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Regulation of Expression Driven by Human Immunodeficiency Virus Type 1 and Human T-Cell Leukemia Virus Type I Long Terminal Repeats in Pluripotential Human Embryonic Cells

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Human pluripotential embryonic teratocarcinoma cells differentially expressed gene activity controlled by the human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type I (HTLV-I) long terminal repeats (LTRs) when differentiation was induced by the morphogen all-*trans* retinoic acid. The alterations occurred after commitment and before the appearance of the multiple cell types characteristic of these pluripotential cells. After commitment, gene activity controlled by the HIV-1 LTR markedly increased, whereas that controlled by the HTLV-I LTR decreased. Steady-state mRNA levels and nuclear run-on transcription indicated that the increased HIV-1-directed activity during differentiation occurred posttranscriptionally, whereas the decreased HTLV-I activity was at the transcriptional level. Phorbol esters did not cause commitment but strongly enhanced expression by both viral LTRs at the transcriptional level. A specific inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, indicated that the enhanced activity involved the activation of protein kinase(s) C; altered cyclic nucleotide metabolism was apparently not involved. Differentiating cells gradually lost the ability to respond to phorbol ester stimulation. Experiments with a deletion mutant of the HIV-1 LTR suggested that this was due to imposition of negative regulation during differentiation that was not reversed by phorbol ester induction. Cycloheximide, with or without phorbol ester, slightly stimulated HIV-1-directed activity at the transcriptional level and massively increased the amounts of steady-state mRNA by posttranscriptional superinduction. It appeared, however, that new nuclear protein synthesis was required for maximal transcriptional stimulation by phorbol esters. Thus, changing cellular regulatory mechanisms influenced human retrovirus expression during human embryonic cell differentiation.

The human immunodeficiency virus type 1 (HIV-1) (6, 34) has been described as one of the most complex retroviruses (7), encoding *gag*, *pol*, and *env*, three known autoregulatory genes, and coding potential for others (12, 26, 42, 43). Many complex cell-virus and virus-virus interactions are possible through the agency of these genes once they are expressed in infected cells. A crucial event in the pathogenesis of acquired immunodeficiency syndrome is the initial activation of control sequences residing in the long terminal repeat (LTR). The long latency period of the disease, the paucity of demonstrably infected cells, and uncertainties as to types of cells susceptible to infection have made the activation process difficult to understand in latently infected individuals. The virus, in turn, is itself capable of modifying the growth, differentiation, or function of a host cell (39).

HIV-1 was originally described as a T-lymphotropic virus, but more recent work indicates that the virus is capable of replicating in a variety of cell types, including macrophages and cells of the central nervous system (8, 14, 23, 24). Acquired immunodeficiency syndrome infection also occurs in utero and is frequently accompanied by developmental abnormalities (31). Little is known, however, about the mechanisms of HIV-1 infection, latency, and activation during human embryogenesis or the effects of cellular differentiation and development on expression of the virus. Expression of endogenous and exogenous retroviruses has been extensively studied in mouse embryos and mouse embryonic teratocarcinoma cells, but fewer studies have been performed with the limited number of pluripotential human embryonic cell lines available (25).

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Under the influence of the naturally occurring morphogen all-*trans* retinoic acid (45), the human embryonal carcinoma clone NT2/D1 differentiates into a range of somatic cell types in vitro (1, 2). That these cells may be related to normal human embryonic cells was suggested by appropriate changes of the cell surface phenotype and the expression of human homeobox sequence Hu1 upon induction of differentiation (1, 20). We used different reporter gene constructs linked to the LTR of HIV-1 (43) to investigate the mechanisms of HIV-1 latency and activation during differentiation in these pluripotential cells. The effects of the viral autoregulatory genes in the analysis of the transient gene expression were avoided. As reference plasmids, we used similar constructs in which gene activities were controlled by the human adult T-cell leukemia virus type I (HTLV-I) (33, 44) and the simian virus 40 (SV40) promoter-enhancer sequences (15). The results reveal different mechanisms of control of gene expression used by the two retroviruses in differentiating cells and link such mechanisms to biochemical and nuclear events coordinating a basic cellular differentiation program.

MATERIALS AND METHODS

Cell cultures and induction of differentiation. NT2/D1 cells derived from the human embryonic teratocarcinoma cell line Tera-2 were a gift from P. Andrews and were received at about passage 64 in vitro. The cells were cultured in Dulbecco modified Eagle medium supplemented with 15% heat-inactivated fetal bovine serum. Cells were transferred at high densities by using the glass bead method previously described (1, 2) and were not used beyond about 100 passages in vitro.

Differentiation was induced by addition of all-*trans* retinoic acid (RA; Eastman Kodak Co.) to 2 μM. After initiation of induction, cells were transferred at 1:4 dilutions by trypsinization. RA induction was started at staggered intervals such that all of the separately induced cultures were plated and transfected at the same time. RA, phorbol 12-myristate-13-acetate (PMA), phorbol 12,13-dibutyrate, phorbol didecanoate, 1-oleoyl-2-acetyl-*rac*-glycerol, calcium ionophore A23187, and 1,25-dihydroxyvitamin D_3 were stored at −20°C as concentrated stock solutions in dimethyl sulfoxide and protected from light.

Cell transfection and enzyme assays. Cells were transfected with plasmid DNAs by a modification of the calcium phosphate coprecipitation technique (15, 16). Cells were plated at 10^6 per dish in 100-mm (diameter) petri dishes 20 h before transfection, and the medium was replaced 3 h before transfection. Ten micrograms of DNA per dish was used in the standard experiments. DNA uptake proceeded for 6 h and was followed by 2 min of glycerol shock treatment at 37°C. Extracts were prepared 24 or 48 h later as previously described (15), and protein concentrations were determined by the Lowry method. Standard chloramphenicol acetyltransferase (CAT) assay mixtures contained 0.45 M Tris hydrochloride (pH 7.8), 1.9 nmol of [^{14}C]chloramphenicol (Amersham Corp.), 0.6 mM acetyl coenzyme A, and 30 to 80 μg of extract protein in a total volume of 150 μl. Reaction mixtures were resolved by silica gel chromatography, followed by liquid scintillation counting of the gel to quantitate conversion of the chloramphenicol to its acetylated derivatives. Relative specific activities were calculated from the slopes of the curves in the linear response range of time and protein concentration and expressed as nanomoles of chloramphenicol converted per minute per milligram of protein. β-Galactosidase activities in extracts of cells transfected with plasmids containing the *Escherichia coli lacZ* gene were determined by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate, and the relative specific enzyme activity was calculated as nanomoles of *o*-nitrophenol released per minute per milligram of protein (27).

RNA purification and Northern (RNA) blot analysis. Total cytoplasmic RNA was isolated by the guanidine thiocyanate method (9) and overnight precipitation in 2 M LiCl as previously described (40). Cytoplasmic poly(A)$^+$ and poly(A)$^-$ fractions were separated by oligo(dT) column chromatography. Agarose gels (0.8 to 1.0%) were cast in borate buffer (50 mM H_3BO_3, 5 mM $Na_2B_4O_7$, 10 mM Na_2SO_4, 1 mM EDTA) containing 3% (wt/vol) formaldehyde. Before loading, the RNA was denatured at 65°C for 5 min in 50% (vol/vol) formamide–5.6% (wt/vol) formaldehyde in borate buffer. The current was maintained at about 100 mA during electrophoresis, and the borate buffer was recirculated. RNA was transferred to nitrocellulose filters and hybridized with nick-translated probes which consisted of the coding regions of the bacterial CAT or *lacZ* gene or those of the rabbit β-globin gene.

Nuclear run-on transcription. Isolated nuclei from the transfected cells were suspended in 1-ml reaction mixtures containing 10^7 nuclei (determined by direct hemacytometer counting) and 33 μCi of [α-^{32}P]UTP (400 Ci/mmol), in addition to the three unlabeled nucleoside triphosphates. Run-on transcription buffer, incubation conditions, and purification of nuclear RNA by the hot phenol method were as previously described (40). The radioactive RNA was hybridized to isolated insert DNAs by using a Schleicher & Schuell blot apparatus with nitrocellulose. The autoradiograms were exposed at −70°C by using preflashed Kodak XAR-5 film with an intensifying screen and scanned in the linear range of the film response with a Joyce-Loebl microdensitometer.

Plasmids and plasmid constructions. pU3R-III contains the entire U3 region and about 75 nucleotides of the R region of the HIV-1 (HTLV-III) 3′ LTR linked 5′ to the bacterial CAT gene. The construction and properties of this plasmid are described elsewhere (43). For convenience in identification, this plasmid is referred to here as pHIVCat. pU3R-I (pHTLVICat) is similar to pHIVCat but contains the U3 and R regions and 105 base pairs of the U5 region of the 3′ LTR of HTLV-I (44). Plasmid pPL-12 expresses the *trans*-acting factor encoded by the HIV-1 *tat* gene (4). pSV2Cat and pCH110 contain the bacterial CAT and *lacZ* genes, respectively, linked to the SV40 promoter-enhancer early gene transcription control region (15, 18). pSVβ-globin is a similar construct containing the rabbit β-globin sequences and was a generous gift from L. Laimins. Plasmids whose inserts were used as controls in several experiments included pM1B1, containing an Hu1 human homeobox sequence (20); pLK215, containing human γ-actin cDNA sequences (17); and pRI-7.4, containing human rDNA 28S, 18S, and transcribed spacer sequences (3).

pHIVlacZ was constructed by removing the 3.2-kilobase *lacZ* gene from pCH110 by *Hin*dIII-*Bam*HI cleavage and inserting it in place of the 1.6-kilobase CAT gene of pHIVCat. Similar strategies were used to insert the rabbit β-globin gene from pSVβ-globin in place of the CAT gene of pHIVCat and pHTLVICat to make pHIVβ-globin and pHTLVIβ-globin. All constructs were transfected into *E. coli* HB101, and plasmid DNAs were isolated by ethidium bromide-CsCl density centrifugation.

RESULTS

Altered gene expression driven by HIV-1 and HTLV-I LTRs in RA-induced NT2/D1 cells. NT2/D1 cells were induced by addition of 2 μM RA, and the growth and morphological differentiation of the cells were monitored for up to 3 months. The differentiation process was roughly divided into three stages. In the first 3 days, the cells exhibited the characteristically polygonal morphology and granular cytoplasm of undifferentiated embryonic teratocarcinoma cells. In accord with previous observations (1, 2), there was no commitment to differentiation during this time. After 3 days of growth in RA, the cells became irreversibly committed, and during the following 2 weeks (stage 2) they gradually appeared more flattened and morphologically more homogeneous. They also increased their generation time from about 24 h to about 72 h. Stage 3 extended beyond about 2.5 weeks. During this time, growth virtually ceased and the cells acquired the distinctive and various phenotypes previously described (1, 2).

NT2/D1 cells were induced by addition of RA to 2 μM at various intervals before transfection. The cultures were subdivided and transfected with 10 μg of pHIVCat, pHTLVICat, or pSV2Cat for transient gene expression assays. Figure 1 shows the results of CAT assays carried out with cell extracts prepared after transfection with pHIVCat, in which the gene is controlled by the HIV-1 3′ LTR (43), and pHTLVICat, in which the gene is controlled by the HTLV-I 3′ LTR (44). There was little change in the CAT activity levels obtained with either plasmid in cells induced for 2 days with RA. After 3 days, CAT activity controlled by the HTLV-I LTR dropped to about 30% of the control values, and thereafter it fell to about 10 to 20% of the values obtained with undifferentiated cells. In cells transfected with

pHTLVIβ-globin, similar decreases with time of exposure to RA were observed in steady-state rabbit β-globin mRNA levels (see below).

In contrast, there was little change in CAT activity levels obtained with pHIVCat until 4 or 5 days after RA induction, whereupon activity increased 8- to 14-fold above control levels (Fig. 1). The activity in stage 3 cultures then declined to control levels as the cells underwent terminal differentiation. Similar increases in expression with time of exposure to RA were obtained with pHIVlacZ and pHIVβ-globin (see below).

Effect of the *tat* gene on CAT activity driven by the HIV-1 LTR. The specificity of induction of pHIVCat activity suggested that differentiation of NT2/D1 cells was accompanied by expression of an unknown cellular factor with *trans*-activating activity similar to that encoded by the *tat* gene of HIV-1 (35). Figure 2 shows a time course of differentiation of NT2/D1 cells in which the cells at each time point were divided into four aliquots. One set was transfected with pHIVCat, another was transfected with pHIVCat plus pPL-12 (a plasmid encoding the HIV-1 *tat* gene) (4), a third set was transfected with pSV2Cat, and a fourth set was transfected with pCH110 (a control expressing the bacterial *lacZ* gene under control of the SV40 promotor-enhancer). Cotransfection of the uninduced cultures with pPL-12 increased CAT activity 56-fold over the basal level obtained with pHIVCat alone (Fig. 2). There was an approximate 10-fold increase in activity after induction with RA and about a 147-fold increase in the induced cells cotransfected with pHIVCat and pPL-12. The effects of *tat* expression and RA induction were thus slightly more than additive but not strongly synergistic. Figure 2 also shows that gene activity

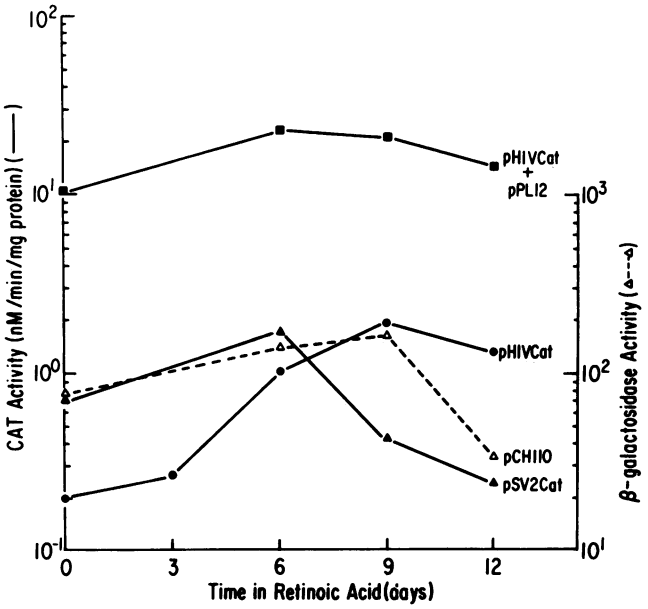

FIG. 2. Effect of *tat* gene expression on HIV-1-directed CAT activity in differentiating NT2/D1 cells. NT2/D1 cells were induced with RA as described in the legend to Fig. 1 and transfected with 7.5 μg of pHIVCat, 7.5 μg of pHIVCat plus 7.5 μg of pPL-12, 7.5 μg of pSV2Cat, or 7.5 μg of pCH110. DNA concentrations were kept constant at 15 μg per dish by addition of carrier pBR322 DNA. CAT activities in nanomoles of chloramphenicol acetylated per minute per milligram of protein are indicated for pHIVCat and pSV2Cat on the left ordinate, and β-galactosidase activity levels in nanomoles of *o*-nitrophenol released per minute per milligram of protein are indicated for pCH110 on the right.

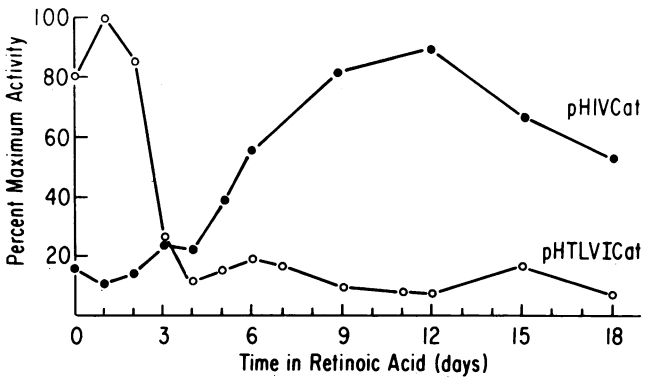

FIG. 1. Expression of HIV-1- and HTLV-I-directed CAT activity in differentiating NT2/D1 cells. Cells were induced by the addition of 2 μM RA to separate cultures at staggered intervals up to 18 days in advance of plating. Extracts were prepared 48 h after transfection, and CAT assays were performed as described in Materials and Methods. The data are expressed as averages from 12 different induction experiments with pHIVCat and 3 experiments with pHTLVICat. The average enzyme activity levels, in nanomoles per minute per milligram of protein, of chloramphenicol converted to acetylated derivatives in these experiments were 0.218 with pHIVCat and 1.07 with pHTLVICat. CAT activities in NT2/D1 cells were also compared with all three viral promoters in human U-87 MG (glioblastoma), U-373 (astrocytoma), MSN (adrenergic neuroblastoma), MC9C (cholinergic neuroblastoma), HEpG2 (hepatoma), and HeLa (cervical carcinoma) cells (see also Fig. 3B and C) and monkey CV-1 and COS-7 (kidney) cells. With the exception of U-87 MG glioblastoma cells, none of these cells showed more than one-third to one-seventh the activity obtained with NT2/D1 cells in replicate determinations.

controlled by SV40 promotor-enhancer sequences was induced by RA differentiation, although to a lesser degree than with pHIVCat. The specificity of RA induction for NT2/D1 cells was indicated by experiments in which eight different human cell lines, including HeLa and HEpG2 cells, were subjected to 15-day time course experiments with RA (2 μM). These cells, which did not terminally differentiate, showed only random and slight variations (≤twofold) with all three viral promoters in the course of RA treatment.

Induction of viral promoter activities by phorbol esters in NT2/D1 cells. Phorbol esters have been widely studied for their ability to promote differentiation in many cell types (46) and their ability to stimulate or inhibit DNA and RNA virus expression (47). It was therefore of interest to determine whether these compounds induced differentiation or affected human retrovirus expression in NT2/D1 cells. The cells were cultured in the presence of PMA at 32 and 96 nM concentrations for 3 and 7 days before removal of the PMA and subsequent subculture. Unlike RA, PMA did not induce differentiation. At 32 nM PMA, cells remained nearly unaffected for over 4 weeks. At 96 nM PMA, there was inhibition of growth between 3 and 7 days of treatment, as well as transient alterations in cell morphology. However, the cells resumed their normal growth and morphological characteristics upon removal of the PMA. Although the cells did not commit, differentiation could be induced in PMA-treated cells by subsequent addition of RA (data not shown).

Figure 3A shows a time course experiment in which NT2/D1 cells were treated with 32 nM PMA and transfected with pHIVCat, pHTLVICat, and pSV2Cat. CAT activity driven by all three promotors increased within 24 h, peaked at 48 to 72 h, and then declined to basal levels. In other

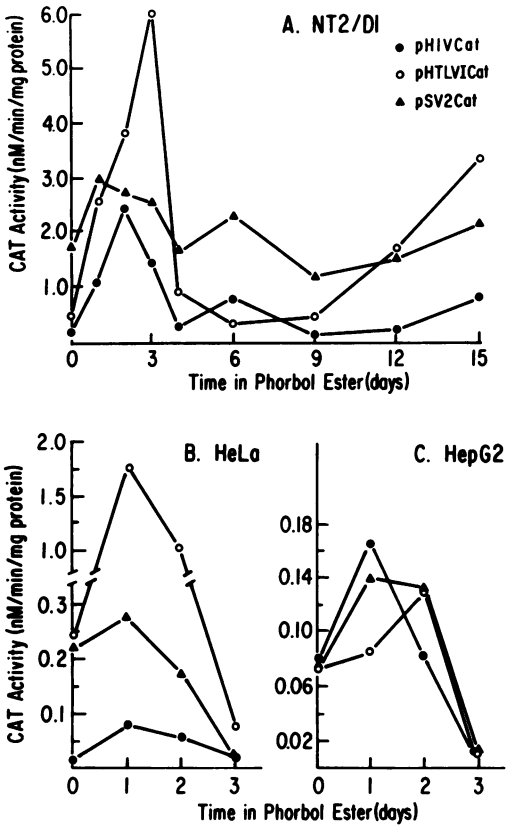

FIG. 3. Induction of viral promoter activity in NT2/D1 cells treated with PMA. (A) PMA at 32 nM (final concentration) was added to separate cultures of NT2/D1 cells at staggered intervals, beginning 15 days before harvesting of the cells. At $t = -1$ day, all cultures were trypsinized, counted, and plated. At $t = 0$ day, each culture was transfected with 10 μg of pHIVCat, pHTLVICat, or pSV2Cat, as indicated. Extracts were prepared for CAT assay at 48 h after transfection. (B and C) Results of similar experiments with HeLa and HEpG2 cells, respectively.

experiments with 96 nM PMA, there were about 30-fold increases in activity over the basal levels with pHIVCat and pHTLVICat and three- to fivefold increases with pSV2Cat. Figures 3B and C show similar PMA induction experiments with HeLa and HEpG2 cells. With the exception of a 7.4-fold induction of pHTLVICat activity in HeLa cells, PMA did not markedly enhance expression of CAT activity in these cells. With the input amounts of DNA used (10 μg per dish), the 2-fold enhancement of pSV2Cat activity by PMA in HEpG2 cells was lower than that previously reported (3.7-fold) in similar experiments with these cells (22).

PMA induction and *tat* gene expression. Enhancement of pHIVCat CAT activity in cells simultaneously expressing *tat* activity and stimulated with PMA is shown in Table 1. The combined effects of PMA stimulation and *tat* gene expression appeared to be synergistic, because the CAT activity of pHIVCat under these conditions was greater than the sum of the activities obtained with PMA or *tat*. Controls in which cells were cotransfected with pHTLVICat or pSV2Cat together with pPL-12 did not show this synergistic effect. The deletion mutant p-167 lacks a negative regulatory element (NRE) upstream of the promoter sequences in the HIV-1 LTR. In agreement with previous studies (37), this construct exhibited about four times the basal levels of activity obtained with pHIVCat. However, PMA induction of cells

TABLE 1. Effects of *tat* gene expression and PMA induction

Transfected plasmid(s)[a]	Fold induction	
	Control	PMA added
pHIVCat + pBR322	1.0	12.9
pHIVCat + pPL-12	31.0	149.0
p-167	1.0	5.3
pHTLVICat + pBR322	1.0	11.6
pHTLVICat + pPL-12[b]	0.20	4.7
pSV2Cat + pBR322	1.0	2.3
pSV2Cat + pPL-12[b]	0.47	0.79

[a] NT2/D1 cells were transfected with 7.5 μg of pHIVCat, pHTLVICat, or pSV2Cat per dish and 7.5 μg of pPL-12 encoding *tat* activity. Total DNA was kept constant at 15 μg per dish by addition of pBR322 as carrier DNA. Induction ratios are set at 1.0 for control dishes for each CAT plasmid and do not reflect absolute enzyme activities.

[b] Note the reduced expression of pSV2Cat and pHTLVICat in the presence of pPL-12, which contains SV40 promoter-enhancer sequences (4). Competition expression experiments indicated that promoter-enhancer sequences from all three viruses (HIV-1, HTLV-I, and SV40) competed with one another and that the HIV-1–SV40 pair cross-competed most effectively (data not shown).

transfected with p-167 consistently yielded only 5-fold enhancement of activity, in contrast to the 10- to 28-fold inductions observed with pHIVCat (Table 1; Fig. 4).

Loss of PMA-inducible activity as NT2/D1 cells differentiate. PMA stimulated some of the effects observed with pHIVCat and pSV2Cat during RA-induced differentiation. The question arose of whether prior differentiation influenced activation of the various viral promoters by PMA. NT2/D1 cells were induced for up to 26 days in RA. The cultures were then divided into four parts and transfected with pHIVCat, p-167, pHTLVICat, and pSV2Cat. Basal enzyme activity levels and induced activity levels in the presence of 96 nM PMA were then determined for each

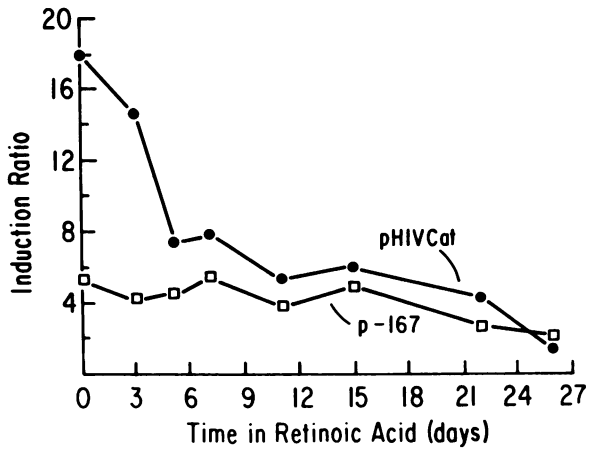

FIG. 4. Responses of differentiating NT2/D1 cells to induction by PMA. Differentiation of NT2/D1 cells was initiated by addition of 2 μM RA, and the cells were subcultured in RA for the periods indicated. Cells from each culture were transfected with 10 μg of pHIVCat or p-167 per dish. One set of plates from each culture was treated with 96 nM PMA immediately after transfection, and another set was left untreated (controls). Enzyme extracts were prepared 48 h later. Induction ratios were calculated as the specific CAT activities of cells treated with PMA divided by the specific activities of control cells.

culture at 48 h after transfection. In Fig. 4, the data for pHIVCat and p-167 are plotted as induction ratios, i.e., the ratios of CAT activity in extracts of cells treated with PMA to that in cells not treated with PMA. The ratios are plotted as a function of time of differentiation in RA. There was a progressive decrease in the ability of PMA to stimulate activity over the levels induced by RA itself (Fig. 4). As the cultures approached terminal differentiation (stage 3), there was little or no stimulation by PMA. It should be noted that the RA-induced cells at this late stage also expressed low levels of activity comparable to those of stage 1 or undifferentiated cells. PMA induction ratios of cells transfected with p-167 remained constant at about 5.0 throughout stage 2 (Fig. 4). This result suggested that as the cells differentiated, the activity of the HIV-1 LTR progressively came under the influence of a cellular negative regulatory factor interacting with the NRE of pHIVCat (37). This interaction could not be reversed by PMA induction and could not take place with p-167, from which the NRE was deleted. pHTLVICat and pSV2Cat showed similar declines in induction ratios with time of differentiation in RA (data not shown).

Role of PKC in induction of transcriptional activity in NT2/D1 cells. Phorbol esters are believed to mediate their effects by direct activation of protein kinase(s) C (PKC) (30). Table 2 summarizes the results of several experiments in which NT2/D1 cells were transfected with pHIVCat and treated with other phorbol derivatives and compounds that reportedly activate PKC. Phorbol 12,13-dibutyrate was less effective than PMA as an inducer of CAT activity, and the inactive analog phorbol 12,13-didecanoate had little or no effect. Except for some inhibition of activity, other compounds that reportedly affect PKC activity had no stimulatory effect on HIV-1-directed CAT activity in NT2/D1 cells.

TABLE 2. Reagents affecting PKC activity and expression of pHIVCat

Addition and concn[a]	Fold induction at:	
	24 h	48 h
None	1.0 (0.171)	1.0 (0.217)
PMA	5.9	14.4
Phorbol 12,13-dibutyrate	4.5	3.8
Phorbol didecanoate	2.0	1.1
1-Oleoyl-2-acetyl-*rac*-glycerol (0.14 mM)	NT[b]	0.80
Ca^{2+} ionophore A23187		
0.3 μM	0.84	0.94
1.0 μM[c]	1.0	1.2
1,25-Dihydroxyvitamin D_3		
2.4 μM	NT	0.83
7.2 μM	NT	0.79
24.0 μM[c]	NT	0.37

[a] The following compounds affecting cyclic nucleotide metabolism were tested with pHIVCat transfected into NT2/D1 cells (the highest concentrations tested are indicated in parentheses): N^6-2′-*O*-dibutyryl cyclic AMP (0.25 mM), 8-bromocyclic AMP (0.3 mM), N^2-monobutyrylcyclic GMP (0.4 mM), N^2-2′-*O*-dibutyrylcyclic GMP (0.4 mM), cholera toxin (25 ng/ml), and forskolin (10^{-6} M). In addition, the above compounds were tested in combination with the phosphodiesterase inhibitor IMX at 0.1 and 0.3 mM.

[b] NT, Not tested.

[c] Slightly higher concentrations of Ca^{2+} ionophore (3 μM) and 1,25-dihydroxyvitamin D_3 (36 μM) showed cytotoxic effects in 24 h.

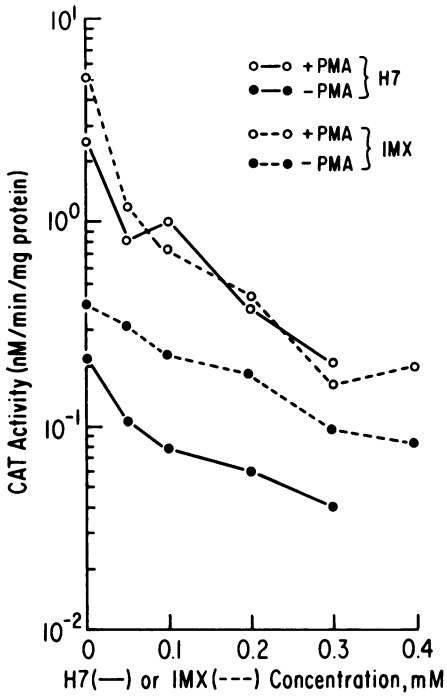

FIG. 5. Inhibition by H7 and IMX of pHIVCat activity in PMA-induced and uninduced cells. NT2/D1 cells were transfected with 10 μg of pHIVCat. After glycerol shock, H7 or IMX was added to the concentration indicated. PMA (96 nM) was added 30 min later, and extracts were prepared for CAT assay 48 h later. CAT activity (nanomoles of chloramphenicol converted per minute per milligram of extract protein) was plotted on semilog paper as a function of inhibitor concentration. Averaged data from two separate experiments with each drug are shown.

These compounds included the calcium ionophore A23187 and 1,25-dihydroxyvitamin D_3, which alter intracellular levels of Ca^{2+} (a cofactor of PKC activity). 1-Oleoyl-2-acetyl-*rac*-glycerol, like PMA itself, is an analog of diacylglycerol and can act to stimulate PKC activity independently of a net increase in intracellular Ca^{2+} concentration (29). This compound was also ineffective in altering levels of CAT activity. Thus, the only significant activators of HIV-1 expression in these experiments were PMA and phorbol 12,13-dibutyrate.

In in vitro studies, the compound 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) has been described as a highly specific and potent inhibitor (K_i = 6 × 10^{-6} M) of PKC (21). With increasing concentrations of H7 in the growth medium, the basal levels of expression of pHIVCat activity were gradually reduced, and at 0.3 mM H7 there was about 80% inhibition of activity (Fig. 5). H7, which did not appear to be toxic to the cells at these concentrations, also inhibited PMA activation in a dose-dependent manner (Fig. 5). Similar results were obtained with pHTLVICat and pSV2Cat. It appeared that the basal levels of LTR-driven activity in untreated cells, as well as the increased levels after PMA stimulation, were maintained by the activity of PKC.

Cellular and molecular responses to activation of PKC may be mediated in some instances by altered levels of cyclic nucleotides (38, 48). Conceivably, the responses of the HIV-1 promoter to PMA treatment were directly related to altered adenyl cyclase activity or increased intracellular levels of cyclic nucleotides induced by PMA treatment.

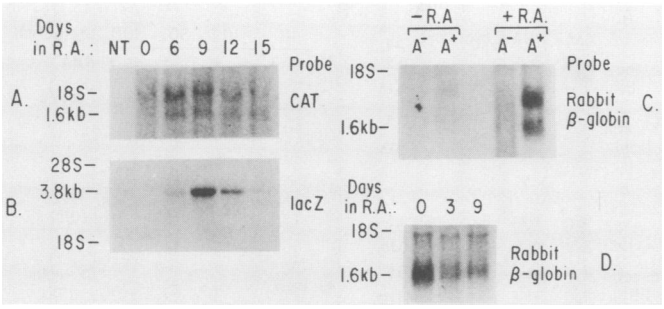

FIG. 6. HIV-1- and HTLV-I-directed mRNA in NT2/D1 cells induced with RA. NT2/D1 cells were induced with RA for various times, as shown, and then transfected with pHIVCat (panel A), pHIVlacZ (panel B), pHIVβ-globin (panel C), or pHTLVIβ-globin (panel D). Cytoplasmic RNA was prepared 48 h later, and 20 μg from each sample was electrophoresed in agarose denaturing gels for Northern blot analysis. Poly(A)$^+$ and poly(A)$^-$ RNAs were isolated from 100 μg of total cytoplasmic RNA by oligo(dT) chromatography (panel C). Nick-translated probes were prepared from the isolated inserts of plasmids containing the coding regions from the bacterial CAT (panel A) or *lacZ* (panel B) gene or the rabbit β-globin gene (panels C and D). The lanes marked NT contained 20 μg of cytoplasmic RNA from cells that were not transfected. kb, Kilobases.

Several compounds that affect cellular levels or activities of cyclic nucleotides were tested with pHIVCat at subtoxic concentrations. These compounds had little or no ability to inhibit or stimulate HIV-1-directed CAT activity in NT2/D1 cells (Table 2). However, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IMX) at 0.05 to 0.4 mM reduced activity by about 50 to 80% when tested alone or in combination with the cyclic nucleotides, cholera toxin, or forskolin. IMX also inhibited the PMA-stimulated activity of pHIVCat by over 90%. On an equimolar basis, IMX was at least as efficient an inhibitor of this activity as was H7 (Fig. 5). IMX has the effect of increasing intracellular levels of cyclic nucleotides by inhibiting their hydrolysis by phosphodiesterases. This effect, which is augmented by cholera toxin or forskolin (11), apparently was not responsible for decreased expression of CAT activity in these experiments. However, the results did suggest that cleavage of a phosphodiester bond(s) may be an important step in activation of the viral LTR.

RA-induced differentiation and steady-state levels of mRNA. Time course experiments were carried out in which NT2/D1 cells were induced with RA and transfected with plasmids containing genes driven by the HIV-1 or HTLV-1 LTR. At 48 h later, total cytoplasmic or poly(A)$^+$ RNA was prepared from each culture and analyzed by Northern blotting. Transcripts from pHIVCat, pHTLVICat, pHIVβ-globin, pHTLVIβ-globin, and pHIVlacZ were probed with the appropriate radioactively labeled insert DNAs.

Increases in CAT or *lacZ* gene activity in differentiating cells in which these genes were controlled by the HIV-1 LTR were paralleled by about a 7-fold increase in steady-state levels of CAT gene mRNA and an 11-fold increase in *lacZ* mRNA (Fig. 6A and B). Such increases over the basal levels of mRNA from the undifferentiated cells were also reflected in the steady-state levels of rabbit poly(A)$^+$ β-globin mRNA (Fig. 6C). The period (stage 2 cells) in which increased levels of HIV-1-directed mRNA were present roughly coincided with the period of turning on and maximal expression of the human homeobox Hu1 gene in NT2/D1 cells (20). Control Northern blots with a human γ-actin gene probe showed that actin transcripts remained constant throughout this period. In contrast, steady-state levels of rabbit β-globin transcripts controlled by the HTLV-I promoter decreased in differentiated cells to about 25% of the control values (Fig. 6D). This result was consistent with the decreased levels of CAT gene activity in differentiated cells (Fig. 1) and with the run-on transcription experiments (see below).

Nuclear run-on transcription. To determine whether the altered levels of gene activity and cytoplasmic levels of the corresponding mRNAs were due to transcriptional or post-transcriptional events, a series of nuclear run-on experiments were performed. In these experiments, NT2/D1 cells were transfected with plasmids in which gene activities were controlled by the HIV-1 or HTLV-I LTR. The nuclei used in these experiments were isolated from cells induced with RA and cells induced with PMA. The run-on transcription rate of the CAT gene in cells induced for 9 days with RA and transfected with pHIVCat was about equal to that of control uninduced cells (Fig. 7). Thus, the increased pHIVCat gene expression, which was about eightfold in this experiment, appeared to be due to posttranscriptional regulation of CAT mRNA and not to an increased transcription rate in RA-induced cells. In contrast with this result, the transcription of the CAT gene directed by the HTLV-I LTR was markedly reduced in RA-induced cells when compared with that of control uninduced cells (Fig. 7B). Transcription of the γ-actin and rDNA sequences which served as controls was relatively little affected by RA induction.

On the other hand, PMA treatment of cells transfected with constructs driven by the HIV-1 or HTLV-I LTR

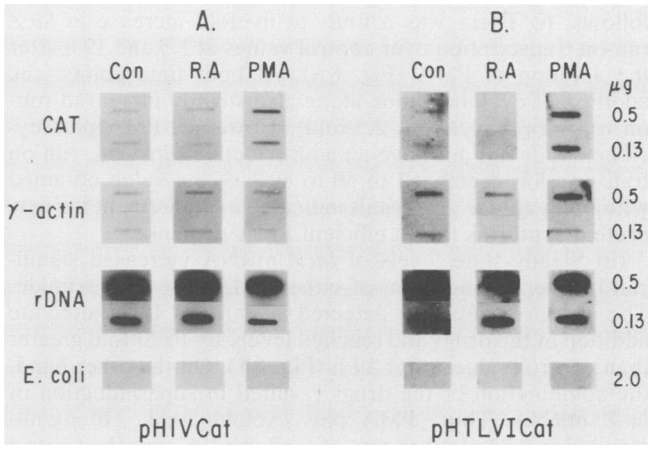

FIG. 7. Nuclear run-on transcription of pHIVCat and pHTLVI-Cat in cells induced with RA and PMA. RA-induced (9 days) and uninduced (control [Con]) NT2/D1 cells were transfected with pHIVCat (panel A) or pHTLVICat (panel B). Uninduced cultures were similarly transfected and divided into two parts; one part served as a control, and the other received 96 nM PMA. At 19 h later, nuclei were isolated from the cells and incubated in run-on transcription buffer in the presence of [^{32}P]UTP. The radioactive RNA from each sample was then hybridized to insert DNAs bound to nitrocellulose filters by slot blotting. The insert DNAs were excised from plasmids containing the bacterial CAT gene, the human γ-actin gene, or human rDNA (18S and 28S sequences), and the DNA concentrations were determined by a spectrofluorometric assay with diaminobenzoic acid. Slots containing either 0.5 or 0.125 μg of insert DNA are shown. The total DNA applied to each slot was kept constant at 2 μg by addition of *E. coli* DNA. The filters were exposed to preflashed Kodak AR-5 film at −70°C, and the autoradiograms were scanned with a Joyce-Loebl microdensitometer in the linear range of the film response.

resulted in four- to eightfold increases in the rate of CAT or *lacZ* gene transcription over the control cell values (Fig. 7A and B). There were also consistent increases in γ-actin gene transcription and essentially no change in rDNA transcription under these conditions. It appeared from these experiments that increased transcription was a major component of the enhanced gene expression induced by PMA treatment of NT2/D1 cells and that both the HIV-1 and HTLV-I promoters responded to such activation.

Effect of cycloheximide on PMA induction. The question arose of whether PMA-induced activation of the HIV control sequences requires the synthesis of new, *trans*-acting nuclear proteins or whether PMA initiates reactions leading to modification and activation of preexisting nuclear proteins. Nuclear run-on experiments, together with measurements of steady-state mRNA levels, were carried out with NT2/D1 cells transfected with pHIVlacZ and treated with PMA or cycloheximide, or both. The experiments included the following procedures. (i) Cycloheximide was added to the cell culture medium at 10, 30, and 100 μg/ml in the presence or absence of PMA. (ii) In addition to measurements of steady-state mRNA, *lacZ* enzyme activity was measured. (iii) Net protein synthesis was monitored by the uptake of [^{14}C]leucine in 1-h pulses at the beginning and end and during the course of the experiments. (iv) The above-described measurements were made with cells during the 1.5-h period immediately after the addition of cycloheximide, PMA, or both, when little or no change in gene expression had yet occurred. Measurements were also made 19 h later, when there were high levels of PMA-induced enzyme activity.

The results shown in Fig. 8 and Table 3 are summarized as follows. (i) There was a four- to fivefold increase in *lacZ* run-on transcription over control values at 1.5 and 19 h after the addition of PMA (Fig. 8A). At both time points, the addition of cycloheximide alone also slightly increased run-on transcription (about 2.5-fold). However, PMA plus cycloheximide did not have an additive effect; instead, run-on transcription decreased to 50 to 60% of the value obtained with PMA alone. This result indicated a requirement for new protein synthesis for an efficient PMA response.

(ii) Steady-state levels of *lacZ* mRNA increased significantly after the addition of either PMA or cycloheximide. The increases could be detected as early as 1.5 h after the addition of the drugs and reached levels 10- to 20-fold greater than control values after 19 h (Fig. 8B). On the other hand, the combination of the drugs resulted in superinduction of *lacZ* mRNA. Thus, PMA plus cycloheximide (10 μg/ml) resulted in a 345-fold excess of *lacZ* mRNA over the control value (Fig. 8B).

(iii) Under conditions in which cycloheximide inhibited net protein synthesis 90 to 95%, *lacZ* enzyme activity was not proportionately inhibited (Table 3). For example, in the presence of PMA plus 10 μg of cycloheximide per ml, *lacZ* enzyme activity increased slightly (1.5-fold) over the control value (Table 3). It therefore seems likely that the increase in enzyme activity in the face of over 90% inhibition of net protein synthesis was due to superinduction of *lacZ* mRNA as described above.

It should be noted that PMA also stimulated γ-actin mRNA run-on transcription and steady-state mRNA levels, whereas cycloheximide markedly inhibited both (Fig. 8A and B). rRNA transcription was essentially unchanged by the above treatments (data not shown).

It appears from these experiments that cycloheximide alone is capable of both slightly enhancing run-on transcription and greatly increasing steady-state mRNA levels. Cycloheximide plus PMA did not exert a synergistic effect on run-on transcription; instead, there was consistently less transcription in the presence of both compounds than in the presence of PMA alone. However, the two drugs exerted a synergistic effect in greatly augmenting steady-state mRNA levels (superinduction). It is probable that continued expression of *lacZ* enzyme activity under these conditions was due to the presence of massive amounts of mRNA compensating for the inhibition, but not total elimination, of protein synthesis by cycloheximide (Table 3). Superinduction made unclear the extent to which new protein synthesis is required for stimulated expression by PMA. However, the decreased run-on transcription consistently observed in the presence of cycloheximide plus PMA compared with that observed with PMA alone indicated that nuclear protein synthesis is required for maximum stimulation of pHIVlacZ expression by PMA. This interpretation did not rule out the possibility that PMA-induced modification of preexisting nuclear *trans*-acting factors is an additional requirement of enhanced transcription.

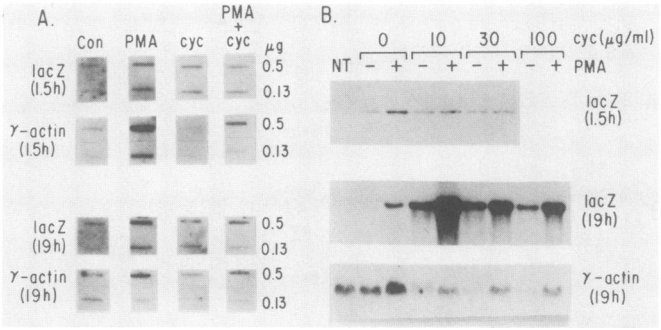

FIG. 8. Nuclear run-on transcription and steady-state levels of *lacZ* mRNA in the presence or absence (control [Con]) of PMA and cycloheximide (cyc). NT2/D1 cells were transfected with pHIVlacZ. Immediately after glycerol shock, cycloheximide was added to a portion of the cultures at the indicated concentrations, followed 30 min later by addition of PMA. The cells were harvested 19 h later for preparation of nuclei for run-on transcription (panel A) and for measurements of steady-state mRNA levels (panel B). These samples are designated as 19 h in the figure. Another portion of the cultures was allowed to incubate for 18 h after transfection and before addition of cycloheximide, PMA, or both. These cells were harvested 1.5 h after addition of the drugs for measurements of run-on transcription and steady-state mRNA levels (labeled 1.5 h in the figure). Parallel measurements of [^{3}H]leucine incorporation and β-galactosidase activity in these experiments are presented in Table 3. Run-on transcription was performed by using isolated *lacZ*, human γ-actin, and rDNA insert DNAs immobilized on the filters. For panel B, 20 μg of cytoplasmic RNA was applied to each well for electrophoresis in an agarose denaturing gel. Nick-translated *lacZ* insert DNA isolated from pCH110 served as a probe. The lanes marked NT contained 20 μg of cytoplasmic RNA from cells not transfected with pHIVlacZ.

DISCUSSION

Of several human cell lines tested, NT2/D1 cells showed activities with all three viral promoters that were about an order of magnitude higher than those obtained with other human cell lines, including myeloid and lymphoid cells (unpublished data). Upon induction of differentiation, there were pronounced changes in the levels of gene expression controlled by these promoters. These changes were mediated by both transcriptional and posttranscriptional events in

TABLE 3. [^{3}H]leucine incorporation and HIV-1-directed β-galactosidase activity in NT2/D1 cells treated with cycloheximide and PMA

Addition (concn)[a]	[^{3}H]leucine incorporation (% of maximum) at[b]:			β-Galactosidase activity at:	
	0–1 h	18–19 h	0–19 h	1.5 h	19 h
None	98	99	100	1.0	1.0
PMA (96 nM)	100	100	38	1.1	22.7
Cycloheximide (10 μg/ml)	9.7	6.8	4.1	0.31	0.25
Cycloheximide (10 μg/ml) + PMA	4.0	2.3	5.8	0.32	1.5
Cycloheximide (30 μg/ml)	3.0	1.2	4.8	1.1	0.04
Cycloheximide (30 μg/ml) + PMA	4.4	2.5	4.0	0.53	0.59
Cycloheximide (100 μg/ml)	0.7	0.9	1.9	0.04	NT[c]
Cycloheximide (100 μg/ml) + PMA	3.3	1.5	2.1	0.15	NT

[a] These experiments were carried out in parallel with measurements of β-galactosidase steady-state mRNA and nuclear run-on transcription (Fig. 8). After transfection with pHIVlacZ, NT2/D1 cells were treated with cycloheximide, PMA, or both either immediately after transfection (19-h samples) or for 1.5 h at 18 h after transfection (1.5-h samples).

[b] PMA was added after cells had been in cycloheximide for 30 min. Leucine uptake was measured during 1-h pulses immediately after transfection (column 1) or after the cells had been in cycloheximide, PMA, or both for 18 h (column 2). Uptake was also measured with the drugs and [^{3}H]leucine continuously present for 19 h (column 3). In the 1-h pulse experiments, L-[4,5-^{3}H]leucine (70 Ci/mmol) was added to 0.55 μCi/ml in leucine-free medium with dialyzed fetal bovine serum. Relative [^{3}H]leucine incorporation was measured as counts per minute per milligram of trichloroacetic acid-precipitable protein. The actual counts per minute per milligram of protein × 10^{-4} of cells that received no additions were as follows: column 1, 112; column 2, 193; column 3, 108.

[c] NT, Not tested.

response to RA. The effects of retinoids in promoting or inhibiting differentiation and specific gene expression are manifold and have been studied in many cell systems, but there appears to be no general consensus as to the mechanism by which these compounds exert their effects (41). However, recent work indicates that the RA receptor is homologous to the receptors for steroid hormones, thyroid hormones, and vitamin D_3—compounds that exhibit selective transcriptional regulation of many target genes (32). RA-induced differentiation of NT2/D1 cells revealed contrasting modes in the cellular control of gene expression driven by the HIV-1 and HTLV-I LTRs.

RA induction of differentiation resulted in increased HIV-1-directed expression of all three receptor genes used in this study (CAT, *lacZ*, and rabbit β-globin genes), as revealed by measurements of enzyme activity and mRNA levels. Nuclear run-on experiments and steady-state levels of the mRNA indicated that posttranscriptional regulation accounted for the increased gene activity; however, the data did not enable us to distinguish whether this regulation was at the level of mRNA processing, transport, or stability. The enhanced expression extended over about a 2.5-week period (stage 2) before it declined with the appearance of terminally differentiated stage 3 cells. As these cells appeared, the activity approached levels equal to or lower than those of uninduced NT2/D1 cells.

These results suggested a process of continuous replacement or modification of intranuclear transcriptional factors with altered transcriptional specificities as the cells differentiated. The altered transcriptional responses during differentiation were revealed in three ways. (i) RA induction led to a decrease in transcription and, consequently, steady-state mRNA levels of constructs directed by the HTLV-I LTR. (ii) As the cells continued to differentiate in RA, the response to PMA induction of the HIV-1 construct was gradually lost. This property was also shared by constructs containing the SV40 and HTLV-I control elements. (iii) Deletion of the HIV-1 NRE in p-167 resulted in a fourfold increase in expression in uninduced cells and continued responsiveness to PMA stimulation in differentiating cells. It should be noted that the p-167 construct exhibited about four times the basal activity levels of pHIVCat but only about fivefold induction of activity in the presence of PMA. In contrast, pHIVCat showed about 20-fold increases over basal levels in the presence of PMA (Fig. 4). Thus, in absolute enzyme activity units, the two constructs yielded about equal amounts of enzyme. A model that may explain these results proposes that the NRE binds a negatively regulating *trans*-acting factor. Nearly half of the PMA response may then consist of overcoming this negative regulation, and the remaining response can be ascribed to positive regulation induced by PMA.

The increased expression of the HIV-1-directed mRNA in stage 2 cells was enhanced by simultaneous expression of the *trans*-acting factor encoded by the HIV-1 *tat* gene; however, the two effects were not strongly synergistic when compared with the effect of *tat* activity on HIV-1 expression in uninduced cells. The enhanced gene activity driven by the SV40 promoter-enhancer during RA induction suggested posttranscriptional regulation similar to that controlling the expression of the HIV-1 constructs. However, the induction ratios were seldom more than about two- or threefold, and there was an early decline in enzyme activities as the cells continued along the differentiation pathways. The SV40 genome does not contain *trans*-activation response sequences responsive to *tat* (35), and cotransfection with the *tat* expression vector pPL-12 did not enhance the SV40-driven activity. We conclude that RA induction of NT2/D1 cells leads to expression of a cellular activity that enhances expression but is distinct from HIV-1 *tat*.

There was a relatively rapid decrease in gene expression controlled by the HTLV-I LTR after RA induction. This decrease was coincident with the period of commitment to differentiation. Measurements of nuclear run-on transcription and mRNA steady-state levels indicated that the cellular control exerted here was primarily at the transcriptional level. Although HTLV-I-directed transcription in induced cells was difficult to detect in nuclear run-on experiments, enzyme activities and corresponding steady-state mRNAs were detected at about 10 to 25% of the levels in uninduced cells. These results suggested that, as with the HIV-1-directed genes, posttranscriptional regulation operated to enhance expression of the limited amounts of HTLV-I-directed mRNAs available in the induced cells. In view of the marked sequence differences between the HIV-1 and HTLV-I LTRs, it is not unexpected that they recognize different sets of nuclear *trans*-acting factors or respond differently to the same sets of factors in induced cells. These experiments further indicated that the differentiated cellular phenotype influences the responses of the viral LTRs to activating cofactors.

In contrast with the effects of RA, PMA did not induce commitment, but it did lead to marked and relatively rapid stimulation of gene activity driven by all three virus promoters. This stimulation was also transient and could be related to the desensitization of cells or the net loss of PKC activity

upon prolonged exposure to PMA (5, 10). Nuclear run-on experiments showed that most, and perhaps all, of the stimulation of gene expression by PMA was transcriptional, but an additional posttranscriptional component was not ruled out.

While this work was in progress, several papers appeared which are relevant to some of the observations presented here. It was shown that the phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) induced the activity of the SV40 enhancer in human HEpG2 hepatoma cells (22). The induction, as measured by steady-state mRNA levels, took place in the presence of 30 μg of cycloheximide per ml, and it was concluded that activation took place in the absence of de novo protein synthesis. Similarly, TPA induced about a fourfold increase in HIV-1 replication and cytotoxicity when added to infected Molt-4 human T-lymphocyte cultures (19). It was recently shown that the specific targets of phorbol ester induction are the enhancerlike sequences in the U3 region of the HIV-1 LTR (37), which share regions of nearly perfect homology with the SV40 and immunoglobulin κ light-chain enhancers (28). Stimulation of the HIV-1 LTR by PMA in Jurkat T-lymphocytic cells and expression of the HIV-1 *tat* gene yielded synergistic responses similar to those reported here with NT2/D1 cells.

The HTLV-I LTR contains several 21- and 42-base-pair repeats that apparently function most efficiently as enhancers in response to a product of the viral $p40^x$ gene (13, 36). However, the HTLV-I LTR does not contain core enhancer sequences similar to those in SV40, HIV-1, or other known cellular enhancer elements, nor is it likely that NT2/D1 cells express $p40^x$. Nonetheless, PMA induction of the HTLV-I LTR in NT2/D1 cells equaled or surpassed that of the HIV-1 LTR and was stronger than that obtained with the SV40 promoter-enhancer constructs. These observations and the contrasting responses to RA of the HTLV-I and HIV-1 LTRs in differentiating NT2/D1 cells suggested multiple nuclear *trans*-acting factors in these cells with different enhancer specificities.

In lymphoid cells, phorbol esters are believed to activate directly members of the PKC family (30) and thus mimic membrane signal transduction phenomena normally triggered by presentation of antigen or growth factors. The cyclic AMP-adenyl cyclase system appears to be involved in a regulatory link with the PKC-diacylglycerol system (48). The cyclic AMP-adenyl cyclase system did not seem to be involved in the effects noted here. The experiments with phorbol esters and the specific PKC inhibitor H7 implicated activation of PKC as the initiating event in transcriptional stimulation. However, it is not certain whether the molecular events leading to the expression of HIV-1 will prove similar in detail in all cell types implicated in the pathogenesis of acquired immunodeficiency syndrome.

Induction in the presence of a protein synthesis inhibitor raised the intriguing and attractive possibility that PKC activation resulted in the modification (e.g., phosphorylation) of a preexisting nuclear *trans*-acting factor(s). De novo protein synthesis may not be required. However, the nuclear run-on experiments described here indicated that cycloheximide at concentrations effecting about 95% inhibition of protein synthesis slightly stimulated transcription of the HIV-1 LTR in cells not treated with PMA. On the other hand, cycloheximide significantly diminished the transcriptional stimulation induced by PMA. The increased expression of the HIV-1-driven CAT or *lacZ* activity in the presence of cycloheximide and PMA could be accounted for entirely by the marked increases in the steady-state levels of the mRNA, in other words, by superinduction. These increases were marked in the presence of cycloheximide alone and reached as high as 345 times the basal mRNA levels in the presence of cycloheximide and PMA. Such increases could arise from posttranscriptional stabilization of mRNA, presumably by inhibition by cycloheximide of cellular processes that normally degrade mRNA. Thus, steady-state mRNA levels in the presence of cycloheximide did not accurately reflect rates of transcription. It appears from these results that, in the absence of protein synthesis, binding of nuclear *trans*-acting factors to the HIV-1 LTR may be stimulated by PMA; however, maximum RNA transcription required protein synthesis.

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