and U1 cells. As described above, in U1 cells the only detectable perturbation in DMS sensitivity of the LTR upon transcriptional activation occurs at the downstream-positioned enhancer repeat sequence (κB box). In order to substantiate this result, we studied the kinetics of induction by PMA of nuclear proteins interacting with this enhancer box in nuclear extracts of U1 and U937 promonocytic cells (U937 is the parental line from which the U1 clone was originally derived [50]). Gel retardation assays were performed with nuclear extracts from stimulated and unstimulated cells, using oligonucleotide probes corresponding to the downstream LTR enhancer sequence and to the USF/MLTF box (26) (Fig. 1).

Three major retarded complexes result from the interaction of the κB box oligonucleotide with nuclear extracts prepared from U937 cells at 1, 3, 6, and 24 h after PMA induction (complex a, which at closer resolution could possibly be composed of two closely migrating bands; complex b; and

complex c [Fig. 7A, lanes 4 to 8]). The specificity of these complexes was proven by competition studies. The formation of both complex a and complex b can be blocked by a 100- or 200-fold excess of the same cold oligonucleotide (Fig. 7B, lanes 2 and 3, respectively), while it is unaffected by the addition of a 200-fold excess of an oligonucleotide corresponding to the USF/MLTF site (lane 4). In contrast, the formation of complex c is blocked by the addition of both competitor species (lanes 2 to 4), suggesting that it is not specific. Complex a is likely to correspond to the p50-p65 heterodimer (NF-κB). In fact, it has the same apparent mobility as the complex induced in nuclear extracts from PMA-stimulated Jurkat cells (Fig. 7A, lane 3), which has been shown to be NF-κB (53), and it is resistant to mild denaturation with formamide and deoxycholate (Fig. 7B, lane 5), as has been described for NF-κB (47).

The two complexes, which are almost undetectable in unstimulated cells, start to increase in the first hour after PMA addition, peak at 6 h, and are again very low at 24 h (Fig. 7A, lanes 5 to 8). The kinetics of induction during the first 6 h correlates with the kinetics of HIV-1 transcriptional activation and with the alteration of the in vivo footprinting pattern over the enhancer sequence. Interestingly, however, at 24 h after induction, while transcription is still increasing and the in vivo footprinting pattern remains unchanged, the NF-κB-like (complex a) and complex b binding activities are considerably reduced.

The same nuclear extracts were challenged also with an oligonucleotide corresponding to the USF/MLTF-binding site in gel retardation assays. As shown in Fig. 7C, lanes 1 to 7, in accordance with what has been reported for other experimental systems (4, 17a, 43), the binding activity corresponding to USF/MLTF is constitutively present in these cells independently from the activation state.

The induction of enhancer-binding proteins by PMA was also directly studied by gel retardation assays with U1 cells at 0, 1, 3, 6, 12, and 24 h after PMA addition. As shown in Fig. 8, the kinetics of appearance of specifically retarded complexes resembles that observed for uninfected U937 cells: κB box-binding proteins are almost undetectable in unstimulated cells, start to increase progressively, thereafter peak at 12 h after PMA addition (the same time point was not previously investigated with U937 cells), and decrease at 24 h (Fig. 8, lanes 1 to 6). The residual binding activity at 24 h appears to be higher in infected U1 cells than in uninfected U937 cells. In fact, the quantity of specifically retarded bands at 24 h is decreased to a level comparable to that obtained at 6 h in infected U1 cells (Fig. 8, lanes 4 and 6, respectively), while the corresponding amount at 24 h in uninfected U937 cells is markedly reduced in comparison to that present at 6 h (Fig. 7A, lanes 7 and 8). It is unclear at present whether this discrepancy is due to a clonal difference between the U1 clone and the parental U937 line or whether HIV-1 replication can play a direct role in the regulation of κB-binding proteins, as already proposed (1). We are currently investigating the latter hypothesis.

The same amounts of nuclear extracts used for gel retardation assays with the enhancer probe were also challenged with the USF/MLTF oligonucleotide. The results obtained indicate that this binding activity is constitutively present also in unstimulated U1 cells and that it does not respond to PMA treatment (Fig. 8B), as already observed in U937 cells.

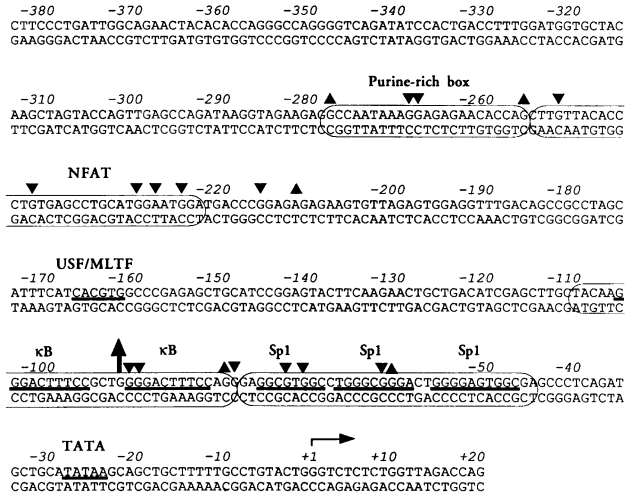


FIG. 6. Summary of in vivo footprinting data for U1 cells. The LTR sequence is that of the LAV strain of HIV-1. Protection of specific guanines on the upper or lower strand is indicated by arrowheads pointing down. Hyperreactive sites are marked by arrowheads pointing up. The regions where specific protein-DNA contacts were detected are boxed, and the consensus sequences for factor binding are underlined, where appropriate. The consensus sequences for USF/MLTF and TATA-binding factors are also underlined. Open boxes indicate the locations of some of the relevant regions protected by in vitro footprinting experiments reported in the literature (26); consensus sequences are underlined. The arrow at position −92 on the coding strand represents the guanine with strong hypersensitivity upon PMA stimulation.

DISCUSSION

Although recent evidence that we and others have obtained indicates that HIV-1 is expressed at all stages of natural infection (3, 41, 48), several observations suggest that the transcriptional activation of the virus is likely to play a role in the pathogenesis of the disease. First, the levels of transcription, measured as the ratio between the amounts of proviral DNA and unspliced RNAs, directly correlate with disease progression (3). Second, recent studies of lymph nodes of infected individuals suggest that a large number of CD4 lymphocytes and macrophages are latently infected and constitute an intracellular reservoir in which replication is activated concomitantly with antigen stimulation (18). Third, the LTR of HIV-1 appears to be highly sensitive to induction by several stimuli that trigger cell proliferation or activation (12). For all these reasons, it appears that the study of the molecular interactions controlling basal HIV-1 transcription, and particularly of those responsible for transcriptional activation, could shed light on the mechanisms of disease progression.

In this work, we have studied the protein-DNA interactions occurring in vivo at the LTR in the U1 cell line. This clone, which was derived from the population of U937 cells surviving acute infection with HIV-1 (21), constitutively expresses low-to-undetectable levels of virus; phorbol esters, certain cytokines, and cytokine-enriched mononuclear cell supernatants are strong activators of viral expression (20, 32, 49, 60). In these cells, however, also in the absence of any stimulation, a low level of viral transcription can be observed. Most of these constitutive transcripts do not result in detectable levels of virus production and are probably processed to fully spliced mRNAs for regulatory proteins (32, 40, 50). In fact, although we could detect a low amount of unspliced transcripts in Northern blotting experiments, the amount of p24 in the culture supernatant is extremely low under basal conditions.

Two remarkable changes in viral transcription occur upon PMA stimulation of U1 cells. The first is a dramatic increase in

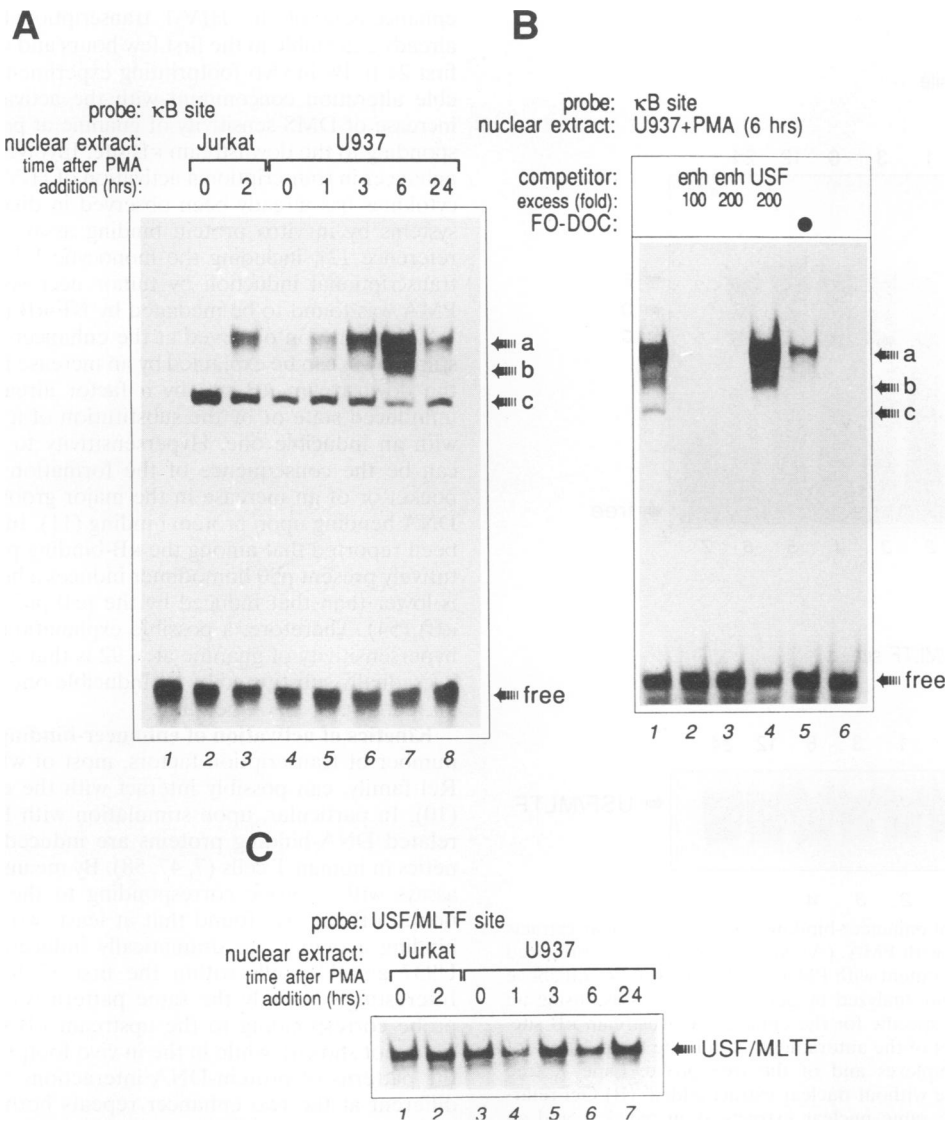


FIG. 7. Transient induction of enhancer-binding activities in nuclear extracts of U937 cells stimulated with PMA. (A) Nuclear extracts were prepared from Jurkat (lanes 2 and 3) and U937 (lanes 4 to 8) cell lines after treatment with PMA for the times indicated above the autoradiograms and analyzed by gel retardation assays using an oligonucleotide probe specific for the enhancer downstream κB site. The arrows on the right of the autoradiogram indicate the positions of the protein-DNA complexes and of the free probe. Lane 1, free labeled oligonucleotide without nuclear extract added. (B) The specificity of the protein-DNA complexes detected in PMA-stimulated U1 cells was tested by competition experiments with cold oligonucleotide competitors. Nuclear extracts from U1 cells prepared 6 h after PMA addition were incubated with the κB box oligonucleotide without competitor (lane 1), with the addition of a 100-fold (lane 2) and 200-fold (lane 3) excess of the same cold oligonucleotide, or with the addition of a 200-fold excess of an oligonucleotide corresponding to the USF/MLTF site (lane 4) and resolved by gel electrophoresis. Lane 5 shows the effect of mild denaturation of the same nuclear extract with formamide and deoxycholate (FO/DOC) to assess sensitivity of the protein-DNA complexes to this treatment. Lane 6, free labeled oligonucleotide without nuclear extract added. (C) Gel retardation assays with an oligonucleotide corresponding to the USF/MLTF site and the same nuclear extracts as in panel A; only the retarded complex is shown.

the total amount of transcripts, starting a few hours after addition of the stimulus and rising until 24 h, and the second is an increase in the ratio between unspliced and spliced transcripts. These changes are followed, although with a certain delay, by the accumulation of viral products in the medium (40, 60). Since this pattern of mRNA accumulation faithfully reflects the kinetics of acute HIV-1 infection (31), this cell line represents a suitable model to study the mechanisms of viral induction and escape from latency (50).

Constitutive protein-DNA interactions. In uninduced U1 cells, in vivo protein occupancy was observed for the two Sp1 sites closer to the enhancer, the downstream κB site of the enhancer, and the NFAT site (all of which have been shown to exert a positive function on LTR-mediated transcription) and a purine-rich site of the NRE. These constitutive interactions could be responsible for the low levels of transcription detectable also under basal conditions. Protein binding in the absence of any stimulation has also been detected in U1 cells by studying the differential accessibility of DNA to nucleases (59), although this approach is less specific and offers far lower resolution than ligation-mediated PCR.

Occupancy of target sites in the absence of transcriptional

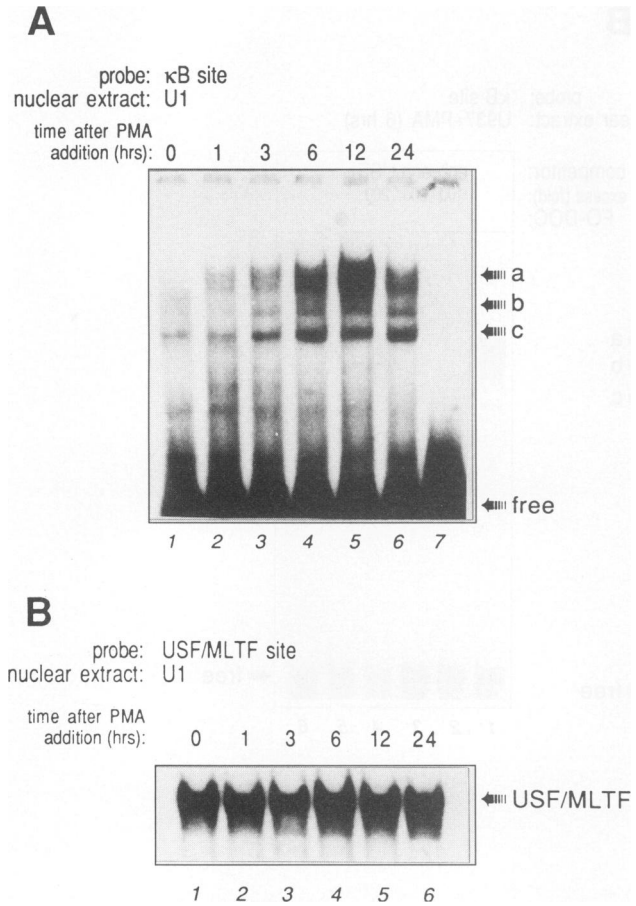


FIG. 8. Induction of enhancer-binding activities in nuclear extracts of U1 cells stimulated with PMA. (A) Nuclear extracts were prepared from U1 cells after treatment with PMA for the times indicated above the autoradiograms and analyzed by gel retardation assays using an oligonucleotide probe specific for the enhancer downstream κB site. The arrows on the right of the autoradiogram indicate the positions of the protein-DNA complexes and of the free probe. Lane 7, free labeled oligonucleotide without nuclear extract added. (B) Gel retardation assays with the same nuclear extracts as in panel A and an oligonucleotide corresponding to the USF/MLTF site; only the retarded complex is shown.

activation has also been described for other systems (33, 44), and it suggests the existence of further levels of regulation. This regulation may be achieved by direct modifications of the proteins constitutively bound to the promoter or by the appearance of ancillary factors, which connect these proteins to the basic transcription machinery or other transcriptional activators (9, 19, 29).

Compared with our previous data on the DNA sites involved in protein-DNA interactions in chronically HIV-1-infected H9 cells (17), results for the U1 cell line show a considerably lower number of LTR sites engaged in protein interactions. This difference could reflect some specific characteristics of the cell lines analyzed or a more general difference between cells of the monocyte and lymphocyte lineages. If the latter hypothesis is correct, in vivo footprinting should offer a useful tool to further investigate the molecular mechanisms determining the different behavior of HIV-1 in different cellular targets.

Genetic elements involved in PMA-induced transcriptional activation. Treatment of U1 cells with PMA triggers a dramatic enhancement of the HIV-1 transcriptional activity, which is already detectable in the first few hours and increases within the first 24 h. By in vivo footprinting experiments, the only detectable alteration concomitant with the activation process is an increase of DMS sensitivity of guanine at position −92, corresponding to the downstream κB site. Involvement of the HIV-1 enhancer in transcriptional activation of HIV-1 by mitogens and cytokines has already been observed in different experimental systems by in vitro protein binding assays (for a review, see reference 12), including the monocytic U1 cell line, in which transcriptional induction by tumor necrosis factor alpha and PMA was found to be mediated by NF-κB (28).

The alteration observed at the enhancer region upon PMA stimulation can be explained by an increase in the occupancy of the downstream κB site by a factor already present in the uninduced state or by the substitution of a constitutive factor with an inducible one. Hypersensitivity to DMS methylation can be the consequence of the formation of a hydrophobic pocket or of an increase in the major groove width typical of DNA bending upon protein binding (11). In this respect, it has been reported that among the κB-binding proteins, the constitutively present p50 homodimer induces a bending angle which is lower than that induced by the p50-p65 heterodimer (NF-κB) (54). Therefore, a possible explanation for the increased hypersensitivity of guanine at −92 is that a constitutive factor is gradually substituted by an inducible one, which determines an increase in DNA bending.

Kinetics of activation of enhancer-binding proteins. A large number of transcription factors, most of which belong to the Rel family, can possibly interact with the enhancer elements (10). In particular, upon stimulation with PMA, several Rel-related DNA-binding proteins are induced with different kinetics in human T cells (7, 47, 58). By means of gel retardation assays with a probe corresponding to the downstream-positioned κB site, we found that at least two specific enhancer-binding activities are dramatically induced by PMA in both U937 and U1 cells within the first 12 h after stimulation. Interestingly, exactly the same pattern was obtained when a probe corresponding to the upstream κB site was used (47) (data not shown), while in the in vivo footprinting experiments the patterns of protein-DNA interactions appear to be quite different at the two enhancer repeats both in the uninduced and in the PMA-induced states. This observation suggests that the pattern of protein binding in vivo is dictated not only by the primary sequence of the binding site, like in the in vitro binding experiments, but also by the context in which the site is positioned within a complex promoter.

Although in this work we do not report any definitive data about the characterization of the proteins interacting with the enhancer, it is very interesting that these κB-binding activities are considerably reduced at 24 h in both cell lines, in agreement with what has been recently observed in PMA-stimulated T cells (56). In contrast, the change observed at the level of the in vivo protein-DNA interactions at the enhancer box appears to persist at 24 h after PMA addition and parallels the further increase in viral transcription which occurs at this time.

Altogether, these observations allow depiction of a complex picture about the involvement of the enhancer element in the transcriptional activation of U1 cells. In the uninduced state, a basal level of transcription is maintained by a constitutively present enhancer-binding factor; upon PMA stimulation, this binding activity is substituted by an inducible factor (28), with a kinetics of induction compatible with the mechanisms proposed for the activation of NF-κB. Subsequently, this NF-κB-like binding activity rapidly disappears from the nucleus (56), while there is a further increase of viral transcription. The

absence of a direct correlation between the levels of κB-binding proteins and the levels of virus expression at 24 h after addition of PMA, as also pointed out by Vlach and Pitha (60), suggests that the maintenance of the induced state is probably due to different mechanisms and that the rapid induction of NF-κB is used only as a trigger for transcriptional activation. These further mechanisms can involve the binding of other factors or the persistence of some induced structural changes, such as an open chromatin structure. Alternatively, only residual low amounts of NF-κB could be sufficient for the maintenance of a sustained rate of transcription, because of its synergetic action with the newly synthesized Tat protein (36).

In conclusion, this study indicates that the utilization of in vivo footprinting techniques is very informative in the analysis of the *cis*-acting elements involved in transcriptional regulation of HIV-1. The combination of in vitro and in vivo binding studies also allows one to address new questions concerning the regulation of the factors involved in the binding to the LTR and the potential cross-talk between these factors and the Tat protein. Overall, the observations presented here suggest that the regulation of HIV-1 transcription in vivo is even more complex than would have been predicted on the basis of in vitro studies.

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