

# Regulation of transcription of immunoglobulin germ-line $\gamma 1$ RNA: analysis of the promoter/enhancer

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**Antibody class switching is achieved by recombinations between switch (S) regions which consist of tandemly repeated sequences located 5' to Ig heavy chain constant (C<sub>H</sub>) region genes. RNA transcripts from specific unrearranged or germ-line Ig C<sub>H</sub> genes are induced in IgM<sup>+</sup> B cells prior to their undergoing class switch recombination to the same C<sub>H</sub> genes. Thus, the antibody class switch appears to be directed by induction of accessibility, as assayed by transcription of germ line C<sub>H</sub> genes. For example, IL-4 induces transcripts from the mouse germ-line C $\gamma$ 1 and C $\epsilon$  genes to which it also directs switch recombination. We report here that the 150 bp region upstream of the first initiation site of RNA transcribed from the murine germ-line C $\gamma$ 1 gene, contains promoter and enhancer elements responsible for basal level transcription and inducibility by anti-Ig phorbol myristate acetate (PMA) and for synergy of these inducers with IL-4 in a surface IgM<sup>+</sup> B cell line, L10A6.2 and a surface IgG<sub>2a</sub><sup>+</sup> B cell line, A20.3. Linker-scanning mutations demonstrated that multiple interdependent elements are required for inducibility by PMA and also for synergy with IL-4. Within the 150 bp region are several consensus sequences that bind known or putative transcription factors, including a C/EBP binding site—IL-4 responsive element, four CACCC boxes, a PU box, a TGF $\beta$  inhibitory element (TIE), an  $\alpha\beta$ -interferon response element ( $\alpha\beta$ -IRE) and an AP-3 site. The relationship between transcription regulated by these elements and the regulation of endogenous germ-line  $\gamma 1$  transcripts and switching to IgG<sub>1</sub> is discussed.**

**Key words:** antibody class switch/cell transfection/enhancer/germ-line  $\gamma 1$  RNA/promoter/regulation of transcription

## Introduction

Antibody class switching occurs by DNA recombination mediated by repetitive segments known as the switch (S) regions that lie a few kilobases upstream of each heavy chain constant region (C<sub>H</sub>) gene, except C $\delta$ . A recombined VDJ gene, which encodes the variable region of an antibody heavy chain, is initially associated with the C $\mu$  gene and can be subsequently translocated to a downstream C<sub>H</sub> gene by switch recombination occurring between the S $\mu$  region and the S region of a target C<sub>H</sub> gene.

Class switching is directed by cytokines. IgM<sup>+</sup> splenic

B cells from mice will switch to IgG<sub>3</sub> and IgG<sub>2b</sub> expression after polyclonal stimulation in culture with lipopolysaccharide (LPS). Lymphokines can alter this isotype specificity. For example, the addition of IL-4 to LPS induced spleen B cells stimulates switching to IgG<sub>1</sub> and IgE and suppresses switching to IgG<sub>3</sub> and IgG<sub>2b</sub> (Isakson *et al.*, 1982; Bergstedt-Lindqvist *et al.*, 1988; Coffman *et al.*, 1988; Lebman and Coffman, 1988). Interferon- $\gamma$  (IFN $\gamma$ ) inhibits the switching to IgG<sub>1</sub> and IgE induced by IL-4 (Snapper and Paul, 1987). Addition of transforming growth factor- $\beta$  (TGF $\beta$ ) stimulates switching to IgA (Coffman *et al.*, 1989; Lebman *et al.*, 1990).

The mechanism underlying the choice of isotype remains unclear. Results of many studies support an accessibility model, which holds that the specificity of switching is directed by regulation of the accessibility of C<sub>H</sub> genes and their S regions to recombinase (Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos *et al.*, 1986). Part of the evidence supporting this model is the fact that transcripts of specific unrearranged (germ-line) C<sub>H</sub> genes are induced prior to switching to these same isotypes. For example, IL-4 appears to direct the switching to  $\gamma 1$  and  $\epsilon$  by increasing the accessibility of  $\gamma 1$  and  $\epsilon$  genes, as shown by IL-4 induced transcription of unrearranged  $\gamma 1$  and  $\epsilon$  genes (Rothman *et al.*, 1988; Stavnezer *et al.*, 1988; Berton *et al.*, 1989; Esser and Radbruch, 1989; Gauchat *et al.*, 1990; Severinson *et al.*, 1990). In addition, the I.29 $\mu$  B lymphoma cell line, which switches from IgM to IgA upon treatment with LPS, has hypomethylated and transcriptionally active C $\alpha$  genes; whereas C<sub>H</sub> genes to which I.29 $\mu$  does not switch are hypermethylated and transcriptionally inactive (Stavnezer-Nordgren and Sirlin, 1986). Additional correlative evidence from other systems also indicates that only transcriptionally active C<sub>H</sub> genes are capable of undergoing switch recombination (Yancopoulos *et al.*, 1986; Lutzker and Alt, 1988; Stavnezer *et al.*, 1988; Sideras *et al.*, 1989; Lebman *et al.*, 1990; Severinson *et al.*, 1990).

We hypothesize that the accessibility of C<sub>H</sub> genes to switch recombination enzymes is determined by regulation of their transcription. Therefore, we decided to determine if the DNA region situated 5' to and surrounding the initiation sites for germ-line  $\gamma 1$  RNA functions as a promoter and also to attempt to identify DNA sequences within this region which respond to stimuli that regulate the transcription of germ-line  $\gamma 1$  RNA. We report here that a 150 bp region 5' to the first initiation site of germ-line  $\gamma 1$  RNA (Xu and Stavnezer, 1990), contains several functional interdependent elements which are responsible for basal level expression and inducibility by phorbol myristate acetate (PMA) and for synergy with IL-4 in an IgM<sup>+</sup> cell line, L10A6.2, and in an IgG<sub>2a</sub><sup>+</sup> cell line, A20.3. A 134 bp segment of DNA containing these elements is sufficient to confer inducibility by PMA and by PMA plus IL-4 to a heterologous promoter, that for thymidine kinase, in L10A6.2 cells. Furthermore,

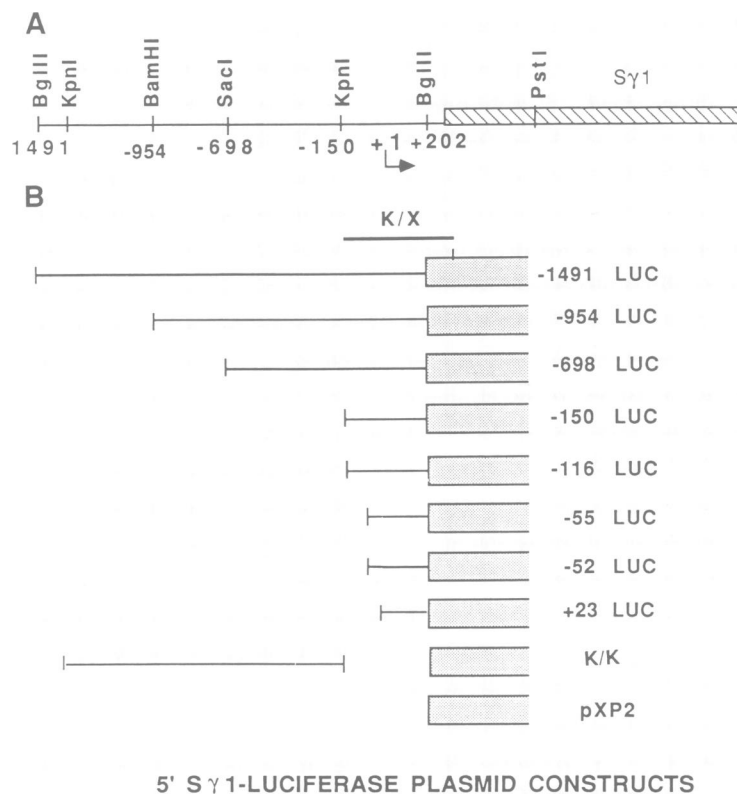
linker-scanning mutations demonstrated that individual elements of this DNA segment function cooperatively, as this segment does not confer inducibility if any one of several individual elements has been mutated.

## Results

### 5'S $\gamma$ 1-LUC plasmids are expressed in L10A6.2 and A20.3 cells

To analyze the promoter for germ-line  $\gamma$ 1 RNA transcripts, we constructed a series of 5'S $\gamma$ 1-LUC plasmids, ligating various lengths of DNA including the  $\gamma$ 1 RNA initiation sites and the 5' flanking segment to the firefly luciferase reporter gene (Figure 1). After screening a number of B and pre-B cell lines by transient transfection experiments, two B lymphoma cell lines, L10A6.2 and A20.3, were found to express 5'S $\gamma$ 1-LUC plasmids. L10A6.2 and A20.3 cells are IgM and IgG<sub>2a</sub> surface positive, respectively (Kim *et al.*, 1979 and data not shown). Figure 2 shows the luciferase activity of cell extracts obtained after transient transfection of  $\gamma$ 1 promoter-luciferase plasmids into four cell lines. The expression pattern of 5'S $\gamma$ 1-LUC in L10A6.2 and A20.3 cells differs. In L10A6.2 cells, -150LUC expresses as much luciferase as -954LUC. Further deletion (-116LUC) reduces the expression of luciferase activity to 23% of that of -954LUC. The +23LUC plasmid [which does not contain the first RNA initiation site but does contain initiation sites for other abundant species of germ-line  $\gamma$ 1 RNAs (Xu and Stavnezer,

1990)] and plasmids containing 55 bp or less 5' to the first initiation site express luciferase equally well as -116LUC. In A20.3 cells, upstream sequences appear to be more important for the function of the  $\gamma$ 1 promoter and sequences between -150 and -116 are less important since -150LUC expresses only ~ 30% of luciferase compared with that of -1491LUC. -116LUC expresses almost as much luciferase as -150LUC. However, the activities of promoters with 55 nt or less are as low as the activities of the pXP2 vector, which contains the luciferase gene without any promoter. These results indicate that 150 bp 5' to the first start site contain almost all the sequences needed for basal expression of the germ-line  $\gamma$ 1 promoter in L10A6.2 cells; however, this segment does lack some sequences important for basal level expression in A20.3 cells. Apparently, at least one transcription factor differs between these two cell lines. Figure 2 also shows that the  $\gamma$ 1 5' flanking segment did not promote luciferase expression in the B cell line, 22A10, a clone of the 1.29 $\mu$  B cell lymphoma that switches to IgA upon stimulation by LPS. The  $\gamma$ 1 promoter also did not function in a T-cell line, EL-4. Also we did not detect expression of the  $\gamma$ 1 promoter in several other B and pre-B cell lines (data not shown). Thus, 5'S $\gamma$ 1-LUC plasmids appear to be expressed in the absence of induction in a subset of B cell lines but not in the one T cell line tested. The initiation sites for germ-line  $\gamma$ 1 RNA are necessary for the expression of 5'S $\gamma$ 1-LUC plasmids in both B cell lines as transfection with a construct in which the start sites have been deleted (contains  $\gamma$ 1 sequence from -954 to -32),



**Fig. 1.** Restriction map of germ-line 5'S $\gamma$ 1 region and 5'S $\gamma$ 1-luciferase (LUC) plasmid constructs. (A) Turned arrow indicates first initiation site of germ-line  $\gamma$ 1 RNA. Germ-line  $\gamma$ 1 RNA is spliced to the C $\gamma$ 1 exon at +484 (Xu and Stavnezer, 1990). (B) Maps of  $\gamma$ 1 promoter-LUC fusion constructs in pXP2. The 3' end of inserted 5'S $\gamma$ 1 DNA is at the BglIII site (+202). Above the promoter-LUC fusion constructs is indicated the KpnI-XbaI probe (K/X) used for RNase protection to detect initiation sites of RNA transcribed from transfected plasmids and from the endogenous gene (Figure 5).

resulted in levels of luciferase activity equal to that produced by pXP2 (data not shown). Transfection with the K/K construct (Figure 1) gave the same result (Figure 2).

**The germ-line  $\gamma$ 1 promoter is induced by anti-Ig antibody and PMA; IL-4 synergizes with anti-Ig antibody and PMA**

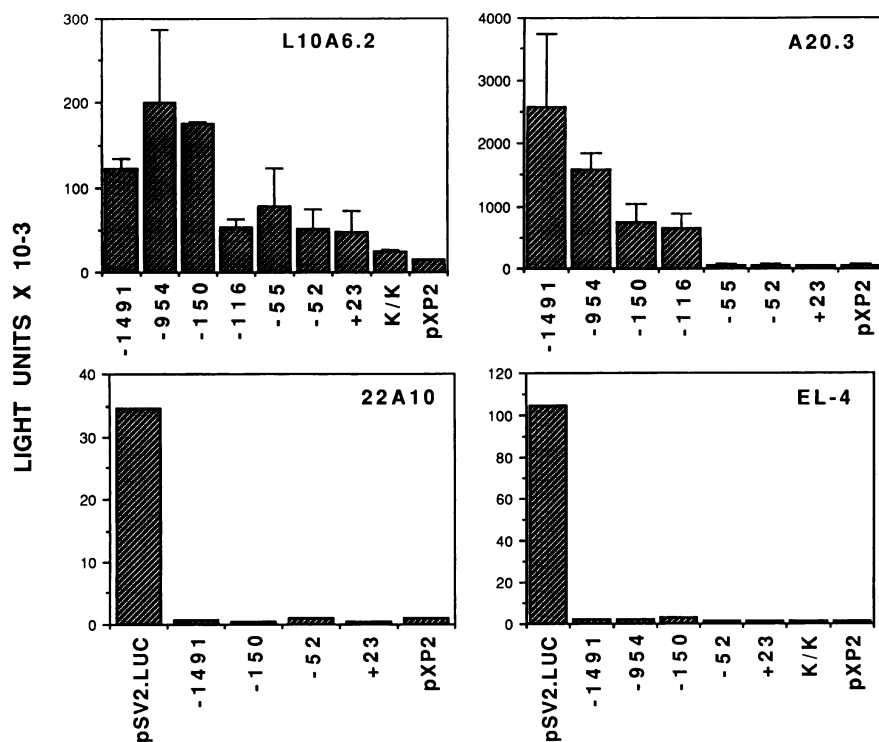
To study the inducibility of the  $\gamma$ 1 promoter, L10A6.2 and A20.3 cells were stably transfected with -1491LUC, co-transfected with the plasmid Homer 6, which confers resistance to G418 (Spandidos and Wilkie, 1984). Stably transfected lines were treated with various inducers and/or cytokines and luciferase activity was measured. Figure 3A shows that luciferase activity is increased in both cell lines by treatment with goat F(ab')<sub>2</sub> anti-mouse Ig or in A20.3 cells with whole goat anti-mouse Ig antibody (L10A6.2 cells were not tested). IL-4, which induces  $\gamma$ 1 germ-line transcripts in spleen cells (Stavnezer *et al.*, 1988; Berton *et al.*, 1989; Esser and Radbruch, 1989; Severinson *et al.*, 1990), synergizes with F(ab')<sub>2</sub> anti-Ig, resulting in a 7- and 127-fold induction in L10A6.2 and A20.3 cells, respectively. IL-4 also synergizes with whole anti-Ig antibody, although numerous experiments showed that IL-4 alone does not induce expression of luciferase in these cell lines. Anti-Ig is known to signal via protein kinase C (PKC) and also to induce a calcium flux and protein tyrosine kinase (Coggeshall and Cambier, 1984; Cambier and Ransom, 1987; Gold *et al.*, 1990). We tested whether PMA, which activates PKC (Nishizuka, 1984), could mimic the effect of anti-Ig treatment. Figure 3B shows that PMA induces expression of the 5'S $\gamma$ 1-LUC plasmids compared with untreated controls by

16- and 21-fold in L10A6.2 and A20.3 cells, respectively, and that IL-4 synergizes with PMA, resulting in increases of expression 87- and 149-fold, respectively.

Purified rIL-4 is saturated at about 300 U/ml for synergy with PMA in both cell lines and, at 100 U/ml, gives ~ 75% of maximal synergy with PMA (Figure 4 and data not shown). These results agree with those of Severinson *et al.* (1990) who found that IL-4 at 20 U/ml was saturating for induction of endogenous germ-line  $\gamma$ 1 RNA in spleen cells (the IL-4 units of Severinson *et al.* are approximately ten times larger than those in the assay used by Immunex). These results are also consistent with experiments demonstrating that 100 U/ml of IL-4 is saturating for induction of IgG<sub>1</sub> expression by splenic B cells (Snapper *et al.*, 1988).

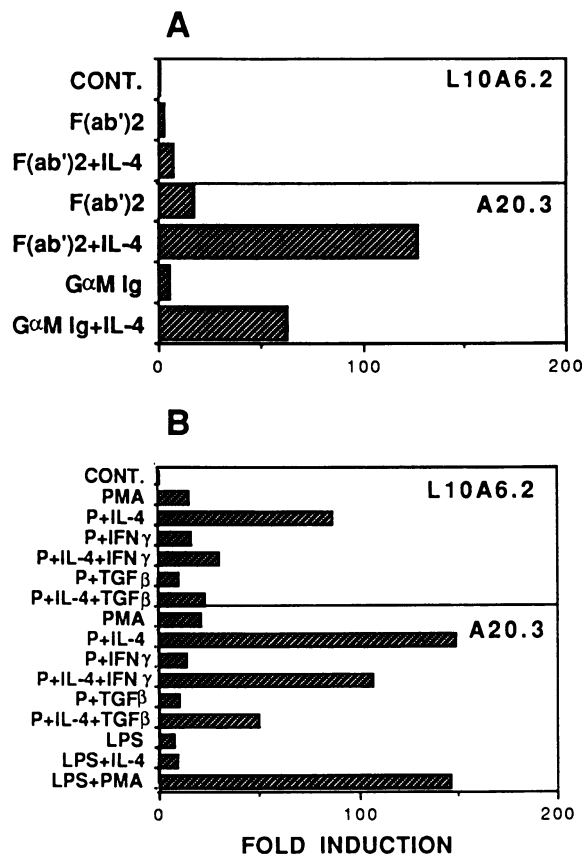
LPS appears to induce low levels of germ-line  $\gamma$ 1 RNA in spleen cells (Severinson *et al.*, 1990; Stavnezer *et al.*, 1988). LPS induces the  $\gamma$ 1 promoter 7-fold in A20.3 cells (Figure 3B), but has no effect in L10A6.2 cells (data not shown). LPS synergizes with PMA to give a similar increase in induction as PMA plus IL-4 (146-fold); however, LPS cannot synergize with IL-4 (Figure 3B).

IFN $\gamma$  has been demonstrated to inhibit the IL-4 induced expression of IgG<sub>1</sub> and germ-line  $\gamma$ 1 RNA (Snapper and Paul, 1987; Berton *et al.*, 1989; Severinson *et al.*, 1990). TGF $\beta$ 1 reduces the expression of all antibody classes although it does induce the expression of IgA and germ-line  $\alpha$  RNA in spleen (Coffman *et al.*, 1989; Lebman *et al.*, 1990) and 1.29 $\mu$  cells (Stavnezer *et al.*, 1990; Shockett and Stavnezer, 1991). We examined the effect of these two cytokines on luciferase expression (Figure 3B). Results



**Fig. 2.** Luciferase activities obtained from four cell lines transiently transfected with 5'S $\gamma$ 1-LUC plasmids.  $30 \times 10^6$  cells were transfected with  $30 \mu\text{g}$  of the indicated plasmid DNA. Cells were harvested at 36 h. Results were normalized to mean CAT activity obtained from a co-transfected pSV2CAT plasmid ( $7.5 \mu\text{g}$  DNA per  $30 \times 10^6$  cells; Gorman *et al.*, 1982). For L10A6.2 and A20.3 cells, data presented are the mean obtained from two representative experiments with the range indicated by error bars. For 22A10 and EL-4 cells, data are from one experiment in which all internal controls were positive. All 5'S $\gamma$ 1-LUC plasmids were linearized immediately 5' to the inserted  $\gamma$ 1 sequences by restriction enzyme to prevent any signal from the upstream vector sequences.

showed that at concentrations of 30–50 U/ml, IFN $\gamma$  inhibited the IL-4 synergy with PMA by 77% in L10A6.2 cells and by 26% in A20.3 cells, but had no effect on the PMA induction itself in L10A6.2 cells and inhibited PMA induction by 30% in A20.3 cells. TGF $\beta$ 1 inhibited PMA induction by 37% and 48% and inhibited synergy with IL-4 by 90% and 78% in L10A6.2 and A20.3 cells, respectively. The incomplete inhibition by IFN $\gamma$  is consistent with the partial inhibition of germ-line  $\gamma$ 1 transcripts by IFN $\gamma$  in spleen cells treated with LPS+IL-4 (Severinson *et al.*, 1990). The inhibitory effects of IFN $\gamma$  and TGF $\beta$  appear to require formation of chromatin structure in L10A6.2 cells as in transient transfection experiments performed by the same protocol, IFN $\gamma$  and TGF $\beta$  induced the  $\gamma$ 1 promoter rather than inhibiting it, whereas if IFN $\gamma$  and TGF $\beta$  were not added until 36 h after transfection, they were inhibitory (data not shown). Furthermore, IFN $\gamma$  and TGF $\beta$  were also inhibitory in stably transfected L10A6.2 cells which were mock electroporated and treated with cytokines by the



**Fig. 3.** Regulation of expression of luciferase in cells stably transfected with the -1491LUC plasmid. (A) Transfected cells ( $10^6$ /ml in 2 ml) were treated with goat anti-mouse Ig F(ab')<sub>2</sub> (30  $\mu$ g/ml for L10A6.2 cells and 10  $\mu$ g/ml for A20.3 cells) or with goat anti-mouse Ig antibody (5  $\mu$ g/ml) with or without IL-4 (2.5% of X63 SN). The A20.3 cells were incubated for 6 h and L10A6.2 cells were incubated for 12 h before harvest. (B) Effects of PMA, LPS, TGF $\beta$ , and IFN $\gamma$ . Cells were treated with PMA (60 ng/ml), purified rIL-4 (400 U/ml for L10A6.2 and 1000 U/ml for A20.3 cells), or with LPS (25  $\mu$ g/ml). LPS had no effect on luciferase activity in L10A6.2 cells. IFN $\gamma$  was used at 30–50 U/ml in both lines. TGF $\beta$  was used at 2 ng/ml for L10A6.2 and 4 ng/ml for A20.3 cells. In the presence of PMA, IFN $\gamma$  and TGF $\beta$  had no effect on viable cell recovery at these doses and at these times. The luciferase is about 5000 and 30 000 light units for uninduced L10A6.2 and A20.3 cells, respectively. P = PMA.

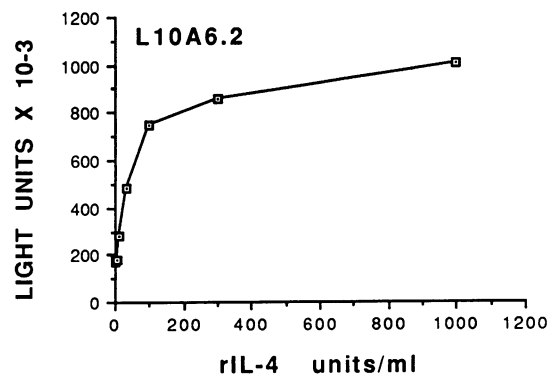
protocol used for transient transfection, suggesting that signal transduction of IFN $\gamma$  and TGF $\beta$  is not perturbed by electroporation. However, IFN $\gamma$  and TGF $\beta$  were inhibitory in A20.3 cells both stably and transiently transfected with -1491LUC. This again reflects the difference in regulation of the germ-line  $\gamma$ 1 promoter between these two cell lines.

Three transfected lines from each cell line were tested for inducibility by PMA, synergy with IL-4 and inhibition by IFN $\gamma$  and TGF $\beta$ . All three transfected lines from each cell line responded similarly to each reagent. Furthermore, the basal level expression was always found to be higher in the A20.3 than in the L10A6.2 lines (data not shown).

rIL-1 $\alpha$ , rIL-2, rIL-5, and rIL-6 had no effect on the expression of -1491LUC and did not synergize with PMA in either of the stably transfected lines (data not shown). Cholera toxin (1  $\mu$ g/ml), which has been reported to induce germ-line  $\gamma$ 1 RNA in spleen cells and to synergize with IL-4 in this induction (Lycke *et al.*, 1990), did not induce the promoter but did augment PMA induction by 40% and by 74% in stably transfected L10A6.2 and A20.3 cells, respectively. However, cholera toxin did not synergize with IL-4 in either transfected cell line (data not shown). At a concentration of 100 ng/ml, the calcium ionophore, A23187, inhibited the PMA induction of -1491LUC by 37% in L10A6.2 cells and by 15% in A20.3 cells (data not shown). This is consistent with the finding that PMA is a more efficient inducer of the germ-line  $\gamma$ 1 promoter than is anti-Ig in these two cell lines.

#### Transcripts from transfected 5'S $\gamma$ -LUC plasmids are correctly initiated

To determine if transcription from the 5'S $\gamma$ -LUC constructs is initiated at the correct sites, RNase protection experiments were performed on RNA from stably transfected A20.3 and L10A6.2 cells which were untreated or treated with PMA or PMA plus IL-4 for 10 h. In both cell lines, correctly initiated transcripts are highly induced by PMA plus IL-4 but are barely detected under all other conditions (identified by the sizes of protected bands: 284, 241, 236 and 229 nt in Figure 5). L10A6.2 cells have significant amounts of read-through transcripts, but they are not inducible. As luciferase expression is highly inducible, it may



**Fig. 4.** Titration of IL-4 synergy with PMA in stably transfected L10A6.2 cells. Experimental procedures are the same as in Figure 3. PMA was included in all incubations at 60 ng/ml. The concentration of purified IL-4 was determined by a co-stimulation assay performed by Immunex. Although a complete dose titration was not performed in A20.3 cells, results at doses greater than 300 U/ml suggested the dose response was similar in A20.3 and L10A6.2 cells.

be possible that the read-through transcripts are not translated. A20.3 cells treated with PMA plus IL-4 also express endogenous germ-line  $\gamma$ 1 RNA (Figure 5, A20.3 panel, lane U), but we have not detected endogenous germ-line  $\gamma$ 1 RNA in A20.3 cells uninduced or treated with PMA or IL-4 alone (data not shown). The sizes of the RNase-resistant fragments obtained from endogenous  $\gamma$ 1 RNA in A20.3 cells induced with PMA plus IL-4 are 81 nt shorter than those obtained from the transfected LUC plasmids as the probe contains 81 nt of the luciferase gene (see Figure 1B). The sizes of RNase-resistant fragments obtained from endogenous transcripts in A30.3 cells are mostly identical to those obtained from RNA isolated from spleen cells treated with LPS plus IL-4 (Xu and Stavnezer, 1990), indicating that most of the initiation sites for germ-line  $\gamma$ 1 RNA in

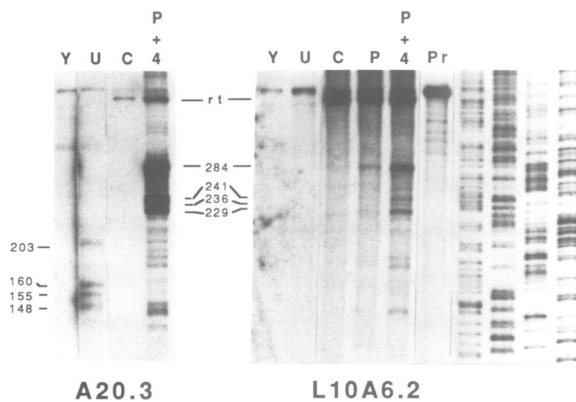
spleen and in A20.3 cells are identical. Presumably there are several initiation sites because this promoter has no TATA box. We have not detected endogenous germ-line  $\gamma$ 1 RNA in induced L10A6.2 cells (Figure 5, L10A6.2, panel, lane U; data not shown).

**Sequences responsible for inducibility by PMA and synergy with IL-4 are located within 150 bp 5' to the first start site of germ-line  $\gamma$ 1 RNA**

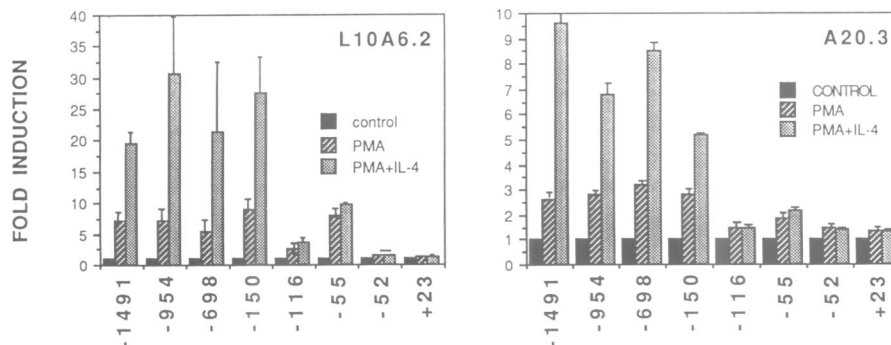
In order to identify DNA sequence elements responsible for induction of the germ-line  $\gamma$ 1 promoter by PMA and IL-4, both cell lines were transiently transfected with the 5'S $\gamma$ 1-LUC plasmids shown in Figure 1. Results shown in Figure 6 indicate that in L10A6.2 cells, the -150LUC plasmid has the same inducibility by PMA (6.9 fold) and synergy with IL-4 (3.5-fold) as -954LUC. In A20.3 cells, -150LUC maintains full PMA inducibility but loses half of the IL-4 synergy, 1.8-fold compared with 3.6-fold synergy of -1491LUC plasmids. These data indicate that most DNA sequences responsible for PMA and PMA plus IL-4 inducibility in L10A6.2 cells and some of the sequences in A20.3 cells, are located within 150 nt 5' to the first initiation site of germ-line  $\gamma$ 1 RNA.

**Linker-scanning mutational analysis of germ-line  $\gamma$ 1 promoter**

In order to define elements responsible for basal level expression of the 5'S $\gamma$ 1-LUC plasmids and their inducibility by PMA and PMA plus IL-4, a series of linker-scanning mutations extending from -160 to -3 relative to the first start site was created (Figure 7A). Transient transfection of these mutated  $\gamma$ 1 promoter-luciferase constructs demonstrated that both the basal level expression and induction by PMA and PMA plus IL-4 of 5'S $\gamma$ 1-LUC plasmids are controlled by multiple elements (Figure 7A, B and C). If any one of several elements is mutated, both the level of expression and inducibility of the 5'S $\gamma$ 1-LUC plasmids are decreased (see the Figure legend for information about the absolute levels of luciferase activity measured and the reproducibility of these experiments). The expression pattern of linker-scanning mutations is similar but not identical in the two cell lines. Mutations in any one of several consensus sequence elements which have been shown to bind transcription factors and/or to be required for the expression



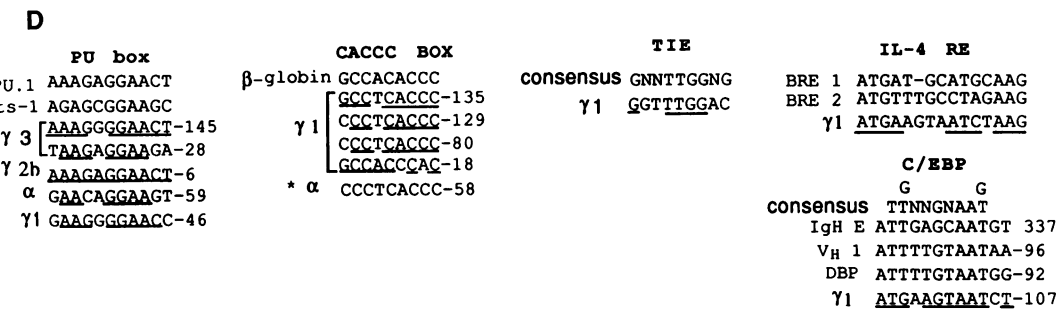
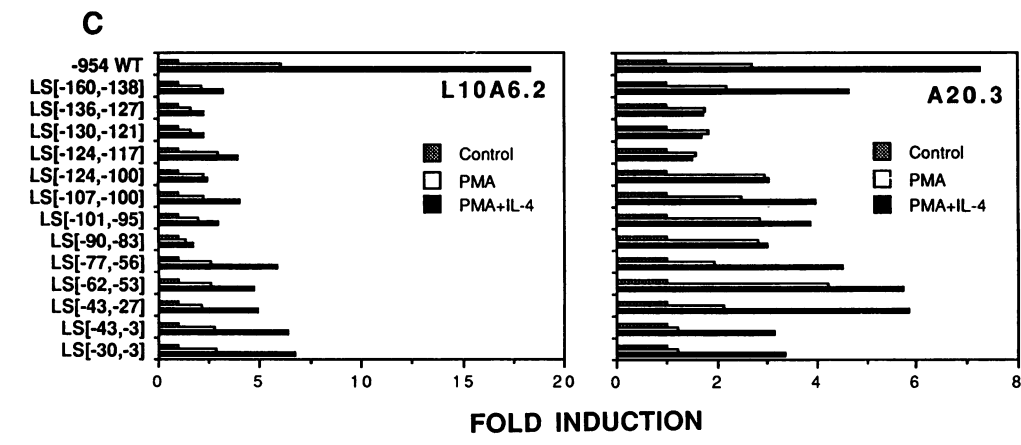
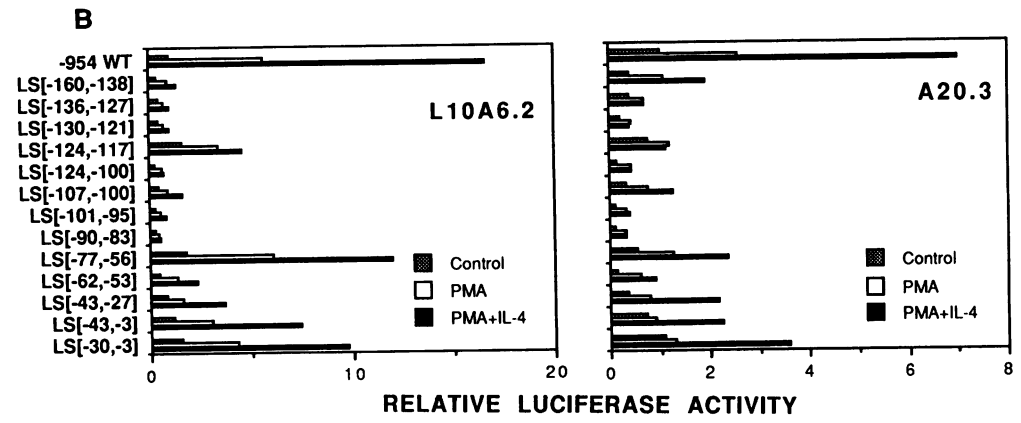
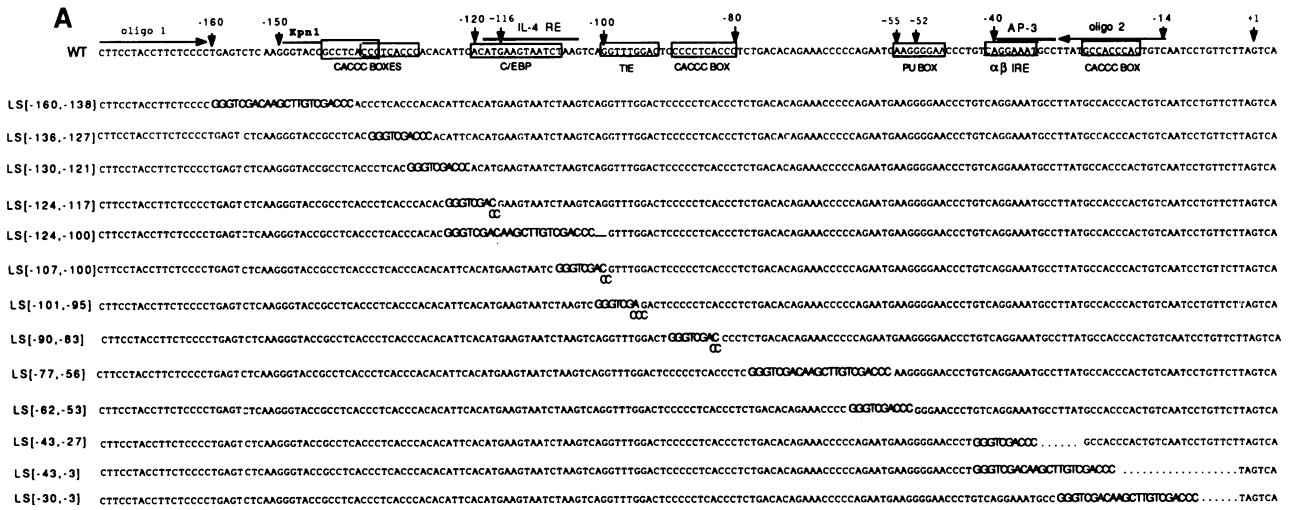
**Fig. 5.** RNase protection experiments to detect RNA transcribed from transfected -1491LUC and the endogenous C $\gamma$ 1 genes. RNase-resistant fragments obtained after hybridization of total cell RNA from L10A6.2 and A20.3 cells with an antisense RNA probe transcribed from the *KpnI*-*XbaI* (K/X) segment. This probe contains the *KpnI*-*BglIII* fragment from 5'S $\gamma$ 1 sequences and 81 nt of the luciferase gene (see Figure 1). Lanes show RNase resistant fragments obtained after hybridization of the probe with 20  $\mu$ g of: Y, yeast RNA; U, total cell RNA from untransfected cells treated with PMA (60 ng/ml) plus rIL-4 (1000 U/ml); C, total cell RNA from untreated cells which were stably transfected with -1491LUC plasmids; P, same as C except cells were treated with PMA; P+4, same as C except cells were treated with PMA plus rIL-4; Pr, lane contains probe alone without RNase digestion. rt denotes read-through transcripts. The RNA was electrophoresed in a 6% polyacrylamide-urea gel alongside a DNA-sequencing ladder.



**Fig. 6.** Inducibility of various 5'S $\gamma$ 1-LUC plasmids in L10A6.2 and A20.3 cells.  $30 \times 10^6$  cells were transiently transfected with 30  $\mu$ g supercoiled plasmid DNA (L10A6.2) or 15  $\mu$ g DNA (A20.3). After transfection, cells were aliquoted into three flasks in 7 ml of medium and treated with nothing (control), PMA (60 ng/ml), or PMA + IL-4 (2.5% X63 supernatant for L10A6.2 cells and 1000 U/ml purified rIL-4 for A20.3 cells). After 12 h, cells were harvested and luciferase activity was assayed. For both cell lines, the data are the mean of two representative experiments except for -954LUC and -150LUC which are from four experiments. The error bars indicate the range (or S.E. for -954LUC and -150LUC).

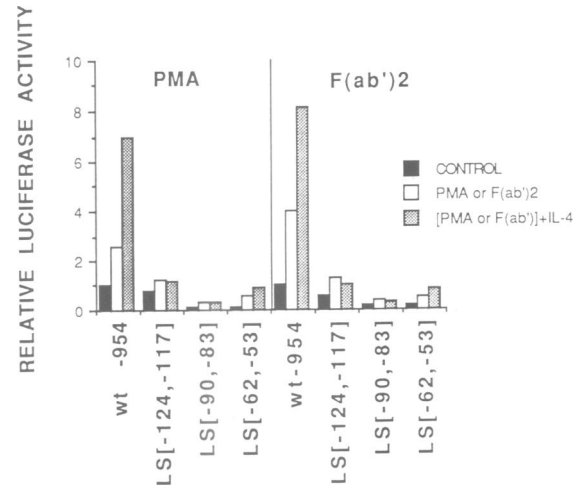
of other genes, reduces expression and inducibility of the 5'S $\gamma$ 1-LUC plasmids. Firstly, CACCC boxes (Dierks *et al.*, 1983; Cowie and Myers, 1988) contribute to the basal

level expression of PMA and PMA plus IL-4 inducibility in both cell lines. There are four CACCC boxes in the germ-line  $\gamma$ 1 promoter, all residing within the 150 bp 5' to the



first start site. Mutations in the first three boxes markedly reduce basal level expression and IL-4 synergy in both cell lines and PMA inducibility in L10A6.2 but not A20.3 cells. The other CACCC box, located at  $-26/-18$ , is less homologous with the CACCC consensus sequence than the others (Figure 7D) and is less important in L10A6.2 cells, but may contribute to PMA inducibility in A20.3 cells as LS $[-43, -3]$  and LS $[-30, -3]$  are not induced by PMA. Secondly, the DNA sequence located between  $-120$  and  $-104$  is homologous to the binding sites for two transcription factors; it perfectly matches the consensus sequence for the C/EBP family of transcription factors (Akira *et al.*, 1990) and it also imperfectly matches two elements found in the promoter of the class II A $\alpha^k$  gene which bind an IL-4 inducible nuclear factor (labeled IL-4 RE in Figures 7A and D; Boothby *et al.*, 1988). One LS mutation which affects only this region LS $[-124, -117]$ , eliminates synergy with IL-4, but also reduces PMA inducibility by 63% and 71% in L10A6.2 and A20.3 cells, respectively. However, if additional nucleotides are mutated (LS $[-124, -100]$ ), PMA inducibility but not IL-4 synergy, is restored in A20.3 cells. Thirdly, the mutation LS $[-101, 95]$  destroyed a putative TGF $\beta$  inhibitory element (TIE) that is responsible for negative regulation of the transin/stromelysin genes (Kerr *et al.*, 1990). This LS mutation results in 70% and 90% loss of basal level expression in L10A6.2 and A20.3 cells, respectively. The inducibility by PMA in L10A6.2 cells and synergy with IL-4 in both cell lines are markedly reduced by this LS mutant (Figures 7B and C). Fourthly, a PU box (Pettersson and Schaffner, 1987) located between  $-55$  and  $-48$  is also necessary for basal level expression and contributes to synergy with IL-4 in both cell lines (see LS $[-62, -53]$ ). In L10A6.2 cells, mutation of the PU box also reduces PMA inducibility. Lastly, the mutation at a putative AP-3 binding site/ $\alpha\beta$ -interferon response element (IRE) (LS $[-43, -27]$ ) also reduces PMA and PMA plus IL-4 inducibility in L10A6.2 but not in A20.3 cells. Taken together, results using this series of mutations demonstrate that the DNA sequence elements residing between  $-150$  and  $-3$  function interdependently to confer basal level expression and inducibility by PMA and PMA plus IL-4.

We also tested the F(ab')<sub>2</sub> inducibility of LS mutants in A20.3 cells. Results shown in Figure 8 suggest, as expected, that the same elements are used to respond to both F(ab')<sub>2</sub>



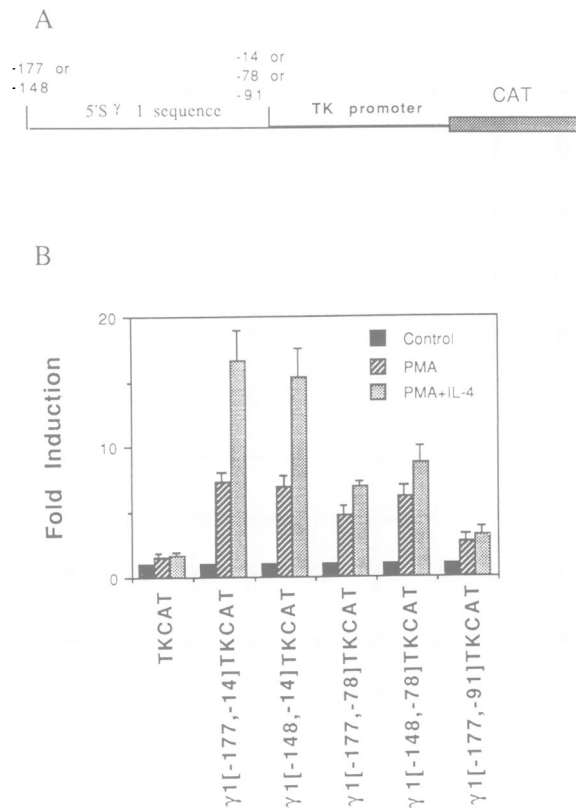
**Fig. 8.** Comparison of inducibility of some LUC mutants by PMA and F(ab')<sub>2</sub> in A20.3 cells. The PMA data presented are re-plotted from Figure 7B. Experimental procedures are the same as in Figure 7B. IL-4 was used at 1000 U/ml, and F(ab')<sub>2</sub> at 10  $\mu$ g/ml. Luciferase activity from the control flask of cells transfected with wild-type promoter-luciferase plasmids was set equal to 1. Average of the results obtained from two experiments are shown. The average range ( $\pm$  S.D.) is 18% ( $\pm$  13%).

and PMA in A20.3 cells. We have not tested the F(ab')<sub>2</sub> inducibility of LS mutants in L10A6.2 cells because of the lower F(ab')<sub>2</sub> inducibility in this cell line.

#### **A segment of the germ-line $\gamma 1$ promoter can confer inducibility by PMA and PMA plus IL-4 to a heterologous promoter**

To ascertain if the DNA segment demonstrated by linker-scanning mutations to be necessary for inducibility by PMA and synergy with IL-4 is also sufficient to confer inducibility, we transferred segments of the  $\gamma 1$  promoter to a plasmid containing a minimal thymidine kinase (TK) promoter ligated to the gene for chloramphenicol acetyl transferase (CAT) (Figure 9A). This plasmid is reported to be unable to direct CAT expression unless enhancer sequences are added (Cato *et al.*, 1986). Various lengths of 5'S $\gamma 1$  fragments were inserted into the TKCAT plasmid. Results of transient transfection of these 5'S $\gamma 1$ -TKCAT plasmids into L10A6.2

**Fig. 7.** DNA sequences and relative luciferase activity of linker-scanning mutation plasmids. (A) Genomic DNA sequences from  $-177$  to  $+4$  relative to the first initiation site for  $\gamma 1$  germ-line RNA and linker-scanning mutations of this region. WT = wild type and LS = linker-scanning mutation. Wild-type is  $-954$ LUC and thus all the mutations are within the  $-954$ LUC plasmid. Bold letters indicate linker sequences which are either 10 bp or 22 bp in length. Boxed sequences indicate consensus sequence motifs for binding sites for known transcription factors taken from the literature except IL-4 RE and AP-3 are indicated by lines above the sequence. Two horizontal arrows denoted two oligonucleotides used for PCR. The 5' end of oligo 1 has an added *Hind*III site and the 3' end of oligo 2 has an added *Sa*I site. (B) Relative luciferase activity obtained from L10A6.2 and A20.3 cells transiently transfected with the wild-type and LS mutant plasmids. 20  $\mu$ g supercoiled plasmid DNA (L10A6.2 cells) and 15  $\mu$ g DNA (A20.3 cells) was used to transfect  $40 \times 10^6$  cells. CAT activity obtained from 2  $\mu$ g of pSV2CAT which was co-transfected was used to normalize the luciferase activity between transfections. After transfection cells were aliquoted into four flasks in 7 ml of medium and treated with nothing (control), PMA (60 ng/ml), or PMA + IL-4 (400 U/ml for L10A6.2 cells and 1000 U/ml for A20.3 cells). After 12 h incubation, cells were harvested for luciferase and CAT assays. Luciferase activity from the control flask of wild-type was set equal to 1 which was about 6000 and 44 000 light units for L10A6.2 and A20.3 cells, respectively. Background in the luciferase assay is about 300 light units. For both cell lines, average of the results obtained from two representative experiments are shown. For data presented in (B), the average range of the mean ( $\pm$  S.D.) is 17% ( $\pm$  14%) for both cell lines. (C) The data in (B) are plotted as fold induction for data points presented in (C), the average range ( $\pm$  S.D.) is 12% ( $\pm$  7.7%) of the mean for L10A.2 cells and 8.6% ( $\pm$  7.1%) of the mean for A20.3 cells. (D) Comparison of  $\gamma 1$  sequence elements with binding sites for known transcription factors (consensus sequence and specific sequences found in other promoters). Nucleotides which are identical with those in the consensus sequences or with those in one of the known functional elements are underlined. References: PU box (Pettersson and Schaffner, 1987; Karim *et al.*, 1990; Klemsz *et al.*, 1990); CACCC box (Dierks *et al.*, 1983; Cowie and Myers, 1988); C/EBP (Akira *et al.*, 1990); Ig/EBP (Roman *et al.*, 1990); IL-4 RE (Boothby *et al.*, 1988); TIE (Kerr *et al.*, 1990);  $\alpha\beta$ -IRE (Yang *et al.*, 1990); and AP-3 (Chiu *et al.*, 1987); IgH E and V<sub>H</sub> 1 (Peterson *et al.*, 1988); germ-line  $\gamma 2b$  (Lutzker and Alt, 1988); germ-line  $\gamma 3$  (Rothman *et al.*, 1990); and DBP (Lichtsteiner *et al.*, 1987). The numbers to the right of the sequences indicate the position of the most 3' nucleotide of the element. \*Denotes an inverted CACCC box in the germ-line  $\alpha$  promoter (Y.-C.A.Lin and J.Stavnezer, in preparation).



**Fig. 9.** Structure of 5'S $\gamma$ 1TKCAT constructs and CAT activity in L0A6.2 cells transiently transfected with TKCAT and 5'S $\gamma$ 1-TKCAT plasmids. (A) The thick line represents TK promoter sequence which includes nucleotides from -105 to +58 relative to the TK mRNA start site (10). The thin line represents germ-line  $\gamma$ 1 promoter sequence and numbers above the line indicate position of the  $\gamma$ 1 sequence relative to the first initiation site of the germ-line  $\gamma$ 1 RNA. (B) 25  $\mu$ g supercoiled plasmid DNA was used to transfect  $30 \times 10^6$  cells. After transfection cells were aliquoted into three flasks and untreated (control), treated with PMA (60 ng/ml) or with PMA+IL-4 (400 U/ml). After 12 h, cells were harvested and assayed for CAT activity. The results shown are the average of two experiments, except those of  $\gamma 1[-177,-14]$ TKCAT which are from four experiments. Data presented are mean with range (S.E. for  $\gamma 1[-177,-14]$ TKCAT) indicated by error bars.

are shown in Figure 9B  $\gamma 1[-177,-14]$ TKCAT and  $\gamma 1[-148,-14]$ TKCAT have full PMA inducibility and about two-thirds of the IL-4 synergistic response of -150LUC (Figure 6).  $\gamma 1[-177,-78]$ TKCAT and  $\gamma 1[-148,-78]$ TKCAT are fully induced by PMA but have lost most of synergy with IL-4.  $\gamma 1[-177,-91]$ TKCAT, which differs from  $\gamma 1[-177,-78]$ TKCAT only by a CACCC box, is only slightly inducible by PMA or by PMA plus IL-4. The TKCAT plasmid itself is not expressed and is not inducible in L10A6.2 cells but is expressed and induced by PMA in A20.3 cells;  $\gamma 1$  promoter-TKCAT plasmids are not inducible above this background (Figure 9 and data not shown).

## Discussion

Germ-line  $\gamma$ 1 RNA can be induced in spleen cells by treatment with IL-4 (Stavnezer *et al.*, 1988; Berton *et al.*, 1989; Severinson *et al.*, 1990). In order to develop a system in which to define DNA sequences involved in regulation of transcription of germ-line  $\gamma$ 1 RNA, we tested a number

of B cell lines for their ability to express the -1491LUC plasmid. Two B cell lines, L10A6.2 and A20.3, were found that demonstrated inducible expression of the germ-line  $\gamma$ 1 promoter-luciferase plasmids. Furthermore, endogenous germ-line  $\gamma$ 1 RNA can be induced in A20.3 cells by PMA plus IL-4, although we have been unable to detect endogenous  $\gamma$ 1 RNA in L10A6.2 cells. It is possible that the endogenous  $\gamma$ 1 gene is methylated in L10A6.2 cells. The regulation of germ-line  $\gamma$ 1 RNA appears to differ in these two lines from that in splenic B cells in that IL-4 alone induces germ-line  $\gamma$ 1 RNA in spleen cells but not in L10A6.2 and A20.3 cells. However, IL-4 is able to synergize with anti-Ig or with PMA to induce transcription directed by the germ-line  $\gamma$ 1 promoter in these two cell lines. Furthermore, the sites of initiation of transcription from the transfected  $\gamma$ 1 germ-line LUC plasmids, are nearly identical to those used in spleen cells.

Cross-linking of the membrane Ig receptor has multiple effects on B cells, including increased expression of class II genes and induction of DNA synthesis (Mond *et al.*, 1981; Cambier and Ransom, 1987). Cross-linking of membrane Ig induces protein tyrosine kinase activity and hydrolysis of phosphatidyl inositol, resulting in formation of diacylglycerol (DAG) and in a calcium flux (Coggeshall and Cambier, 1984; Campbell and Sefton, 1990; Gold *et al.*, 1990). DAG activates PKC, the subsequent effects of which are unknown. Treatment with the phorbol ester PMA also activates PKC. We found that treatment with anti-Ig or PMA induces expression of the 5'S $\gamma$ 1-LUC plasmids and IL-4 can synergize with these inducers. Since PMA induces more luciferase activity than does anti-Ig in L10A6.2 cells, it is possible that membrane IgM is uncoupled from the PKC signaling pathway in this cell line.

The finding that anti-Ig and/or PMA treatment induces the germ-line  $\gamma$ 1 promoter in the two cell lines is intriguing since it has been shown that a T cell supernatant containing IL-4 induces about eight times more IgG<sub>1</sub> secretion from splenic B cells, treated with anti- $\mu$  antibody coupled to Sepharose beads, than from splenic B cells treated with LPS (Isakson, 1986). The effect appeared to be isotype specific since LPS induced secretion of IgG<sub>3</sub> whereas anti- $\mu$  did not. Furthermore, anti-idiotypic antibody has been found to augment LPS-induced switching to IgA in the I.29 $\mu$  lymphoma cell line (Stavnezer *et al.*, 1985). In preliminary experiments we found that expression of 5'S $\gamma$ 1-LUC plasmids transfected into LPS-treated spleen cells, was increased 2-fold by anti-Ig or PMA (unpublished data). Interestingly, it has been shown that injection of anti-IgD antibody into mice greatly increases the serum level of IgG<sub>1</sub> and IgE (Finkelman *et al.*, 1986, 1988). The increase of IgG<sub>1</sub> cannot be explained solely by the effect of IL-4, which is induced by injection of anti-IgD antibody, because co-injection of a large excess of anti-IL-4 antibody (11B11) effectively blocks the IgE production but does not significantly inhibit IgG<sub>1</sub> production. Furthermore, Goroff *et al.* (1991) recently demonstrated that injection of anti-IgD, which can cross-link sIgD, induces much more IgG<sub>1</sub> than non cross-linking antibodies. We hypothesize that anti-IgD activates the PKC pathway which induces transcription of germ-line  $\gamma$ 1 genes and thereby directs switching to IgG<sub>1</sub> in these mice.

Linker-scanning analyses indicated that multiple elements are required for  $\gamma$ 1 promoter activity and for inducibility



by PMA and synergy with IL-4. We noted several sequence elements that matched known transcription factor binding sites in the 150 nt region 5' to the first initiation site. A CACCC box is repeated four times in this region (Figure 7A). The first three of these repeats contribute to basal level expression and are essential for synergy with IL-4. All four of these elements contribute to PMA inducibility except the one at -89/-80 which does not contribute in A20.3 cells. A CACCC box is required for transcription of the mouse  $\beta$ -globin gene (Dierks *et al.*, 1983; Cowie and Myers, 1988). An inverted CACCC box has also been found in the germ-line  $\alpha$  promoter (Y.-C.A. Lin and J. Stavnezer, in preparation). The putative CACCC transcription factor has not been identified.

A TIE-like element also contributes significantly to the basal level expression and to IL-4 synergy in both cell lines and to PMA inducibility in L10A6.2 cells. TIEs are involved in TGF $\beta$ -inhibition of several genes and bind a protein complex containing c-Fos (Kerr *et al.*, 1990).

The sequences located between -120 and -104 perfectly match a C/EBP binding site whose core sequence, TT/GNNGNAAT/G, is present in many viral enhancers and mammalian promoters, including the Ig heavy chain enhancer and Ig heavy chain variable region promoters (Peterson *et al.*, 1988; Akira *et al.*, 1990). The C/EBP site is recognized by a protein which was originally found in liver cells (Johnson *et al.*, 1987) but also exists in many other cell types (Lichtsteiner *et al.*, 1987; Maire *et al.*, 1989; Akira *et al.*, 1990). Recently, Roman *et al.* (1990) characterized a new member of the C/EBP family, Ig/EBP, which is expressed in all cell lines and tissues examined but is most abundant in pre-B cells. Both Ig/EBP and C/EBP bind the same DNA element and this element is essential for *in vitro* transcription of the mouse albumin gene using nuclear extracts from either liver or spleen as transcription machinery (Maire *et al.*, 1989). Mutations of the C/EBP binding site in the  $\gamma$ 1 promoter abolish synergy with IL-4 but have a less severe effect on PMA inducibility. Whether Ig/EBP, C/EBP or another member of this family contributes to IL-4 synergy is unknown. Overlapping the C/EBP site is an element homologous to two binding sites for an IL-4 inducible protein in the promoter for the class II A $\alpha^k$  gene (Boothby *et al.*, 1988). These binding sites have not been previously demonstrated to be necessary for IL-4 inducible gene expression.

Another important element is the PU box, which is present in the enhancers of SV40 and lymphotropic papovavirus (LPV), and has been shown to contribute to SV40 enhancer activity in lymphoid cells and to be a major determinant of the lymphotropic host range of LPV (Petersson and Schaffner, 1987; Karim *et al.*, 1990). The PU box is recognized by murine Ets-1, a product of the proto-oncogene, *ets-1* and by a macrophage and B cell specific transcription factor, PU.1, whose amino acid sequence in the binding domain has considerable homology with proteins belonging to the *ets* oncogene family (Gunther *et al.*, 1990; Klemsz *et al.*, 1990). Mutation of the PU box reduces basal level expression and reduces the IL-4 synergistic response in both cell lines, but reduces PMA inducibility only in L10A6.2 cells. The PU box is also found 5' to the initiation sites for germ-line  $\gamma$ 2b,  $\gamma$ 3, and  $\alpha$  RNAs (at -16/-6 relative to the first initiation site of germ-line  $\gamma$ 2b RNA, at two sites located at -155/-145 and at -38/-28 relative

to the 5' end of germ-line  $\gamma$ 3 cDNA, and at -69/59 5' to first start of germ-line  $\alpha$  RNA; Figure 7D; Lutzker and Alt, 1988; Rothman *et al.*, 1990; Y.-C.A. Lin and J.S., in preparation). We did not find any other of the putative  $\gamma$ 1 promoter elements in the published sequences of the region 5' to the start sites of the germ-line  $\gamma$ 3 and  $\gamma$ 2b transcripts.

The sequences located between -41 and -31 match with two elements: one is a perfect  $\alpha\beta$ -interferon response element ( $\alpha\beta$ -IRE; Yang *et al.*, 1990) and another an imperfect AP-3 element (Chiu *et al.*, 1987). This region of the promoter appears to be less important than other regions but is required for optimal induction of the  $\gamma$ 1 promoter.

The -177/-14 and -148/-14 fragments are sufficient to confer full PMA inducibility and about two-thirds of the synergy with IL-4 of the -150LUC plasmid (which contains the  $\gamma$ 1 RNA initiation sites), whereas the -177/-78 and -148/78 segments only confer PMA inducibility and the -177/-91 fragment confers very little inducibility. This indicates that sequences within -148/-78 are sufficient for PMA inducibility but sequences located within -78/-14 are required to obtain IL-4 synergy in L10A6.2 cells. These results, combined with our other analyses, demonstrate that synergy with IL-4 is mediated by multiple interdependent elements.

It has been shown previously that either an AP-1 or AP-2 element (Angel *et al.*, 1987; Imawaga *et al.*, 1987; Mitchell *et al.*, 1987) or an AP-3 element (Chiu *et al.*, 1987) can confer inducibility by the phorbol ester-activated and PKC-dependent signal transduction pathway. None of these sequence elements (except an imperfectly matched AP-3 site which makes only a minor contribution to  $\gamma$ 1 promoter activity) are located in the 150 bp region that confers PMA inducibility to the  $\gamma$ 1 promoter. It is possible that PMA induces transcription of other genes whose products induce the  $\gamma$ 1 promoter since the earliest time we have demonstrated PMA inducibility is at 3 h, and the maximal response is not achieved until 12-24 h after addition of PMA. We have not examined earlier times. The signaling mechanisms underlying the IL-4 synergy with PMA and LPS synergy with PMA are completely unknown. This is the first case of which we are aware in which multiple functionally interdependent elements are needed to respond to PMA.

In conclusion, we find that cytokines which have been shown to regulate switching to IgG<sub>1</sub> regulate the germ-line  $\gamma$ 1 promoter, thus indicating that much of the regulation of switching to IgG<sub>1</sub> can be explained by regulation of transcription directed by the germ-line  $\gamma$ 1 promoter. IL-4, which induces switching to IgG<sub>1</sub> in the presence of LPS and also induces germ-line  $\gamma$ 1 RNA, induces expression of the germ-line  $\gamma$ 1 promoter in the presence of PMA. IFN $\gamma$  and TGF $\beta$ , which down-regulate switching to IgG<sub>1</sub>, inhibit the IL-4 activation of the germ-line  $\gamma$ 1 promoter. Furthermore, our results suggest that cross-linking the antigen receptor on B cells may be involved in inducing accessibility of the C $\gamma$  gene, and thereby, directing the switch to IgG<sub>1</sub>.

## Materials and methods

### Cell lines

Cell lines L10A6.2, A20.3 (Kim *et al.*, 1979), and EL-4 (T lymphoma cell line, TIB 40) were maintained in RPMI 1640 with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) in a 5% CO<sub>2</sub> incubator. A20.3 cells were given by Dr David Parker (University of Massachusetts, Worcester, MA) and L10A6.2 cells were provided by Dr

Richard Asofsky (NIH, Bethesda, MD). 22A10, a subclone of 1.29  $\mu$  Alberini *et al.*, 1987), was maintained in RPMI 1640 with 20% FBS in an 8% CO<sub>2</sub> incubator.

### Reagents and lymphokines

Goat anti-mouse Ig antibody (Southern Biotech. Associates, Birmingham, AL) and goat anti-Ig F(ab')<sub>2</sub> (Organon Teknika Corporation, West Chester, PA) were purchased. PMA, LPS, and calcium ionophore A23187 were purchased from Sigma (St. Louis, MO). Cholera toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). IFN $\gamma$  was donated by Schering Corporation, USA as part of the American Cancer Society's program on interferon. TGF $\beta$ 1 was purchased from R & D Systems, Inc. (Minneapolis, MN). Purified recombinant human IL-1 $\alpha$  (rhIL-1 $\alpha$ ; Boehringer Mannheim, Indianapolis, IN) was given by Dr Robert Woodland (University of Massachusetts, Worcester, MA). Purified recombinant IL-4 (rIL-4) was produced by Immunex (Seattle, WA) and provided by Sterling Research group (Malvern, PA). Mouse IL-2, IL-4, and IL-5 were supernatants of a plasmacytoma X63-Ag8-653 (X63SN) transfected with IL-2, IL-4, IL-5 cDNA clones, respectively (Karasuyama and Melchers, 1988). We obtained the highest IL-4 synergy on both cell lines by using 2.5% of one batch of X63SN which was used for all the experiments shown. An anti-IL-4 antibody, 11B11 (Ohara and Paul, 1985) which was given by Dr David Parker (University of Massachusetts, Worcester, MA), could eliminate the X63SN synergy when added to the X63SN for 15 min at RT before use (data not shown). Purified IL-6 (Jambou *et al.*, 1988) was given by Dr Dana Fowlkes (University of North Carolina). In both cell lines the PMA induction was maximal 12 h after addition. IL-4 synergy was maximal at 12 h in L10A6.2 cells and at 24 h in A20.3 cells (data not shown).

### Plasmid construction

The molecular techniques were adapted from Sambrook *et al.* (1989). Different lengths of 5'S $\gamma$ 1 DNA fragments (Figure 1) were cloned into promoterless luciferase plasmids (pXP1 or pXP2) (De Wet *et al.*, 1987; Nordeen, 1988) using appropriate enzyme sites. The 5' deletions having < 150 bp flank were created by *Bal31* digestion from the *KpnI* site (Figure 1). After the ends were filled with T4 polymerase and Klenow enzyme, *BglII* digestion was performed and the isolated fragments were ligated into the *SmaI* and *BglII* digested pXP2 plasmid. The 3' deletions were created by *Bal31* digestion from the *BglII* site (at +202) of -954LUC (Figure 1) and filled in with T4 polymerase and Klenow enzyme. After digestion with *BamHI*, the isolated fragments were ligated into the *BamHI* and *SmaI* digested pXP1 plasmid.

The linker-scanning mutants were constructed by combining appropriate 5' and 3' deletion mutants. Briefly, the 3' deletion plasmids were digested with *BamHI*+*Sall* or *BamHI*+*HindIII* and the isolated fragments were ligated into *BamHI*+*Sall* or *BamHI*+*HindIII*-digested 5' deletion plasmids. *BamHI*+*Sall* digestion creates a 10 bp linker while *BamHI*+*HindIII* digestion creates a 22 bp linker. All deletions and linker-scanning mutants were verified by sequencing (Tabor and Richardson, 1987).

Heterologous promoter constructs (5'S $\gamma$ 1-TKCAT) were created by two methods: firstly, PCR was used to amplify the fragments with two oligos (Figure 7A; Saiki *et al.*, 1988). The 5' oligo has a *HindIII* site and the 3' oligo has a *Sall* site. The digested fragments were cloned into *HindIII*+*Sall* digested TKCAT plasmid (Cato *et al.*, 1986); secondly, Asp718 (filled in using T4 polymerase and Klenow enzyme) plus *Sall* digested fragments, which were directly isolated from certain LS mutants, were cloned into *HindIII*- (filled in using T4 polymerase and Klenow enzyme) plus *Sall*-digested TKCAT plasmid.

### RNase protection

RNase protection was performed as previously (Xu and Stavnezer, 1990). The probe (K/X) used for RNase protection (Figure 1B) contains the *KpnI*-*BglII* fragment from 5'S $\gamma$ 1 DNA and 81 bp of the luciferase gene.

### Transfection and assays

Transfection was performed by electroporation (Maxwell and Maxwell, 1988) using Cell ZapII (Anderson Electronics Brookline, MA). Briefly, the cells were diluted 4- to 5-fold and then cultured for 2-2.5 days before collection and washed once with warmed RPMI 1640 without serum. Cells were resuspended in RPMI 1640 without serum and electroporated at 1250  $\mu$ F/740 V per cm (1 ml volume) or 625  $\mu$ F/750 V per cm (0.5 ml volume). After transfection, cells were kept at RT for 10 min and then were resuspended in 10% FBS/RPMI 1640 and aliquoted into flasks at a concentration of 1-1.3  $\times 10^6$ /ml (counted before transfection) in 7 ml. After addition of inducers, cells were incubated for 12 h (except where indicated) and then harvested, washing once with PBS (without calcium and magnesium) and assayed for luciferase or CAT activity. To establish stably transfected

lines, cells were co-transfected with linearized -1491LUC and Homer 6 plasmid (Spandidos and Wilkie, 1984) at a ratio of 5:1. Two days after transfection, cells were subjected to G418 selection (1 mg/ml for A20.3 cells and 1.2 mg/ml for L10A6.2 cells) for at least 2 weeks in 24-well tissue culture plates (Costar, Cambridge, MA). Cells recovered from one well were maintained as a line and were not subjected to further cloning. Luciferase assays were performed according to Brasier *et al.* (1989). Briefly, cells from one flask were lysed by adding 100  $\mu$ l of 0.1% Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM MgSO<sub>4</sub> and 4 mM EGTA pH 7.8. After vigorous pipetting, the lysate was centrifuged for 2 min in a microcentrifuge. Supernatant was collected and added into 360  $\mu$ l of 25 mM glycylglycine pH 7.8, 15 mM MgSO<sub>4</sub>, 15 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.8, 4 mM EGTA pH 7.8, 2 mM ATP, and 1.27 mM DTT. Luciferase assays were performed on a luminometer (Analytical Luminescence Laboratory, San Diego, CA) immediately after injecting 100  $\mu$ l of 1 mM luciferin (Analytical Luminescence Laboratory, San Diego, CA) into lysate mixture.

CAT assays were performed as described by Neumann *et al.* (1987). Briefly, cells were lysed with 50  $\mu$ l of the lysing buffer used for luciferase assay. After incubation at 70°C for 15 min, the lysate was combined with 200  $\mu$ l of 125 mM Tris-HCl pH 7.8, 1.25 mM chloramphenicol (Sigma, St. Louis, MO) and 0.1  $\mu$ Ci of [<sup>3</sup>H] acetyl CoA (New England Nuclear, Boston, MA) in a 7 ml glass scintillation vial. 5 ml of Econofluor (Biotechnology Systems, Boston, MA) was carefully layered on top of the reaction mixture and the vial was incubated at 37°C for 2-3 h. The samples were counter in a liquid scintillation counter.

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### Note added in proof

We have been unable to induce L10A6.2 cells to undergo class switching by treatment with LPS  $\pm$  IL-4. The DNA sequence between –119 and –109 relative to the initiation site for germ-line  $\gamma$ 1 RNA, has a 10/11 nt match with a sequence surrounding the first initiation site for murine germ-line  $\epsilon$  RNA; this has been shown to bind an IL-4-inducible protein [Rothman, P., Li, S.C., Gorham, B., Glimcher, L., Alt, F. and Boothby, M. (1991) *Mol. Cell. Biol.*, **11**, 5551–5561].