Alternative Pathway for Induction of Human Immunodeficiency Virus Gene Expression: Involvement of the General Transcription Machinery

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Human immunodeficiency virus type 1 (HIV-1) is viable and mitogen inducible in the absence of its binding sites for the inducible transcription factor NF- κ B. We have investigated alternative mechanisms for induction of HIV-1 transcription. Using transient transfection assays, we found that transcription from an HIV-1 LTR containing mutant κ B sites was activated 10- to 20-fold in a variety of human cell types by the phorbol ester phorbol myristate acetate (PMA). The promoter elements conferring this inducibility were localized to the region downstream of nucleotide -70, which contains the TATA and TAR elements and binding sites for transcription factors Sp1 and LBP-1. Synthetic promoters containing only Sp1 sites and a TATA element were also induced in transfection experiments as well as in *in vitro* transcription experiments with T-cell nuclear extracts. Moreover, promoters containing a TATA box in the absence of Sp1 sites or Sp1 sites in the absence of a TATA box were equally inducible *in vitro*, as was an RNA polymerase III promoter. The activities of RNA polymerases II and III and of the 38-kDa TATA-binding protein transcription factor IID (TFIID), were not induced by PMA, but electrophoretic mobility shift assays revealed a highly inducible protein-DNA complex that interacted specifically with the TATA sequence. This protein-DNA complex appeared to be much larger than that found with the 38-kDa human TFIID expressed in bacteria. Taken together, these data suggest that a component of the general transcription machinery, and possibly a TFIID-associated protein, is induced in T cells by PMA. This induction may be important for augmenting HIV expression and for the pathogenesis of AIDS.

Gene expression of human immunodeficiency virus type 1 (HIV-1) is regulated both by cellular factors and by its own viral gene products, including the *tat* and *rev* proteins (reviewed in references 10 and 29). One of the major routes for modulating HIV-1 expression appears to be through the control of transcription initiation from the long terminal repeat (LTR). Transcriptional control sequences in the LTR interact with several cellular DNA-binding proteins, including Sp1, NF- κ B, LBP-1, NFAT-1, and the TATA-binding protein, transcription factor IID (TFIID) (reviewed in reference 16). Indeed, genetic and virologic studies of HIV strains containing mutations in the binding sites for some of these proteins have confirmed that they influence viral growth and replication (13a, 21, 25).

The accepted model of viral kinetics in HIV-infected individuals predicts that during the early phase of infection, virus replicates rapidly in the absence of antibodies to HIV, causing an early viremia (reviewed in reference 22). This stage is followed by a long period of latency, clinically characterized as the asymptomatic phase, during which virus production is very low or undetectable. In general, the progression to AIDS coincides with an increase in virus titer in the peripheral blood. It is not clear whether this is caused by a sudden insurgence of virus *in vivo* or whether the dramatic rise in virus titer is due to a weakening of the immune system. Whatever the mechanism, it appears that

there is a tight correlation between viral replication and the development of AIDS. Therefore, study of the transition from nonproductive to productive (or low- to high-level) replication is important for understanding how latent virus is activated and thus for unraveling the pathogenetic mechanism of HIV. For this reason, identification and characterization of factors that activate viral gene expression, especially upon cellular induction, have been major components of the molecular studies of HIV.

Among the many cellular transcription factors, NF- κ B (20, 40) has been shown to be a potent activator of HIV transcription and to be highly inducible in T cells stimulated with a variety of agents, including phorbol ester (20, 27). Therefore, it has been proposed that NF- κ B or related proteins (4) might be involved in the activation of dormant HIV-1 provirus *in vivo*, resulting in the transition from latent to productive infection. However, it has recently been shown that the DNA sequence elements in the LTR that interact with NF- κ B sites are not essential for the growth or infectivity of HIV in certain cell lines and that virus lacking these sites could still be induced by mitogen (13a, 21, 25). These studies suggest that factors other than NF- κ B play a role in induction of HIV expression.

In this report, we have examined the possibility that inducible cellular factors other than NF- κ B might regulate HIV transcription. Our data demonstrate that transcription from an LTR containing mutated or no κ B sites was highly induced by treatment of cells with phorbol ester. The induced activator appears to be a component of the general

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transcription machinery and possibly a protein that interacts with either the TATA box or TFIID.

MATERIALS AND METHODS

Plasmids and construction of LTR mutants. The wild-type LTR-chloramphenicol acetyltransferase (CAT) plasmid, p938, contains the HIV-1 LTR from pUR-III (34) and was described previously (19). Plasmid p942 is identical to p938 but contains point mutations at both of the κ B sites of the LTR. This was constructed by replacing the *Bgl*II fragment of p938 with that of pUR-III containing mutations at both κ B enhancer sites (19, 27). The *tat* expression vector, pCMV-Tat, was provided by D. Trono (Salk Institute) and consists of the first exon of *tat* on a *Sall*-*Kpn*I fragment from HXB2 cloned into the polylinker linker site (*Xho*I) of the expression vector pCPLK (45).

Deletion mutations were constructed by standard methods as described by Maniatis et al. (26). All plasmids used in these experiments had an SP65 backbone and were identical except for various mutations or deletions described. To construct deletion derivatives, *Taq*I (−120), *Hae*III (−70), *Ban*II (−46), and *Pvu*II (−20) sites of the LTR region were converted to *Bam*HI sites, and then *Bam*HI-*Hind*III fragments of the LTR were ligated into the *Bgl*II-*Hind*III backbone of p942. Therefore, the 5' endpoint of all of the deletions is the *Bgl*II site (−488) of the HIV LTR.

The CAT plasmids containing the herpes simplex virus *tk* gene TATA box with or without multimerized Sp1-binding sites were described previously (7) (obtained from Al Courey, University of California, Los Angeles).

Plasmids IV, V, and VI were described previously (42, 43). (The inductions shown with plasmids V and VI were also observed with similar plasmids that delete a binding site for transcription factor AP-1, which is located adjacent to the Sp1 sites in V and VI. This single AP-1 site did not influence the levels of either uninduced or induced transcription in vitro [data not shown]. It was not surprising that a single AP-1 site had no influence on the strong in vitro transcription activated by five Sp1 sites. However, this result served as an important control because it also ruled out the possibility that a cryptic AP-1 site elsewhere in the plasmid was influencing the induction observed.)

Transfections and CAT assays. Cells (1×10^7 to 2×10^7) were transfected with 1 μ g of HIV LTR-CAT and 2 μ g of *tat* expression plasmids (for cotransfection), using DEAE-dextran (13, 44). Cells were grown in RPMI 1640 containing 10% (CMK) or 20% (H9) fetal bovine serum; 12 to 15 h later, half of the cell population was treated with phorbol myristate acetate (PMA; Sigma) at 50 ng/ml. Cell extracts were prepared 15 to 18 h postinduction, and CAT activity was determined according to standard methods (12), using equivalent amounts of protein. The degree of conversion of [14 C]chloramphenicol to its acetylated forms was determined by cutting out appropriate spots and quantitating the amount of radioactivity by liquid scintillation.

Gel retardation assays. Cellular extracts were prepared as described by Baeuerle and Baltimore (2). Approximately 15×10^6 cells were harvested, washed with ice-cold phosphate-buffered saline, and transferred to Eppendorf tubes. Cells were then lysed in a *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer containing 20mM (HEPES; pH 7.9), 0.35 M NaCl, 20% glycerol, 1% Nonidet P-40, 1 mM $MgCl_2$, 1mM dithiothreitol (DTT), 0.5 mM EDTA, 0.1 mM EGTA, 1% aprotinin (Sigma), and 1 mM phenylmethylsulfonyl fluoride. After lysis and extraction for 10 min on ice,

particulate material was removed by centrifugation (Microfuge) for 15 min at 4°C. Amounts of protein in the resulting supernatants were quantitated by using bicinchoninic acid (Micro BCA protein assay reagent 23235; Pierce).

Two binding conditions for the electrophoretic mobility shift assay (EMSA) were used. For Sp1 and the inducible TATA factor(s), the binding reaction mixture contained, in a total volume of 15 μ l, 3 μ g of poly(dI-dC), the radioactive probe (5,000 to 10,000 cpm in Tris-EDTA, and 5 to 10 μ g of cellular extracts (generally 0.5 to 1 μ l) in the binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 20 μ g of bovine serum albumin, 1 mM EDTA [pH 8.5], 5% glycerol) (1). After incubation for 30 min at room temperature, samples were loaded onto 5% polyacrylamide gels and run with 0.5 $\times$ TBE (0.045 M Tris, 0.045M borate, 0.001 M EDTA) at 150 V for 1.5 to 2 h. For purified TFIID, binding was carried out according to the method of Kao et al. (18). Basically, the reaction mixture contained, in a total volume of 15 μ l, 3 μ g of poly(dI-dC), the radioactive probe (5,000 to 10,000 cpm in Tris-EDTA), and 5 to 10 μ g of cellular extracts (generally 0.5 to 1 μ l) in the binding buffer (12 mM HEPES [pH 7.9], 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 50 μ g of bovine serum albumin, 0.5 mM EDTA, 0.05% Nonidet P-40). After incubation for 30 min at 30°C, the binding reaction mixtures were loaded onto 5% polyacrylamide gels and run with 0.5 $\times$ TBE at 100 V for 2 to 3 h.

Radioactive probes were prepared from oligonucleotides as described by Sen and Baltimore (40). The nucleotide sequences of the oligonucleotides (synthesized by Genosys, The Woodlands, Tex.) used were as follows: wild-type Sp1 oligonucleotide:

GATCTGCCTGGGCGGGACTGGGGAGTGGCG
ACGGACCCGCCCTGACCCCTACCGCCTAG

mutant Sp1 oligonucleotide:

GATCTGCCTGTTTCGGGACTGGTTAGTGGCG
ACGGACAAGCCCTGACCAATCACC GCCTAG

wild-type TATA oligonucleotide:

GATCTGCATATAAGCAG
ACGTATATTCGTCTAG

mutant TATA oligonucleotide:

GATCTGCAGAGAAGCAG
ACGTCTCTTCGTCTAG.

In vitro transcription reactions and analysis of RNA polymerase and TFIID activities. Extract preparation and in vitro transcription reactions were as described previously (42, 43). A total of 10^9 CEM T cells (at 5×10^5 cells/ml) were induced for 24 h with PMA (50 ng/ml) in 1-liter roller bottles. A similar number of cells were not induced. Extracts were prepared simultaneously. Protein pellets following ammonium sulfate precipitation were suspended in 2.4 ml of HM.1 (25 mM HEPES [pH 7.9], 20% glycerol, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 100 mM KCl) and dialyzed against HM.1. Protein concentrations were determined by Bradford assay and were found to be 1.8 mg/ml for the uninduced extract and 2.3 mg/ml for the induced extract. Equal amounts of protein were used for each comparison. TFIID activity was heat inactivated by the method of Nakajima et al. (28). HeLa TFIID was partially purified by chromatography on phosphocellulose as described previously (38). RNA polymerase III transcription with the adenovirus VAI gene (plasmid pVAI) was performed by the method of Clark and Dasgupta (6), using a runoff transcription assay with

FIG. 1. Induction of HIV-1 transcription in the absence of binding sites for NF-κB. (A) CMK or H9 cells were transfected with HIV-1 LTR-CAT constructs containing wild type (+) or mutated (-) κB sites in the presence (+) or absence (-) of a *tat* expression vector. One half of each transfected population was induced with PMA (50 ng/ml) for 15 to 18 h (+ lanes). CAT activity was measured by determining the amount of acetylated chloramphenicol (AC) produced from [¹⁴C]chloramphenicol (C). The values shown are from one representative of at least three independent assays (also see Fig. 2). (B) CMK cells were transfected and induced as described above. When cells were transfected only with the HIV LTR-CAT plasmid containing mutated κB sites, 100 μg of protein was incubated with [¹⁴C]chloramphenicol for 3 h at 37°C (lanes 1 and 2); 40 μg of protein was incubated for 40 min when a *tat* expression vector was cotransfected (lanes 3 and 4).

[α-³²P]GTP. The RNA product of 247 nucleotides was analyzed by electrophoresis on an 8% denaturing acrylamide gel. Western immunoblot analysis of TFIID was performed with a rabbit anti-human TFIID primary antibody, a biotinylated anti-rabbit immunoglobulin G secondary antibody, and then peroxidase-conjugated streptavidin (BioGenex, Inc.). Chemiluminescence was detected with the Amersham ECL gene detection reagent. Nonspecific RNA polymerase activities were determined by the method of Schwartz et al. (39), using α-amanitin (Sigma) at 2 μg/ml to inhibit RNA polymerase II activity and at 100 μg/ml to inhibit RNA polymerase III.

RESULTS

Induction of HIV-1 transcription in the absence of binding sites for NF-κB. To investigate NF-κB-independent mechanisms for induction of HIV-1 transcription, we performed transient transfections with HIV LTR-CAT fusion constructs containing wild-type or mutated κB sites (Fig. 1A). It previously was shown that the mutations used in these constructs abolish NF-κB binding (27). After transfection into the human T-lymphoid cell line H9 (31) or the human megakaryocytic cell line CMK (36), both constructs resulted in low or undetectable levels of CAT activity (Fig. 1A, lanes 2, 4, 6, and 8). However, treatment with the phorbol ester PMA following transfection induced readily detectable CAT activity with the wild-type but not the mutant LTR construct (lanes 1, 3, 5, and 7).

As expected, cotransfection with a *tat* expression vector resulted in a dramatic increase in CAT activity from both constructs, although the mutations in the κB sites significantly decreased overall promoter strength (Fig. 1A; compare lanes 2 and 10 and lanes 6 and 14 for the wild type and lanes 4 and 12 and lanes 8 and 16 for the mutant construct). Treatment with PMA following cotransfection with *tat* produced higher levels of CAT activity with the wild-type construct (lanes 9 and 13) and also increased the level of expression from the mutant LTR (lanes 11 and 15). This induction, of at least 10- to 20-fold, was observed in other

human cell lines tested, including the T-lymphoid cell lines CEM and the monocytic cell line U937 (data not shown). In the T-lymphoid line Jurkat, which is frequently used for studies of HIV expression, the degree of induction was marginal. This increase in LTR activity, despite the absence of functional κB sites, implied that factors other than NF-κB were induced in these cells by treatment with PMA.

tat is not involved in the observed transcriptional induction.

It has been reported that transactivation of the LTR by *tat* requires protein kinase C, raising the possibility that the increase in CAT activity of the mutant LTR might be due simply to an increase in *tat* activity rather than to the induction of cellular transcription factors (15). However, because we treated the transfected cells with PMA (50 ng/ml) for relatively short periods (16 to 18 h), *tat* is unlikely to be responsible. Under these conditions, *tat* transactivation of the LTR has been reported to be unaffected by PMA (15). To confirm this hypothesis, we tested whether CAT activity was induced when cells were transfected only with the mutant LTR-CAT construct and without a *tat* expression vector. Because the level of CAT activity was very low in these circumstances, we used higher amounts of protein than usual and extended reaction times for the CAT enzyme assay (Fig. 1B, lanes 1 and 2). PMA treatment of cells transfected with only the mutant LTR construct increased CAT activity by at least sixfold. (The background level of activity usually resulted in 0.1% conversion of [¹⁴C]chloramphenicol to its acetylated forms with our experimental conditions. Thus, the induction actually may be greater than sixfold.) This result demonstrates that the induction observed was not a result of increased *tat* activity, although a minor influence of *tat* cannot be ruled out. This conclusion is supported by the results presented below.

Localization of the cis-acting element conferring PMA inducibility. To localize the DNA sequence element responsive to PMA in the κB mutant plasmid, deletions of the LTR were tested for their abilities to direct PMA-inducible transcription (Fig. 2A). Deletion of the nucleotide sequences upstream from the κB sites did not significantly affect inducibility (data not shown). Mutations or complete dele-

FIG. 2. Localization of the DNA sequence element responsive to phorbol ester. (A) Each mutant DNA (1 μ g) was cotransfected into CMK cells with 2 μ g of *tat* expression plasmid, treated with PMA, and subjected to CAT analysis as described for Fig. 1. The autoradiograph shown is one representative transfection, and the absence (-) or presence (+) of the major transcription elements of the HIV LTR such as κ B sites, Sp1 sites, and TATA box is indicated (also see Fig. 2B). There are three Sp1-binding sites in the HIV LTR, and +2 indicates the deletion of one Sp1 site (the most distal from the mRNA start site). (B) Sequences deleted from the LTR are indicated by the shading. The 5' endpoint of all of the deletions is the *Bgl*III site of the LTR, and their 3' endpoints are as described below. Asterisks indicate point mutations introduced into the κ B sites as described by Nabel and Baltimore (27). NM, the magnitude of stimulation could not be calculated because of undetectable levels of CAT activity. The relative stimulation (fold) is the ratio of CAT conversion following PMA induction to that without induction. Means and standard deviations are based on three or more independent transfection assays.

tion of the κ B sites significantly lowered LTR activity relative to the wild-type plasmid (Fig. 2A; lane 4 for point mutations and lane 6 for deletion), but CAT activity remained highly inducible by PMA (compare lane 2 with lanes 3 and 5). Removal of one of the three Sp1 sites significantly decreased basal CAT activity (compare lanes 6 and 8), but the remaining activity was still inducible by 10-fold (lanes 7 and 8). When all three Sp1 sites were deleted, CAT activity was undetectable (compare lanes 9 and 10). Overall, non-NF- κ B factors activated the HIV LTR 10- to 15-fold upon PMA induction (Fig. 2B). Similar results were obtained for CEM and H9 cells (data not shown). This analysis indicates that Sp1 itself or proteins interacting with the nucleotide sequences downstream from the Sp1 sites are induced by PMA.

Sp1 is not induced by mitogen. The minimal region of the LTR responding to PMA induction contained two Sp1 sites, a TATA box, and regions near the transcription start site and within the transcribed leader that are important for HIV-1 expression. None of these regions bind proteins known to be induced by PMA (16). However, it was recently reported that Sp1 can be induced following infection with simian virus

40 (35) and that Sp1 can be phosphorylated (14). To our knowledge, Sp1 has not been tested for inducibility by mitogen. Therefore, we investigated this possibility. We transfected cells with an Sp1 test plasmid containing a CAT gene fused to a promoter incorporating multimerized consensus Sp1-binding sites upstream from the herpes simplex virus *tk* gene TATA box (7). It has previously been shown that the activity of this promoter depends on the presence of Sp1 (7). As a negative control, a similar plasmid lacking the Sp1 sites was used. Both plasmids were transfected into CMK cells, and transient expression of the CAT enzyme was used to measure the inducibility by PMA. In the absence of PMA induction, both plasmids gave a background conversion of chloramphenicol to its acetylated forms of 0.1% (Fig. 3A, lane 2 and 4). In most human lymphoid or myeloid cell lines tested, these promoters resulted in similarly low levels of CAT activity (lane 2). However, when cells were treated with PMA, CAT activity was induced to detectable levels with the test plasmid containing the Sp1 site (Fig. 3A; compare lanes 1 and 2). Only background levels were detected in the absence of the Sp1 sites. Similar results were obtained for the human T-cell line H9. These data suggested

FIG. 3. Effect of PMA induction on Sp1. (A) Transient transfection assay with Sp1 test plasmids. CMK cells were transfected with Sp1 test plasmids carrying the truncated promoter of the herpes simplex virus *tk* gene with (+) or without (-) inserted Sp1-binding-site oligonucleotides. (B) EMSA with 32 P-labeled Sp1 oligonucleotide. Nuclear extracts from a variety of human cell lines, either uninduced (day 0) or stimulated (days 1 and 2) with PMA, were incubated with a 32 P-labeled oligonucleotide whose sequence was taken from the Sp1-binding sites of the HIV LTR (-70 to -46). Sp1-specific DNA-protein complexes were verified by competition assay which involved incubating the protein sample from lane 10 with unlabeled wild-type (W) or mutated (M) oligonucleotide (lanes 13 and 14). S, specific DNA-protein complex; FP, free probe.

that the PMA induced either Sp1 activity or the activity of a protein required for basal transcription from a TATA box. Also, because this experiment did not involve a *tat* expression vector, it confirmed our observation that induction of transcription from an LTR containing defective κ B sites did not result simply from increased *tat* activity.

To test whether Sp1-binding activity was induced by PMA, we performed EMSA. Nuclear extracts from a variety of cell lines, either prior to or following stimulation with PMA, were incubated with a double-stranded 32 P-labeled oligonucleotide probe, corresponding to the -70 to -46 region of the HIV-1 LTR. The same sequence has been shown to interact with Sp1 by DNase I footprinting analysis (17). As expected, there were significant basal levels of Sp1 in uninduced CMK, H9, and HeLa cells (Fig. 3B, lanes 1, 4, 7, and 10). Induction of these cells with PMA did not yield significant increases in amounts of electrophoretically retarded DNA-protein complexes. On the contrary, amounts of DNA-protein complexes decreased upon induction in

most cell types. This result suggested that the DNA-binding activity of Sp1 was not increased by treatment with phorbol ester. Thus, the transcriptional induction observed might be due to induction of a component of the general transcription machinery.

In vitro analysis of PMA-inducible transcription. In vitro transcription experiments were used to further analyze transcriptional induction by PMA. These experiments were necessary because we could not detect transcription in vivo from a TATA box in the absence of Sp1 sites. Moreover, in vitro analyses allowed us to assay for activities of individual components of the general transcription machinery.

Nuclear extracts were prepared from CEM cells either with or without a 24-h induction by PMA. The protein concentrations of the extracts were similar and were adjusted to equivalence with buffer. In vitro transcription experiments were performed with three different synthetic promoters to define the DNA sequence elements responsible for PMA inducibility. Plasmid V (Fig. 4A, lanes 1 to 4) contained Sp1 sites upstream from a consensus TATA element derived from the adenovirus major late promoter. Plasmid IV (lanes 5 to 8) contained the TATA box in the absence of Sp1 sites but in the presence of a transcriptional initiator (Inr) element at the start site (42). This element enhances transcription from the TATA element and is thought to interact with a known component of the general transcription machinery (e.g., TFIID, TFIIB, or RNA polymerase II [32, 43]). Plasmid VI (lanes 9 to 12) contained the Sp1 sites and the Inr element but lacked a TATA element. Detailed characterization of these promoters and of the proteins required for their activities have been reported (32, 43) (see also Materials and Methods).

Comparison of the activities of these promoters in the CEM nuclear extracts showed that transcription from all three was induced to a similar degree by PMA (Fig. 4A). Laser densitometry revealed that transcription from plasmid V was induced by fivefold, from plasmid IV by fourfold, and from plasmid VI by sixfold. Somewhat stronger inductions were observed with CMK cells, but no inductions were found with HeLa cells (data not shown). These results confirm that Sp1 is not responsible for the observed PMA induction and also demonstrate that a TATA box is not required. However, these results do not rule out the induction of the TATA-binding protein, TFIID, because TFIID is known to be required for transcription from plasmid VI as well as from other promoters that lack TATA elements (32, 43).

Because of the general nature of the PMA inducibility observed, we tested in the CEM extracts the RNA polymerase III-transcribed adenovirus VAI gene (11). We found that specific RNA polymerase III transcription was also strongly induced (ninefold) by PMA in CEM cells (Fig. 4B). These results demonstrate that one or more general factors required for specific transcription by RNA polymerases II and III are induced in PMA-treated T cells. Based on the multiplicity of events that occur in activated T cells (8), these results may not be surprising (see Discussion).

Analysis of general transcription factors. The induction of RNA polymerase II transcription could result from the induction of a number of general transcription factors. These include RNA polymerase II itself or TFIID. Alternatively, other general transcription factors (5), such as TFIIB, TFIIE, or TFIIIF, might be induced by PMA. Finally, a general effect on elongation of transcription by RNA polymerase II might be responsible. To begin to identify the

FIG. 4. In vitro analysis of PMA-inducible transcription. (A) Three different plasmids were tested for promoter activity and inducibility in nuclear extracts prepared from CEM cells (lanes 1, 2, 5, 6, 9, and 10) or from CEM cells induced for 24 h with PMA (lanes 3, 4, 7, 8, 11, and 12). Characterization of these plasmids was described previously (43) (see also Materials and Methods), and the promoters contain either Sp1 sites and a TATA box (plasmid V; lanes 1 to 4), a TATA box and an Inr element (plasmid IV; lanes 5 to 8), or Sp1 sites and an Inr element (plasmid VI; lanes 9 to 12). Reaction mixtures contained 900 ng of template DNA and 5 or 15 μ g of nuclear extract, as indicated. RNA synthesized in vitro was analyzed by primer extension with an SP6 promoter primer. cDNA products were either 79 (lanes 5 to 12) or 70 (lanes 1 to 4) nucleotides. (B) Specific transcription of the adenovirus VAI gene, transcribed by RNA polymerase III (Ad VA Trxn RNA Pol. III), was tested by a runoff transcription assay. The reactions included 300 ng of template DNA and either no extract (lane 1) or 2 μ g (lanes 2, 5, and 8), 10 μ g (lanes 3, 6, and 9), or 20 μ g (lanes 4, 7, and 10) of nuclear extract. Extracts were from untreated CEM cells, PMA-treated CEM cells, or HeLa cells, as indicated. The labeled RNA product was 247 nucleotides and was analyzed on an 8% denaturing polyacrylamide gel.

inducible protein(s), we have begun to analyze the levels and activities of individual components.

We first determined the relative levels of RNA polymerase activities in the uninduced and induced extracts (Table 1). These experiments measured the incorporation of tritiated nucleotides into RNA synthesized from total herring sperm DNA (39). Because the three RNA polymerases efficiently begin transcription from single-stranded nicks and gaps, or from double-stranded breaks in the absence of other general transcription factors, this assay could be used to determine

TABLE 1. Total RNA polymerase activities in uninduced and PMA-induced CEM cells

Prepn	Incorporation of [3 H]UTP into RNA (cpm)	
	Uninduced	PMA induced
No protein	180	180
4 μ g of CEM extract	1,340	1,250
10 μ g of CEM extract	4,170	3,020
10 μ g of CEM extract + 2 μ g of α -amanitin per ml	3,560	2,750
10 μ g of CEM extract + 100 μ g of α -amanitin per ml	3,380	2,250

the total RNA polymerase activity within the extracts. The results from duplicate experiments (Table 1) demonstrate that the total RNA polymerase activity from the induced extract was significantly lower (8 to 30%, depending on whether 4 or 10 μ g was used) than from the uninduced extract. In addition, inhibition experiments with α -amanitin revealed that the individual activities of RNA polymerases I, II, and III were not significantly different in the two extracts. α -Amanitin at 2 μ g/ml is known to specifically inhibit RNA polymerase II activity, and 100 μ g/ml inhibits both RNA polymerases II and III. RNA polymerase I is resistant to α -amanitin. Thus, neither RNA polymerase II nor III appears to be responsible for the induced specific transcription observed. These results additionally rule out a general effect on transcriptional elongation, which would have been revealed in this assay as well.

We next tested for the levels of TFIID, the TATA-binding protein (37). As mentioned above, this protein is absolutely required for transcription from promoters that both contain and lack TATA boxes (32, 37, 43). By Western blot analysis with rabbit antisera directed against the human 38-kDa TFIID protein (18, 30) (Fig. 5A), no induction of TFIID protein was observed. Moreover, in vitro transcription experiments showed that the activity of TFIID was not induced (Fig. 5B). Depletion of TFIID activity by heat treatment of the nuclear extracts resulted in strong decreases in transcriptional activity from plasmid IV (Fig. 5B, lanes 2 and 6). When a partially purified HeLa TFIID fraction was added back to the heat-treated extracts, the degree of induction was similar to that before heat treatment (lanes 3, 4, 7, and 8). If the heat-labile TFIID were responsible for the induction, HeLa TFIID would have increased transcription by similar amounts from both the induced and uninduced heat-treated extracts. Thus, the protein responsible for PMA induction did not correspond to the heat-labile TFIID.

PMA induction of a protein or protein complex interacting with the TATA box. Despite the foregoing evidence that TFIID is not induced by PMA, EMSA studies revealed a surprising result. We directly tested by EMSA whether a protein or protein complex interacting with the TATA sequence was induced by PMA. Cellular extracts from human cells (Fig. 6A, lanes 2 to 4) or cloned, 38-kDa human TFIID purified from *Escherichia coli* (lane 1) were incubated with an oligonucleotide probe corresponding to the TATA box (−33 to −20) of the HIV LTR. The bacterially expressed TFIID protein formed a specific band (S2) with the TATA oligonucleotide, whereas crude cellular extracts formed a DNA-protein complex (S1) whose electrophoretic mobility was slower. This result is consistent with a current hypoth-

FIG. 5. Analysis of TFIID in uninduced and PMA-induced extracts. (A) The amounts of the 38-kDa TFIID protein in the uninduced and induced CEM extracts was analyzed by Western blot, with rabbit antisera directed against the human TFIID protein expressed in bacteria (see Materials and Methods). The lane labeled cTFIID contains an *E. coli* extract expressing this protein. The largest band corresponds to the full-length protein, and the lower-molecular-weight bands are thought to be degradation products. Increasing amounts of uninduced and induced extract were tested. (B) In vitro transcription experiments with TATA/Inr plasmid IV were performed to measure the TFIID activities in the uninduced and PMA-induced extracts. This experiment was performed by heat-treating the extracts to inactivate the labile TFIID, followed by reconstitution with 1 or 2 μ l of partially purified TFIID from HeLa cells, as indicated. If the heat-labile TFIID in the CEM extracts was responsible for the PMA induction, the signal in lane 8 would have been equivalent to the sum of the signals from lanes 4 and 6.

esis that TFIID may interact with other proteins in vivo (23, 32, 43), slowing down its mobility in gel shift analyses. These complexes were specific for the TATA element because they were abolished by the unlabeled wild-type TATA sequence but not by a mutant oligonucleotide which was identical to the wild-type probe used except for two G's substituting the T's of TATA (lanes 5 to 8). The amounts of these DNA-protein complexes did not increase with PMA induction in most cells that we have tested (lanes 2 to 4). Similar results were obtained with human T-cell lines such as CEM and H9.

We also performed EMSA with different binding conditions (see Materials and Methods), the most significant differences being the inclusion of 5 mM $MgCl_2$ in the binding reaction and incubation of the binding reaction at 30°C rather than at room temperature (Fig. 6B). With these conditions, even high concentrations of cloned TFIID protein did not bind to the TATA oligonucleotide (data not shown). However, an electrophoretically retarded DNA-protein complex was readily observed with crude extracts, and this complex was specifically abolished by the unlabeled wild-type TATA sequence but not by a mutant oligonucleotide (Fig. 6B, lanes 7 and 8). Most interestingly, the amounts of this DNA-protein complex increased more than 10-fold upon treatment of cells with PMA, as determined by laser densitometry analysis (lanes 1 to 3 for CMK and lanes 4 to 6 for CEM). These results suggest that cellular proteins interacting with the TATA sequence are highly inducible and that activation of an LTR containing defective κ B sites by phorbol ester might be due to the increase in this factor(s). This protein-DNA complex may or may not contain the 38-kDa TATA-binding protein.

DISCUSSION

We have analyzed NF- κ B-independent pathways for induction of HIV-1 transcription. These studies were neces-

FIG. 6. EMSA with ^{32}P -labeled TATA oligonucleotide. (A) Cloned TFIID purified from *E. coli* (lane 1) and nuclear extracts prepared from CMK cells (lanes 2 to 8) were analyzed by EMSA. The cloned TFIID protein forms a single specific band (S2) with the TATA oligonucleotide, whereas crude nuclear extract formed a DNA-protein complex (S1) whose electrophoretic mobility was slower. Specificities of DNA-protein complexes were verified by competition assays, using the protein sample from lane 2 with two different concentrations (30 and 50 ng) of unlabeled wild-type (W) or mutated (M) oligonucleotide. (B) Inducible factors other than TFIID interact with the TATA box. Nuclear extracts from CMK cells, either uninduced (day 0) or stimulated (days 1 and 2) with PMA, were incubated with a ^{32}P -labeled TATA oligonucleotide whose sequence corresponds to the TATA box of the HIV LTR (−33 to −20). Specificities of DNA-protein complexes were verified by competition assays by incubating the protein sample used in lane 2 with unlabeled wild-type (W) or mutated (M) oligonucleotide (lanes 7 and 8).

sary because HIV strains with mutations in the binding sites for the inducible transcription factor NF- κ B were found to be viable and mitogen inducible (13a, 21, 25). Transient transfection assays in both myeloid and T-cell lines demonstrated that PMA strongly induced transcription from an HIV LTR lacking κ B sites. The DNA sequence elements responsive to PMA were localized to the region downstream of nucleotide −70. In vitro transcription experiments suggested that no particular sequence element was responsible for the PMA induction of RNA polymerase II transcription and that specific transcription by RNA polymerase III was also PMA inducible. These results suggest that the activities of components of the general transcription machinery are responsible for the observed inductions. These components do not appear to include an RNA polymerase or the 38-kDa TATA-binding protein, TFIID. However, our studies revealed that a protein or protein complex interacting with the TATA box is highly inducible by PMA. This complex might be responsible for the observed inducible transcription by RNA polymerase II.

Although the data implicated a TATA-associated protein in the PMA induction, we do not know what protein(s) are involved in this protein-DNA complex. It might include the 38-kDa TFIID protein in association with another protein or might instead be a novel TATA-binding protein. The latter possibility is unlikely because no functional alternative TATA-binding proteins have been identified. By contrast, the idea that the 38-kDa TFIID is part of a multisubunit protein or multiprotein complex is already widely accepted.

Not only does TFIID from cellular extracts migrate as a very high molecular weight protein when analyzed by gel filtration chromatography (33), but functional studies have demonstrated that the cloned TATA-binding protein could only partially substitute for extensively purified TFIID from HeLa cells (30, 32, 43). Moreover, TFIID is one target for transcriptional transactivation by the adenovirus E1a protein, which recently was found to form a tight complex with TFIID (3). The proteins in the EMSA complex will need to be purified to determine whether TFIID is included, because the purified anti-TFIID immunoglobulin G used for the Western analysis could not inhibit or supershift the EMSA complex observed even with bacterially expressed TFIID (data not shown).

The induction of transcription from a promoter lacking a TATA box is consistent with the hypothesis that a TFIID-associated protein is responsible. It has been established that TFIID is essential for transcription from promoters that lack TATA elements and that subunits in addition to the 38-kDa TATA-binding subunit are likely to be required for complete TFIID function (32, 43).

Although the EMSA data implicated a putative TATA-binding protein in PMA induction, the functionally induced protein could instead be one of the other general transcription factors (5), such as TFIIA, TFIIB, TFIIE, or TFIIIF. In addition, although nonspecific RNA polymerase II activity decreased upon induction, a polymerase subunit that is required only for specific initiation might be induced. The RNA polymerase III transcription factor responsible for induction of adenovirus VAI transcription must also be defined. Because nonspecific RNA polymerase III activity was not induced, the most likely candidates are TFIIB and TFIIC (reviewed in reference 11).

Our data demonstrating the induction of an LTR with mutated or deleted κ B sites was somewhat unexpected in view of previous transfection analyses reporting that similar constructs did not respond to PMA (27). One possible explanation for this discrepancy is that different cell lines were used for the experiments. We have found that the activity of the mutant LTR was indeed very weak and, at best, marginally inducible by mitogen in the human T-lymphoid Jurkat cell line (data not shown), which is widely used for HIV transcriptional studies. Recently, *in vitro* transcription experiments with extracts from Jurkat cells revealed an approximately threefold PMA-induction of HIV transcription in the absence of the binding sites for NF- κ B (24). In our transfection experiments, we have found the mutant HIV LTR to be inducible by mitogen in multiple human cell lines, indicating that our observation is not restricted to a particular cell line.

This induction of the general transcription machinery might not be surprising, considering the events that occur during T-cell activation (reviewed in reference 8). It has been reported that this process induces more than 70 genes. Transcription factors, such as NF- κ B, NFAT, c-Fos, and c-Myc, are induced in activated T cells, but a contribution by the general transcription machinery seems reasonable in light of the extent of transcriptional activation. Moreover, induction of RNA polymerase III transcription may be necessary to allow for increased processing and translation of mRNA.

If a component involved in transcription of all protein-coding genes is induced, why is the transcription of some genes not induced? The interleukin-2 (IL-2) gene as well as other genes appear to be unaffected by PMA (8, 41). Possibly, the degree of induction is determined by which tran-

scription factor is rate limiting for each promoter. For example, an increase in a putative TFIID-associated protein may not increase IL-2 transcription upon PMA induction because another required factor, NFAT-1, has not yet been induced (8). This putative general factor may not even be limiting for basal IL-2 transcription in the absence of the NFAT-1 sites.

Our findings are consistent with studies of IL-2 receptor transcription, which is inducible by PMA in T cells (9). The element responsible for this induction has not been easily defined. Although a region containing an NF- κ B site has been found in the IL-2 receptor enhancer, no mutation has been found to eliminate induction. It must be considered that these results may be due, at least in part, to the fact that a general transcription factor is PMA inducible.

The observation that our inducible factor(s) interacts with nucleotide sequences downstream from the HIV κ B sites has implications for the role of NF- κ B in HIV gene expression. The reported 50-fold induction of LTR activity upon PMA treatment has been attributed to an increase in NF- κ B activity (27). However, since PMA appears to induce another factor, which we have shown to increase LTR activity by more than 10-fold, a significant portion of this increase might be due to this other inducible factor. The relative contributions of these two factors will need to be addressed with the intact virus in primary cells. It has been found that mutations in the NF- κ B sites do not significantly affect virus replication or induction (13a, 21, 25). If a general transcription factor is indeed important for induction, we might expect that no mutations will inhibit induction unless they abolish transcriptional activity.

These possibilities have implications for the pathogenesis of AIDS. We have found NF- κ B-independent induction in a variety of cell types, including T cells, megakaryocytes, and early myeloid cells, all of which have been shown to be infected with HIV *in vivo*. The possible induction of a general RNA polymerase II transcription factor by immune stimulation or cell differentiation might activate virus in infected cells, causing accelerated viral spread or switching of the virus from a latent state to a productive infection.

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