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Inducible gene expression from multiple promoters by the tumor-promoting agent, PMA

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ABSTRACT

Phorbol ester tumor promoters affect a broad scope of changes in mammalian cells. This report describes the activation of expression of an introduced chloramphenicol acetyltransferase (CAT) reporter gene by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), in a variety of fibroblast and hematopoietic cell lines. PMA-mediated activation appears to be promoter region specific, yet widespread. Enhanced gene expression is observed for four out of five promoter systems tested, and, in some cases, is dependent on the cellular environment. Further experiments indicate that PMA mediates elevated gene expression by rapidly increasing steady state levels of CAT mRNA. The broad range of promoters affected by PMA may help explain the high potency of this agent in tumor production.

INTRODUCTION

Phorbol ester tumor promoters induce numerous responses in mammalian cells. These processes include terminal differentiation of human myeloid leukemia lines (1, 2, 3), enhanced transformation of rat embryo fibroblasts (4, 5), inhibition of phosphoenol pyruvate carboxykinase gene activity (6), altered phosphorylation of specific cellular proteins (7), and increased transcription of certain cellular genes (8, 9, 10). This latter response has been especially well documented for the c-fos gene in many cell lines (9, 10) and for the prolactin gene in rat hepatoma cells. In both cases, phorbol 12-myristate 13-acetate, PMA, activates mRNA transcription by mimicking diacylglycerol, thereby stimulating protein kinase C. In this report, we investigate PMA stimulation of the chloramphenicol acetyltransferase (CAT) gene (11) under the control of five different enhancer-promoter systems. In a wide variety of both fibroblast and hematopoietic human cells, PMA induces CAT expression from four of

the five promoters. Activation is especially strong from the cytomegalovirus immediate early gene promoter and from the murine sarcoma virus LTR. In these two cases, the activation is due to increased levels of steady state mRNA. The results indicate that phorbol esters can induce increased gene activity from many expression units in multiple cell types and may account for the potency of these agents in tumor promotion.

MATERIALS AND METHODS

Cell Lines And Cultures

HL60, U937, KG1a, KG1, and NC37 cells were all maintained in RPMI 1640 medium supplemented with 20% fetal calf serum. Cell lines 293 and HepG2 were grown in DMEM containing 10% fetal calf serum. Penicillin and streptomycin were added to all cultures.

Transfection And Establishment Of Permanent Cell Lines

The plasmids of Figure 1 were linearized with XmnI. Twenty ug of each plasmid were cotransfected individually into each cell line, along with 2 ug XmnI linearized pHeBo.1, a plasmid containing the hygromycin resistance gene (29). To mediate transfection, calcium phosphate coprecipitation was used for adherent lines, and electroporation was used for hematopoietic lines. For electroporation, a single 500 usec pulse was delivered to the cells at a field strength of 1.6kv/cm for HL60 cells and 2.7kv/cm for all other lines. Three days after transfection 200 ug/ml hygromycin B was added to the cellular medium. Four weeks later hygromycin B resistant clones were pooled to create individual lines containing the CAT gene driven by a specific promoter.

Analysis Of CAT Gene Activity

$1X10^7$ cells of a given line were seeded into duplicate cultures. One culture was treated wtih 16nM PMA. Seventy-two hours later, $1X10^7$ cells were taken from each culture and extracts were made for CAT assay (28). CAT assays were performed at 37°C for two hours and analyzed by thin layer chromatography. CAT activity levels were quantitated by scanning densitometry of short exposure autoradiographs to most accurately determine percent CAT conversion.

Transient Short-Term Transfection Studies

Supercoiled plasmids were introduced by calcium phosphate

precipitation or electroporation into the above-described cell lines. Twenty ug of plasmid DNA were used for each sample. Twenty-four hours after transfection, the cellular medium was changed and 16nM PMA was added. Forty-eight hours after PMA addition, the cells were harvested and used for CAT assay.

RNA Analysis

Cytoplasmic RNA was isolated (30) from $1X10^7$ viable control or PMA-treated cells and electrophoresed on 6% formaldehyde, 1% agarose gels. Viability was approximately 95% for both untreated and PMA-treated cells at the time of harvest. After gel electrophoresis, the RNA was transferred to nitrocellulose and hybridized with the ^{32}P-labeled 1.6 kb HindIII-BamHl CAT gene fragment of pSV2cat. Autoradiograms of filters were quantitated by densitometry.

RESULTS

In the original experimental plan, we wanted to examine expression of a reporter gene in human cells as they differentiate in culture. To this end, the bacterial chloramphenicol acetyltransferase gene (CAT) was introduced into HL60, KG1, and U937 cels, three human myeloid leukemia lines that express macrophage markers upon PMA treatment. To drive CAT transcription, five enhancer promoter systems were used: 1) the Rous Sarcoma Virus long terminal repeat (RSV), 2) the Murine Sarcoma Virus long terminal repeat (MSV), 3) the cytomegalovirus immediate early gene promoter and upstream elements (CMV-IE), 4) the SV40 early gene promoter and enhancer (SV2), and 5) the human heat shock promoter-regulatory region (HB). To establish permanent cell lines containing these promoter-CAT constructs, appropriate plasmids (Figure 1) were linearized and electroporated along with the hygromycin B resistance gene into the above leukemia lines. Hygromycin B resistant cells were selected, and, for each construct, greater than 100 clones were pooled. Populations of hyg^R clones were chosen to negate any effects of integration position on CAT expression.

In the initial experiments, CAT expression was analyzed from the CMV-IE, HB and MSV promoters in HL60 cells by determining CAT enzyme activity. When lysates from $5X10^7$ cells were assayed, CAT

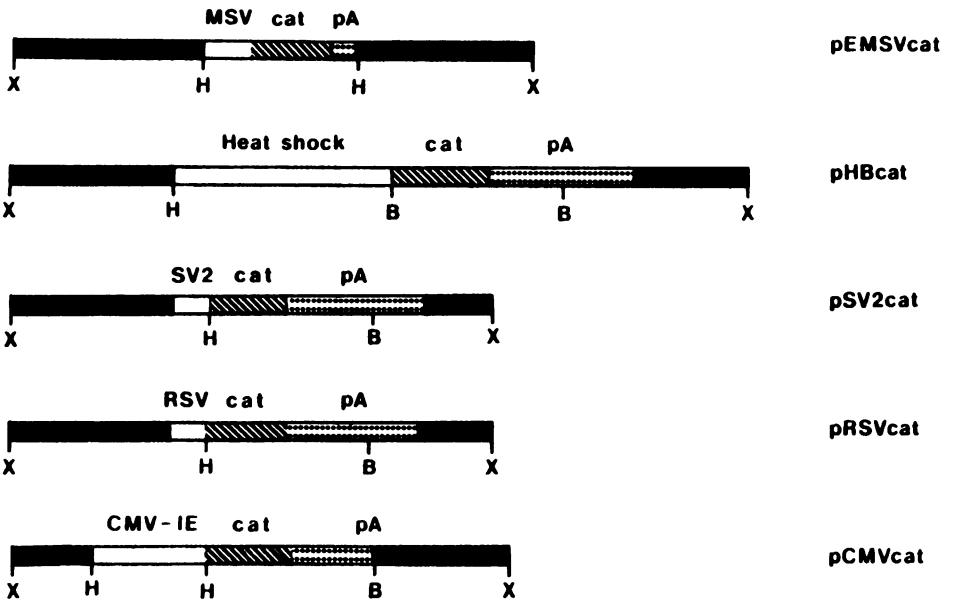

Figure 1. Plasmids used to express the chloramphenicol acetyltransferase gene. pEMSVcat was the gift of Richard Harland and contains the MSV LTR (430 bp) in front of the 773 base pair CAT gene. In this plasmid, the SV40 late polyadenylation site is used. pHBcat (27), pSV2cat (28), and pRSVcat (29) have been described elsewhere and contain the 2600 base pair human heat shock promoter, the SV40 early promoter and the Rous Sarcoma virus LTR, respectively. pCMVcat contains the 1150 base pair PstI-SacI fragment of the CMV-IE gene controlling region cloned by H3 linkers in front of the CAT gene in pUC 18. pHBcat, pSV2cat, pRSVcat and pCMVcat all use the SV40 early region small t antigen splice signals and polyadenylation site. The sequences are represented as 1) solid bars, pBR233, or pUC 18 sequences; 2) hatched bars, the CAT gene; 3) open bars, the promoter fragment; 4) dotted bars, the polyadenylation signals.

activity produced from all three promoters was negligable (Figure 2; HB data not shown). To induce differentiation, these lines were treated for 72 hours with 16nM PMA. After that period, CAT activity was assayed. For comparative purposes, activity was measured from equal numbers of viable PMA-treated and untreated cells. In HL60 lines constructed with the CMV-IE or MSV promoters, CAT activity dramatically increased after PMA treatment (Figure 2). As quantitated by densitometry, PMA stimulated CAT protein levels 5.5-fold and 21.4-fold from the CMV-IE and the MSV

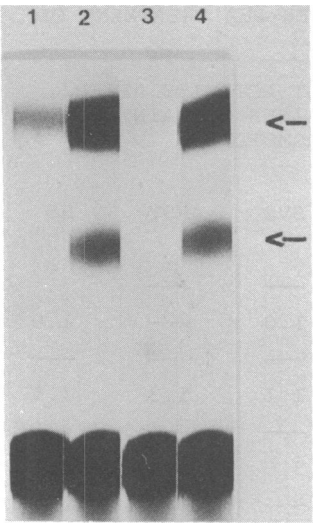

Figure 2. PMA stimulates CAT activity in HL60 cells. Lane 1, HL60 CMV-IE CAT cells, no PMA treatment; lane 2, HL60 CMV-IE CAT cells, 16nM PMA treatment; lane 3, HL60 MSV CAT cells, no PMA treatment; lane 4, HL60 MSV CAT cells, 16nM PMA treatment. The arrows indicate the two monoacetylated species.

promoters, respectively. Activity from the HB promoter remained undetectable (Table 1).

PMA induced similar increases in CAT activity in KG1 and U937 lines. Seventy-two-hour treatments with PMA produced 3.6 - 7.1-fold increases from the CMV-IE promoter in these lines, and 3.9-fold increases from the MSV promoter in KG1 cells (Table 1). In KG1 cells, PMA did not stimulate CAT activity produced from the SV2 or HB promoter (Table 1), although basal activities from all four promoters in untreated KG1 cells were at least 20 times higher than observed in HL60 or U937 cells (data not shown).

Since HL60, KG1, and U937 cells all produce macrophage markers upon PMA treatment, we wanted to determine if the observed stimulation of CAT expression was a manifestion of cellular differentiation or was due to some more direct effect of PMA. To this end, we constructed similar CAT gene lines using four other cell types which do not display macrophage surface markers upon PMA treatment: 1) K562 cells (16), a chronic myelogeneous leukemia line; 2) NC37 cells, a human lymphoblastoid line; 3) KG1a cells (18), a clonal derivative of KG1 cells, and 293 cells (18), an adenovirus 5 human embryonic kidney fibroblast line. Again, a variety of CAT lines were created from these cells using the individual promoters described above. Pools of hygromycin B

TABLE 1. INCREASE IN CAT EXPRESSION AFTER PMA TREATMENT OF STABLY TRANSFECTED CELLS

CELL LINE	FOLD INCREASE IN CAT ACTIVITY AFTER PMA TREATMENT OF LINES CONTAINING VARIOUS PROMOTERS				
	CMV-IE	MSV	SV2	RSV	HB
HL60	5.5	21.4	——	——	*
KG1	3.6	3.9	1.0	——	1.0
U937	7.1	——	——	——	——
K562	3.5	8.9	1.1	1.3	1.5
KG1a	2.7	4.0	——	——	——
NC37	6.9	7.8	3.0	——	4.1
293	4.0	2.5	——	0.9	1.7

The data in this table are exemplified by Fig. 2 for HL60 cells. The numbers tabulated here represent the averages of at least two experiments performed for each line. For most lines, multiple determinations were made. In every instance, the induction of CAT activity by PMA was very reproducible, both qualitatively and quantitatively.

resistant clones were selected, and CAT enzyme activity was measured after PMA treatment of these populations.

CAT levels in all four lines constructed with either the CMV-IE or the MSV promoter increased substantially upon 72-hour exposure to PMA. Increases ranged from 2.5-fold for the MSV promoter in 293 cells to 8.9-fold from the same expression unit in K562 cells (Table 1). The effect of PMA on expression from the SV2 and HB promoters was more variable, being dependent on the cell line used. Less than 2-fold increases in CAT activity were detected from these promoters in K562 lines, whereas 3 - 4-fold activations were observed from the HB promoter in 293 cells. In the two cell lines constructed with the RSV promoter, PMA never stimulated CAT activity. Therefore, these results suggest that induction by PMA is a promoter control region specific phenomenon. Promoters such as the CMV-IE and the MSV promoters are stimulated by 16nM PMA in every cell line tested. Activation from the SV2, RSV and HB promoters is either weaker or non-existent and is more dependent on the cellular environment. In addition, activation

TABLE 2. FOLD INCREASED CAT ACTIVITY IN PMA-TREATED SHORT-TERM TRANSFECTED CELLS

CELL LINE	CMV-IE PROMOTER	MSV PROMOTER
HL60	5.9	6.0
U937	8.3	——
KG1a	3.6	4.6
KG1	1.9	1.9
NC37	4.9	2.0
293	3.2	4.3
HepG2	5.0	3.3

The experiments performed here were as those in Table 1, except that 20 ug pCMVcat or pEMSVcat were transfected by calcium precipitation or electroporation. Twenty-four hours after transfection, the cellular medium was changed and 16nM PMA was added. Forty-eight hours after PMA addition, the cells were harvested and used for CAT assay.

is independent of the differentiation process. Comparable increases in CAT activity are seen with PMA in differentiating and non-differentiating lines. Therefore, the observed increases of CAT expression are not a function of differentiation; rather, they are responses to PMA activation.

In the experiments described above, PMA treatment leads to increased CAT enzyme levels in a variety of cell constructs. However, it was still important to determine whether these responses occur as a result of universal activation of our introduced constructs or as a result of activation in a few cell clones carrying specific integration positions. To address this question, transient expression experiments were performed to measure CAT levels prior to stable DNA integration. pEMSVcat and pCMVcat were transfected by either calcium phosphate coprecipitation (20) or electroporation (21) into the parental lines described above, as well as into HepG2 cells, a human hepatoma line. Twenty-four hours later, the cells were treated with 16nmM PMA and CAT activity was measured after an additional 48 hours. Again, in every cell line tested, PMA stimulated CAT levels from both pCMVcat and pEMSVcat (Table 2). Increases were similar to those determined in the stably transfected populations and ranged from 2-fold for

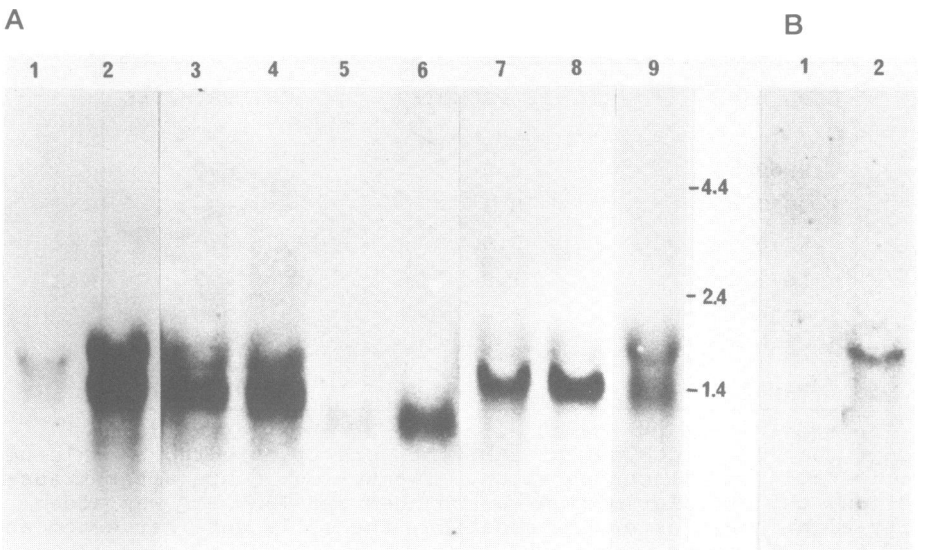

Figure 3. Northern blots of CAT mRNA from 293 and NC37 cells treated with PMA. Panel A) 293 cells stably transfected with pCMVcat lanes 1, 2, and 9; pRSVcat lanes 3 and 4; pEMSVcat lanes 5 and 6; and pHBcat lanes 7 and 8. RNA in lanes 1, 3, 5, 7, and 9 were from untreated cells, whereas lanes 2, 4, 6, and 8 were prepared from cells treated for 48 hours with 16nM PMA. Note that lanes 3 - 6 are photographed from a more exposed autoradiogram. Lane 9 is a long exposure from a comparable experiment and is included to demonstrate two CAT transcripts from untreated 293 CMV-IE cells. (B) NC37 cells stably transfected with pCMVcat. Lane 1, untreated cells; lane 2, RNA from an equal number of cells treated for 48 hours with 16nM PMA.

both promoters in KG1 cells to 8-fold for the CMV-IE promoter in U937 cells (Table 2). Therefore, PMA stimulation of expression is not dependent upon DNA integration. Rather, it can occur when transfected DNA is transiently expressed or when it is stably integrated in the host genome. As a corollary, it appears that all cis acting factors required for PMA activation are contained within the introduced DNA constructs.

Since PMA acts to increase CAT protein levels, we postulated that this phorbol ester activated CAT production by stimulating steady state levels of CAT transcripts. To test this hypothesis, CAT mRNA levels were examined in the presence and absence of PMA. The stably integrated 293 populations containing the CMV-IE, MSV, HB or RSV promoter/CAT constructs were used for this experiment.

The cells were treated with PMA for 48 hours and cytoplasmic RNA was subjected to Northern analysis and probed with a ^{32}P-labeled CAT restriction fragment.

Transcripts of the expected size are observed in lines using the RSV and HB promoters (Figure 3A). Upon PMA treatment, the steady state levels of these transcripts remain constant, concurrent with the minimal increases in CAT enzyme activity induced by PMA. On the other hand, dramatic increases in CAT mRNA levels are observed upon PMA treatment of the 293 CMV-IE CAT and 293 MSVcat lines. With the MSV promoter, the 1.3 kb transcript increases 6-fold, whereas in the 293 CMV-IE CAT line, two transcripts of 1.4 and 1.7 kb increase 6-fold and 8-fold, respectively. Both of the CMV-IE CAT transcripts are seen in untreated controls (Figure 3A, Lane 9 for a longer exposure) and in transient assays in 293 cells using these exact constructs. Only the larger 1.7 kb transcript is observed from the CMV-IE promoter in NC37 cells (Figure 3B). The level of this transcript also increases 15-fold upon PMA treatment. Therefore, PMA stimulates CAT enzyme activity in these lines by increasing steady state levels of CAT mRNA. The increases in CAT transcript levels are comparable to the increases in CAT activity observed, suggesting that the elevated levels of translational substrate can account for the entire PMA effect. Furthermore, since there is promoter specificity of this activation, it is likely that this increase is due to elevated transcriptional activity.

To further characterize these effects of PMA, we examined the kinetics of induction of gene expression. The induction of CAT enzyme activity from the CMV-IE promoter in NC37 cells is very rapid (Figure 4A). Elevations of CAT activity are evident as early as six hours after PMA treatment and continue to increase until approximately 69 hours. CAT levels do not increase further after this time (data not shown). Similar time courses are observed for the MSV promoter and for both promoters in 293 cells. In an analogous experiment, we measured both CAT mRNA and protein levels at various time points after PMA addition to NC37 CMV-IE CAT cells (Figure 4B). After as little as six hours, CAT mRNA levels increased 7-fold and continued to increase more slowly to a final 15-fold stimulation at 48 hours. Initially, the induction

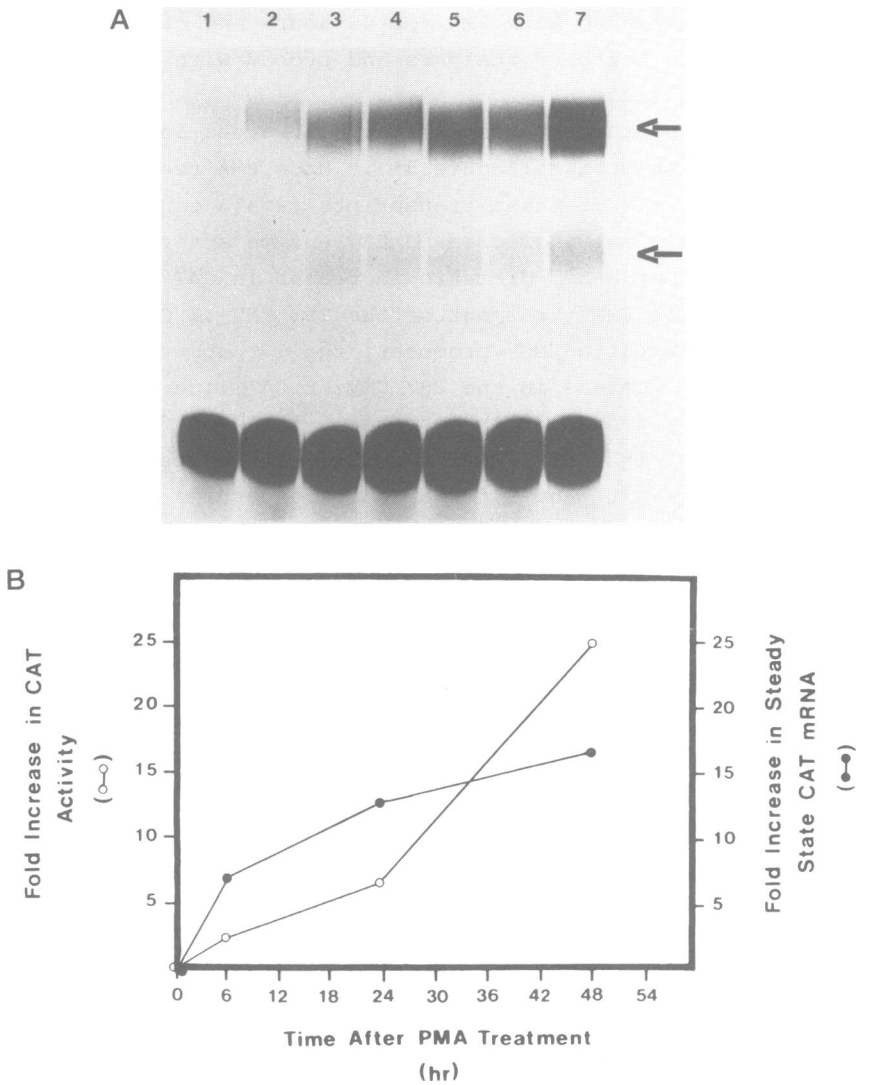

Figure 4. Kinetics of induction of CAT mRNA and protein. Panel A) $3X10^6$ CMV-IE CAT cells were seeded into seven wells and at various times 16nM PMA was added. Lane 1, no exposure to 16nM PMA; lane 3, 17-hour exposure; lane 4, 23-hour exposure; lane 7, 69-hour exposure. The arrows denote the monoacetylated chloramphenicol species. Panel B) Plot of increasing CAT mRNA and protein levels in PMA-treated NC37 CMV-IE CAT cells. The experiment was performed as in A, except that $3X10^6$ cells were used for the CAT assay and $1X10^7$ cells from the same culture were used for Northern analysis of CAT mRNA. mRNA and CAT levels were quantitated by densitometry.

of CAT protein levels lagged behind the increases in CAT mRNA but eventually accelerated and reached a 25-fold stimulation at 48 hours. This lag of induction of CAT protein levels with respect to mRNA levels suggests that PMA mediates increases in expression by primarily influencing the accumulation of steady state CAT mRNA. Furthermore, the degree of increase of both mRNA and CAT activity are approximately the same. This observation again indicates that PMA affects a cellular pathway whose fundamental mode of action is to enhance transcriptional activity and/or mRNA stability.

DISCUSSION

We have shown that PMA can induce increased expression of CAT protein levels in a wide variety of cell types and from a number of promoters. Induction is especially significant from the MSV and CMV-IE promoters in every line tested. This enhancement of activity is independent of the differentiation effects of PMA and is the result of an elevation of steady state mRNA levels.

The effects of PMA observed here are very similar to those seen with the c-fos gene in Balb/c 3T3 cells (9, 10), the prolactin gene in GH_1 rat pituitary cells (8, 22), and the human mettallothionein IIA gene (23) in HeLa and HepG2 cells. Stimulation has also been observed from the MLV LTR in GH_1 cells (24) and from the SV40 promoter in human HepG2 cells (25). Where analyzed, induction in these systems appears to be mediated by the protein kinase C pathway, and in the case of c-fos, is independent of cellular differentiation (26). The kinetics of induction in this system as compared to that of other systems is slightly different. In all cases, induction of mRNA is very rapid. However, in these other system, especially in the case of c-fos, mRNA levels decay almost as quickly as they are induced. Elevated CAT mRNA levels in this system persist for many hours. This difference may stem from the inherent variations in stability of these mRNAs and/or the cell lines chosen for these experiments.

In conclusion, the results presented here expand the number of known PMA responsive expression systems, PMA can induce expression from multiple promoters in a wide variety of cell types.

The cellular milieu can also influence the responsiveness of an expression element to PMA. This broad range of targets for PMA activation may explain the high potency of this phorbol ester as a tumor-promoting agent.

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