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Phorbol Ester Enhances Human Immunodeficiency Virus-Promoted Gene Expression and Acts on a Repeated 10-Base-Pair Functional Enhancer Element

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T-cell activation pathways are involved in the regulation of human immunodeficiency virus (HIV) expression. Phorbol 12-myristate 13-acetate (PMA) is a potent inducer of T-cell immune functions and has recently been demonstrated to increase viral replication in cell lines infected with HIV. To define sequences required for viral induction by PMA, T-cell lines were transiently transfected with viral long terminal repeat (LTR) sequences directing chloramphenicol acetyltransferase (CAT) gene expression. PMA added to transfected cell cultures 24 h before harvest reproducibly increased both CAT mRNA and enzyme expression 2- to 20-fold. Sequences necessary for basal and PMA-induced levels of CAT expression were determined by deletion and enhancer reconstitution constructs with fragments and oligonucleotides from the original LTR-CAT expression plasmid. PMA-inducible and basal activity required tandem repeats of a core enhancer element (GGGACTTTCC) located in the LTR between −105 and −82 relative to the RNA start site. The enhancerlike sequence could be inserted at a site distant to the CAT gene open reading frame and functioned in a position- and orientation-independent manner. The data thus define a transcriptionally active regulatory-enhancer element critical to the control of HIV gene expression.

Human immunodeficiency virus (HIV) is the etiologic agent of acquired immune deficiency syndrome. Clinical manifestation of HIV infection characteristically occurs after a prolonged period of latency (9, 11, 26). A model of latency has been described for chronically infected normal T-cell lines in vitro. These cells can be propagated in interleukin-2 with little evidence of viral replication. Addition of phytohemagglutinin, a T-cell-activating agent, stimulates intense viral replication and cytopathic effects (34). Another T-cell-activating agent, phorbol 12-myristate 13-acetate (PMA), has been shown to induce intense viral replication in chronically infected Molt-4 cells (13). These two studies suggest that T-cell activation pathways have direct regulatory influence on HIV-directed gene expression and viral latency.

Regulation of HIV-directed gene expression appears to involve both transcriptional and translational control mechanisms. The virus-encoded *trans*-activator gene (TAT) has been implicated in both transcriptional and translational regulation of HIV gene expression (7, 20, 23). The TAT protein functions on sequences in the viral long terminal repeat (LTR) (see Fig. 1A) referred to as the *trans*-acting responsive element (25). Other transcriptionally important LTR regions identified include a repressor region (−340 to −185 relative to the mRNA start site), an enhancer region (−137 to +17 [25] and −121 to −20 [20]), sequences responsive to the transcriptional factor Sp1 (−79 to −48) (15), and a TATA box sequence (−28 to −24).

This study was undertaken to examine the effect of T-cell activation by phorbol ester treatment on the function of the HIV LTR as measured by LTR-directed chloramphenicol acetyltransferase (CAT) expression. The work reported here demonstrated that gene expression under the control of the HIV LTR can be regulated in transformed T-cell lines by a PMA-responsive pathway. Deletion mapping and enhancer reconstitution experiments mapped the genetic site of activation to a 10-base-pair (bp) repeated enhancer element located in the LTR, 5′ to the Sp1-binding sites.

MATERIALS AND METHODS

Plasmid construction. HIV LTR-directed CAT constructs are detailed in Fig. 1. Plasmid pLTR-cat replaces the simian virus 40 (SV40) promoter sequence of pSV2-cat (12) with a 723-bp *Xho*I-to-*Hin*dIII U3/R LTR fragment subcloned from the HIV isolate HXB2 (29, 31). Deletion mutants were constructed with the *Sca*I site at −139 bp (p-139-cat), the *Taq*I site at −119 bp (p-119-cat), and the *Hae*III site located at −69 bp (p-69-cat) upstream from the mRNA cap site (+1). Plasmid p-69E3S contains the same promoter element as p-69-cat except that a 90-bp *Hae*III LTR fragment from −160 to −70 was inserted in the sense orientation with respect to the CAT open reading frame at the *Bam*HI site distal to the CAT gene. Plasmid p-69E3A contains the same insert in the antisense orientation. Plasmids p-69E1S, -E1A, -E1.2A, -E2S, and -E2A have oligonucleotides containing either single (E1, −94 to −80) or tandem (E1.2, −94 to −80; E2, −106 to −80) copies of the enhancer repeat inserted in the sense (S) and antisense (A) orientation at the *Bam*HI site of p-69-cat as indicated in Fig. 1B. The plasmid pA10-cat2 contains the SV40 early promoter without enhancer regions and has been described elsewhere (17). Plasmids pA10E1A and pA10E1.2A-cat have one and two inserts, respectively, of the same oligonucleotide (−94 to −80) in the antisense orientation in the *Bam*HI site of pA10-cat2 (see Fig. 5A). All

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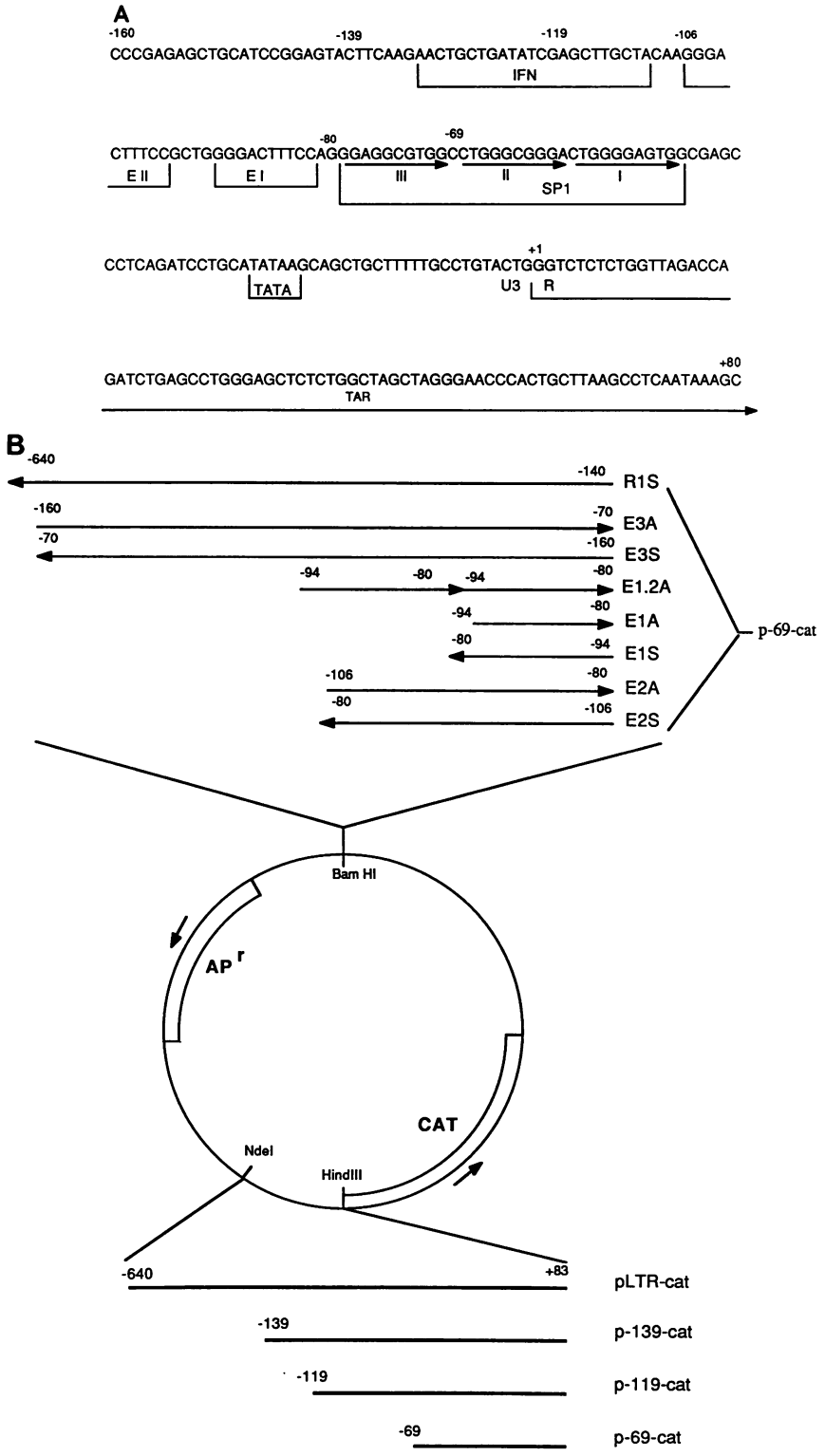

FIG. 1. DNA sequence of the HIV LTR and LTR-directed CAT gene expression constructs. (A) LTR sequence from −160 to +80 (31). U3 and R region proviral sequences with transcriptional and translational regulatory elements are detailed. Interferon (IFN) homologous region is from reference 31. TAR, *Trans*-acting responsive element. (B) Construction of pLTR-cat, deletion derivatives, and enhancer reconstitution plasmids. AP, Ampicillin.

oligonucleotides have *Bam*HI- and *Bgl*II-compatible but asymmetric ends.

Plasmid DNA transfections and CAT assays. Adult T-cell leukemic lines H-9, Molt-4, and Hut-78 (10, 19, 24) were grown in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum. A total of 10^7 cells were transfected by a variation of the DEAE-dextran method (2, 30). Cells were incubated for 45 min in 2 ml of 250-μg/ml DEAE-dextran–5-μg/ml plasmid DNA–0.05 M Tris hydrochloride (pH 7.3) in serum-free RPMI 1640 medium. Cells were then washed once in serum-free medium and suspended in medium containing 20% fetal calf serum. PMA (10 ng/ml; Sigma Chemical Co., St. Louis, Mo.) in 100% dimethyl sulfoxide was added, and cultures were incubated for 24 h.

CAT assays. Cells were harvested and assayed essentially as described previously (12). CAT assay reaction mixtures typically contained 20 to 58 μl of lysate, 70 μl of 0.25 M Tris (pH 7.8), 0.2 μCi of [^{14}C]chloramphenicol (New England Nuclear Corp., Boston, Mass.), and 20 μl of 0.4 mM acetyl coenzyme A (Pharmacia Fine Chemicals, Piscataway, N.J.). Reaction mixtures were incubated for 0.5 to 4 h at 37°C. Specific percent conversion was calculated by the formula: specific percent conversion = [(dpm of acetylated chloramphenicol/total dpm) × 100]/milligrams of protein in the reaction. Specific percent conversions were normalized to a 4-h assay.

RNA preparation and dot-blot hybridization. RNA was prepared from 5 × 10^7 transfected H-9 cells. Cells were harvested 24 h after transfection, washed twice in ice-cold phosphate-buffered saline, lysed in guanidine isothiocyanate, and then centrifuged through a 5.7 M CsCl cushion (5). Dot-blot hybridizations were performed by filtering 50 μg of whole-cell RNA onto nitrocellulose (BA 85; Schleicher & Schuell, Inc., Keene, N.H.) and then hybridizing to a 784-bp *Hin*dIII-*Sau*3A1 ^{32}P-labeled nick-translated CAT gene DNA probe. Filters were hybridized for 12 to 18 h at 42°C (18), washed at high stringency (60°C, 0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate), and exposed to X-ray film.

RESULTS

Activation of HIV LTR-directed CAT gene expression by phorbol esters. To test the effect of T-cell activation on LTR-mediated gene expression, the plasmid pLTR-cat (Detailed in Fig. 1) was transiently transfected into the lymphoid cell lines H-9, Molt-4, and Hut-78. The cell lines were stimulated by the addition of PMA directly after transfection and then incubated for 24 h. All the T-cell lines expressed the LTR-directed CAT gene at moderate levels that increased upon the addition of PMA (Fig. 2). Overall, PMA stimulation of LTR-directed CAT gene expression varied in a range of 2- to 20-fold, with an average induction of 10-fold. H-9 cells gave the best response and were used for all subsequent experiments.

PMA activation functions at transcriptional level. Several experiments were performed to determine whether PMA activation was occurring at the transcriptional or posttranscriptional level of gene expression. To block mRNA synthesis, we pretreated transfected H-9 cells with the transcriptional inhibitor actinomycin D before the addition of PMA. To avoid the toxic effects of actinomycin D, cells were transfected, cultured for 18 h, and then stimulated with PMA

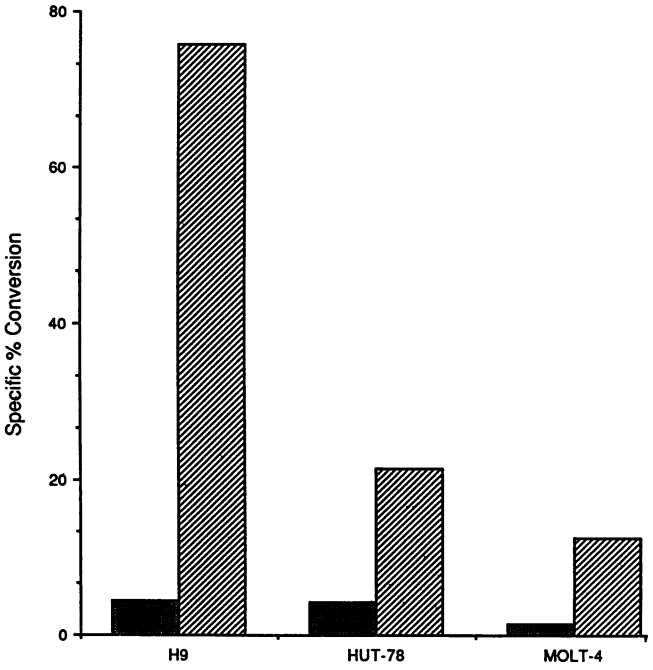

FIG. 2. PMA induction of pLTR-cat expression in three T-cell lymphoma lines. Cell lines H-9, Hut-78, and Molt-4 were transfected with (▨) or without (■) the addition of PMA. Specific percent conversion = percent conversion per milligram of protein in a 4-h assay.

for 6 h in the presence of actinomycin D. This protocol permitted the endogenous expression of CAT protein before PMA addition. PMA stimulated the plasmid p-139-cat cultures over a short 6-h incubation (Fig. 3A, bar 3), although at a lower level than the 24-h-stimulated culture (bar 2). Actinomycin D completely blocked PMA induction (Fig. 3A, bar 5), but allowed CAT expression approximately equal to that of the untreated and actinomycin D-treated control cultures (Fig. 3A, bars 1 and 4). This demonstrates that (i) mRNA synthesis is required for PMA effects; (ii) induction occurs rapidly, at least in less than 6 h; and (iii) stimulation is not solely dependent on posttranscriptional or translational effects.

To verify that PMA enhanced transcription of the CAT constructs, we prepared RNA from whole-cell lysates from transfections of several LTR-cat plasmids. Plasmids p-139-cat and p-69-cat (Fig. 1) were constructed to map regions of the HXB2 LTR necessary for PMA induction. A 50-μg sample of RNA was probed with a labeled fragment containing the CAT gene. pLTR-cat and p-139-cat both exhibited increases (2.5- to 3-fold) in CAT RNA levels 24 h after the addition of PMA (Fig. 3B). The p-69-cat plasmid had low levels of RNA, which did not detectably increase with PMA. CAT assays were used for all subsequent experiments because of the sensitivity of the technique.

Mapping of PMA-inducible sequence. Deletion mutants of the pLTR-cat plasmid were constructed to map regions of the HIV LTR necessary for PMA induction. Previously published data indicate that enhancer sequences are essential to transcriptional induction of SV40 virus, prolactin, immunoglobulin κ, and Moloney murine leukemia virus by phorbol esters (8, 14, 27, 28). Several regions within the LTR have been identified that contain enhancer sequences (20, 25). Rosen et al. (25) mapped the enhancer between −137

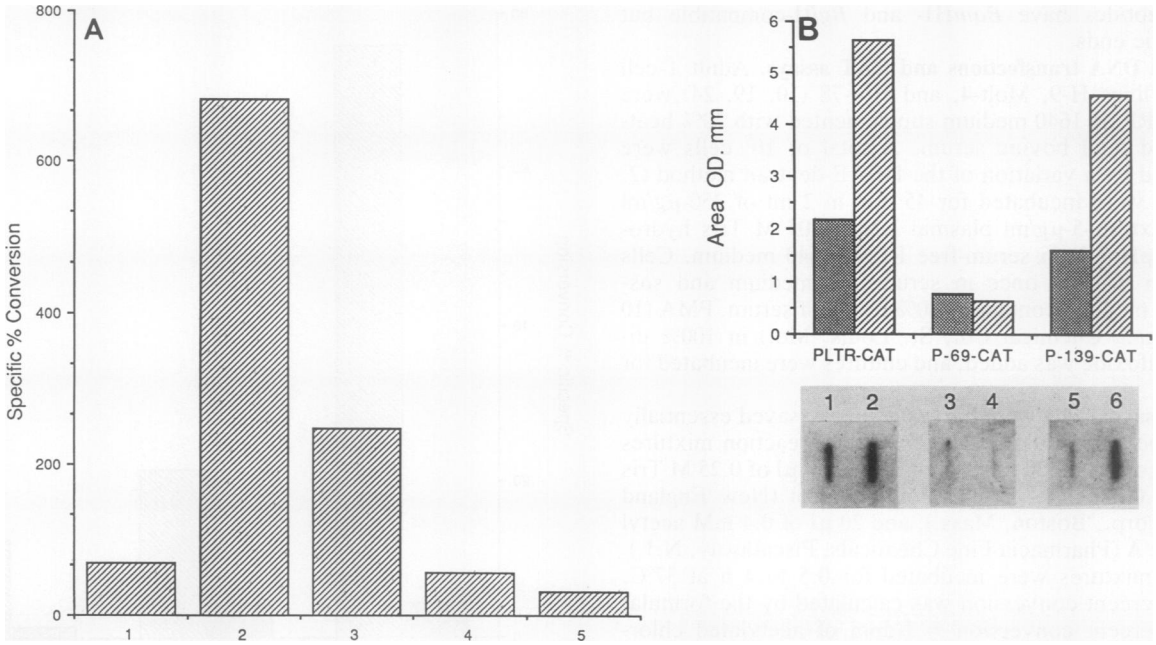

FIG. 3. PMA functions at the transcriptional level. (A) Actinomycin D blocks PMA induction of P-139-cat expression in H-9 cells. Shown are basal CAT expression level (bar 1) and PMA-induced expression levels over 24 h (bar 2) or 6 h (bar 3) of treatment before harvest. Actinomycin was added 6.5 h before cell harvest (bar 4) followed by PMA 6 h before harvest (bar 5). (B) CAT RNA levels measured in transfected H-9 cells 24 h after PMA induction. Slot blots of 50 μg of whole-cell RNA were probed with labeled CAT DNA. Shown are RNA levels for plasmid pLTR-cat without PMA (slot 1) and with PMA (slot 2), plasmid p-69-cat without PMA (slot 3) and with PMA (slot 4), and plasmid p-139-cat without PMA (slot 5) and with PMA (slot 6). RNA levels as measured with a scanning densitometer (Bio-Rad model 620) are reported as the area of optical density (OD) above each slot.

and +17, while Meusing et al. (20) showed orientation-independent enhancer activity between −121 and −20. Basal levels in the latter study were dependent on sequences 5′ to −86. Wright et al. (33) showed basal levels requiring sequences 5′ to −33. We constructed the plasmid p-139-cat to delete sequences known to repress LTR-directed gene expression, but possibly involved in phorbol induction. The plasmid p-119-cat partially deleted sequences homologous to the gamma interferon gene (31), within previously defined enhancer regions. Plasmid p-69-cat deleted these upstream regions and one Sp1 site, leaving two Sp1-binding sequences.

Both p-139-cat (41-fold) and p-119-cat (15-fold) remained PMA inducible and retained basal activity (Fig. 4A). The plasmid p-69-cat lost base-line CAT activity and was no longer PMA inducible. These experiments show that the LTR region bounded by −119 and −69 contains sequences necessary for PMA-induced and basal levels of CAT expression.

Position and orientation independence of reconstitution of PMA inducibility. To test whether HIV PMA inducibility mapped to an enhancerlike element, we inserted fragments and oligonucleotides from the HIV LTR into the p-69-cat plasmid at a site distant to the CAT gene. Constructs p-69E3A and p69E3S-cat contain a 91-bp *Hae*III fragment from −160 to −70, while p-69R1S contains the rest of the LTR from −140 to −640 inserted into the *Bam*HI site. Plasmids p-69E3A and p69E3S-cat expressed the CAT gene at levels considerably higher than p-69-cat and were PMA inducible (25-fold and 17-fold, respectively) (Fig. 4B). The p-69R1S construct had slightly more basal activity than p-69-cat (fivefold) but was not inducible. Because the segment of DNA from −160 to −70 functioned at a site remote to the CAT promoter elements and was orientation independent, we concluded that this DNA fragment contains a functional enhancer. Together, the deletion and reconstitution experiments indicate that an enhancer–PMA-inducible element is contained in the region between −119 and −69.

PMA activation maps to 10-bp repeated sequence. Visual examination of the sequence between −119 and −69 revealed a directly repeated 10-bp sequence between −104 and −84 (Fig. 1). To test whether this sequence contained enhancer and PMA-inducible properties, synthetic oligonucleotides E2 (−106 to −80) and E1 (−94 to −80) containing this sequence with *Bam*HI- and *Bgl*II-compatible ends were cloned into the *Bam*HI site of p-69-cat and into the enhancerless vector pA10-cat2 in several combinations and orientations. Plasmids p-69E1S and p-69E1A contain one copy of the repeated sequence in opposite orientations compared with the CAT gene reading frame (Fig. 1). Plasmids p-69E2S, -E2A, and -E1.2A contain tandem repeats of the sequence. pA10E1A and pA10E1.2A-cat contain one and two copies, respectively, of the sequence in the antisense orientation.

All the p-69-cat constructs with tandem repeats were PMA inducible (5- to 10-fold) and expressed elevated basal levels (38- to 73-fold) compared with those of the p-69-cat plasmid (Fig. 4C). With one repeat in the antisense orientation (p-69E1A), basal (5-fold) and inducible (2.5-fold) activities were present but reduced compared with those of tandem repeats. A single core element in the sense orientation was without activity. The activity of the enhancer elements in the heterologous promoter construct pA10-cat2 verified the requirement for tandem repeats in reconstituting basal and PMA-inducible function (Fig. 5). Both pA10-cat2 and the single-oligonucleotide insert (pA10E1A) were slightly induc-

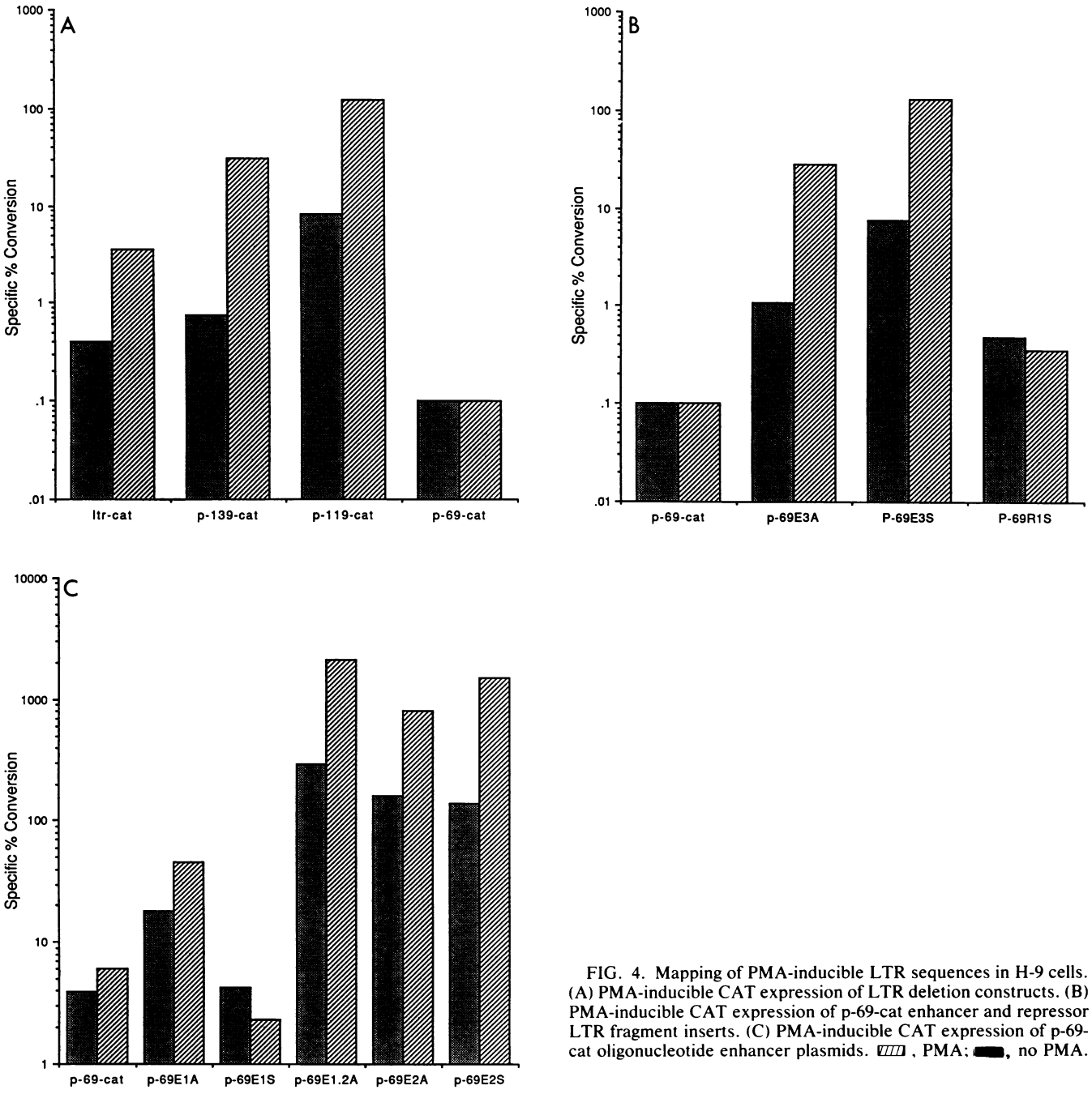

FIG. 4. Mapping of PMA-inducible LTR sequences in H-9 cells. (A) PMA-inducible CAT expression of LTR deletion constructs. (B) PMA-inducible CAT expression of p-69-cat enhancer and repressor LTR fragment inserts. (C) PMA-inducible CAT expression of p-69-cat oligonucleotide enhancer plasmids. ▨, PMA; ■, no PMA.

ible, in contrast to p-69E1A, which showed increased activity compared with p-69-cat (see Discussion).

These data demonstrate that the oligonucleotide −94 to −80 contains an inducible core enhancer element which can act both in the context of the LTR promoter–*trans*-acting responsive element sequences and on an heterologous enhancerless construct. Tandem repeats of the 10-bp core sequence are required for optimal activity.

DISCUSSION

The data presented in this paper demonstrate several elements important to the regulation of HIV expression and possibly viral latency. First, activation of protein kinase C by phorbol esters stimulates the enhancer-promoter function of the HIV LTR. This inducible activity is seen in a variety of T-cell lines and in some monocyte lines (data not shown). Protein kinase C activation is associated with cell activation in response to a variety of ligands (22). Antigen stimulation of T cells through the T-cell receptor-T3 complex has been shown to activate protein kinase C through the generation of diacylglycerol (16). Phorbol ester stimulation may therefore mimic the normal activation pathway and provide a good model for studying host cell regulation of HIV expression. In the context of viral latency, our data suggest that reexpression of virus is a direct effect of cellular regulatory mechanisms functioning on viral regulatory elements contained in the LTR.

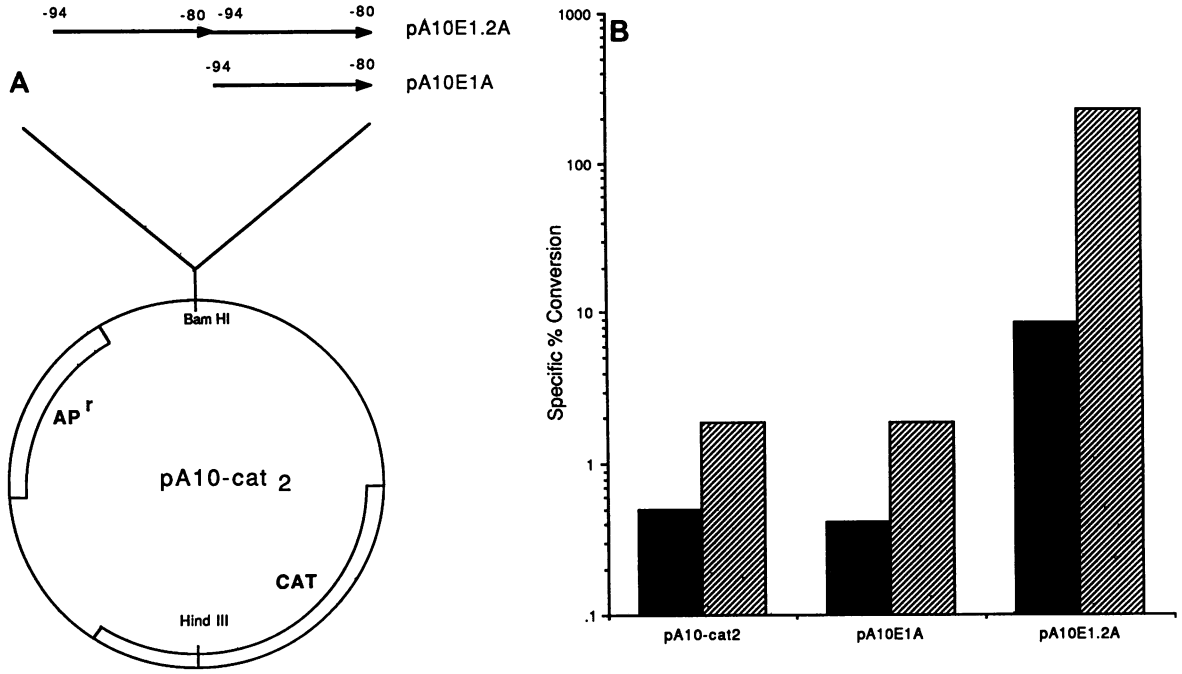

FIG. 5. Reconstitution of basal and PMA-induced levels of CAT expression in the plasmid pA10-cat2. (A) Construction of plasmids pA10E1A and pA10E1.2A. AP, Ampicillin. (B) Basal and PMA-induced CAT expression of pA10-cat2, pA10E1A, and pA10E1.2A. ▨, PMA; ■, no PMA.

Cellular activation via PMA induces transcriptional activation in the HIV LTR. This is shown by actinomycin D blocking, which totally eliminates induction by PMA but does not affect background CAT expression. Correlating with actinomycin D blocking are RNA dot blots showing PMA-induced increases in CAT-specific mRNA.

The genetic site of PMA stimulation is a 10-bp directly repeated element 5′ to the Sp1-binding sites. This sequence can transfer both basal and PMA responsiveness to the p-69-cat construct in an orientation- and position-independent manner, thus defining it as an enhancer element. The location of the HIV enhancer function has been studied by Rosen et al. (25) who mapped activity between −137 and +17 and showed TAT-dependent activity in LTR constructs deleted to position −104. Recently, Meusing et al. (20) showed activity between −121 and −20 and noted the duplicated sequence between −105 and −82.

More recently, Nable and Baltimore (21) observed a similar inducible activity of the HIV LTR in response to mitogen plus phorbol ester. Mutation of the repeated elements between −105 and −86 in the context of the LTR depleted inducible activity and eliminated DNA-protein interactions of the LTR. Our study with a deletion and reconstitution analysis confirmed these results and further demonstrated that the enhancer functions in a position- and orientation-independent manner, requires a tandem repeat to be functional, acts at the transcriptional level, is responsive to phorbol ester directly, and can function in a heterologous enhancerless vector.

The repeated HIV enhancer sequence GGGACTTTCC is similar to sequences in the SV40 72-bp enhancer (32), the human cytomegalovirus enhancer (4), and the B element of the κ immunoglobulin enhancer (27). The core enhancer sequence TGGAAAG as reported by Weiher et al. (32) appears necessary for SV40 enhancer function and is conserved in the HIV most 3′ enhancer repeat. This homology would also explain the lack of tissue specificity for viral growth once HIV is internalized, as demonstrated by DNA transfection experiments (1). Because of the potent effect of these sequences on PMA inducibility of the HIV LTR and the sequence homology with SV40, the data suggest a similar role for this sequence in phorbol stimulation of SV40 enhancers (8).

Maximal basal and phorbol-stimulated expression levels require that the enhancer core sequence be tandemly repeated. Full reconstitution of the p-69-cat and pA10-cat2 constructs required the insertion of either tandem repeats of the single oligonucleotide or single inserts of the tandem core sequence oligonucleotide. However, a single antisense insert was found to restore inducible function to p-69-cat and not pA10-cat2. This phenomenon is probably related to the presence of an highly homologous sequence (GGAGCTTCC) derived from SV40 DNA (3) adjacent to the 5′ side of the *Bam*HI site in p-69-cat and not pA10-cat2. The activity of the antisense orientation single insert in p-69-cat suggests that tandem repeats must be oriented to each other in head-to-tail fashion.

The construct p-69-cat deletes one Sp1-binding site along with the enhancer regions. The ability to reconstitute full activity to p-69-cat with the enhancer sequence suggests that two Sp1 sites are sufficient for LTR function. This is consistent with other studies showing that the two most 3′ Sp1 sites are most critical to in vitro transcriptional activity (15).

Phorbol ester stimulation of LTR-mediated gene expression can occur in the absence of the viral *trans*-activator gene, although with TAT, the phorbol ester effect is greatly amplified (data not shown). In experiments not shown, the enhancer element was not absolutely required for expression if sufficient TAT expression was present. This was found by transfection of p-69-cat into virus-infected cells in which the −69 deletion had strong promoter activity. Again, this is

consistent with the TAT product having transcriptional regulating activity independent of other LTR-contained regulatory regions (20, 23). This would support a model of latent HIV expressing no transcripts or protein, which then becomes activated, first by direct LTR stimulation through cellular activation mediators, followed by the generation of autoamplifying viral *trans*-activators.

PMA stimulation of enhancer function has previously been described in other systems. The SV40, Moloney murine leukemia virus LTR, κ immunoglobulin, and prolactin gene enhancers all respond directly to phorbol treatment. All these enhancers presumably serve as sites for DNA-binding regulatory proteins. With the SV40 and κ enhancer, cycloheximide treatment does not block transcriptional induction (14) or the appearance of a DNA-binding protein (28). This suggests that induction is mediated by a constitutive regulatory protein presumably modified by phosphorylation events. Current studies are directed toward defining whether phorbol induction of the HIV LTR is dependent on a constitutive DNA-binding phosphoprotein.

Scanning sequences 5′ to promoters and within introns from the interleukin-2 receptor and gamma interferon gene revealed numerous sequences related to the HIV enhancer. For example, in the interleukin-2 receptor gene, a sequence GGAATCTCC appears at −265, a region which may be involved in interleukin-2 receptor gene regulation (6). Whether these endogenous sites serve as enhancer-binding sites for proteins which cross-react with the HIV enhancer and function as normal regulatory elements requires further study.

The critical role of this enhancer sequence to the stimulation of LTR function in vitro would imply a similar role in vivo, not only in T cells, but also in monocyte viral replication. The identification of this regulatory site, in addition to establishing new insight into the process of viral latency, may allow other molecular approaches to controlling and treating HIV infections.

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