Activation of T cell-derived lymphokine genes in T cells and fibroblasts: effects of human T cell leukemia virus type I p40^x protein and bovine papilloma virus encoded E2 protein

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ABSTRACT

The effects of p40^x, a product of an human T cell leukemia virus type I, on the activation of lymphokine genes were examined. The mouse GM-CSF and IL-3 genes were activated by cotransfection with a pX containing plasmid both in Jurkat and CV1 cells. Mouse GM-CSF gene was also activated by phytohaemagglutinin A (PHA)/phorbol myristate acetate (PMA) or PMA/calcium ionophore A23187 stimulation. The 5'-flanking region of the mouse GM-CSF gene which is required for activation by pX or mitogen was mapped within 226 bp upstream from the transcription initiation site. Action of pX was not restricted to T cells. pX activated exogenously added GM-CSF, IL-2, IL-3 and IL-4 genes in fibroblasts. Activation of the GM-CSF gene in fibroblasts appears to require the same regulatory region as in T cells. Similar results were obtained using bovine papilloma virus encoded E2 protein. We propose that pX or E2 protein, both in T cells and fibroblasts, activates cellular component(s) in the signal transduction pathway which results in the activation of lymphokine genes in the absence of extracellular stimuli.

INTRODUCTION

T cells coordinately express a battery of lymphokines such as IL-2, IL-3, IL-4, IL-5, GM-CSF and IFN- γ when the T cell antigen receptor recognizes antigen and a major histocompatibility complex (MHC) molecule presented on antigen presenting cells. It has been proposed that the signal, generated by interaction of antigen and MHC with the T cell receptor, is transmitted to the cytoplasm via stimulation of phosphatidyl inositol turnover resulting in increased concentrations of cytoplasmic free calcium and activation of protein kinase C (1). PMA and calcium ionophore can substitute for the antigen stimulus and directly activate production of lymphokines. Activation of lymphokine genes initiated by antigen or lectin stimulation is temporal and is regulated at the transcriptional level.

Some lymphokine genes are expressed only by certain T cell subsets. For example, two types of helper T cell clones have been recognized in the mouse on the basis of a differential pattern of lymphokine production. Both types (T_H1 and T_H2) produce IL-3 and GM-CSF, whereas IL-2 and INF- γ are produced by

T_H1 cells but not by T_H2 cells. In contrast, IL 4 and IL-5 are produced by T_H2 cells but not by T_H1 cells. However, results from somatic cell hybrids generated by fusion of T_H1 and T_H2 cells indicate that the expression of T_H1-specific and T_H2-specific lymphokines is not always mutually exclusive (2). Unlike the mouse system, there is no clear separation of human clones into two subsets and different modes of activation such as antigen, lectin or anti-CD2 stimulation can result in both qualitative and quantitative differences in lymphokine production (for a review see reference 3).

In order to study the molecular mechanisms that lead to activation of lymphokine genes, we have determined the genomic sequences encoding mouse IL-3 (4), mouse and human GM-CSF (6) and mouse (7) and human IL-4 (8). Many lymphokine genes including GM-CSF, IFN- γ , IL-2, IL-4 and IL-5 exist as single copies in the haploid genome and are composed of 4 exons and 3 introns whereas IL-3 (4,9,10), IL-6 (11) and G-CSF (12) are composed of 5 exons and 4 introns. 5' flanking regions of GM-CSF (5,6), IL-2 (13,14), IL-4 (7,8) or IL-3 genes (4,9,10), which are highly conserved between mouse and human extending 200 to 500 bp upstream of TATA boxes, may be important for regulated expression during T cell activation. In contrast, there is no obvious homology among different lymphokine genes except for about a 10 bp consensus sequence which is present in the 5' flanking region of several lymphokine genes (5,6). Although IL-2 and IL-4 are expressed in different T cell subsets in the mouse system, the two genes share significant sequence homology in the regions covering more than 200 bp upstream from "TATA" box (3,7).

Human T-cell leukemia virus type I (HTLV-I), etiologic agent of adult T cell leukemia (ATL) (15), transformed T cell lines were shown to constitutively produce various lymphokines such as GM-CSF (16), IFN- γ (17), IL-1 α (18), ATL derived factor (ADF) (19), IL-5 (20) and IL-6 (21) and to express elevated levels of the low affinity form of the IL-2 receptor (Tac antigen) (22). These observations suggest a possible relationship between HTLV-I infection and T cell activation. The genome of HTLV I contains the "pX" sequence in addition to gag, pol and env genes found in replication competent retroviruses. The "pX" sequence encodes for three proteins, p40 $\times$, p27 $\times$ -iii and p21 $\times$ -iii from overlapping open reading frames. p40 $\times$ (pX) acts as a positive transcriptional regulator of the HTLV I LTR promoter in various mammalian cell lines (for a review see reference 15). Therefore, it has been speculated that p40 $\times$ may also activate certain cellular genes which are activated in antigen stimulated T cells. In fact, both HTLV I- and HTLV II-

encoded pX activated IL-2 and IL-2 receptor genes in the Jurkat human T cell leukemia line (23-25).

We found that pX can activate exogenously added lymphokine genes (26) and that the bovine papilloma virus (BPV) encoded E2 protein also activates the mouse GM-CSF gene (27). This paper describes the effect of HTLV I pX and other viral transactivators on the expression of various lymphokine genes in transient cotransfection experiments. Our results, which indicate that the activation of lymphokine genes by pX is neither T cell-specific nor species-specific, strongly suggest that pX promotes the expression of transiently introduced lymphokine genes by activating a pathway common to T cells and fibroblasts.

MATERIALS AND METHODS

Cell lines.

The cell lines used were; Jurkat, an human T cell leukemia line (supplied by R. Levy, Stanford University); UC, an EBV transformed human B cell line (supplied by H. Hayakawa, Stanford University); MD13-5.1, a mouse T cell clone (obtained from T. Mosmann, DNAX); BW5147, a mouse thymoma line (obtained from A. Zlotnik, DNAX); HT-2, a mouse IL-2 dependent T cell line (gift from S. Strober, Stanford University); HeLa, human epitheloid carcinoma line; M1, SV40 transformed human skin fibroblast line; Ltk⁻, a mouse subcutaneous fibroblast line; CV1, African green monkey kidney cell line, monolayer fibroblast-like cell. Lymphocyte cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 0.05 mM 2-mercaptoethanol. Recombinant mouse IL-2 was added to grow MD13-5.1 and HT-2. Every two weeks, MD13-5.1 was stimulated by chicken red blood cells in the presence of irradiated Balb/C mouse spleen cells. Ltk⁻ and CV1 cells were maintained in Dulbecco's modified Eagle (DME) medium and HeLa cells were maintained in minimum essential medium (MEM) with 10% FCS.

Plasmid construction.

A 3.6 kb XmnI-BalI fragment containing the entire mouse GM-CSF gene was inserted between HincII and BamHI site of pUC13 plasmid to yield pGMXB. Construction of pMGM12-4 is as described (4). A 636bp AvaII fragment of the mouse IL-3 gene (4) (positions -609 to +27, with respect to the cap site), 253 bp StuI-MstII (positions -226 to +27) or 87 bp BstEII-MstII (positions -60 to +27) fragments of the mouse GM-CSF gene (6), and a 474 bp SacI fragment of human GM-CSF gene (positions -458 to +16), mouse IL-2 gene (positions--2300 to

+40), human IL-3 gene (positions --1100 to +44) and human IL-5 gene (positions -506 to +1) were inserted into the HindIII site of pSVOCAT (29) to yield pmoIL-3-CAT, pmoGM-CSF-CAT-226, pmoGM-CSF-CAT-60, phuGM-CSF-CAT, pmoIL-2-CAT, phuIL-3-CAT and phuIL-5-CAT. phuIL2-CAT was kindly supplied by J. Crabtree, Stanford University. The SV40 promoter region of pSV2CAT plasmid was replaced with an approximate 2.3 kb SacI-EcoRV fragment of the human IL-4 gene (positions about -2300 to -12) to yield phuIL-4-CAT and 800 bp HindIII-BglIII fragment of the mouse IL-4 gene (7) and 57 bp of synthetic oligonucleotides contained transcription initiation site (position about -800 to +36) to yield pmoIL-4-CAT. pHTLVI-CAT is identical to pLTR-CAT (30). pSV2CAT and pA₁₀-CAT are gifts from B. Howard (NIH). The structures of plasmids carrying transactivators are shown in Figure 1. To achieve high level expression of pX protein, a BamHI-NdeI fragment of pMTPX (31) was placed downstream of a strong promoter, SR α (28), to yield the pX plasmid, pcDSR α -pX. A plasmid carrying BPV-encoded E2 clone (pcDSR α -E2) was constructed by inserting the E2 open reading frame (ORF) (position 2113 to 4451) of the BPV genome into the EcoRI site of pcDL-SR α 296 (28). An adenovirus E1A 13S cDNA containing fragment (position 493 to 1571) from pMT13S (32) (kind gift from K. Maruyama) was ligated through the EcoRI site of pcDL-SR α 296 to yield E1A 13S expression plasmid (pcDSR α -E1A).

Transfection, CAT, and proliferation assays.

A DEAE-dextran method with modifications was used for transfection (33). Electroporation was used to introduce plasmid DNA into UC cells (43). Cell extracts were prepared by three cycles of freezing and thawing as described (29). CAT enzyme activity was measured after 6-10 hrs incubation at 37°C with 20-400 μ g of cell extracts and was shown by percent conversion of [¹⁴C]chloramphenicol to 1- or 3-acetylated chloramphenicol. For mouse GM-CSF and IL-3 assays, a subline of mouse NFS60 (provided by D. Rennick, DNAX) and mouse MC/9 mast cell line were used. Colorimetric proliferation assays were performed as described (33).

RESULTS

pX activates whole mouse GM-CSF and IL-3 genes in human T cell leukemia line Jurkat and CV1 monkey kidney fibroblasts.

pGMXB plasmid, which contains about 1.1 kb of 5' flanking region and the entire coding region of the mouse GM-CSF gene, and pcDSR α -pX, which allows high level expression of pX (Fig. 1A), were cotransfected into human T cell leukemia line Jurkat or CV1, monkey kidney fibroblasts, and the supernatants

Fig. 1 A. Structure of pcDSRα-pX which contains the pX gene. SRα promoter contains the SV40 early region promoter (SV40 ori) and R-U5 of HTLV I LTR, HTLV I pX gene coding region; hatched box, and poly A represents SV40 late transcription termination region. B. Structure of pcDSRα-E2 which contains BPVI E2 open reading frame (ORF) represented by hatched box. C. Structure of pcDSRα-E1A (13S) which contains adenovirus E1A 13S cDNA. Detailed description is given in "Materials and Methods".

were assayed for mouse GM-CSF activity. Since activities of mouse and human GM-CSF are species-specific (33), mouse GM-CSF activity directed by the transfected gene could be selectively assayed in the human background. The supernatant cotransfected with both the mouse GM-CSF gene (pGMXB) and pX showed significantly higher levels of mouse GM-CSF activity and its activity was comparable to that obtained by transfection with SV40 promoter driven mouse GM-CSF cDNA, E1-11 in the presence of pcDSRα-pX (Table I column A). Likewise, high levels of mouse IL-3 activity were detected in the supernatant of Jurkat cells when pMGM12-4 which contains the entire mouse IL-3 gene was cotransfected with pX plasmid whereas almost no IL-3 activity could be detected without pX plasmid (Table I column A). Similarly, both mouse IL-3 and GM-CSF genomic sequences were activated in CV1 cells by cotransfection with pX plasmid (Table I column B). These results indicate that pX activates

TABLE I
Biological activity in supernatants of cells
transfected with whole mouse GM-CSF or IL-3 genes

Transfected plasmid	A Jurkat		B CV1	
	-pX	+pX	-pX	+pX
Activity (units/ml) mouse GM-CSF				
mock	<40	<40	<40	<40
pGMXB	<40	160	<40	160
E1-11 (GM-CSF cDNA)	<40	100	ND	ND
mouse IL-3				
mock	<40	<40	<40	<40
pλMGM12-4	<40	160	<40	640
b9 (IL-3 cDNA)	60	700	320	320

Cells were transfected with pGMXB carrying the mouse GM-CSF gene or pλMGM12-4 carrying the mouse IL-3 gene with or without pcDSRα-pX.

E1-11 (33) and b9 (4) are cDNA clones whose expression is under control of SV40 promotor

GM-CSF and IL-3 activities were determined using NFS60-GM and MC/9 cells, respectively.

transiently transfected mouse GM-CSF or IL-3 genes in Jurkat cells and CV1 cells.

5' flanking region of GM-CSF gene is required for activation by pX in Jurkat cells.

To determine the mouse GM-CSF genomic region required for pX action, CAT gene fusion plasmids, with 5' flanking region of the mouse GM-CSF gene having different 5' end points and a fixed 3' end point at 27 bp downstream of the transcription initiation site, were introduced into Jurkat cells. pX dependent induction of CAT activity was observed with 5' flanking sequence of the GM-CSF gene shortened to -226, although pX independent expression increased as we shortened the 5' sequence (Table II). Jurkat cells are known to be activated by treatment with PMA/PHA or PMA/calcium ionophore, A23187

TABLE II

Effect of cotransfected pX gene on the expression of CAT activity of mouse GM-CSF promoter-CAT fusion plasmids having various 5' end points of 5' flanking region

5' end (bp)	pX gene	CAT activity % conversion	Stimulation fold
-2400	-	0.04	35.0
	+	1.40	
-1100	-	0.07	47.1
	+	3.30	
-750	-	0.08	87.5
	+	7.00	
-400	-	0.10	95.0
	+	9.50	
-226	-	0.21	52.9
	+	11.10	
-60	-	0.39	1.0
	+	0.38	
-19	-	0.50	0.9
	+	0.44	

Mouse GM-CSF promoter-CAT fusion plasmids having various 5' deletion end points were transfected into Jurkat cells with or without pcDSR α -PX plasmid containing pX gene. CAT activity in the cell extracts was determined as described in "Materials and Methods". The number represents the nucleotide length of the insert from transcription initiation site.

(34). Exposure of Jurkat cells to these stimulants also induced CAT activity (Fig. 2). However, enhancement by pX was much stronger than PHA/PMA stimulation. Combination of both pX and PHA/PMA stimuli further augmented CAT activity. These results indicated that the 5' flanking region of mouse GM-CSF gene contains a nucleotide sequence required for activation by pX or mitogens. Similarly, the human GM-CSF promoter was strongly enhanced by pX and was augmented by PHA/PMA stimulation (Table III). Activation of the mouse GM-CSF gene in Jurkat cells is dependent on the expression of p40^x since mutant pX plasmid which expresses only p40^x by deleting ATG start codon on frame III in the pX cDNA clone can support activation of mouse GM-CSF gene. In contrast,

Fig. 2 Expression of CAT activity of mouse GM-CSF-CAT fusion plasmid in Jurkat cells. Plasmids pmoGM-CAT-226 or pmoGM-CAT-60 were transfected with or without pcDSR α -pX. 40 hrs after transfection, half of the transfected cells were stimulated with PMA (50 ng/ml) and PHA (27 μ g/ml) for 8 hrs. After incubation with 300 μ g of extract for 6 hrs at 37°C, conversion of [14 C]chloramphenicol to 1- or 3-acetylated chloramphenicol (e.g., upper two spots) was measured.

mutant plasmids which express p21 α -iii or p21 α iii and p27 α -iii, constructed by introducing a stop codon into frame IV in the pX cDNA clone (24), failed to activate the mouse GM-CSF gene (data not shown). Neither pX nor PHA/PMA treatment could stimulate CAT activity of pmoGM CSF-CAT-60, which contains 60 bp upstream of the transcription initiation site, in Jurkat cells (Fig. 2, Table II). These results indicated that the 5' boundary of the cis-acting element required for pX activation and PHA/PMA stimulation is located between positions -226 and -60.

TABLE III

Effect of cotransfected pX gene on the expression of CAT activity of various lymphokine promoter CAT fusion plasmids

PROMOTER CAT PLASMID	JURKAT					CV1					LTK-				
		A	B	C	D		A	B	C	D		A	B	C	D
mGCMCSF	3)	1.4	32.1	85.4	80.7	4)	0.69	1.6	2.7	9.0	4)	0.19	0.21	2.1	N.D.
hGCMCSF	1)	0.9	0.9	12.8	32.0	4)	0.28	0.25	0.44	2.3	1)	0.44	N.D.	2.81	N.D.
mIL-3	1)	0.85	0.5	9.15	17.5	4)	0.64	0.45	3.0	4.5	1)	4.1	4.3	42.6	56.7
hIL-3	1)	0.4	0.4	4.1	15.7	4)	0.2	0.4	3.6	4.6	1)	4.1	4.2	45.9	46.3
mIL-2	3)	0.4	2.8	0.6	N.D.	4)	0.33	0.39	0.47	0.95	1)	0.16	N.D.	0.64	N.D.
hIL-2	3)	0.1	1.4	0.2	4.7	3)	0.09	N.D.	0.11	N.D.	3)	0.01	N.D.	0.1	N.D.
mIL-4	1)	1.0	1.2	1.0	1.0	4)	0.39	0.29	0.46	0.95	1)	0.08	N.D.	0.4	N.D.
hIL-4	3)	0.01	N.D.	0.01	N.D.	4)	0.01	N.D.	0.05	N.D.	3)	0.01	N.D.	2.20	N.D.
hIL-5	1)	0.2	0.2	0.2	0.2	4)	0.6	0.6	0.6	0.6	1)	0.4	0.35	0.7	0.6
HTLV(II)LTR	3)	2.9	N.D.	>80	N.D.	3)	0.15	N.D.	9.3	N.D.	3)	1.0	N.D.	>80	N.D.

PROMOTER CAT PLASMID	UC		HELA		M1		MD 13-5*1		BW5147		HT-2							
	A	C	A	C	A	C	A	C	A	C	A	C						
mGMC5F	3)	0.28	0.28	3)	0.01	32.0	3)	0.01	0.01	3)	1.8	8.3	2)	0.32	0.44	3)	0.05	0.02
mIL-3	3)	0.05	0.03	3)	0.01	9.6	N.D.	N.D.	3)	0.82	2.8	2)	0.15	0.25	3)	0.17	0.23	
hIL-2		N.D.	N.D.	3)	0.02	2.3	3)	0.01	0.01	3)	0.01	0.01	2)	0.01	0.01	3)	N.D.	N.D.
mIL-4	3)	0.01	0.01	3)	0.13	3.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.		N.D.	N.D.	N.D.	N.D.	
hIL-4		N.D.	N.D.	3)	0.01	3.2	N.D.	N.D.	N.D.	N.D.	2)	0.01	0.01	3)	0.01	0.01		
HTLV(I)LTR	3)	0.28	22.0	3)	1.1	>80	3)	1.1	>80	3)	17	>80	1)	1.0	>80	3)	0.29	>80

CAT enzyme activities shown on this table indicate % conversion of [14 C] chloramphenicol to acetylated form. 5 μ g of lymphokine CAT gene fusion plasmids were cotransfected with 5 μ g of pcDSRa-296, which has no cDNA insert, without stimulant (column A), with PMA and A23187 (column B). pcDSRa-pX (5 μ g) was cotransfected with various lymphokine-CAT plasmids without stimulant (column C) and with PMA and A23187 (column D). Detailed transfection conditions are described under "Materials and Methods". For CAT enzyme activity measurement 1) 40 μ g, 2) 100 μ g, 3) 200 μ g, and 4) 400 μ g of cell extracts were used and were incubated for 10 hrs at 37°C. N.D.; not determined.

pX activates the GM-CSF promoter in a wide variety of cells.

In addition to Jurkat cells, mouse GM-CSF-CAT is activated by cotransfected pX plasmid in mouse helper T cell clone MD13-5.1 (Table III). In contrast, the mouse GM-CSF promoter was not activated in human T cell clone HG120 (R.D.M. unpublished, data not shown), mouse thymoma line BW5147 or mouse T cell line HT2 although the HTLV I LTR was strongly activated by pX in all cases examined (Table III). Consistent with the results seen with whole the GM-CSF gene, pX activates pmoGM-CSF-CAT-226 in CV1 cells, indicating that the action of pX is not restricted to T cells. To study further the tissue specificity of the effect of pX, mouse GM-CSF promoter-CAT and pX plasmids were cotransfected into three non T cell human lines. Although the HTLV I LTR promoter was strongly activated by pX in an EBV-transformed B cell line (UC) and SV40 transformed skin fibroblast line (M1) (Table III), the mouse GM-CSF

Fig. 3 Expression of mouse GM-CSF-CAT and mouse IL-3-CAT fusion plasmids in Ltk⁻ (A) and HeLa cells (B). Plasmids pmoGM-CAT-226, pmo-CAT-60 and pmoIL-3-CAT were transfected with or without pcDSR α -pX. For measurement of CAT activity, 300 μ g of cell extracts were incubated at 37°C for 10 hrs (A) and 200 μ g of cell extracts were incubated for 10 hrs (B) at 37°C.

promoter was not affected by pX. In contrast, in HeLa cells, the mouse GM-CSF promoter was strongly activated in the presence of pX whereas no appreciable expression of CAT activity was observed without pX (Fig. 3 right, Table III). To further test the species specificity of the host cells in the activation of the GM-CSF promoter by pX, mouse fibroblast lines were used for transfection experiments. In Ltk⁻ mouse fibroblast cells, mouse or human GM-CSF promoter-CAT fusion gene were activated in the presence of pX whereas CAT activity was not appreciably expressed without pX (Fig. 3 left, Table III). These results clearly show that activation of an exogenously introduced GM-CSF promoter by pX is not restricted to T cells nor to human cells. Similar to results with

Jurkat cells, activation of CAT activity by pX was not observed in HeLa, CV1 (data not shown) and in Ltk⁻ cells (Fig. 3), when pmoGM-CSF-CAT-60 was cotransfected with pX plasmid. These results indicate that the 5' flanking region covering positions -226 to -60 is essential for activation of the mouse GM-CSF gene in CV1, L, HeLa and Jurkat cells.

Calcium ionophore and PMA augment pX-dependent activation of the mouse GM-CSF gene in CV1 fibroblasts.

In Jurkat cells, certain lymphokine genes were activated by pX alone or by PHA/PMA (A23187/PMA) and the combined action of both were more than additive (Table III). To examine whether the mouse GM-CSF promoter could be affected by intracellular calcium and protein kinase C in cells other than T cells, the plasmid pmoGM-CSF-CAT-226 was transfected into CV1 cells with or without pX plasmid and the cells were stimulated with PMA and A23187. As shown in Figure 4, A23187 and PMA alone weakly enhance the level of pX-dependent activation of the mouse GM-CSF promoter. The combined action of both A23187 and PMA further enhanced the level of pX-dependent activation of the mouse GM-CSF promoter whereas no effect was seen without added pX.

Effect of pX on the IL-3 promoter.

The effects of pX or PHA/PMA on the activity of various lymphokine promoters were examined by transfecting Jurkat cells or fibroblasts with a plasmid carrying 5' flanking regions of lymphokine genes placed upstream of the CAT gene. In Jurkat cells, pX stimulated the mouse IL-3 promoter and the human IL-3 promoter about 10 fold (Table III), whereas PHA/PMA or PMA/A23187 did not appreciably affect these promoters (Table III). However, PMA/A23187 enhanced pX-dependent activation of mouse and human IL-3 promoters. pX also activated the mouse IL-3 promoter in mouse T cell clone MD13-5.1 and mouse and human IL-3 promoters in cells other than T cells such as HeLa cells, L cells and CV1 cells. All these data suggest that pX activation of the IL-3 promoter is not restricted to human cells or T cells.

Effects of pX on the IL-2 promoter.

In contrast to the strong activation of GM-CSF (as much as 60 fold) or IL-3 promoters by pX, human and mouse IL-2 promoters were activated only two fold by pX in Jurkat cells (Table III) (24). However, the human IL-2 promoter was activated as much as 15 fold by PHA/PMA (Table III). The human IL-2 promoter was activated by pX in HeLa cells but was only slightly affected by pX in Ltk⁻ cells.

Effect of pX on IL-4 or IL-5 promoters.

Activation of mouse or human IL-4 promoters by pX (Table III) or anti

Fig. 4 Effect of calcium ionophore A23187 and PMA on the expression of mouse GM-CSF-CAT fusion plasmid cotransfected with pX plasmid in CV1 cells. pmoGM-CAT-226 was transfected into CV1 cells either with pcDSRα-pX or pcDL-SRα296 carrying no cDNA insert (28). Transfected cells were stimulated with PMA (30 ng/ml) and A23187 (0.2 - 1.0 μM). CAT activity was measured by incubating for 6 hrs with 300 μg of cell extracts. Percent conversion of [¹⁴C]chloramphenicol to 1- or 3-acetylated chloramphenicol is shown at the bottom.

CD3 antibody/PMA treatment was under detection (data not shown). However, the human IL-4 promoter was activated by PMA/A23187 in Jurkat cells (8). The combination of pX and PMA/A23187 was not stimulatory. Mouse and human IL-4 promoters were activated by pX in L cells and HeLa cells. In contrast, the Ltk⁻ cell is the only cell line we found in which pX or PMA/A23187 slightly activated the human IL-5 promoter (Table III).

Effect of pX on SV40 or other viral promoters.

In the course of transfection experiments, we noticed that the SV40 promoter was also activated strongly by pX in Jurkat cells but not in CV 1 cells (Table I and Fig. 5). This activation was not observed with other viral promoters, such as Rous sarcoma virus LTR, herpes simplex virus thymidine

Fig. 5 Effect of HTLVI pX (A) or BPV-encoded E2 protein (B) on the expression of SV40 early region promoter in Jurkat cells. (A) pcDSRα-pX was cotransfected either with pSV2CAT or pSVOCAT and 300 μ g of cell extracts were incubated for 6 hrs for CAT assay. (B) pcDSRα-E2 was cotransfected with pSV2CAT and incubated 7 hrs with 100 μ g of cell extract for CAT enzyme measurement.

kinase, mouse mammary tumor virus LTR and SRα promoter. We recognized that the plasmid pSV2CAT carrying the SV40 early region promoter fused to the CAT gene, which is inefficient in Jurkat cells, is strongly activated by pX or PHA/PMA treatment. We then introduced plasmid pA10CAT carrying an enhancer-deleted SV40 early region promoter. This plasmid failed to respond to either pX or PHA/PMA (Fig. 5) although combination of both pX and PHA/PMA is slightly stimulatory. These results suggest that the cis element required for pX or PHA/PMA stimulation is functionally equivalent to the enhancer sequence in SV40 promoter.

Bovine papilloma virus encoded E2 protein or adenovirus E1A protein activates the mouse GM-CSF gene.

We examined whether other viral transactivators such as BPV-encoded E2 protein (35), adenovirus-encoded E1A protein or EBV-encoded EBNA1 protein

Fig. 6 Effect of HTLVI pX, BPV-encoded E2 and adenovirus encoded E1A protein on the expression of mouse GM-CSF-CAT fusion plasmid in Jurkat cells. For CAT enzyme assay, 300 μ g of cell extract was incubated at 37°C for 7 hrs with [C^{14}]chloramphenicol and the formation of acetylated chloramphenicol was measured.

(36), which can activate certain genes of their own genomes, can activate lymphokine genes. We found that monkey CV1 cells transfected with the whole mouse GM-CSF gene and a plasmid carrying the E2 gene produce mouse GM-CSF activity in culture supernatant in a manner similar to pX (data not shown). This was further confirmed by using a mouse GM-CSF-CAT fusion plasmid. As shown in Figure 6, pmoGM-CSF-CAT-226 was activated by cotransfected plasmids carrying pX, E2 ORF or E1A cDNA sequences whereas EBNA1 cDNA did not have any measurable effect in Jurkat cells (data not shown). In contrast, mouse GM-CSF-CAT-60 was not stimulated by E2 (data not shown). E2 protein was also capable of activating the SV40 early promoter (Fig. 5) in Jurkat cells although E2 alone shows much weaker activity compared to pX. Activation of pmoGM-CSF-226 promoter in CV1 or Jurkat cells by E2 protein was strongly enhanced by

PMA/A23187. Certain nuclear onco genes such as c-fos, c-myc, and p53 are activated concomitant with the expression of lymphokines during T cell activation. However, none of these appreciably affected the expression of mouse GM-CSF gene by themselves or in combination with PMA/A23187 in Jurkat cells or in L cells (data not shown).

DISCUSSION

The results described in this paper indicate that the HTLV I-encoded pX gene activates GM-CSF, IL-3 and IL-2 promoters exogenously introduced into Jurkat cells and fibroblasts in transient transfection assay and that 5' flanking regions of these lymphokine genes are required for pX activation. Deletion analysis showed that the cis acting element required for pX activation of mouse GM-CSF is localized within 226 bp upstream of the transcription initiation site. It appears that sequences required for activation by PHA/PMA or PMA/A23187 are mapped on the same region. A plasmid carrying only 60 bp upstream from the transcription initiation site failed to respond to either pX or PHA/PMA. These results suggest that the 5' boundary of the cis acting element required for pX activation or PHA/PMA stimulation is localized between 226 bp and 60 bp upstream of the transcription initiation site. As we pointed out previously (6), several patches of regions homologous to the IL-3 gene were found in the GM-CSF gene at positions -129 to -123 (element A), -108 to -99 (element B), -94 to -88 (element C) and -81 to -76 (element D). Among them, element B is well conserved among several lymphokine genes (5,6,8). Further analysis of the minimum region required for pX or PHA/PMA stimulation will establish whether pX and PHA/PMA activation pathways share exactly the same cis acting element. Chan *et al.* also described the 5' flanking region required for inducible expression of human GM-CSF gene in HTLV I-transformed T cells (16).

We also found that the SV40 early region promoter was activated in enhancer-dependent manner in Jurkat cells by pX, E2 or by PHA/PMA (Fig. 5). Since the SV40 enhancer appears to augment the expression of pmGM-CSF-CAT-60 in response to pX or PHA/PMA stimulation (S. Miyatake, manuscript in preparation), it is likely that the 5' flanking region of the mouse GM-CSF gene covering positions -226 and -60 may contain an enhancer like element which ultimately responds to signals generated by pX or PHA/PMA stimulation. However, no convincing sequence homology was found between the 226 bp mouse GM-CSF 5' flanking region, SV40 enhancer and HTLV I LTR U3 region which contains a pX-dependent enhancer element (37). Therefore, it is unlikely that

Fig. 7 Possible role of pX or E2 protein in signal transduction pathway leading to the activation of transfected lymphokine genes in T cells or in fibroblasts. TcR, T cell antigen receptor. GFR, growth factor receptor, PLC, phospholipase C. PKC, protein kinase C, E, enhancer. In T cells, an antigenic signal recognized by the T cell receptor is transmitted via the CD3 complex and activates phospholipase C which generates intracellular signals (1) and results in the activation of protein kinase C and in increased intracellular calcium ions. In fibroblasts, growth factor-receptor systems such as PDGF or FGF can activate protein kinase C (40,41). Protein kinase C, possibly through protein phosphorylation and along with a Ca^{2+} -dependent step yet to be identified, activates the signal transduction pathway which ultimately activates transcription of transfected lymphokine genes. pX or E2 may interact with component(s) of signal transduction proteins and activate lymphokine genes in the absence of extracellular stimuli. It should be noted that activation of protein kinase C and increase in intracellular calcium ion are sufficient to activate endogenous chromosomal lymphokine genes in T cells (shown by solid arrows) but are not sufficient in fibroblasts (shown by dotted arrows).

transactivation by pX in Jurkat cells is mediated by pX interaction with host protein(s) which mediate PHA/PMA stimulation signals rather than sequence specific direct interaction (Fig. 7).

We observed that the response of exogenously introduced lymphokine genes to pX, PHA/PMA or a combination of both varied from lymphokine to lymphokine in Jurkat cells. Both GM-CSF and IL-3 promoters, which are expressed both in

T_H1 and T_H2 subsets in the mouse system, respond to pX stimulation. However, the mouse GM-CSF gene but not the IL-3 gene is stimulated by PMA/A23187. In contrast, the IL-2 promoter, which is expressed only in the T_H1 subset but not in the T_H2 subset, responds to PMA/A23187 or pX and the combination of both PMA/A23187 and pX promotes more efficient expression. These results may reflect an additional regulatory mechanism which may be unique to each set of lymphokine genes. Further analysis of the cis-acting regulatory element of each lymphokine gene will help to clarify the mechanism of coordinated induction of a set of lymphokine genes during T cell activation.

Some lymphokines or cytokines such as GM-CSF, G-CSF and IL-6 are produced in fibroblasts and expression of their genes is stimulated by IL-1 (38), while the expression of IL-2, IL-3, IL-4, IL-5 genes is restricted to T cells (39).

It is well established that combinations of calcium ionophore and PMA can substitute for antigen stimulus in T cells and lead to the induction of lymphokine genes. The same treatment also activates certain cellular genes such as c-myc and c-fos in T cells and fibroblasts (40,41). These results suggest that a signal transduction pathway, employing protein kinase C and intracellular calcium may activate a set of genes in T cells and fibroblasts. We showed that the mouse GM-CSF promoter exogenously introduced into CV1 fibroblasts was enhanced by PMA and A23187 only when pX is present. The region between positions -226 and -60 of the GM-CSF promoter, which is required for activation in T cells by pX, is also required for activation of the GM-CSF promoter by pX in L cells. These results suggest that pX can activate many lymphokine promoters which have been transiently transfected into T cells or non-T cells through a common transcription activation mechanism that exists in various cell types. Since growth factors such as PDGF activate phospholipase C in fibroblasts and generate signals similar to that of antigen stimulation in T cells (40), we speculate that a set of host proteins, which are activated either by growth signals or by antigen activation signals via a general signal transduction pathway involving protein kinase C and calcium, are present in various tissues and cooperate with pX in the activation of lymphokine genes (Fig. 7).

Among other viral transactivators examined, BPV-encoded E2, which activates long control region (LCR, positions 6958 to 7945) in BPV genome (35) and adenovirus-encoded E1A protein, which activates adenovirus early promoters as well as a number of cellular genes (42) also activated the mouse GM-CSF promoter in fibroblasts and Jurkat cells (Fig. 6). E2 protein seems to

activate the GM-CSF gene in a way analogous to pX. Activation of the GM-CSF promoter by pX and E2 appears to share the same 5' flanking region and actions of both pX and E2 proteins are augmented by PMA/A23187. Since pX does not activate the BPV-LCR sequence, responsive sequence(s) seems to be different in GM-CSF and BPV-LTR. In fact, the E2 binding sequence found in LCR does not have any homology with the GM-CSF regulatory region. In contrast, the regions of the mouse GM-CSF gene required for activation by E1A are distinct from that for E2 or pX (T. Heike, unpublished observation). These results suggest that E1A protein activates the GM-CSF gene by employing a mechanism which is different from those of pX or E2. However, the physiological significance of the stimulation of lymphokine genes by E2 or E1A remains to be determined.

It should be noted that the results described in this paper were obtained using transient transfection experiments. It is not yet clear whether the endogenous lymphokine genes respond to pX similar to the transfected ones. Since pX activates the expression of transiently transfected, as well as endogenous IL-2 receptor (p55) gene in Jurkat cells (24), the expression of some lymphokine genes exogenously introduced into Jurkat cells may be regulated by pX in a manner similar to endogenous lymphokine genes. However, there were a number of instances where pX failed to activate endogenous lymphokine genes or exogenous genes integrated into the chromosome while strongly activating exogenously added genes. For example, although pX activated exogenously introduced GM-CSF promoters (Table III), the endogenous GM-CSF gene was not stimulated by pX (R.D. Malefijt and S. Miyatake, unpublished results) in Jurkat, mouse T cell clone MD13-5.1 or various fibroblast lines. pX also failed to activate the mouse GM-CSF promoter CAT fusion gene integrated into the chromosome in 12 different Jurkat stable transformants tested (S.M. unpublished result). These results may imply that endogenous genes in the chromosome are more tightly regulated and that localization of the genes within the chromosome is crucial for proper response to signals for gene activation. Nevertheless, the transient transfection system described in this paper provides an opportunity to delineate cis acting sequences which are responsible for activation of each lymphokine gene by pX, E2, E1A or PMA/calcium ionophore in different cell types and will help to elucidate the set of signal transduction pathways in T cells and non T cells.

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