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Bovine papilloma virus encoded E2 protein activates lymphokine genes through DNA elements, distinct from the consensus motif, in the long control region of its own genome

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Activation of T cells by antigen, lectin or a combination of phorbol ester (PMA) and calcium ionophore (A23187) leads to the induction of a set of lymphokine genes. Transfection of a human T cell leukemia cell line, Jurkat, or an African green monkey kidney cell line, CV1, with a cDNA encoding E2 protein, a *trans*-activator of bovine papilloma virus type 1, results in activation of interleukin 2 (IL-2), interleukin 3 (IL-3) and granuloycte/macrophage colony-stimulating factor (GM-CSF) genes in a transient transfection assay. 5′ deletion and mutation analyses showed that the sequence between positions −60 and a TATA-like sequence is required for basic promoter function and that the sequence between positions −95 and −73 containing conserved lymphokine element 2 (CLE2) and a GC box (CLE2/GC box) mediates the positive response to E2 protein. The latter has been previously shown to respond to PMA/A23187 stimulation or to p40^{tax}, a *trans*-activator encoded by human T cell leukemia virus type 1 (HTLV-I). The sequence located between −108 and −99 (CLE1) is inhibitory to E2 protein or PMA/A23187 stimulation. The combination of E2 protein and PMA/A23187 appears to eliminate an inhibitory effect of the upstream region. However, E2 protein, like p40^{tax}, mediates a positive response through CLE1 alone linked to the basic promoter sequence. The level of activation of the long control region (LCR) by E2 protein is unaffected by the number of CLE2/GC box sequences. These results indicate that E2 protein, similar to p40^{tax}, activates the GM-CSF gene by interacting with a component(s) of the signal transduction pathway without involving the consensus DNA motif, ACCN$_6$GGT, present in the long control region of the papilloma genome.

Key words: BPV E2/GM-CSF/HTLV-I p40^{tax}/lymphokine/T cell activation

Introduction

Various hormones and growth factors regulate eukaryotic gene expression. These signals are transmitted to cytoplasm through specific receptors to induce various intracellular signals. Activation of protein kinase C and increased Ca^{2+} mobilization are involved in signal transduction processes in many different types of cells (Nishizuka, 1984). One such example is helper T cells, which produce a battery of lymphokines when exposed to antigens. The external signals, which are recognized by a T cell antigen receptor–CD3 complex, are transduced across the plasma membrane to stimulate phosphoinositide turnover that results in the production of diacylglycerol and inositoltrisphosphate (IP3). Diacylglycerol activates protein kinase C and IP3 increases Ca^{2+} influx and/or mobilization (Kishimoto *et al.*, 1980; Berridge and Irvine, 1984). Signal transduction events further downstream trigger a series of biochemical changes in the nucleus and induce production of a battery of lymphokines. However, very little is known about downstream events in the signal transduction pathway. Activation of T cells by antigen can be mimicked by lectins, and anti-T cell receptor and anti-CD3 antibodies. Combinations of PMA and Ca^{2+} ionophore also directly stimulate T cells to produce lymphokines (Weiss *et al.*, 1984).

Human T cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T cell leukemia (ATL). ATL cells are known to produce high levels of the IL-2 receptor (p55 chain) (Depper *et al.*, 1984) and constitutively produce several lymphokines such as GM-CSF, IL-5, IL-6 and IFN-γ (Gasson *et al.*, 1984; Salahuddin *et al.*, 1984; Shimizu *et al.*, 1985). We have recently shown that p40^{tax}, a *trans*-activator encoded by the HTLV-I genome which activates its own LTR (Chen *et al.*, 1985; Yoshida and Seiki, 1987) and the IL-2 receptor (p55) gene (Inoue *et al.*, 1986; Cross *et al.*, 1987; Maruyama *et al.*, 1987), also activates transfected lymphokine genes in Jurkat cells and fibroblasts (Miyatake *et al.*, 1988a). The CLE1 motif located between −108 and −99 of mouse GM-CSF gene, which is conserved among many lymphokine/cytokine genes (Stanley *et al.*, 1985; Yokota *et al.*, 1988), mediates response to p40^{tax}. Likewise, the CLE2 motif, which also appears in the IL-3 gene followed by a GC box, mediates response to p40^{tax} and PMA/A23187 stimulation (Miyatake *et al.*, 1988b). The HTLV-1 LTR contains a transcriptional enhancer consisting of three 21-bp direct repeats which is responsible for *trans*-activation by p40^{tax}. However, so far there is no evidence for DNA binding activity of p40^{tax}. On the basis of these observations, we proposed that p40^{tax} promotes the expression of the GM-CSF gene through interaction with a component(s) of the signal transduction machinery (Yokota *et al.*, 1988; Miyatake *et al.*, 1988a).

We have also reported that E2 protein, a *trans*-activator encoded by the bovine papilloma virus type 1 (BPV-1) genome, can also activate lymphokine genes (Miyatake *et al.*, 1988a). Papilloma viruses, a group of small DNA tumor viruses, induce both benign and malignant epithelial proliferations and are responsible for a variety of pathologic conditions in humans and cattle. In a 69% subgenomic fragment of BPV, which is sufficient for transformation function, eight open reading frames (ORFs) were deduced

from DNA sequence data (Chen *et al.*, 1982). The transcription control elements (Sarver, 1984) and the sequence required for plasmid maintenance or replication (Lusky and Botchan, 1984; Waldeck *et al.*, 1984) were mapped on a non-coding region between *Hin*dIII (position 6958) and *Hpa*I sites (position 7975/1) to the upstream of these ORFs. E2 protein activates BPV genes through a conditional enhancer sequence (Spalholz *et al.*, 1985) also referred to as the long control region (LCR) (Rabson *et al.*, 1986) or upstream regulatory region (Androphy *et al.*, 1987). E2 protein is a DNA binding protein with a C-terminal domain involved in this binding (McBride *et al.*, 1988; Giri and Yaniv, 1988) and recognizes $ACCN_6GGT$ sequences which are clustered in the LCR region (Spalholz *et al.*, 1987; McBride *et al.*, 1988). Our finding that E2 protein, like $p40^{tax}$, can activate many lymphokine genes in both T cells and fibroblasts raised an interesting question on the mechanism of its action since no obvious consensus motif similar to $ACCN_6GGT$ of the LCR was found in the 5′ flanking region of lymphokine genes.

In this paper we extend these observations and define *cis*-acting DNA elements within the 5′ flanking region of the GM-CSF gene that mediate response to E2 protein in the T cell leukemia cell line, Jurkat, and the CV1 monkey kidney cell line. Our results indicate that the major E2 responsive element contains a CLE2/GC box which is also responsible for activation by $p40^{tax}$. The results suggest that E2 protein, like $p40^{tax}$, activates the GM-CSF gene by interacting with a component(s) in the signal transduction machinery without involving the consensus DNA motif, $ACCN_6GGT$.

Results

Activation of lymphokine genes by BPV-encoded E2 protein in T cells and fibroblasts

We previously showed that BPV-E2 protein activates the GM-CSF promoter in Jurkat cells in a transient transfection assay. The action of E2 proteins was further enhanced by treatment of transfected cells with PMA/A23187 (Miyatake *et al.*, 1988a). As shown in Table I, E2 protein, in the absence of PMA/A23187, activates the mouse GM-CSF promoter and other lymphokine promoters, such as that of mouse IL-2 and IL-3, in both Jurkat and CV1 cells. Treatment of Jurkat cells with PMA/A23187, which mimics T cell activation signals via a T cell receptor CD3 complex (Weiss *et al.*, 1984), strongly stimulated the mouse GM-CSF and IL-2 promoter. Combinations of PMA/A23187 stimulation and E2-protein-activated GM-CSF and IL-4 promoters were more than additive in Jurkat and CV1 cells. PMA/A23187 treatment did not significantly enhance IL-3 and IL-5 promoters in either Jurkat or CV1 cells. Interestingly, human IL-5 gene did not appreciably respond to the various stimulations tested. Likewise, transfection of Jurkat cells or CV1 cells with $p40^{tax}$ alone enhanced the mouse GM-CSF and IL-3 promoters (Table I; Miyatake *et al.*, 1988a).

E2 ORF is responsible for activation of the GM-CSF promoter

To confirm that the E2 protein is responsible for activation of lymphokine genes, a frameshift mutation was introduced into the E2 ORF by cleaving and filling-in a *Nar*I site followed by ligation. As shown in Figure 1, pcDSRα-cE2 (Nar), which encodes the N-terminal 117 amino acids of full-length E2 protein (410 amino acids), did not support activation of the GM-CSF gene. This result indicated that activation of the GM-CSF is dependent on the E2 ORF.

Table I. Effect of BPV E2 protein and HTLV-I $p40^{tax}$ protein on lymphokine promoters in Jurkat and CV1 cells

Lymphokine plasmid	*Trans*-activators PMA/A23187	Jurkat			CV1		
		–	E2	$p40^{tax}$	–	E2	$p40^{tax}$
pmGMCAT	–	1.7	4.4	47.2	0.7	2.5	2.7
	+	20.8	87.6	89.4	1.6	21.0	9.0
pmIL-2CAT	–	0.2	4.7	0.9	0.3	0.9	0.5
	+	51.1	52.9	50.0	0.4	0.8	1.0
pmIL-3CAT	–	0.7	20.6	4.7	0.6	1.2	3.0
	+	0.6	10.0	6.8	0.5	1.0	4.5
pmIL-4CAT	–	0.2	0.2	0.2	0.2	0.8	0.2
	+	0.5	6.9	2.7	0.3	4.9	0.8
phIL-5CAT	–	0.6	0.2	0.2	0.6	0.6	0.6
	+	0.2	0.6	0.2	0.6	0.6	0.6

CAT enzyme activities shown in this table indicate percentage conversion of [^{14}C]chloramphenicol to the acetylated form. Five micrograms of lymphokine CAT gene fusion plasmids were co-transfected with 5 μg of either pcDSRα-296, which has no cDNA insert, pcDSRα-pX ($p40^{tax}$) or pcDSRα-cE2 (E2) plasmid DNA. Detailed transfection conditions and structure of plasmids are given in Materials and methods. For CAT assays, 50 μg of Jurkat cell extract, except for mGM CAT (30 μg), or 300 μg of CV1 cell extract was incubated with [^{14}C]chloramphenicol at 37°C for 6 h.

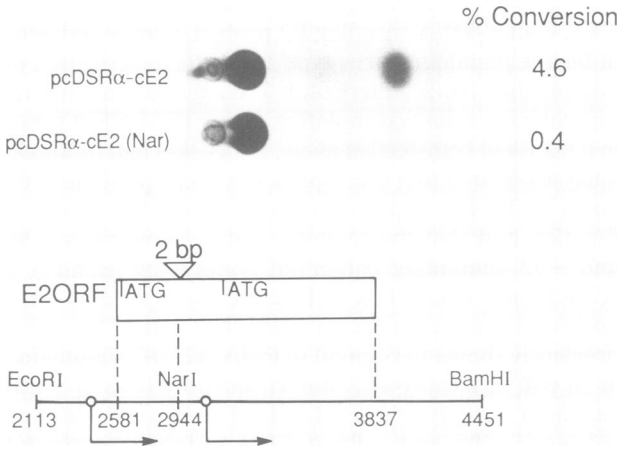


Fig. 1. Involvement of E2 ORF in expression of the GM-CSF gene. pmGMCAT-740 was co-transfected with pcDSRα-cE2(Nar), which produces truncated protein due to a frameshift mutation, or pcDSRα-cE2 into Jurkat cells. Transfected cells were stimulated with PMA (30 ng/ml) and A23187 (1 μM) for 8 h before harvest. PMA did not show any profound effect on the expression of $p40^{tax}$ and E2 driven by SRα promoter (Takebe *et al.*, 1987), although SV40 promoter was highly enhanced by PMA in Jurkat cells (Miyatake *et al.*, 1988). CAT activities were evaluated using 300 μg of cell extract with 6 h incubation. Circles and arrows represent promoters and directions of transcription respectively.

5′ deletion analysis of the mouse GM-CSF promoter

To map the region required for activation of the mouse GM-CSF promoter by E2 protein, a series of deletions, generated from the 5′ end by using *Bal*31 nuclease, were examined in Jurkat cells in a transient transfection assay (Figure 2). As reported previously (Miyatake *et al.*, 1988b), PMA/A23187 activated GM-CSF promoters carrying

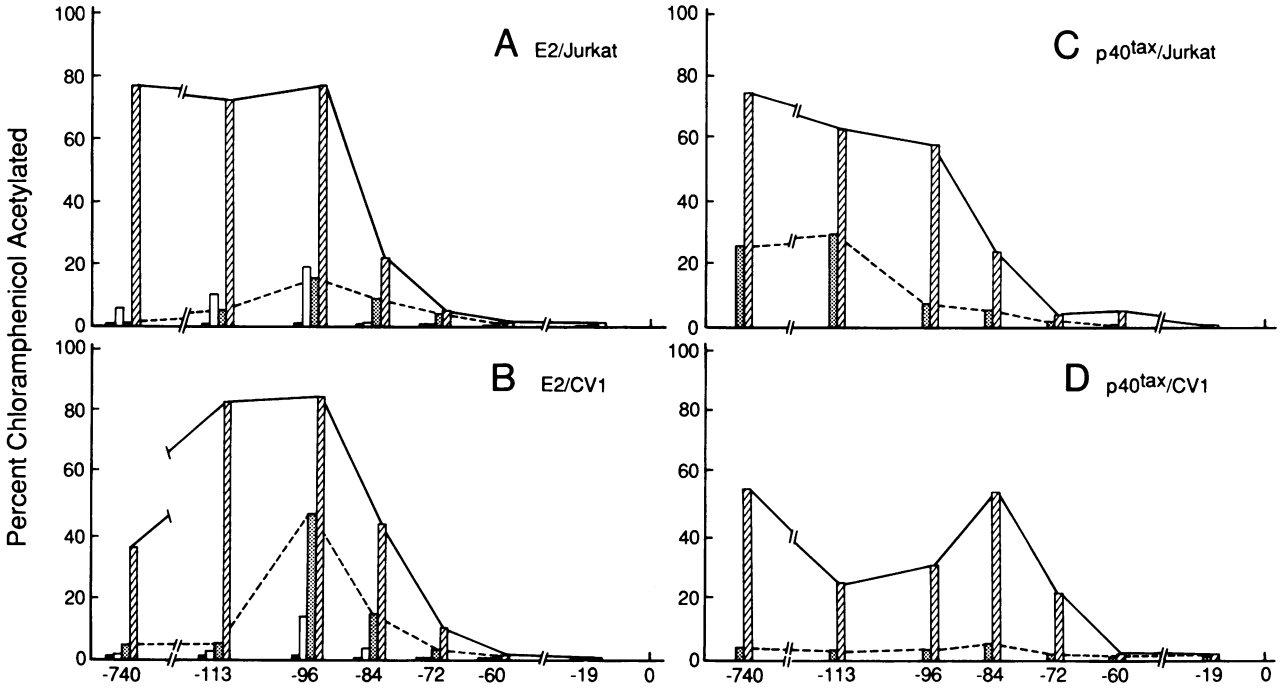
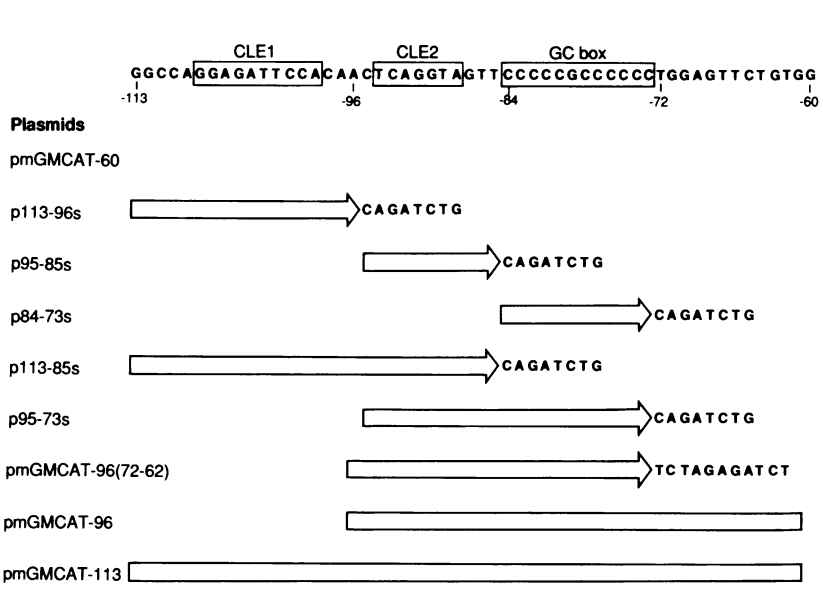


Fig. 2. 5′ deletion analysis of mouse GM-CSF promoter in Jurkat or CV1 cells. Plasmids having various lengths of mGM-CSF gene upstream sequence were co-transfected with pcDSRα-cE2 (**A,B**) or pcDSRα-p40^{tax} (**C,D**) into Jurkat (**upper panels**) or CV1 (**lower panels**) cells. When no *trans*-activator cDNAs were added, pcDL-SRα296, which has no cDNA insert, was added in order to adjust the amount of DNA transfected into cells. Closed bar, no cDNA with no stimulation; open bar, no cDNA with PMA/A23187 stimulation; stippled bar with dashed line, E2 or p40^{tax} without stimulation; hatched bar with solid line, E2 or p40^{tax} with PMA/A23187 stimulation. The numbers indicate relative positions from the transcription initiation site.



% Chloramphenicol Acetylated

PMA/A23187	trans. activator	A. Jurkat -	A. Jurkat E2	A. Jurkat p40tax	B. CV1 -	B. CV1 E2	B. CV1 p40tax
-		0.6	1.1	0.4	0.1	1.3	0.3
+		0.7	1.4	2.4	0.2	1.7	0.8
-		0.9	0.7	2.4	0.3	3.1	1.5
+		0.7	1.8	11.8	0.5	46.2	2.7
-		0.6	1.3	0.4	0.2	1.9	0.3
+		0.6	1.0	1.2	0.2	5.2	0.6
-		0.6	1.0	0.9	0.2	0.2	0.4
+		0.9	3.6	5.8	0.2	7.1	0.9
-		0.2	0.4	0.5	0.2	1.0	0.3
+		0.1	0.8	1.6	0.2	33.4	0.4
-		0.7	0.7	1.7	0.2	0.8	0.4
+		3.4	23.0	13.2	0.5	25.3	1.0
-		0.5	1.1	2.4	0.2	4.4	1.2
+		5.8	32.3	23.4	0.3	20.7	5.9
-		0.9	5.1	3.2	0.3	28.8	2.3
+		10.7	62.9	18.7	2.1	68.6	10.0
-		0.7	2.6	2.8	0.3	4.6	2.0
+		10.5	70.5	15.0	0.6	22.8	3.9

Fig. 3. Identification of DNA elements which are required for the activation by various stimulants. Plasmids carrying various segments were co-transfected with either pcDL-SRα296 (no cDNA insert), pcDSRα-cE2 or pcDSRα-pX as indicated (see Materials and methods for plasmid constructions). Arrows indicate orientation and length of fragments. CAT activities were measured by incubation for 6 h with [^{14}C]chloramphenicol and extracts of Jurkat (30 μg) or CV1 (300 μg) cells respectively.

sequences up to position −96 but failed to activate the construct with a deletion extended to position −84 (Figure 2). E2 protein activated the GM-CSF promoter if a sequence encompassing 96 bp upstream of the transcription initiation site was present. A combination of E2 protein with PMA/A23187 further enhanced the level of activation. When the deletion was extended to positions −84 and −72, the stimulatory effect of E2 protein, either by itself or in combination with PMA/A23187, was significantly diminished, and at −60, the stimulatory effect of E2 protein was almost completely abolished. These results indicate that the 5′ boundary of the DNA element required for

activation lies between positions −96 and −60 of the GM-CSF promoter. The basal level of CAT activity did not change even if the conventional 'TATA' box was deleted (pmGMCAT-19). The region upstream of position −96 appears inhibitory since the presence of sequences covering positions −96 to −740 markedly diminished the response to either E2 protein or PMA/A23187 stimulation. However, when both E2 protein and PMA/A23187 stimulation were combined, the inhibitory effect of the sequence upstream of −96 was overcome and the response became even greater than that of the −96 construct. In contrast, the region upstream of −96 did not show any inhibitory effect on activation by $p40^{tax}$ (Figure 2C; Miyatake *et al.*, 1988b). In CV1 cells (Figure 2B and D) constructions carrying the sequence up to −96 responded to PMA/A23187, E2 protein or $p40^{tax}$, as in Jurkat cells. In contrast, constructs carrying the sequence >96 bp from the transcription initiation site barely responded to PMA/A23187 or E2 protein (Figure 2B). These results suggest that the sequence between −113 and −96, which contains CLE1, may be an inhibitory element. Combination of PMA/A23187 and E2 protein appears to overcome the inhibitory action of the upstream sequence (Figure 2B).

Sequence between positions −54 and −31 of the GM-CSF promoter is required for basic promoter function

To determine the 3′ boundary of the 5′ flanking sequence required for activation by *trans*-activators and PMA/A23187 stimulation, sequences between positions −72 and −62 [pmGMCAT-96(72−62)], −54 and −43 [pmGMCAT-96(54−43)] and −43 and −32 [pmGMCAT-96(43−32)] were replaced with unrelated sequences of the same length. pmGMCAT-96(54−43) and pmGMCAT-96(43−32) lost the ability to respond to E2 protein or $p40^{tax}$ and/or PMA/A23187 stimulation (data not shown). In contrast, pmGMCAT-96(72−62) retained a significant portion of its ability to respond to $p40^{tax}$ or E2 protein in combination with PMA/A23187, although the ability to respond to E2 protein alone was substantially impaired (Figure 3, lane 7). On the other hand, replacement of the sequence between −72 and −62 [pmGMCAT-96(72−62)] did not appreciably affect response to $p40^{tax}$ in the presence or absence of PMA/A23187 (80−100% of pmGMCAT-96). These data suggest that the region downstream of −60 is required for basic promoter function and the region between −96 to −72 is essential for activation by *trans*-activators and for PMA/A23187 stimulation, although full activation by E2 protein either with or without PMA/A23187 stimulation was accomplished only when the entire sequence covering −96 to −60 was present.

Identification of the DNA element responsible for activation of the GM-CSF gene

To identify the minimum region that mediates the response to E2 protein with or without PMA/A23187 stimulation, subfragments of the region −113 to −73 were inserted into pmGMCAT-60 using a *Bgl*II linker (CAGATCTG). In Jurkat cells (Figure 3, column A), pmGMCAT-96(72−62) or p95-73s plasmids carrying the intact sequence between −95 and −73 (CLE2 and GC box) responded to PMA/A23187 stimulation and the E2 protein only partially: however, combination of E2 protein with PMA/A23187 enhanced CAT activity synergistically. The same sequence is also activated by $p40^{tax}$, PMA/A23187 or a combination of $p40^{tax}$ and PMA/A23187 stimulation (Figure 3; Miyatake *et al.*, 1988b). The sequence covering CLE1 (p113-96s), which responded to $p40^{tax}$, did not significantly respond to E2 protein in Jurkat cells. Similarly, in CV1 cells (Figure 3, column B) the sequence containing both CLE2 and the GC box [p95-73s and pmGMCAT-96(72−62)] responded to E2 protein or $p40^{tax}$ and this stimulatory effect was further enhanced by combination with PMA/A23187. However, this sequence hardly responded to PMA/A23187 stimulation alone. Although the sequence between −113 and −96 containing CLE1 has been suggested as an inhibitory element in CV1 cells, this sequence, when inserted into pmGMCAT-60, effectively mediated the response to E2 protein or $p40^{tax}$ either with or without PMA/A23187 stimulus. As with $p40^{tax}$, the fragment covering CLE2 (p95-85s) or the GC box (p84-73s) alone barely responded to E2 protein.

Interaction of E2 protein with GM-CSF regulatory element

E2 protein activates an enhancer sequence (LCR) through the consensus sequence motif $ACCN_6GGT$ (Spalholz *et al.*, 1985, 1987). Furthermore, direct interaction between the motif and E2 protein has been demonstrated by various procedures (Androphy *et al.*, 1987; Hauley-Nelson *et al.*, 1988; McBride *et al.*, 1988). Since the CLE2/GC box of the GM-CSF promoter shares no homology with the consensus motif of LCR, it is important to know whether action of E2 protein on the GM-CSF promoter also involves a direct protein−DNA interaction similar to BPV LCR or an indirect one. In order to examine these possibilities, a competition experiment using the LCR motif and the CLE2/GC box motif was carried out. As expected from the strong affinity of E2 protein for the LCR motif, addition of increasing amounts of BPV LCR sequence profoundly inhibited expression of the GM-CSF gene either with or without PMA/A23187 (Table IIA). On the contrary, addition of increasing amounts of the GM-CSF regulatory region containing the CLE2/GC box motif hardly affected the conditional enhancer activity of BPV LCR (Table IIB). These results indicate that the interaction, if any, between the GM-CSF control region and E2 protein is much weaker than that of E2 protein and LCR or that activation of the CLE2/GC box motif by E2 protein may involve an indirect mechanism.

Discussion

In this paper we show in a transient transfection assay that BPV-encoded E2 protein activates several transfected lymphokine promoters, including those for GM-CSF, IL-2 and IL-3. As with HTLV-I-encoded $p40^{tax}$, activation of lymphokine genes by E2 protein requires the 5′ flanking region and is enhanced by PMA/A23187 treatment. We also demonstrated that the 5′ flanking region of the mouse GM-CSF gene contains several DNA elements that specify the ability to respond to E2 protein, $p40^{tax}$ or PMA/A23187 stimulation. Mutations introduced at positions −54 to −43 or −43 to −32 of pmGMCAT-96 markedly diminished response to any of these stimuli, PMA/A23187, $p40^{tax}$ or E2 protein, although the resulting constructs contained the

Table II. The GM-CSF regulatory sequence does not interfere with the BPV LCR conditional enhancer activity by E2 protein

(**A**) Luciferase activity

	Competitor DNA			
	−PMA/A23187		+PMA/A23187	
pBPVLCR-CAT (μg)	Relative light units	Relative %	Relative light units	Relative %
−	1003	100	34706	100
2	960	95.7	23379	67.3
4	658	65.6	12988	37.4
6	398	39.7	6577	19.0

(**B**) CAT activity

	Competitor DNA			
	−PMA/A23187		+PMA/A23187	
pmGMLuc-96 (μg)	Conversion %	Relative %	Converseion %	Relative %
−	11.6	100	22.1	100
2	12.3	106	21.7	98.2
4	10.5	90.5	17.5	79.2
6	13.8	119	22.1	100

(**A**) pmGMLuc-96 (2 μg) was co-transfected with pcDSRα-cE2 (2 μg) and an increasing amount of pBPVLCR-CAT (0−6 μg) in Jurkat cells. The amount of plasmid DNA was adjusted to 10 μg total with pcDL-SRα296 plasmid DNA. After 40 h, cells were treated with PMA (30 ng/ml) and A23187 (1 μM) for 8 h as indicated. Luciferase activities were measured using 10 μg of cell extract.
(**B**) pBPVLCR-CAT (2 μg) was co-transfected with pcDSRα-cE2 (2 μg) and an increasing amount of pmGMLuc-96 (0−6 μg) in Jurkat cells. The amount of plasmid DNA was adjusted to 10 μg with pcDL-SRα296 plasmid. CAT activities were measured using 30 μg of cell extract and incubation at 37°C for 6 h.

intact CLE2/GC box. In contrast, mutation at positions −72 to −62 did not show an appreciable effect with the exception of the response to E2 protein alone, which is impaired to some extent. These results extend our previous observations that pmGMCAT-60 does not respond to PMA/A23187 or $p40^{tax}$ and further confirm that the region downstream of −60 is required for basic promoter function.

We have previously reported that, in Jurkat cells, the region required for PMA/A23187 activation spans positions −73 to −95 containing CLE2 and a GC box, which are also found in IL-3 gene. This CLE2/GC box motif also mediates the response to $p40^{tax}$ (Miyatake *et al.*, 1988b). The CLE1 motif (positions −99 to −108), which is conserved in the 5′ flanking region of IL-3 and other lymphokine genes, also responds to $p40^{tax}$ stimulation. Both CLE1 and CLE2/GC box motifs acted like enhancers because they were functional regardless of this orientation. The results described in this paper demonstrate that E2 protein, like $p40^{tax}$, strongly activated the basic promoter through CLE2/GC boxes in both Jurkat and CV1 cells either by itself or in co-operation with PMA and A23187. The GC box alone responded weakly to E2 protein and to $p40^{tax}$ even in combination with PMA/A23187. Furthermore, both E2 protein and $p40^{tax}$ activate CLE1 in CV1 cells. These results suggest that E2 protein and $p40^{tax}$ share common features in their mechanism of action. This notion was further substantiated by the fact that E2 protein activates the SV40 early promoter through the NK-κB binding motif which mediates the response to $p40^{tax}$ in Jurkat cells (Miyatake *et al.*, 1988; T.Heike *et al.*, in preparation). It is tempting to

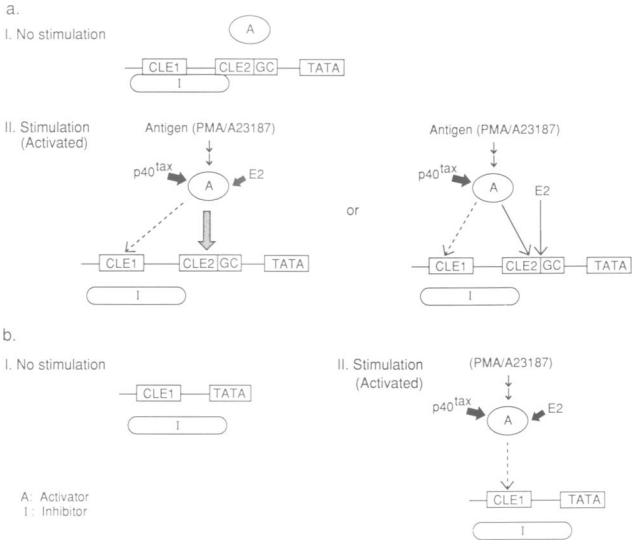


Fig. 4. Model of GM-CSF gene activation by viral *trans*-activators.

speculate that E2 protein and $p40^{tax}$ activate some cellular component(s) involved in the signal transduction pathway, which results in the activation of lymphokine genes in the absence of extracellular stimuli. However, it should be noted that there are several differences between the mode of action of E2 protein and that of $p40^{tax}$. For example, $p40^{tax}$ activated pmGMCAT-96(72−62) lacking the sequence −72 to −60 as efficently as the parent pmGMCAT-96, which had the entire −96 to −60 sequence. In contrast, E2 protein activated the former construct far less efficiently than the latter in Jurkat cells. These results may imply that, although E2 protein and $p40^{tax}$ share a common activation mechanism largely through the CLE2/GC box, the E2-dependent pathway and $p40^{tax}$-dependent pathway may not be identical. Among other viral *trans*-activators, adenovirus-encoded E1A protein activated the transfected mouse GM-CSF promoter in both Jurkat and CV1 cells (Miyatake *et al.*, 1988a). Mapping of the sequences that respond to E1A protein indicated the presence of multiple DNA regions (T.Heike, unpublished results). Even pmGMCAT-60 could be activated by E1A protein, suggesting that the mechanism of action of E1A protein is distinct from that of E2 protein or $p40^{tax}$.

Our results indicate that the sequence upstream of position −113 is inhibitory to PMA/A23187 or E2 protein activation in both Jurkat and CV1 cells. The inhibitory effect was much stronger in CV1 cells than in Jurkat cells. However, the same region did not inhibit activation by $p40^{tax}$. It is possible that some inhibitor(s) are present which interact with the sequence upstream of −113, thereby inhibiting the response to PMA/A23187 or E2 protein. This inhibitor may be removed by $p40^{tax}$ activation or by a combination of PMA/A23187 and E2 protein activation (Figure 4a). Interestingly, however, and contrary to our expectation, the sequence between positions −113 and −96 (CLE1), which inhibited activation of the CLE2/GC box by E2 protein or by PMA/A23187 in the original context, was activated by E2 protein, like $p40^{tax}$, when linked to the basic promoter sequence downstream of −60 (Figure 4b). These results imply a complexity of DNA−protein interactions that may occur in the upstream regions of the GM-CSF promoter.

Our finding that E2 protein and $p40^{tax}$ activate the

GM-CSF promoter through common DNA motifs raises an interesting question regarding the mechanism of the action of E2 protein, which is known to activate its own viral promoter as a sequence-specific DNA binding protein (Howley, 1987). Neither the CLE2/GC box nor CLE1 motif in the GM-CSF promoter have any homology with the consensus motif, $ACCN_6GGT$, of LCR, which directly interacts with E2 protein. Moreover, another difference between BPV-LCR and the GM-CSF promoter in their response to E2 protein is the sensitivity to PMA/A23187 stimulation. Although PMA/A23187 greatly enhanced E2 protein-dependent activation of the GM-CSF promoter, no significant effect was seen with LCR (data not shown). These results suggest that the mechanism of activation by E2 protein of the GM-CSF promoter is different from that of BPV-LCR. Since the CLE2/GC box in the GM-CSF promoter and the NK-κB binding motif in the SV40 promoter have no homology with the consensus $ACCN_6GGT$ motif, E2 protein may activate these sequences without involving direct DNA binding. We would like to speculate that E2 protein, like $p40^{tax}$, interacts with some nuclear protein(s) that has an ability to interact with the CLE2/GC box or CLE1 motif. This protein(s) may be a downstream component in the signal transduction pathway involving protein kinase C and Ca^{2+}. However, it is still possible that E2 protein directly but weakly binds to the CLE2/GC box or CLE1 motif to activate the GM-CSF promoter. Our results indicate that the E2-TR protein, a transcriptional repressor of E2 which shares a common C-terminal region and inhibits E2 *trans*-activation by competing for the same site on DNA (Lambert *et al.*, 1987; McBride *et al.*, 1988), inhibited the action of E2 protein on GM-CSF promoter (data not shown). Furthermore, E2-TR protein also inhibited $p40^{tax}$ action on the GM-CSF promoter in Jurkat cells (data not shown). At present, it is not clear whether E2-TR inhibits the activation step by binding directly to regulatory elements of the GM-CSF promoter, by interacting with a target protein which is common to E2 protein and $p40^{tax}$ or by inactivating E2 or $p40^{tax}$ protein through formation of hetero oligomers. More detailed analysis of proteins that recognize the regulatory elements in the GM-CSF promoter will be needed to understand the mechanisms of action of E2 protein and $p40^{tax}$ and their relationship with the signal transduction pathway in T cells and fibroblasts. In order to examine whether these *trans*-activators regulate the endogenous gene in the same manner as in a transient transfection assay, we measured the induction of endogenous GM-CSF gene by $p40^{tax}$ or E2 expressed from pcDSRα-pX or pcDSRα-cE2 transiently introduced into Jurkat cells. We detected only low levels of human GM-CSF induction (2 pg/ml) by E2 protein, although $p40^{tax}$ showed a reasonably high induction (40 pg/ml) (data not shown). Low induction by E2 protein could be explained by severe inhibitory effects of 5′ upstream sequences on the activation of the GM-CSF gene, as shown in this paper. These results suggest that expression of endogenous genes correlated well with expression of the transfected genes.

It is widely accepted that skin plays an important part in the immune system in addition to serving as a protective barrier. Recently several groups demonstrated that keratinocytes, where papilloma virus can propagate, secrete various cytokines such as IL-1α, IL-1β (Kupper *et al.*, 1986; Bell *et al.*, 1987), IL-3 (Luger *et al.*, 1985) and GM-CSF (Coleman *et al.*, 1987), and may act as important immunoregulatory cells through the secretion of cytokines. However, the physiological significance of E2 action on lymphokine genes during papilloma virus infection and transformation remains to be elucidated.

Note added

Haugen *et al.* (1988) have demonstrated that the N-terminal domain of E2 protein activates heterologous promoters without E2P ($ACCGN_4CGGT$). We have also shown that truncated E2 protein having 273 N-terminal amino acids has the ability to activate the CLE2/GC box of GM-CSF gene. Haugen *et al.*'s observations support our notion that the E2 protein activates the GM-CSF gene without involving binding to the E2 consensus sequence.

Materials and methods

Plasmid construction

pcDSRα-pX was constructed as previously described (Miyatake *et al.*, 1988a). E2 cDNA clone #198 (Yang *et al.*, 1985) was kindly provided by Dr P.Howley. To construct pcDSRα-cE2, which expresses E2 protein at a high level, E2 cDNA was placed downstream of a strong promoter, SRα, by replacing a *Bam*HI fragment containing E2 cDNA with that of pcDL-SRα296 (Takebe *et al.*, 1988). pcDSRα-cE2(Nar) was constructed by cleaving pcDSRα-cE2 with *Nar*I (at position 2944 of the BPV map; Chen *et al.*, 1982) followed by filling-in with DNA polymerase I large fragment and ligation. Construction of pmGMCAT-2400, 1100, 740, 433, 226, 113, 96, 84, 72, 60 or 19 was as previously described (Miyatake *et al.*, 1988b). pmGMCAT-32 was constructed by replacing the −60 to −19 fragment of pmGMCAT-60 with a synthetic oligonucleotide covering positions −32 to −19. p113-96s, p113-85s, p95-85s, p95-73s or p84-73s was constructed by introducing a synthetic oligonucleotide covering positions −113 to −96, −113 to −85, −95 to −85, −95 to −73 or −84 to −73 respectively of the GM-CSF gene into a unique *Bgl*II site of pmGMCAT-60, which is placed upstream of the 5′ flanking region of the GM-CSF gene, in either the sense or antisense orientation. pmGMCAT-96(72−62) was constructed by replacing the sequence −72 to −62 with TCTAGAGATCT using a synthetic oligonucleotide. pmGMCAT-96(54−43) and pmGMCAT-96(43−32) were constructed by replacing sequences at −54 to −43 and −43 to −32 with CTCGAGGCTAGC and ATCGATGGTACC respectively. pBPVLCR-CAT is the same structure as p407-1 (Spalholz *et al.*, 1985). pmGMLuc-96 was constructed by replacing the CAT gene (*Hin*dIII−*Bam*HI) of pmGMCAT-96 with the luciferase gene (*Hin*dIII−*Bam*HI) of L-AΔ5′pJD206 (de Wet *et al.*, 1987). Lymphokine and CAT gene fusion plasmids, pmGMCAT-226, pmIL-2CAT, pmIL-3CAT, pmIL-4CAT and phIL-5CAT contain 226, 2300, 609, 800 and 506 bp upstream sequences respectively, and have been described previously (Miyatake *et al.*, 1988b).

Cell culture

Cell lines used were Jurkat, a human T cell leukemia cell maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, and CVI, an African green monkey kidney fibroblast maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum.

Transfection and CAT assays

A DEAE−dextran method was used for transfections. For Jurkat cells, 5×10^6 cells, washed in PBS (phosphate-buffered saline) were resuspended in 1 ml of PBS containing 0.5 mg/ml DEAE−dextran and 10 μg of DNA and incubated for 25 min at room temperature. After 25 min, the cells were resuspended in tissue culture medium containing 0.1 mM chloroquine diphosphate and incubated for 1 h and then for an additional 40 h in normal medium. For CV1 cells, 1×10^6 cells were plated on a 100-mm tissue culture plate for 24 h before transfection. Cells, washed once with serum-free medium, were incubated in 5 ml of serum-free medium containing 0.4 mg/ml DEAE−dextran and 10 μg of DNA for 4 h in medium containing 0.1 mM chloroquine and 2% fetal calf serum for 2 h, and then in 10% serum containing DMEM medium for 72 h. For activation, cells were incubated with 30 ng/ml PMA and 1 μM A23187 for 8 h and were harvested for chloramphenicol acetyltransferase (CAT) assays. Cell extracts were prepared by three cycles of freezing and thawing and CAT assays

were performed as described (Miyatake *et al.*, 1988a). CAT enzyme activity was measured as percentage conversion of [^{14}C]chloramphenicol to 1- or 3-acetylated chloramphenicol after a 6-h incubation at 37°C with 30 or 50 μg (Jurkat), or 300 μg (CV1) of cell extdracts as described previously (Miyatake *et al.*, 1988a).

Luciferase assay

Luciferase activity was assayed as described previously (Arai *et al.*, 1989).

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