May 1991

Regulation of Transcription of Mouse Immunoglobulin Germ-Line $\gamma_1$ RNA: Structural Characterization of Germ-Line $\gamma_1$ RNA and Molecular Analysis of the Promoter: A Dissertation

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REGULATION OF TRANSCRIPTION OF MOUSE IMMUNOGLOBULIN GERM-LINE $\gamma_1$ RNA: STRUCTURAL CHARACTERIZATION OF GERM-LINE $\gamma_1$ RNA AND MOLECULAR ANALYSIS OF THE PROMOTER

A Dissertation Presented

By

Minzhen Xu

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester, in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCE

May 1991

Immunology and Virology
DEDICATED TO MY FAMILY
REGULATION OF TRANSCRIPTION OF MOUSE IMMUNOGLOBULIN GERM-LINE $\gamma_1$ RNA: STRUCTURAL CHARACTERIZATION OF GERM-LINE $\gamma_1$ RNA AND MOLECULAR ANALYSIS OF THE PROMOTER

A Thesis Presented
by
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ACKNOWLEDGEMENTS

I would first like to thank my friend and advisor, Dr. Janet Stavnezer, for her extreme patience and step-by-step advice over the years. It would have been impossible for me to finish this thesis without her support.

I would also like to thank all the people in the laboratory who helped me. It would also be impossible for me to finish this thesis without such a warm and helpful environment.

I would like to thank Drs. David Parker, Robert Woodland, and Paul Dobner not only for supplying cell lines, plasmids, lymphokines, and antibodies but also for their useful advice, suggestions, and opinions.

I like also to express my gratitude to Dr. Eva Severinson for IL-4 and 2.19 T-cell supernatant, Dr. Wesley Dunnick for cloned fragments of the genomic崔1 gene, and Dr. Richard Asofsky for his wonderful cell line, L10A6.2.

I like to thank my wife and son for their support and cooperation.

And, finally, I wish to express my appreciation for anyone in the department and in the school who has given me a hand through this process.
A simple acknowledgement cannot encompass my gratitude to all the people to whom I owe a debt during this long journey. I would like to say: your help will not be forgotten.
The antibody class switch is achieved by DNA recombination between the sequences called switch (S) regions located 5' to immunoglobulin (Ig) heavy chain constant (CH) region genes. This process can be induced in cultured B cells by polyclonal stimulation and switching can be directed to specific antibody classes by certain lymphokines. These stimuli may regulate the accessibility of CH genes and their S regions to a recombinase as indicated by hypomethylation and transcriptional activity. For example, RNAs transcribed from specific unrearranged (germ-line) CH genes are induced prior to switching under conditions that promote subsequent switching to these same CH genes. The function of transcription of these germ-line CH genes is unknown. How stimuli regulate the accessibility of CH genes is also unclear.

I report in this dissertation the structure of the RNA transcribed from the unrearranged Cy1 gene in mouse spleen cells treated with LPS plus a HeLa cell supernatant containing recombinant interleukin 4 (rIL-4). I will also show that an 150-bp region upstream of the first initiation site of germ-line γ1 RNA contains promoter and enhancer elements responsible for basal level expression and inducibility by phorbol 12-myristate 13-acetate
(PMA) and synergy with IL-4 in an IgM+ B cell line, L10A6.2, and an IgG2a+ B cell line, A20.3.

The germ-line γ1 RNA is initiated at multiple start sites 5' to the tandem repeats of the γ1 switch (Sγ1) region. As is true for analogous RNAs transcribed from other unrearranged genes, the germ-line γ1 RNA has an I exon transcribed from the region 5' to the Sγ1 region. The Iγ1 exon is spliced at a unique site to the Cγ1 gene. The germ-line γ1 RNA has an open-reading frame (ORF) that potentially encodes a small protein 48 amino acids in length.

Elements located within the 150 bp region 5' to the first initiation site of germ-line γ1 RNA are necessary and sufficient to confer inducibility by PMA and synergy with IL-4 to a minimal thymidine kinase (TK) promoter in L10A6.2 cells but are not sufficient to confer this inducibility in A20.3 cells. Linker-scanning mutations demonstrated that these multiple elements function in a mutually dependent manner as indicated by the fact that mutation of any single element will decrease constitutive expression and inducibility by PMA and PMA plus IL-4.

This 150-bp region contains several consensus sequences that bind to known or putative transcription factors, including a C/EBP binding site/IL-4 response element (in the promoter for Ia Aαk gene), four CACCC boxes, a PU box, a TGFβ inhibitory element (TIE), an interferon-αβ response element (αβIRE), and an AP-3 site.

My results begin to provide a description of the mechanism of regulation of the accessibility of unrearranged germ-line Sγ1-Cγ1
gene. By activating the germ-line γ1 promoter, IL-4 induces transcription of germ-line γ1 RNA, thereby inducing accessibility of the Sγ1-Cγ1 gene. By inhibiting expression of the germ-line γ1 promoter, IFNγ and TGFβ down-regulate transcription of germ-line γ1 RNA, thus reducing the accessibility of the Sγ1-Cγ1 gene. My results also suggest that signaling via the antigen receptor on B cells may be involved in induction of switch to IgG1. Furthermore, this is the first case reported in which multiple functionally interdependent elements are needed to respond to PMA.
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INTRODUCTION

The immune system is able to respond to an almost infinite number of antigenic challenges by producing a tremendous diversity of antibody specificities. An antibody consists of two heavy (H) chains and two light (L) chains. The amino terminal domains of these chains contain a region of variable amino acid sequence (variable region) that is usually unique to the chains of an antibody. The carboxy terminal domains of H and L chains contain regions with relatively invariant amino acid sequence (constant region). The variable region interacts with antigens, whereas the constant region is involved in a variety of effector functions, such as Fc-receptor binding and complement fixation. It is clear that a complete gene that encodes an antibody is generated by DNA recombinations (for review, see Yancopoulos and Alt, 1986).

Two DNA Recombinational Events Are Involved in Generating Complete Immunoglobulin Heavy Chain Genes

During B cell development and differentiation, immunoglobulin (Ig) genes undergo two types of rearrangements (Davis et al., 1980; Sakano et al., 1980). The first type of recombination event, called VDJ recombination, occurs at the pre-B cell stage between three separated germ-line DNA segments, VH (for variable), D (for diversity), and JH (for joining) (Yancopoulos and Alt, 1986). This type
of recombination brings one of the V_H segments, one of the D segments, and one of the J_H segments together and results in the deletion of intervening sequences and the formation of a gene which encodes the variable region of the heavy chain of an antibody. A recombined VDJ gene is initially associated with the most 5' constant region gene (C_\mu). The order of Ig heavy chain constant region genes in mouse is: 5'-V/D/J-C_\mu-C_\delta-C_\gamma3-C_\gamma1-C_\gamma2b-C_\gamma2a-C_\epsilon-C_\alpha-3' (Shimizu et al., 1981, 1982; Honjo, 1983). A successful VDJ rearrangement results in expression of cytoplasmic \mu chain in a pre-B cell. Subsequent VJ rearrangement of a light chain gene (\kappa or \lambda) and its expression results in a B lymphocyte which expresses membrane-bound IgM and IgD antibody. Only after a B cell expresses its surface antigen receptor (IgM and IgD) is it susceptible to antigen stimulation, whereupon it undergoes further differentiation. The second type of recombination, which is called heavy chain class switch recombination and occurs at the B cell stage, replaces the C_\mu gene with one of the constant region genes 3' to the C_\mu gene (except the C_\delta gene) and results in deletion of the CH genes located between the C_\mu gene and the target CH gene (Honjo and Kataoka, 1978; Honjo, 1983). A cell that has undergone this type of recombination will produce antibodies of other isotypes, although it maintains identical antigen specificity. Class switch recombination allows the progeny of a single pre-B cell to produce antibodies with the same antigen-binding specificity but with different immunological effector functions.
VDJ recombination is site specific and mediated by recognition sequences that flank all of the VH, D, and JH gene segments. The recognition sequences are two blocks of short, conserved sequences: a palindromic heptamer interrupted by an AT base pair at the middle of symmetry, CACTGTG, and a nonamer, GGTGGTTTGT (Early 1980; Sakano et al., 1980; Honjo, 1983; Yancopoulos and Alt, 1986). The 3' side of each VH segment and the 5' side of each JH segment are flanked by these two conserved sequences separated by 23 base pair spacers. Each D segment is flanked on both the 5' and 3' sides by the same heptamer and nonamer which are separated by 12 base pairs. Thus, according to the 12/23 joining rule, VH-to-D and D-to-JH joins are allowed, whereas VH-to-JH joins are not (Sakano et al., 1980; Early et al., 1980; Alt et al., 1984; Yancopoulos and Alt, 1986). VHDJH formation is accomplished through an ordered two-step process whereby DJH joining occurs first and serves as an intermediate after which VH-DJH joining occurs (Alt et al., 1984). Thus, diversity of antigen recognition results from the recombinational assembly of large numbers of different V, D, and J gene segments including N regions, followed by somatic mutation (Tonegawa, 1983).

Class switch recombination is mediated by the repetitive segments known as the switch (S) regions that lie a few kilobases upstream of each constant (C) gene (except Cδ). A recombined VDJ gene which is initially associated with the Cμ gene can be subsequently translocated to a downstream heavy chain constant
region (CH) gene by switch recombination occurring between the Sμ region and the S region of a target CH gene (Honjo and Kataoka, 1978; Honjo, 1983). S regions are structurally related and consist of tandem repeats of characteristic nucleotide sequences. Both the lengths and sequences of the repeated units differ for each S region, but all contain the short, conserved pentanucleotide sequences, GAGCT and GGGGT (Nikaido et al., 1981, 1982; Kataoka et al., 1981; Obata et al., 1981). The Sμ region was characterized by Nikaido et al. (1981) using restriction endonuclease digestion and sequencing. They found that the Sμ region is comprised of simple tandem repetitions of two kinds of pentanucleotides, GAGCT and GGGGT. The Sε region is the shortest S region and it shares considerable homology with Sμ in having an abundance of the shared common repeats (Nikaido et al., 1982; Gritzmacher, 1987). Sγ sequences contain relative few of these repeats (Kataoka et al., 1981; Wu et al., 1984; Obata et al., 1981), but Sγ regions (Sγ1, Sγ2b and Sγ3) are most homologous with each other (Kataoka et al., 1981). The Sα region also shares homology with Sμ in terms of the abundance of repeating pentamers (Obata et al., 1981). In contrast to VDJ recombination, switch recombination is not site-specific. Recombination sites occur at widely spaced positions within any particular S region and this appears not to be determined by the recombining pair of S regions. Thus, switch recombination generally does not involve extensive homologous pairing between the Sμ and target S regions near the recombination site (Marcu et al., 1982; Eckhardt and Birshtein, 1985). This is particularly apparent in the Sμ
region, but recombination in Syl occurs within the 49-bp unit repeats rather than within the unrelated DR (direct repeat) I and DR II sequences indicating that these 49-bp repeats may have functions in switch recombinations (Mowatt and Dunnick, 1986; Petrini et al., 1987).

Two basic models have been proposed for class switch recombination. The first involves intrachromosomal recombination. In this model, paring of the Sμ and target S region results in looping out of the intervening sequence. Recombination between S regions in the same chromosome results in the formation of hybrid S region (Sμ-target S regions) and a deletion of intervening sequences. The intervening sequence could form a DNA circle which would presumably be lost during cell division. The second model involves unequal sister-chromatid cross-over at S regions. This type of recombination results in a deletion on one chromatid and a duplication on the other chromatid. These two chromatids would segregate at mitosis (Honjo and Kataoka, 1978; Obata et al., 1981). Wabl et al. (1985), however, found that the frequency of unequal sister-chromatid exchange as a mechanism for creating a gene encoding the IgG2b heavy-chain is very low in a pre-B cell line.

Class switch can occur directly from IgM to a target isotype or occur successively. Recently, Yoshida et al. (1990) have characterized some immunoglobulin switch circles in splenocytes from mice infected with the nematode parasite, Nippostrongylus brasilenis. In
one kind of switch DNA circle that contains Cγ1 and 5'Sε and 3'Sγ1 (ε circle), a short piece of Sμ sequence was found to be inserted between Sε and Sγ1 sequences. These findings suggested that the recombination occurred in a successive manner, first from Cμ to Cγ1, and then from Cγ1 to Cε. The basic rule for successive switching is that a cell expressing an isotype encoded by a 5' CH gene can successively switch to an isotype encoded by a 3' CH gene but once switched to an isotype encoded by a 3' CH gene, it can not switch back to a isotype encoded by a 5' CH gene.

The Specificity of Immunoglobulin Class Switching Is Directed.

The regulation of specificity of antibody class switching has been extensively reviewed (Cebra et al., 1984; Bergstedt-Lindqvist et al., 1984; Teale and Abraham, 1987; Coffman et al., 1988; Gritzmacher, 1989; Esser and Radbruch, 1990). A large body of evidence indicates that the specificity of antibody class switching can be influenced by the stimulation of antigens, by T cells, and/or by T cell derived soluble products.

A tremendous amount of work has been invested in attempting to determine whether antigens promote isotype specific switching during normal B lymphocyte differentiation (for review, see Cebra et al., 1984). The conclusions drawn from these earlier experiments are: 1) Mitogen, such as lipopolysaccharide (LPS), can induce B lymphocytes to produce IgG3 and IgG2b and thymus-independent
antigens (type 1 and type 2) give similar results in vivo. 2) Thymus-dependent antigens can promote expression of all isotypes in vivo and the specific isotypes expressed are influenced by T cells. It is now clear that activated T cells can regulate the isotype switching by themselves and secrete lymphokines to direct isotype switching.

Many experiments have shown that T cells play an important role in class switching. Using T cell clones specific to the carrier keyhole limpet hemocyanin (KLH) as the sole source of T helper (TH) cells, it has been shown that a single T-cell clone is able to elicit all heavy chain classes, although some isotypes (IgM, IgG1, IgA) are favored (Teale, 1983). However, Kawanishi et al. (1983) found that T cells derived from Peyer’s patches (PP), a mucosal site important in IgA regulation, selectively stimulated IgA production by LPS-stimulated PP B cells and suppressed the production of IgM, whereas LPS, in the absence of PP T cells, induced abundant production of IgM and IgG. T cell clones derived from spleen also could induce IgA synthesis by LPS-stimulated B cells but did not inhibit IgM and IgG production. Kawanishi et al. (1983) have also found that stimulation of IgA production did not appear to be the result of increased proliferation of IgA-expressing cells because LPS treatment of cultures of purified PP sIgM B cells and cloned PP T cells caused appearance of sIgA-bearing cells.

Cloned mouse helper T cells fall into two subsets, TH1 and TH2, which have different effects on class switching. Stevens et al. (1988) showed that, in the presence of antigen-carrier complex, both
carrier-specific TH1 and TH2 cells are capable of inducing antigen-specific B cells to secrete IgM and IgG3, whereas clones of TH1 and TH2 cells specifically induce these B cells to secrete IgG2a or IgG1, respectively. They claimed that regulation of secretion of a particular Ig isotype occurred at two distinct stages: cognate interaction between T and B cells and interaction between T-cell-derived lymphokines and B cells. They found that in the presence of TH1 cells, which produce IFNγ which inhibits induction by IL-4 (see below), addition of IL-4 induces secretion of IgG1. In contrast, addition of IFNγ to cultures containing TH2 cells, did not induce an IgG2a response but did inhibit the IgG1 response. Their explanation is that either TH2/B cell interaction induces a signal that prevents IFNγ from inducing IgG2a or TH1/B cell contact is necessary for the induction of IgG2a. Lebman and Coffman (1988) also noticed that addition of IL-4 did not affect the proportion of TH2-stimulated B cell clones which secrete IgG1 and they suggested that other consequences of TH2/B cell interaction may play a role in the generation of an IgG1 response, but this can also be explained by the possibility that TH2 cells had already secreted a saturating concentration of IL-4 into the cultures.

Soluble T-cell-derived lymphokines have also been shown to influence the specificity of antibody class switching by LPS activated B cells (Isakson et al., 1982; Bergstedt-Lindqvist et al., 1984; Coffman et al., 1988; Teale and Abraham, 1987; Esser and Radbruch, 1990). Normal IgM+ splenic B cells from mice will switch to IgG3 and IgG2b
expression after polyclonal stimulation in culture with LPS. This
isotype pattern can be altered by addition of interleukins, although
interleukins by themselves cannot induce switching (Bergstedt-
Lindqvist et al., 1984; Isakson et al., 1982).

Effects of IL-4 and IL-5 on class switching

The best studied murine lymphokine that regulates expression of
specific isotypes is IL-4 (BSF-1) (Paul and Ohara, 1987). IL-4 is
produced by TH2 cells. The addition of supernatant from activated
TH2 clones to LPS-stimulated spleen B cells results in a significant
enhancement of the production of IgG1 and IgE (Isakson et al., 1982;
Bergstedt-Lindqvist et al., 1984 and 1988) and a small increase in
IgA (Coffman and Carty, 1986). Using purified IL-4 and a blocking
monoclonal antibody to IL-4, investigators have found that both IgG1
and IgE enhancing activities are due to IL-4 (Vitetta et al., 1985;
Coffman et al., 1986; Bergstedt-Lindqvist et al., 1988; Lebman and
Coffman, 1988) and IgA enhancing activity is due to IL-5 (Bond et
al., 1987). IL-4 does not have to be present from the beginning of the
culture, but must be added by day 2 for maximal IgE and IgG1
enhancement. If IL-4 was added after day 3, no enhancement of
either isotype could be detected (Coffman and Carty, 1986). The IL-4
induced IgG1 and IgE switching can be inhibited by interferon-γ
(IFNγ) and enhanced by IL-5. Although IL-5 itself has a little effect
on the level of IgE and IgG1 produced in response to saturating
concentrations of IL-4, it can substantially enhance IgE and IgG1
production when IL-4 is used at low concentration (Coffman et al.,
1988). For example, in the presence of IL-5 at 5 units/ml, the concentration of IL-4 required for 1/2 maximal enhancement of both IgE and IgG1 was reduced by 10- to 30-fold. Approximately 100 U/ml and 30 U/ml of IL-4 are required for 1/2 maximal IgE and IgG1 production, respectively. By the use of limiting dilution analysis, Bergstedt-Lindqvist et al. (1988) found that IL-4 dramatically increases the precursor frequency of IgG1 and IgE-secreting cells with no significant effect on the clone size, suggesting that IL-4 and LPS are acting directly on uncommitted B cells, directing them to switch to a different isotype, rather than expanding committed subsets of cells. Lebman and Coffman (1988) have also shown that IL-4 increases both the proportion of clones that secrete IgE and the amount of IgE secreted by individual clones in clonal cultures of B cells stimulated by TH2 cell line, indicating IL-4 may have two effects on IgE production by B cells in the presence of TH2 cells.

Some other lymphokines have been reported to induce expression of other isotypes. IL-5, in the absence of IL-4, can increase the IgA response in LPS induced cultures. The IgA enhancing activity of IL-5 could be augmented by IL-4. For example, purified IL-5 induced one-third to one-half the levels of IgA production compared to a TH2 supernatant and addition of IL-4 to the culture restored the IgA production to the same level as induced by TH2 supernatant (Coffman et al., 1987). Both IL-4 and IL-5 are produced by TH2 cells. The combined effect of IL-4 and IL-5 may account for the effect of TH2 cells in isotype switch. IgA production
induced by IL-5, unlike the IgG1 and IgE induced by IL-4, is not inhibited by concentrations of IFNγ that inhibit IL-4 induction of IgG1 and IgE (Coffman et al., 1987; Bond et al., 1987). Harriman et al. (1988) have shown that IL-5 can induce mIgA-positive Peyer's patch (PP) B cell to secrete large amounts of IgA but does not induce significant amounts of IgA secretion from mIgA-negative PP B cells. This result indicates that IL-5 acts on committed IgA B cells, promoting differentiation into IgA-secreting cells with resultant increased IgA secretion.

Effects of IFNγ and TGFβ on class switching

IFNγ, at low dose, has been found to inhibit IgE expression completely and most of the IgG1 expression induced by IL-4 in LPS-stimulated B cells (Coffman and Carty, 1986; Snapper and Paul, 1987). It appears that IFNγ and IL-4 can reciprocally regulate Ig isotype production in T cell-dependent immune responses. IFNγ also inhibits switching to IgG3 and IgG2b. IFNγ, however, can cause an increase in IgG2a expression by murine splenic B cells with a peak at 10 U/ml of IFNγ (Snapper and Paul, 1987). Addition of antibody to IFNγ (anti-IFNγ) to the culture could completely reverse the inhibitory effect of IFNγ on LPS stimulated murine splenic B cells. The reciprocal effects of IL-4 and IFNγ have also been studied in vivo (Finkelman et al., 1988). Injection of mice with anti-IgD induced a large increase in levels of serum IgG1 and IgE and a relatively small increase in serum IgG2a. Multiple injection of these mice with IFNγ substantially inhibited production of IgE and partially inhibited
production of IgG₁. Anti-IFNγ antibody blocked the effects of IFNγ and increased IgG₁ and IgE production and inhibited the IgG₂a response in these mice. Using highly purified splenic B cells, Coffman and Carty (1986) found that both IL-4 and IFNγ acted directly on B cells.

More recently, transforming growth factor-β1 (TGFβ1) has been reported to reduce the expression of all antibody classes except IgA whose expression is increased by TGFβ in LPS-stimulated Peyer's patch (PP) and splenic B cells (Coffman et al., 1989; Kim and Kagnoff, 1990; Lebman et al., 1990a). TGFβ stimulates IgA production by LPS-treated surface IgA⁻ cells and increases the frequency of IgA-secreting B cell clones, indicating that TGFβ promotes switching to IgA by LPS-stimulated IgA⁻ cells rather than expanding IgA⁺ cells or inducing them to secrete more IgA. Although IL-2 alone weakly enhances IgA production in these cultures, it synergizes with TGFβ, resulting in a dramatic increase of IgA secretion. IL-5 also enhances the TGFβ-induced IgA response, but to a lesser extent than IL-2. In these cultures, IL-2 does not increase either the proportion or the total number of sIgA⁺ cells, indicating that IL-2 induces secretion of IgA by sIgA⁺ cells rather than promotes class switching. Thus, the synergistic effect of TGFβ and IL-2 on IgA production is achieved by two different steps: TGFβ promotes IgA⁻ cells switch to IgA⁺ and IL-2 increases the secretion of IgA by sIgA⁺ cells. Recently, Miyajima et al. (1991) found that IL-2 completely inhibits IgE production induced by IL-4 in LPS-stimulated spleen B cells and also inhibited IgG₁
production if IL-2 was added before IL-4, but these authors did not determine whether IL-2 inhibits switching to IgE or inhibits the secretion of IgE by IgE+ cells.

Effect of cholera toxin on class switching
Recently, cholera toxin (CT) has been reported to be a powerful immunogen and adjuvant and when CT is admixed with an antigen with various immunization protocols, it greatly enhances the immune response to that antigen, particularly the IgG1 and IgA responses (Lycke et al., 1989, 1990). This group has also shown that CT promotes IgG1 switch differentiation in spleen B cells stimulated with LPS. CT increases the proportion of IgG1-secreting cells but also synergizes with IL-4 to further increase switching to IgG1. CT plus IL-4 has no effect on the formation of cells producing IgA and suppresses IgM and IgG3 responses.

Normal spleen B cells are multipotent for isotype expression. During clonal expansion, a single precursor can give rise to progeny that produce antibodies with multiple isotypes depending on the stimuli (Cebra et al., 1984). In some B cell or pre-B cell lines, however, class switching appears committed. The murine B-cell lymphoma, I.29μ, is one model system used to investigate the regulation of isotype switching (Stavnezer et al., 1982, 1984, 1985, 1986). Addition of LPS (with or without anti-I.29μ idiotype antibody) to cultured I.29μ (IgM+) cells induces IgA-expressing cells and a very low proportion of IgE-expressing cells. Cytoplasmic staining experiments demonstrating the presence of IgM+IgA+ double-
expressing cells indicated that at least some of the IgA+ cells in the
cultures are a result of isotype switching rather than clonal
expansion. In I.29μ cells isotype switching is accompanied by DNA
recombinations that occur within or immediately 5' to the targeted
switch regions. In cultured Abelson murine leukemia virus (A-
MuLV) transformed pre-B cells, spontaneous isotype switching to
IgG2b occurs at a low frequency (Akira et al., 1983; Burrows et al.,
1983) and this switching can be induced by LPS and inhibited by IL-
4 (Lutzker et al., 1988). Notably, all the switching in A-MuLV-
transformed pre-B cell lines is from IgM to IgG2b and switching is
accompanied by recombination between Sμ and Sy2b regions
although some recombinations occur outside of the switch regions.
Some of these lines do not have their L chains rearranged, indicating
class switch recombination can occur before variable region gene
recombination. It is not clear whether this is a consequence of the
transformation by A-MuLV. Another cell line, CH12LX, which is IgM+
and specific for sheep red blood cells, has increased mIgA expression
and IgA secretion after treatment with LPS and a lymphokine-rich
supernatant of the D10 T cell line, which contains IL-4 and IL-5 but
no IL-2 (Kunimoto et al., 1988). These investigators have also found
that IL-4 induces an increase in the number of mIgA+ cells, whereas
IL-5 has no effect on mIgA expression but increases IgA secretion.
Addition of both IL-4 and IL-5 to the culture produces the same
effect as D10 supernatant.
The common feature of class switching in transformed B and pre-B cell lines is that they have committed isotype switching. The switching patterns in B and pre-B cell lines are different from that in mouse spleen cells. Committed isotype switching in these cell lines may be due to their being transformed. Alternatively, these lines may represent a particular stage of differentiation of B cells at particular locations in the body and/or immediately after receiving a particular signal from a T cell or other cell or from a cytokine.

In summary, specificity of the class switching is regulated by LPS and lymphokines in mouse spleen cells. LPS directs switching to IgG3 and IgG2b. In the presence of LPS, IL-4 induces switching to IgG1 and IgE and inhibits switching to IgG3 and IgG2b. IFNγ inhibits switching induced by IL-4 and induces switching to IgG2a. TGFβ induces switching to IgA but inhibits switching to other isotypes. The effect of IL-5 on IgA production appears to be achieved by enhancing the synthesis and secretion of the IgA. IL-2 increases the secretion of IgA but inhibits production of IgE and IgG1 by an unknown mechanism.

Accessibility Model and Immunoglobulin Germ-line Transcripts
The mechanism underlying specificity of class switching remains unclear. An accessibility model has been proposed. According to this model, specificity of switching is directed by regulation of the accessibility of CH genes and their S regions to recombinase
(Stavnezer-Nordgren and Sirlin, 1986; Yancopoulos et al., 1986; Lutzker et al., 1988). There are three lines of evidence supporting this model. (1) The finding that the I.29μ cell line, which switches from IgM to IgA upon treatment with LPS, has hypomethylated Cα genes. (2) The finding that IL-4 induces DNase I hypersensitive sites 5' to and within the Sγ1 region. (3) The finding that transcripts of specific unrearranged (germ-line) CH genes are induced prior to switching to these same isotypes. All three pieces of evidence are indicators of an activated gene.

The first evidence supporting the accessibility model came from the study of Stavnezer-Nordgren and Sirlin (1986) who found that the I.29μ cell line, which switches from IgM to IgA when treated with LPS, has hypomethylated Cα genes that are transcriptionally active, whereas CH genes to which I.29μ does not switch are hypermethylated and transcriptionally inactive. A large body of data has shown that DNA methylation at sites specific for each gene regulates gene expression in higher eukaryotic cells (Doerfler, 1983).

The second evidence supporting the accessibility model is the finding that IL-4, with or without LPS, induces DNase I hypersensitive sites 5' to and in the Sγ1 region in activated murine B cells (Schmitz and Radbruch, 1989; Berton and Vitetta, 1990). These IL-4 induced DNase I hypersensitive sites may reflect an increase in the accessibility of the Sγ1 chromatin to DNase I and presumably also to other enzymes, such as switch recombinase, but most likely
reflects the binding of IL-4 responsive regulatory proteins, such as transcription factors, within or near the hypersensitive sites.

The best evidence supporting the accessibility model is that in all cases of isotype switching, specific transcripts are induced from particular CH genes prior to switching to this isotype. For example, IL-4 appears to direct the switch to IgG1 and IgE by increasing the accessibility of γ1 and ε genes, as shown by the fact that IL-4 induces transcripts from unrearranged γ1 and ε genes (Stavnezer et al., 1988; Rothman et al., 1988; Berton et al., 1989; Esser and Radbruch, 1989; Gauchat et al., 1990; Severinson et al., 1990). In normal splenic B cells LPS induces germ-line γ2b and γ3 transcripts (Lutzker et al., 1988; Severinson et al., 1990). IL-4 induces germ-line γ1 and ε transcripts and high doses of IL-4 suppresses LPS-induced γ3 and γ2b transcripts (Severinson et al., 1990). IFNγ induces a low level of germ-line γ2a transcripts and profoundly inhibits the germ-line γ1 and ε transcripts. Lutzker et al. (1988) have also found that LPS specifically induces transcription through the germ-line γ2b locus before γ2b class switching occurs in A-MuLV-transformed pre-B cells. Sterile μ RNA has been found (Lennon and Perry, 1985). Germ-line α RNA is also transcribed from the Cα genes in I.29μ cells (Stavnezer-Nordgren and Sirlin, 1986; Stavnezer et al., 1988). TGFβ, which induces switching to IgA, also induces germ-line α transcripts in spleen cells and in I.29μ (Lebman et al., 1990a, 1990b; Stavnezer et al., 1990). Lycke et al. (1990) have shown that cholera toxin (CT), which promotes switching to IgG1, also induces germ-line γ1 RNA in
LPS-stimulated normal B cells. More strikingly, CT enhances by seven-fold the expression of germ-line γ1 RNA in LPS-stimulated spleen cells treated with optimal IgG1-inducing concentrations of IL-4.

The induction of transcription of germ-line RNA is rapid. Berton et al. (1989) showed that germ-line γ1 RNA could be detected as early as 4 hours after treatment with LPS and IL-4. Lutzker et al. (1988) found similar results in A-MuLV transformed pre-B cells. TGFβ induced transcription of germ-line α RNA was also detected by 4 hours of induction (P. Shockett and J.S., in preparation). The pattern of regulation of transcription of germ-line RNAs is consistent with that of regulation of class switching. From the results mentioned above, the correlation between regulation of transcription of germ-line RNAs and that of class switching has been firmly established.

The structures of many germ-line RNAs have been defined by sequencing cDNA clones derived from germ-line RNA, and also by RNase protection, S1 mapping, primer extension, and polymerase chain reaction (PCR) experiments. Among the characterized germ-line RNA species are: μ (Lennon and Perry, 1985), γ2b (Lutzker et al., 1988), α (Radcliffe et al., 1990; Gaff and Gerondakis, 1990; Lebman et al., 1990b), ε (Rothman et al., 1990a; Gerondakis et al., 1990; S. Delphin unpublished data), and γ3 (Rothman et al., 1990b). Determination of the structure of germ-line γ1 RNA was part of my thesis research. The results have been published (Xu and Stavnezer, 1990).
The germ-line RNAs share several common features. (1) All the germ-line RNAs have an exon (I exon, I standing for initiated within the JH-CH intron) (Lennon and Perry, 1985) located 5' to each S region. The sizes of the I exons differ. The Iμ exon is the longest (about 700 bp) and Iγ2b is about 420 bp long. The size of the Iα exons vary from 200 to 460 bp due to the presence of multiple 3' splice sites. The Iε exon is about 80 to 100 bp in length. (2) All germ-line RNAs have multiple initiation sites ranging from two start sites (α) to more than ten start sites (μ and γ1). The initiation sites of germ-line ε and γ3 RNAs have not been defined precisely. All I exons have a unique splice site which marks the 3' border of I exons except for Iα (see above). (3) All germ-line RNAs are transcribed in the sense direction through the S regions and the I exon is spliced to the C region. (4) There are no TATA and CAAT boxes located 5' of initiation sites of germ-line RNAs (except α has an inverted CAAT box located at -54/58 relative to the first initiation site (Lin and Stavnezer, submitted). (5) Germ-line transcripts have been assumed not to encode proteins although several of them have small open reading frames (ORFs) in I exons. Germ-line α RNA appears to be associated with polysomes and can be translated in a cell free system (Radcliffe et al., 1990; D.J. Waite, unpublished data).

The function of germ-line RNA is unknown. Germ-line RNAs might simply be by-products of transcriptionally activated CH genes. Alternatively, transcripts might serve as part of the mechanism of switch recombination or encode a protein involved in switch
recombination. The act of transcription may serve to change chromatin structure, DNA methylation, or supercoiling and thereby direct switch recombination.

Working Hypothesis and Specific Questions
All the results mentioned above suggest that the stimuli that regulate transcription of germ-line RNA also regulate the accessibility of CH genes to recombinase, although germ-line RNAs and/or products of germ-line RNAs may also function in antibody class switching. My working hypothesis is that stimuli that regulate transcription of germ-line RNAs must do so via promoter and enhancer elements many of which will be located 5' to or around the initiation sites of germ-line RNAs. In order to identify DNA sequences necessary for the regulation of transcription of germ-line RNA and to understand the possible functions of germ-line RNA, it is first necessary to define the structure of germ-line RNA. I chose to study regulation of the germ-line γ1 RNA because when I first came to Dr. Janet Stavnezer's laboratory, it was found that germ-line γ1 RNA was induced in IL-4 treated mouse spleen cells and the structure of germ-line γ1 RNA had not yet been defined. Based on these results, my thesis research work attempted to answer the following questions:
1) What is the structure of germ-line γ1 transcripts?
2) Are there functional promoter and/or enhancer elements 5' to or around the start sites of germ-line γ1 RNA?
3) Are these sequence elements responsible for regulation by IL-4 and/or other stimuli?

In this dissertation, I describe results that demonstrate I have achieved the initial goals proposed at the beginning of my thesis research.

1) I have defined the structure of germ-line γl RNA.

2) I have demonstrated the existence of several DNA sequence elements located within an 150-bp region relative to the first initiation site of germ-line γl RNA. These elements function together as a promoter to activate a luciferase gene in γl promoter-luciferase gene fusion plasmids.

3) These functional mutually dependent elements are necessary and sufficient to confer PMA and PMA plus IL-4 inducibility in a B cell line L10A6.2 and are necessary but not sufficient to confer PMA and PMA plus IL-4 inducibility in another B cell line, A20.3.

4) These findings support the accessibility model for regulation of class switching and begin to explain how accessibility is regulated.

5) The γl promoter is so-far unique in that multiple elements are required to obtain a PMA activated signal. Furthermore, multiple elements are required to obtain induction of the γl promoter by IL-4.
The following manuscript has been published


Most of the Introduction and the entire Discussion of this manuscript have been fused into the Introduction and Discussion of the dissertation. The references have been incorporated into bibliography of the dissertation.
MANUSCRIPT 1

Structure of Germ-Line Immunoglobulin Heavy-Chain γ1 Transcripts in Interleukin 4 Treated Mouse Spleen Cells

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ABSTRACT

Antibody class switching is mediated by a DNA recombination event that replaces the $C_\mu$ gene with one of the other heavy (H) chain constant region ($C_H$) genes located 3' to the $C_\mu$ gene. The regulation of this process is essential to the immune response because different $C_H$ regions provide different biological functions. Correlative evidence indicates that the isotype (class) specificity of the switch is determined by the accessibility of specific $C_H$ genes as indicated by hypomethylation and transcriptional activity. For example, RNAs transcribed from specific unrearranged $C_H$ genes are induced prior to switching under conditions that promote subsequent switching to these same $C_H$ genes. The function of transcription of these germ-line $C_H$ genes is unknown. In this report, we describe the structure of RNA transcribed from unrearranged $\gamma_1$ genes in mouse spleen cells treated with LPS plus a HeLa cell supernatant containing recombinant interleukin 4. The germ-line $\gamma_1$ RNA is initiated at multiple start sites 5' to the tandem repeats of the $\gamma_1$ switch ($S_{\gamma_1}$) region. As is true for analogous RNAs transcribed from unrearranged $\gamma_2b$ and $\alpha$ genes the germ-line $\gamma_1$ RNA has an I exon transcribed from the region 5' to the $S_{\gamma_1}$ region, which is spliced at a unique site to the $C_\gamma 1$ gene. The germ-line $\gamma_1$ RNA has an open-reading frame (ORF) that potentially encodes a small protein 48 amino acids in length.
INTRODUCTION

To identify DNA sequences necessary for their regulation and to understand the possible function(s) of germ-line transcripts, it is first necessary to know the structure of the germ-line transcripts. We report here the structure of germ-line \( \gamma 1 \) transcripts induced in mouse spleen cells by treatment with LPS plus a HeLa cell supernatant containing rIL-4. We find that the structure of the germ-line \( \gamma 1 \) RNA is similar to that of RNAs transcribed from unrearranged \( \text{Cy}2b \) and \( \text{C} \alpha \) genes (Lutzker and Alt, 1988; Radcliffe et al., 1990).

RESULTS

LPS and IL-4 Induce Germ-Line \( \gamma 1 \) RNA Transcripts

We have previously shown that treatment of spleen cells with IL-4 or a supernatant from a \( \text{T}_{\text{H}2} \) cell line that contains IL-4 (Noma et al., 1986) (and other factors) in the presence or absence of LPS induces in B cell transcripts from unrearranged \( \text{Cy} \) genes that hybridize with the 5'S\( \gamma 1 \) HindIII-PstI segment (Fig. 1A) as 1.7-kb RNA species (Stavnezer et al., 1988; Severinson et al., 1990). We began to localize the sequences encoding the I exon of germ-line \( \gamma 1 \) RNA by additional RNA blotting experiments. The 2.7 kb HindIII-PstI fragment derived
from clone γ1/EH10 (Mowatt and Dunnick, 1986) was subcloned into three fragments: a 1.2-kb HindIII-BamHI fragment, a 0.8-kb BamHI-KpnI fragment, and 0.7-kb KpnI-PstI fragment (Fig 1A). Labeled RNA probes transcribed from these fragments were hybridized with blots containing poly(A)+ RNA from mouse spleen cells induced for 2 days with LPS and IL-4. Of these three fragments, only the KpnI-PstI probe detected the 1.7-kb and 3.2-kb RNAs (Fig. 1B). No RNA was detected by the HindIII-BamHI or BamHI-KpnI probes (data not shown). No RNA was detected with a KpnI-PstI probe for antisense transcripts (data not shown). This result indicates that the I exon of germ-line γ1 RNA is encoded within the KpnI-PstI fragment.

**Determination of Splice Site of Germ-Line γ1 RNA**

In order to precisely locate the Iγ1 exon, we used the PCR to prepare cDNA clones containing the 3' donor splice site of the Iγ1 exon. Based on the RNA blotting data described above and previous work (Stavnezer et al., 1988), we expected that the germ-line γ1 RNA was initiated within the KpnI-PstI fragment and spliced to the Cy1 gene. To show this, we synthesized a Cy1 oligonucleotide complementary to the 5' end of the Cy1 gene (Figs. 1A and 2C) and an oligonucleotide (oligo 4) containing sequences located from 448 to 465 nucleotides 3' of the KpnI site (Figs. 1A and 2A). Oligo 4 primes DNA synthesis toward the 3' direction and Cy1 oligo primes DNA synthesis toward the 5' direction. Using the PCR to amplify cDNA transcribed from
poly(A)+ RNA from spleen cells treated 2 days with LPS and 15% rIL-4-containing HeLa cell supernatant, we obtained several cDNA clones that contained the splice site of germ-line γ1 RNA. Eight of these clones were sequenced. All eight of these clones had the identical splice donor located between nucleotides 633 and 634 in Fig. 2A and demonstrated that the splice acceptor at the 5' end of the Cγ1 gene which is used in γ1 mRNA is used for germ-line γ1 RNA. The location of the Iγ1 splice donor is consistent with RNase protection experiments in which a predominant protected band of 281 bp was obtained after hybridization of total cell RNA with a RNA probe transcribed from the BglIII-PstI segment (data not shown). These results are also consistent with S1 protection experiments of Berton et al. (1989). The splice junction of the Iγ1 and Cγ1 exons employ consensus donor and acceptor sequences (Figs 2A and 2B) (Shapiro and Senapathy, 1987).

Initiation Sites of Germ-Line γ1 RNA
The initiation sites for germ-line γ1 RNA were determined by RNase protection experiments. Hybridization of RNA from spleen cells (induced with LPS plus 15% HeLa cell supernatant which contains IL-4) with a RNA probe transcribed from the genomic DNA KpnI-PstI segment produced multiple bands after electrophoresis of the RNase resistant products on a sequencing gel (Fig. 1C). The lengths of the predominant bands varied from 387 to 484 nucleotides. Less predominant bands of 139 to 359 nucleotides in length were also
observed. These results indicated that the 5' border of the Iγ1 exon occurred at multiple sites since we had only found a single 3' splice site, suggesting that the germ-line γ1 RNA may have heterogeneous initiation sites. To confirm this and to more precisely locate the initiation sites, primer extension experiments were performed. As the RNase protection experiments indicated the major initiation sites are located 5' of the BglIII site, an oligonucleotide (oligo 2) complementary to the sequence from 8 to 24 nucleotides 3' to the BglIII site was used for primer extension experiments (Fig. 2A). The 3' end of oligo 2 is 262 bp 5' to splice site thus the sizes of bands obtained from primer extension experiment differ from those obtained from RNase protection experiment by 262 base pairs. The sizes of the primer extended products (Fig. 1D) matched with those predicted from RNase protection experiments and indicated that there are multiple initiation sites for germ-line γ1 RNA. The fact that the results from RNase protection and primer extension completely corresponded indicated that the multiple bands on the gels were not due to degradation of the RNA. In addition to the predominant initiation sites located 5' to the BglIII site, there are several initiation sites located 3' to the BglIII site that were detected by the RNase protection assay shown in Fig. 1C and by primer extension experiments (not shown) using an oligonucleotide (oligo 1) complementary to sequences located 75-92 nucleotides 5' to the splice site. Taken together, germ-line γ1 RNA has multiple initiation sites distributed over a 345-nucleotide region, but the predominant
initiation sites are located within a region of about 97 bp near the 5' most start site. The most 5' initiation site of the Iγ1 exon is 484 bp upstream of the Iγ1/Cγ1 splice site. The sizes of the Iγ1 exon determined from these experiments (387 to 484) (predominant) and the Cγ1 exon (1067 bp) (Honjo et al., 1979) would produce a germ-line γ1 RNA of 1.7 kb, assuming a 200-nucleotide poly(A) tail. This corresponds in size to the predominant 1.7 kb germ-line γ1 RNA detected on RNA blot. As is also true for analogous RNAs transcribed form immunoglobulin α (Radcliffe et al., 1990), γ2b (Lutzker and Alt, 1988), and μ (Lennon and Perry, 1985) genes, there are no TATA and CAAT boxes located within 150 nucleotides upstream of the initiation sites of the germ-line γ1 RNA (Fig. 2A).

The Germ-Line γ1 RNA Has an Open-Reading Frame (ORF)
The nucleotide sequence of the γ1 exon indicated the presence of an open-reading frame (ORF) that would be initiated by a Met codon located in such a position that germ-line γ1 RNA initiated at any of the sites we detected would have the Met. Potentially, this ORF would encode a 48 amino acid polypeptide with a termination codon located within the Cγ1 domain (Figs. 2A and 2C). The reading frame that would be used in the Cγ1 exon for the ORF in germ-line RNA differs from that used in the mRNA for γ1 H chains. The predicted amino acid sequence of the ORF is indicated below the nucleotide sequence in Fig. 2C. As is true for the ORF encoded by germ-line α RNA, the nucleotide sequence surrounding the initiator AUG codon should
form a good translation initiation site according to Kozak (1986, 1987) since it has a purine at the -3 and a G at the +4 positions.

MATERIALS AND METHODS

Mice and Cell Culture
BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, Massachusetts). Spleen cells (2x10^6/ml) from mice were cultured for 2 days in RPMI 1640 (GIBCO, Grand Island, New York) in the presence of 10% fetal calf serum (HyClone Laboratories, Logan, Utah). LPS (RIBI Immunochrom Research Inc., Hamilton, Montana) was added at 25 µg/ml. Either 8 units/ml of recombinant IL-4 (Stavnezer et al., 1988) or 15% HeLa cell supernatant which contains IL-4 were added (Noma et al., 1986) (kindly donated by Eva Severinson of the University of Stockholm).

RNA Isolation and Blot Hybridization
Total cell RNA was prepared by the guanidinium isothiocyanate-CsCl protocol and poly(A)+ RNA was isolated by one cycle of
chromatography on oligo(dT)-cellulose. Radioactive RNA probes were transcribed from 5'Sγ1 germ-line DNA fragments cloned into Bluescript plasmid (Stratagene, La Jolla, California) and hybridization was performed as described (Maniatis et al., 1982).

DNA Sequencing
Sequenase (United States Biochemicals Corp., Cleveland, Ohio) was used for sequencing plasmid DNA (Tabor and Richardson, 1987).

Polymerase Chain Reaction (PCR)
PCR was performed according to Frohman et al. (1988). Briefly, 2 μg of poly(A)+ RNA (preheated at 65°C for 3 min and put on ice for 1 min) was reverse transcribed using a Cγ1 oligo (Figs 1A and 2C) complementary to Cγ1 sequence (Honjo et al., 1979) in a 20-μl reaction containing 50 mM Tris-HCl (pH 8.15), 6 mM MgCl2, 40 mM KCl, 1 mM dithiothreitol (DTT), each dNTP at 1.5 mM, 25 μCi of [32]P-dCTP, 25 U of RNase inhibitor (RNasin) (Boehringer Mannheim Biochemicals, Indianapolis, Indiana), and 10 U of avian myeloblastosis virus reverse transcriptase (Life Science, St. Petersburg, Florida) for 2 hours at 42°C. After transcription, the reaction mixture was passed over a 5-ml Sepharose CL-6B column. The first peak (6 drops) was collected and diluted with 500 μl of 10 mM Tris (pH 8.0), 1 mM EDTA and stored at 4°C. The PCR was
performed with 10 µl of above cDNA in 50 µl of 10% (v/v) dimethyl sulfoxide, 67 mM Tris-HCl (pH 8.8), 16.6 mM (NH)₄SO₄, 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol, 100 µg/ml BSA, 1.6 mM each dNTP, with 200 ng Cyl oligo, 200 ng oligo 4 (Figs 1A, 2A, and 2C), and 2.5 U of Thermus aquaticus (Taq) DNA polymerase (Perkin-Elmer-Cetus). Just before adding Taq enzyme, the reaction mixture was denatured at 95°C for 5 min and annealed at 45°C for 2 min. 30 µl of mineral oil (Sigma) was overlaid. The cDNA was amplified at 72°C for 30 min followed by 40 cycle PCR using a Techne programmable Dri-Block machine (American Bioanalytical, Natick, Massachusetts). Each cycle was programmed as 94°C, 1.2 min; 48°C, 2.4 min; and 72°C, 3.6 min. The amplified products were cloned into a Bluescript plasmid with blunt-end ligation and identified using the 5'Syl BamHI-PstI fragment as a probe (Fig. 1A).

**RNase Protection**

Full-length ³²P-labeled antisense RNA probes were transcribed by T7 polymerase from Bluescript plasmids containing various 5'Syl segments. RNase protection analysis was performed using these probes as described (Zinn et al., 1983). Briefly, ³²P-labeled (2 to 5 X 10⁵ cpm) full-length RNA probe produced by transcription with T7 RNA polymerase was hybridized with 20 µg of total cell RNA or yeast RNA in 30 µl of 80% (v/v) formamide, 40 mM PIPES pH 6.7, 0.4 M NaCl, 1 mM EDTA at 45°C overnight. Following hybridization, 300 µl
of RNase digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.3 M NaCl) containing 20 μg/ml of nuclease P1 and 2 μg/ml of RNase T1 was added. After mixing, the reaction was incubated at room temperature for 1 hour and terminated by adding 20 μl of 10% SDS and 50 μg proteinase K and incubating at 37°C for 15 minutes. The reaction was then extracted with phenol/chloroform once and 10 μg of yeast RNA was added. The reaction was precipitated by adding 1 ml of 95% ethanol and centrifuging in a microcentrifuge at 4°C for 20 minutes. The pellet was washed once with 75% ethanol and dried down at room temperature for 30 minutes. The pellet was dissolved in 5 μl of loading buffer (80% formamide, 50 mM Tris-HCl pH 8.2, 1 mM EDTA, 42 mM boric acid, 0.1% xylene and bromphenol blue) and electrophoresed on a sequencing gel.

Primer Extension

Primer extension was performed as described (Ausubel et al., 1987). Five ng 32P-labeled oligo 2 (Figs 1A and 2A) (labeled using T4 polynucleotide kinase) was mixed with 150 μg of total cell RNA in 30 μl of 80% (v/v) deionized formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, and 1 mM EDTA (pH 8.0), and incubated overnight at 30°C. After ethanol precipitation, 25 μl of a mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 5 mM DTT, 50 mM KCl, 560 μM of each dNTP, 50 μg/ml BSA, 1.25 μl of RNasin, and 40 U reverse transcriptase was added and the reaction was incubated for 90 min
at 45°C. 1 μl of 0.5 M EDTA and 1 μl of 1 μg/ml RNase A were added and incubation continued for 30 min at 37°C. After phenol/chloroform extraction and ethanol precipitation the extended products were analyzed on 8 M urea sequencing gels.

ACKNOWLEDGEMENTS

This research is supported by a grant, A123283, from the National Institutes of Health. We thank Dr. Eva Severinson for IL-4.
FIGURE LEGENDS

Figure 1. (A) Restriction map of the unrearranged genomic Cγ1 gene. The RNA probe used for hybridization of the RNA blot shown in B and in the RNase protection experiments shown in C is indicated above the maps and the location of oligonucleotides used for primer extension and PCR are shown below the maps. (B) Blot of poly (A)+ RNA (3 μg) from BALB/c spleen cells treated 2 days with LPS (lane 1) or with LPS plus IL-4 (8 U/ml) (lane 2) (Stavnezer et al., 1988) hybridized with an antisense RNA probe encoding the KpnI-PstI segment. (C) RNase-resistant fragments obtained after hybridization of the RNA probe used in B electrophoresed alongside a sequence ladder. Lanes are P, probe alone; Y, probe hybridized with yeast RNA (10 μg); and S, probe hybridized with total cell RNA (10 μg) from spleen (treated with 15% HeLa cell supernatant). (D) Products of primer extension experiment using oligo 2 electrophoresed alongside a DNA sequencing ladder. Lanes are O, oligonucleotide incubated alone; Y, primer extension with yeast RNA (150 μg); and S, primer extension with total cell RNA (150 μg) from spleen (treated as in C).

Figure 2. (A) DNA sequence of the KpnI-PstI fragment. The sequence was obtained from Genbank (data of Wesley Dunnick) and confirmed by us. Uppercase letters represent Iγ1 exon and lowercase letters represent the 5' and 3' flanking sequences. Turned arrows indicate initiation sites. Triangles indicate the Iγ1/Cγ1 splice site (also in B and
C). Sequence present in oligo 2 (used for primer extension) and oligo 4 (used for PCR) are indicated. The initiator Met codon for the potential ORF is boxed. (B) Cγ1 splice acceptor sequence from Honjo et al. (1979) and found in germ-line γ1 cDNA produced by the PCR. (C) Open-reading frame (ORF) of the germ-line γ1 RNA. The ORF is initiated in the Ig1 and terminated within the first exon of Cγ1. The DNA sequence derived from Cγ1 is underlined. (D) Comparison of the sequences of the RNA initiation sites of γ2b (Lutzker and Alt, 1988), α (Radcliffe et al., 1990), and γ1 RNAs. Thick bars indicate initiation sites of these RNAs.
Figure 1
Figure 2
The following manuscript has been submitted. Figures are renumbered in the dissertation. Figures 6, 8 show supporting data which have not been included in the submitted manuscript. Most of the Introduction and entire Discussion of the manuscript are fused into the Introduction and Discussion of the dissertation and references have been incorporated into the bibliography of the dissertation.
Regulation of Transcription of Immunoglobulin Germ-Line $\gamma$1 RNA: Molecular Analysis of the Promoter

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Running title: Germ-line $\gamma$1 promoter
SUMMARY

Antibody class switching is achieved by recombinations between switch (S) regions which consist of tandemly repeated sequences located 5' to immunoglobulin (Ig) heavy chain constant (CH) region genes. RNA transcripts from specific unrearranged, or germ-line, Ig CH genes are induced in IgM+ B cells prior to their undergoing class switch recombination to the same CH genes. Thus, the antibody class switch appears to be directed by induction of accessibility, as assayed by transcription of germ-line CH genes. For example, interleukin-4 (IL-4) induces transcripts from the germ-line Cγ1 and Cε 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 germ-line Cγ1 gene contains promoter and enhancer elements responsible for basal level transcription and inducibility by anti-immunoglobulin, phorbol 12-myristate 13-acetate (PMA), and for synergy of these inducers with IL-4 in a surface IgM+ B cell line, L10A6.2, and a surface IgG2a+ B cell line, A20.3. Most of this 150-bp segment is necessary and sufficient to confer PMA and PMA plus IL-4 inducibility in L10A6.2 cells but it is not sufficient to confer inducibility in A20.3 cells. Linker-scanning mutations demonstrated that multiple interdependent elements are required for inducibility by PMA and 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 TGFβ inhibitory element (TIE), an αβ-interferon response element (αβ-IRE), and an AP-3 site. The relationship between transcription regulated by these elements and the regulation of endogenous germ-line γ1 transcripts and switching to IgG1 is discussed.

INTRODUCTION

To begin to understand how transcription of germ-line genes and thereby the accessibility of CH genes is regulated, we decided to determine if the DNA region 5' to and surrounding the initiation sites for germ-line γ1 RNA functions as a promoter and to attempt to identify DNA sequences within this region which respond to stimuli which regulate the transcription of germ-line γ1 RNA. We report here that an 150-bp region 5' to the first initiation site of germ-line γ1 RNA (Xu and Stavnezer, 1990) contains several functional interdependent elements which are responsible for basal level expression and inducibility by PMA and by PMA plus IL-4 in an IgM+ cell line, L10A6.2, and in an IgG2a+ cell line, A20.3. An 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 the individual elements has been mutated.

RESULTS

5'Sγ1-LUC Plasmids are Expressed in L10A6.2 and A20.3 Cells

To analyze the promoter for germ-line γ1 RNA transcripts, we constructed a series of 5'Sγ1-LUC plasmids, ligating various lengths of DNA including the γ1 RNA initiation sites and 5' flanking segment to the firefly luciferase reporter gene (Fig. 3). 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'Sγ1-LUC plasmids. L10A6.2 cells are IgM surface-positive and A20.3 cells are IgG2a surface-positive (Kim et al., 1979; data not
shown). Figure 4 shows luciferase activity of cell extracts obtained after transient transfection of γ1 promoter-luciferase plasmids into 4 cell lines. The expression pattern of 5'Sγ1-LUC in L10A6.2 and A20.3 cells differs. In L10A6.2 cells, the -954LUC is the most active plasmid but -150LUC expresses almost as much luciferase activity 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 γ1 RNAs (Xu and Stavnezer, 1990), and plasmids containing 55 bp or fewer 5’ to the first initiation site are expressed as well as -116LUC. In A20.3 cells, upstream sequences appear more important for the function of the γ1 promoter and sequences between -150 and -116 are less important since -150LUC expresses only 28% of the luciferase activity of -1491LUC, and -116LUC is almost as active as -150LUC. However, the activities of promoters with 55 nucleotides or fewer are as low as 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 level expression of the germ-line γ1 promoter in L10A6.2 cells, but lack some sequences important for basal level expression in A20.3 cells. Fig 4 also shows that the γ1 5’ flanking segment did not promote luciferase expression in the B cell line, 22A10, a clone of the I.29μ B cell lymphoma that switches to IgA upon stimulation by LPS. The γ1 promoter also did not function in a T-cell line, EL-4. We also did not
detect expression of the γ1 promoter in several other pre-B and B cell lines (data not shown). These cell lines are: two pre-B cell lines, ABE8.1/2 and 18.81, and four B cell lines, X16C8.5, BAL17.7.7, M1/34/28, and 21M10.4. M1/34/28 and 21M10.4 are subclones of I.29μ. Thus, 5'Sγ1-LUC plasmids appear to be expressed in the absence of induction in a subset of B cell lines and not in the one T cell line tested. The initiation sites for germ-line γ1 RNA are necessary for the expression of 5'Sγ1-LUC plasmids in both B cell lines since transfection with a construct in which the start sites have been deleted (contains γ1 sequence from -954 to -32) resulted in levels of luciferase activity the same as that produced by pXP2 (data not shown). Transfection with K/K (Fig. 3) gave the same result (Fig.4).

The Germ-line γ1 Promoter Is Induced by Anti-Immunoglobulin (Ig) Antibody and by PMA; IL-4 Synergizes with Anti-Ig Antibody and PMA

To study the inducibility of the γ1 promoter, L10A6.2 and A20.3 cells were stably transfected with -1491LUC, co-transfecting with the plasmid Homer 6, which confers resistance to G418 (Spandidos and Wilkie, 1984). Stably transfected uncloned lines were treated with various inducers and/or cytokines and luciferase activity was measured. Luciferase activity is induced in both cell lines by
treatment with goat F(ab')2 anti-mouse Ig (Fig. 5A) or in A20.3 cells with whole goat anti-mouse Ig antibody (L10A6.2 cells were not tested). The induction by F(ab')2 anti-Ig in L10A6.2 cells was 2.3 fold and in A20.3 cells was 17 fold. IL-4, which induces γ1 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')2 anti-Ig, resulting in 7 and 127 fold induction in L10A6.2 and A20.3 cells, respectively. The induction of luciferase expression by F(ab')2 is much lower in L10A6.2.2 cells than in A20.3 cells even the concentration of F(ab')2 used in L10A6.2 cells is higher than used in A20.3 cells. IL-4 also synergizes with whole anti-Ig antibody, although numerous experiments (e.g. Fig. 8) 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 to also induce a calcium flux and protein tyrosine kinase (Coggeshall and Cambier, 1984; Cambier and Ransom, 1987; Gold et al., 1990). We tested whether phorbol 12-myristate 13-acetate (PMA), which activates PKC (Nishizuka, 1984), could mimic the effect of anti-Ig treatment. Fig. 5B shows that PMA is a better inducer of 5'Sγ1-LUC plasmids than anti-Ig in both cell lines and that IL-4 synergizes with PMA. Since PMA is dissolved in dimethyl sulfoxide (DMSO), we tested whether DMSO induces the γ1 promoter. Results showed that DMSO does not induce the γ1 promoter in either stably or transiently transfected cells (Fig. 6).
Purified rIL-4 is saturating at about 300 U/ml for synergy with PMA in both cell lines (Fig. 7 and data not shown) and at 100 U/ml gives about 75% of maximal synergy with PMA in L10A6.2 cells. 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 γ1 RNA in spleen cells (the IL-4 units of Severinson et al. are approximately 10 times larger than in the assay used by Immunex). These results are also consistent with experiments demonstrating that 100 U/ml of IL-4 (Immunex units) is saturating for induction of IgG₁ expression by splenic B cells stimulated by LPS (Snapper et al., 1988). This group found the dose-response curve of IL-4 for IgG₁ production is bimodal with peaks at 100 and 10,000 U/ml. The suppressing effects of IL-4 at concentration between 100 and 10,000 U/ml can be eliminated by adding anti-IL-4 antibody (11B11) into the cultures at day 2. This result indicates that enhancement of IgG₁ production by IL-4 acts during the initial 2 days of culture and the suppressing effect of IL-4 for IgG₁ production acts after day 2.

LPS appears to induce low levels of germ-line γ1 RNA in spleen cells (Stavnezer et al., 1988; Severinson et al., 1990). LPS weakly induces the γ1 promoter (7-fold) in A20.3 cells (Fig. 5B) but has no effect in L10A6.2 cells (Fig. 8). LPS synergizes with PMA to give as large an induction as PMA plus IL-4 (146-fold), but does not synergize with IL-4 (Fig. 5B).
IFNγ has been demonstrated to inhibit the induction by IL-4 of expression of IgG1 and germ-line γ1 RNA (Snapper and Paul, 1987; Berton et al., 1989; Severinson et al., 1990). TGFβ1 reduces the expression of all antibody classes except IgA; it induces the expression of IgA and germ-line α RNA in spleen cells (Coffman et al., 1989; Lebman et al., 1990) and in I.29μ cells (Stavnezer et al., 1990; P. Shockett and J. S., in preparation). We examined the effect of these two cytokines on luciferase expression (Fig. 5B). Results showed that at optimal doses IFNγ 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. In addition, IFNγ inhibited PMA induction by 30% in A20.3 cells. These data are consistent with those of Snapper and Paul (1987) and Severinson et al. (1990) who found that the same dose of IFNγ will inhibit switch to IgG1 induced by IL-4 and the induction of germ-line γ1 RNA by IL-4. The weaker inhibitory effect of IFNγ in A20.3 cells is not due to the high concentration of IL-4 used with A20.3 cells. By testing different concentrations of IFNγ (10-1000 U/ml) and different concentrations of IL-4 (400 U/ml and 1000 U/ml) in both cell lines, we found a similar inhibition by IFNγ to the results shown in Fig. 5. TGFβ1 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 inhibitory effects of IFNγ and TGFβ appear to require formation of chromatin structure in L10A6.2 cells since in transient transfection experiments performed by the same protocol, IFNγ and TGFβ induced
the γ1 promoter rather than inhibiting it. However, if IFNγ and TGFβ were not added until 36 hours after transfection, they were inhibitory (data not shown). IFNγ and TGFβ were also inhibitory in other lines of stably transfected L10A6.2 and A20.3 cells. Furthermore, IFNγ and TGFβ were inhibitory in stably transfected L10A6.2 cells which were mock electroporated and treated with cytokines by the protocol used for transient transfection, suggesting that signal transduction of IFNγ and TGFβ is not disturbed by electroporation. IFNγ and TGFβ, however, were inhibitory in both stably and transiently transfected (with -1491LUC) A20.3 cells. This again reflects the difference in regulation of the germ-line γ1 promoter between these two cell lines.

rIL-1α, rIL-2, rIL-5, and rIL-6 had no effect on the expression of -1491LUC at various concentrations tested and did not synergize with PMA in either of the stably transfected lines (data not shown). Cholera toxin (1 μg/ml), which has been reported to induce germ-line γ1 RNA in spleen cells (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 (Fig. 8). Calcium ionophore, A23187, inhibited the PMA induction of -1491LUC in L10A6.2 cells at various concentrations and in A20.3 cells at concentrations exceeding 100 ng/ml. A23187 either induced expression or had no effect at concentrations less than 100 ng/ml in A20.3 cells (data not shown).
Transcripts From Transfected 5'Sγ1–LUC Plasmids are Correctly Initiated

To determine if transcription from the 5'Sγ1-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 hours. 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 nucleotides in Fig. 9A). L10A6.2 cells have large amounts of read-through transcripts, but they are not inducible. Since luciferase expression is inducible, it appears likely that the read-through transcripts are not translated.

The endogenous germ-line γ1 RNA is also induced in A20.3 cells by treatment with PMA plus IL-4 but we have been unable to detect it by after treatment with IL-4 or PMA alone (Fig. 9B). We have not detected endogenous germ-line γ1 RNA in induced L10A6.2 cells (Fig. 9A, lane U; data not shown). The sizes of the RNase-resistant fragments obtained from endogenous γ1 RNA in A20.3 cells induced with PMA plus IL-4 are the same as those obtained from RNA isolated from spleen cells treated with LPS plus IL-4 (Fig. 9A, lane U; Xu and Stavnezer, 1990), indicating that the initiation sites for germ-line γ1 RNA in spleen and in A20.3 cells are identical.
Sequences Responsible for Inducibility by PMA and PMA Plus IL-4 are Located within 150 bp 5' to the First Start Site of Germ-Line γ1 RNA

To attempt to identify DNA sequence elements responsible for induction of the germ-line γ1 promoter by PMA and IL-4, both cell lines were transiently transfected with the 5'Sγ1-LUC plasmids shown in Fig. 3. Results shown in Fig. 10 indicate that in L10A6.2 cells the -150LUC plasmid has the same inducibility by PMA alone (6.9-fold) and synergy with IL-4 (3.5-fold) as -954LUC. In A20.3 cells -150LUC maintains full PMA inducibility but has lost half 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 nucleotides 5' to the first initiation site of germ-line γ1 RNA.

Linker-Scanning Mutational Analysis of Germ-Line γ1 Promoter

In order to define elements responsible for basal level expression of the 5'Sγ1-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 were created (Fig. 11A). Transient transfection of these mutated γ1 promoter-luciferase constructs demonstrated that both the basal level expression and induction by
PMA and PMA plus IL-4 of 5'Sγ1-LUC plasmids are controlled by multiple elements (Figs. 11A-C). If any one of several elements is mutated, both the basal level of expression and inducibility of the 5'Sγ1-LUC plasmids are decreased. 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 of other genes reduces expression and inducibility of the 5'Sγ1-LUC plasmids. (1) CACCC boxes (Dierks et al., 1983; Cowie and Myers, 1988) contribute to the basal level expression, PMA, and PMA plus IL-4 inducibility in both cell lines. There are 4 CACCC boxes in the germ-line γ1 promoter, all residing within 150 bp 5' to the first start site. Mutations in any one of them (except for the one at -26/-18) reduce the basal level expression and abolish the PMA and PMA plus IL-4 inducibility in both cell lines except the CACCC box located at -89/-80 which appears not important for PMA inducibility in A20.3 cells (Fig. 11C). Another CACCC box located at -26/-18 is less homologous with the CACCC consensus sequence than the others (Fig. 12) and has less effect in L10A6.2 cells, but may contribute to PMA inducibility in A20.3 cells since LS[-43,-3] and LS[-30,-3] are not induced by PMA. (2) The DNA sequence located between -120 and -104 is homologous to the binding sites for two transcription factors. It matches perfectly the consensus sequence for the C/EBP family of transcription factors (Akira et al., 1990) and it also matches imperfectly two IL-4 response elements (labeled IL-4
RE) found 5' to the promoter of class II Aαk gene (Figs. 11A and 12) (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. (3) The mutant LS[-101,-95] destroyed a putative TGFβ inhibitory element (TIE) that is responsible for negative regulation by TGFβ 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 very much reduced by this LS mutant but the inducibility by PMA in A20.3 cells is not affected by this mutant (Fig. 11C). (4) A PU box (Pettersson and Schaffner, 1987) located between -55 and -48 is important for basal level expression in both cell lines, and also contributes to inducibility by PMA and synergy with IL-4 (see LS[-62,-53]). This mutation reduces basal level expression by 54% and 84% in L10A6.2 and A20.3 cells, respectively. Mutation at the PU box reduces PMA inducibility by 57% in L10A6.2 cells but increases PMA inducibility by 56% in A20.3 cells and inhibits synergy with IL-4 by 79%. (5) The mutation at a putative AP-1 binding site/αβ-interferon response element (IRE) (LS[-43,-27]) also reduces PMA and PMA plus IL-4 inducibility by 70% in L10A6.2 cells but has little effect in A20.3 cells. DNA sequences located within -77/-56 do not contribute significantly to the promoter activity in L10A6.2 cells (Fig. 11B) but do contribute to basal level expression, PMA, and PMA plus IL-4
inducibility in A20.3 cells (Figs. 11B and 11C). 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 F(ab’)2 inducibility of some LS mutants in A20.3 cells. Results shown in Fig. 13 suggest that the same elements are used to respond to both F(ab’)2 and PMA in A20.3 cells. We have not tested the F(ab’)2 inducibility of LS mutants in L10A6.2 cells, because of the lower F(ab’)2 inducibility in this cell line.

A Segment of the Germ-line γ1 Promoter Can Confer Inducibility by PMA and PMA Plus IL-4 to an Heterologous Promoter

Linker-scanning experiments showed that the DNA elements located within the 150-bp region 5’ to the first RNA initiation site function together to contribute to the basal level expression and inducibility of 5’Sγ1-LUC plasmids by PMA and PMA plus IL-4. To ascertain if these elements which are necessary for inducibility by PMA and synergy with IL-4 are sufficient to confer same inducibility to another heterologous promoter, we transferred segments of the γ1 promoter to a plasmid containing a minimal thymidine kinase (TK) promoter ligated to the gene for chloramphenicol acetyl transferase (CAT). This plasmid does not direct CAT expression unless enhancer
sequences are added (Cato et al., 1986). Various lengths of 5'Sγ1 fragments were inserted into the TKCAT plasmid. Results of transient transfection of these 5'Sγ1-TKCAT plasmids into L10A6.2 are shown in Fig. 14. γ1[-177,-14]TKCAT and γ1[-148,-14]TKCAT have full PMA inducibility and about 2/3 of the IL-4 synergy of -150LUC (Fig. 10). γ1[-177,-78]TKCAT and γ1[-148,-78]TKCAT are fully induced by PMA but have lost most of synergy with IL-4. γ1[-177,-91]TKCAT, which differs from γ1[-177,-78]TKCAT only by a CACCC box, is only weakly inducible by PMA (2.7-fold) or by PMA plus IL-4 (3.37-fold). This result is consistent with that of LS[-90,-83] and confirmed that the CACCC box is important for basal level expression, PMA, and PMA plus IL-4 inducibility in L10A6.2 cells. We have not determine whether the transcription of these heterologous 5'Sγ1TKCAT constructs is correctly initiated. The TKCAT plasmid itself is not expressed and is not inducible in L10A6.2 cells but it is expressed and induced by PMA in A20.3 cells and γ1 promoter-TKCAT plasmids are not inducible above this background.
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% CO₂ 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 I.29μ (Alberini et al., 1987), was maintained in RPMI 1640 with 20% FBS in an 8% CO₂ incubator.

Reagents and lymphokines:
Goat anti-mouse Ig antibody (Southern Biotech. Associates, Birmingham, AL) and goat anti-Ig F(ab')2 (Organon Teknika Corporation, West Chester, PA) were purchased. Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), and calcium ionophore A23187 were purchased from Sigma (St. Louis MO). Cholera toxin was purchased from List Biological Laboratories, Inc. (Campell, CA). Interferon-γ (IFNγ) was donated by Schering Corporation, USA as part of the American Cancer Society's program on interferon. Transforming growth factor-β1 (TGFβ1) was purchased from R & D Systems, Inc. (Minneapolis, MN). Purified recombinant human IL-1α (rhIL-1α) (Boehringer Mannhein, 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). In both cell lines the PMA induction was maximal at 12 hours after addition. Potentiation of IL-4 was maximal at 12 hours in L10A6.2 cells and at 24 hours in A20.3 cells (data not shown). 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 15 minutes at RT before use (data not shown). Purified IL-6 (Jambou et al., 1988) was given by Dr. Dana Fowlkes, University of North Carolina.

**Plasmid Construction**

The molecular techniques were adapted from Sambrook et al. (1989). Different lengths of 5'Sy1 DNA fragments (Fig. 3) were cloned into promoterless luciferase plasmids (pXP1 or pXP2) (De Wet et al., 1987; Nordeen, 1988) using appropriate enzyme sites. The 5' deletions having less than an 150 bp flank were created by BAL31 digestion from the KpnI site (Fig. 3). After the ends were filled with T4 polymerase and Klenow enzyme, BgIII digestion was performed and
the isolated fragments were ligated into the SmaI/BglII digested pXP2 plasmid. The 3' deletions were created by BAL31 digestion from the BglII site (at +202) of -954LUC (Fig. 3) and filled with T4 polymerase and Klenow enzyme. After digestion by BamHI, the isolated fragments were ligated into the BamHI/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/SalI or BamHI/HindIII and the isolated fragments were ligated into BamHI/SalI or BamHI/HindIII digested 5' deletion plasmids. BamHI/SalI digestion creates a 10-bp linker and 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'Syl-TKCAT) were created by two methods. (1) The polymerase chain reaction (PCR) (Saiki et al., 1988) was used to amplify the fragments with two oligos (Fig. 11A). The 5' oligo has an HindIII site and the 3' oligo has a SalI site. The digested fragments were cloned into HindIII/SalI digested TKCAT plasmid (Cato et al., 1986). (2) Alternatively, Asp718 (filled with T4 polymerase and Klenow enzyme) plus SalI digested fragments, which were directly isolated from certain LS mutants were cloned into HindIII (filled by T4 polymerase and Klenow enzyme) plus SalI digested TKCAT plasmid.
RNase Protection and RNA Blotting

RNase protection and RNA blotting were performed as previously described (Xu and Stavnezer, 1990). The probe (K/X) used for RNase protection (Fig. 3B) contains the KpnI-BglII fragment from 5'Sγl DNA and 81 bp of the luciferase gene cloned in the KpnI/XbaI sites in Bluescript. The probe used for northern blotting was a KpnI-PstI (K/P) segment shown in Fig. 3A.

Transfection and Assays

Transfection was performed by electroporation (Maxwell and Maxwell, 1988) using Cell ZapII (Anderson Electronics Brookline, MA). Briefly, the cells were diluted 4-5 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 μF/750 V per cm for 1 ml volume and 625 μF/750 V per cm for 0.5 ml volume. After transfection, cells were kept at room temperature for 10 min and then were resuspended in 10% FBS/RPMI 1640 and aliquoted into flasks at a concentration of 1-1.3x10^6/ml (counted before transfection) in 7 ml. After addition of inducers, cells were incubated for 12 hours (except where indicated) and then harvested, washing once with phosphate buffered saline (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, respectively.
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. All lines were screened for the luciferase activity in the absence of induction and several lines were then tested for inducibility by PMA and/or IL-4. All lines tested showed similar induction pattern. These lines were not further cloned. One L10A6.2 and one A20.3 lines were used for all experiments except where noted. These two lines maintained luciferase expression during several months of continuous culture in the absence of G418.

Luciferase assays were performed according to Brasier et al. (1989). Briefly, cells from one flask were lysed by adding 100 µl of 0.1% Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM MgSO4, 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 µl of 25 mM glycylglycine pH 7.8, 15 mM MgSO4, 15 mM KH2PO4 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 µl of 1 mM luciferin (Analytical Luminescence Laboratory, San Diego, CA) into the lysate mixture.

CAT assays were performed as described (Neumann et al. 1987). Briefly, cells were lysed with 50 µl of the lysing buffer used for luciferase assay. After incubation at 70°C for 15 minutes, the lysate was combined with 200 µl of 125 mM Tris-HCl pH 7.8, 1.25 mM chloramphenicol (Sigma, St. Louis, MO) and 0.1 µCi of 3H acetyl
CoA (New England Nuclear, Boston, MA) in a 7 ml glass scintillation vial. Five 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 hours. The samples were counted in a liquid scintillation counter.

ACKNOWLEDGEMENTS

We thank Yi-Chaung Lin and Penny Shockett for sharing unpublished data, Drs. Paul Dobner, David Parker, and Robert Woodland for useful suggestions, Dr. Wesley Dunnick for cloned fragments of the genomic Cγ1 gene, Dr. Richard Asofsky for L10A6.2 cells, Dr. Fritz Melchers for X63-Ag8-653 cells expressing rIL-2, rIL-4, or IL-5, Immunex (Seattle, WA) for purified IL-4, Dr. Dana Fowlkes for purified rIL-6, and Jennifer Nietupski for preparation of X63SN. This work was supported by a Public Health Service grant from NIH, AI23283.
FIGURE LEGENDS

Figure 3. Restriction Map of Germ-Line 5'Sγ1 Region and 5'Sγ1-Luciferase (LUC) Plasmid Constructs.

(A) Turned arrow indicates first initiation site of germ-line γ1 RNA. Germ-line γ1 RNA is spliced to the Cγ1 exon at +484 (Xu and Stavnezer, 1990). The thick line above the map indicates the probe (K/P) used for RNA blot analysis. (B) Maps of γ1 promoter-LUC fusion constructs in pXP2. The 3' end of inserted 5'Sγ1 DNA is at the BglII 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 (Fig. 9).

Figure 4. Luciferase Activities Obtained from 4 Cell Lines Transiently Transfected with 5'Sγ1-LUC Plasmids.

30 x 10^6 cells were transfected with 30 μg of the indicated plasmid DNA. Cells were harvested at 36 hours. Results were normalized to CAT activity obtained from a co-transfected pSV2CAT plasmid (7.5 μg DNA per 30 x 10^6 cells) (Gorman et al., 1982). For L10A6.2 and A20.3 cells, data presented were obtained from two representative experiments. Data presented are mean with range indicated by error
bars. For 22A10 and EL-4 cells data were from one experiment in which all internal controls were positive. All 5'Sγ1-LUC plasmids were linearized immediately 5' to the inserted γ1 sequences by restriction enzyme to prevent any signal from the upstream vector sequences.

Figure 5. Induction 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')2 (30 μg/ml for L10A6.2 cells and 10 μg/ml for A20.3 cells) or with goat anti-mouse Ig antibody (5 μg/ml) with or without IL-4 (2.5% of X63 SN which had been determined to be optimal dose). The A20.3 cells were incubated for 6 hours and L10A6.2 cells were incubated for 12 hours before harvest. (B) Effects of PMA, LPS, TGFβ, and IFNγ. 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 μg/ml). LPS had no effect on luciferase activity in L10A6.2 cells. IFNγ was used at 30-50 U/ml in both lines. TGFβ was used at 2 ng/ml for L10A6.2 and 4 ng/ml for A20.3 cells. P=PMA. In the presence of PMA. Both IFNγ and TGFβ have no effect on cell growth at the concentrations used in both cell lines. Data presented are from one experiment and other 2 experiments gave similar results. The luciferase activity in the control well was about 5000 light units in L10A6.2 cells and was about 30,000 light units in A20.3 cells.
Figure 6. Effect of DMSO on Stably (Upper) and Transiently (Lower) Transfected L10A6.2 and A20.3 Cells.

(Upper) Stably transfected cells were treated with PMA (60 ng/ml) or with the indicated concentrations of DMSO. Experimental procedures are the same as in Fig. 5. (Lower) L10A6.2 (40x10^6) were transiently transfected with 30 μg of -698LUC plasmids and A20.3 (40x10^6) cells were transiently transfected with 15 μg of -954LUC plasmids. After transfection, PMA (60 ng/ml) and the indicated concentrations of DMSO were added. Cells were harvested after 12 hours incubation.

Figure 7. Titration of IL-4 Synergy with PMA in Stably Transfected L10A6.2 Cells.

Experimental procedures are the same as in Fig. 5. PMA was included in all incubations at 60 ng/ml. Although a complete dose titration was not performed in A20.3 cells, results at doses great than 300 u/ml suggested the dose response was similar in A20.3 and L10A6.2 cells.
Figure 8. Effect of Cholera Toxin (CT) on Stably Transfected L10A6.2 and A20.3 cells.

Experimental procedures are the same as in Fig. 5. Concentration of cholera toxin used in L10A6.2 cells was 1 μg/ml. In both cell lines, PMA = 60 ng/ml, LPS = 25 μg/ml. IL-4 was given at 400 U/ml in L10A6.2 cells and at 1000 U/ml in A20.3 cells. 4=IL-4. Concentrations of CT tested in both L10A6.2 and A20.3 cells were from 1 ng/ml to 10 μg/ml. CT had no effect on the expression of -1491LUC at concentrations less than 10 μg/ml in both cell lines (data not shown).

Figure 9. RNase Protection (A) and RNA Blot Experiment (B) to Detect RNA Transcribed From Transfected -1491LUC and the Endogenous Cγ1 Genes and their Induction by PMA and IL-4.

(A) 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-BglII fragment from 5'Sγ1 sequences and 81 nucleotides of the luciferase gene (see Fig. 1). Lanes show RNase resistant fragments obtained after hybridization of the probe with 20 μg 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. (B) RNA blot to detect endogenous germ-line γ1 RNA in A20.3 cells. The K/P probe, labeled with 32P by random priming was hybridized with total cell RNA from A20.3 cells (10 μg/ml). Lanes contain RNA from: C, untreated cells; 4, cells treated for 10 hours with IL-4 (1000 U/ml); P, cells treated with PMA (60 ng/ml); P+4, cells treated with PMA+IL-4.

Figure 10. Inducibility of Various 5'Sγ1-LUC Plasmids in L10A6.2 and A20.3 Cells.

30 x 10^6 cells were transiently transfected with 30 μg supercoiled plasmid DNA (L10A6.2) or 15 μg DNA (A20.3). After transfection, cells were aliquoted into 3 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 hours, cells were harvested and luciferase activity was assayed. For both cell lines, data are the average of 2 representative experiments except those of -954LUC and -150LUC which were from 4 experiments. Data presented are the mean with range indicated by error bars (S.E. for -954LUC and -150LUC)
Figure 11. 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 γ1 germ-line RNA and linker-scanning mutations of this region. WT = wild type and LS = linker-scanning mutation. Wild type is -954LUC and all the mutations are within the -954LUC 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 denote two oligonucleotides used for PCR. The 5' end of oligo 1 has an added HindIII site and the 3' end of oligo 2 has an added SalI site.

(B) Relative luciferase activity obtained from L10A6.2 and A20.3 cells transiently transfected with the wild type and LS mutant plasmids. 20 μg supercoiled plasmid DNA (L10A6.2 cells) and 15 μg DNA (A20.3 cells) were used to transfect 40 X 10⁶ cells. CAT activity obtained from 2 μg of pSV2CAT which was co-transfected was used to normalize the luciferase activity between transfections. After transfection, cells were aliquoted into 4 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 hours incubation, cells were harvested for luciferase and CAT assays. Luciferase activity from the control flask of wild-type was set equal
to 1. For both cell lines, mean of the results obtained from 2 representative experiments are shown. For all data points presented in (B), the average range (±S.D.) is 17% (±14%) of the mean for both cell lines.

(C) The data in (B) are plotted as fold induction. For data points presented in (C), the average range (±S.D.) is 12% (±7.7%) of the mean for L10A.2 cells and 8.6% (±7.1%) of the mean for A20.3 cells.

Figure 12. Comparison of γ1 Sequence Elements with Binding Sites for Known Transcription Factors.

The consensus sequence and specific sequences which are found in other promoters are shown. Nucleotides in the γ1 promoter 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); αβ-IRE (Yang et al., 1990); and AP-3 (Chiu et al., 1987); IgH E and VH 1 (Peterson et al., 1988); germ-line γ2b (Lutzker et al., 1988); germ-line γ3 (Rothman et al., 1990); 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 α promoter (Y.-C.A. Lin and J.S., submitted).

Figure 13. Comparison of Inducibility of Some LS Mutants by PMA and F(ab')2 in A20.3 Cells.

Experimental procedures are the same as in Fig. 11B. PMA was used at 60 ng/ml, IL-4 at 1000 U/ml, and F(ab')2 at 10 μg/ml. Luciferase activity from the control flask of cells transfected with wild-type promoter-luciferase plasmids was set equal to 1. Mean of the results obtained from 2 experiments are shown. For data points presented in this figure, the average range (±S.D.) is 18% (±13%) of the mean.

Figure 14. Structure of 5'Sγ1TKCAT Constructs and CAT Activity in L0A6.2 Cells Transiently Transfected with TKCAT and 5'Sγ1-TKCAT Plasmids.

(A) The thick line represents TK promoter sequence which is from -105 to +58 relative to TK RNA (Cato et al., 1986). The thin line represents germ-line γ1 promoter sequence and the number above the line indicates position of the γ1 sequence relative to the first initiation site of germ-line γ1 RNA.
(B) 25 µg supercoiled plasmid DNA was used to transfect 30 x 10⁶ cells. After transfection cells were aliquoted into 3 flasks and untreated (control), treated with PMA (60 ng/ml) or with PMA+IL-4 (400 U/ml). After 12 hours, cells were harvested and assayed for CAT activity. The results shown are the mean of two experiments, except those of γ1[-177,-14]TKCAT which are from 4 experiments. The error bars indicate the range (or the S.E. for γ1[-177,-14]).
A

B

K/P

Sγ1

K/X

LUC

5' Sγ1-LUCIFERASE PLASMID CONSTRUCTS

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 10

Graph showing fold induction with various control and PMA+IL-4 conditions.
Figure 11B
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<th>PMA</th>
<th>PMA+IL-4</th>
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_Figure 11C_
CACCC BOX

β-globin GCCACACCC
γ1 GCCCTCACCC-135
γ1 GCCCTCACCC-129
γ1 GCCCTCACCC-80
γ1 GCCCTCACCC-18
γ1 GCCCTCACCC-58

IL-4 RE

BRE 1 ATGAT-GCATGCAAG
BRE 2 ATGTTTGCTTGAAAG
γ1 ATGAAGTAAATCTAAG

C/EBP binding site

consensus TTNNGNAAAT
IgH E ATTGAGCAATGT 337

VH 1 ATTTGGTAATTA-96
DBP ATTTTGTAATGG-92
γ1 ATGAAGTAAATCT-107

PU box

PU.1 AAAGAGGAAC

ets-1 AGAGCAGGAAGC

γ3 AAAGGGGAAC-145
γ3 TAAGAGGAAGA-28
γ2b AAAGAGGAAC-6
α GAACAGGAAGA-59
γ1 GAAGGGGAAC-46

TIE

consensus GNNNTTGGNG
γ1 GGTNTGGAAC

Figure 12
Figure 13
Figure 14

A

-177 or -148

5'S γ 1 sequence TK promoter CAT

-14 or -78 or -91

B

Fold Induction

Control
PMA
PMA+IL-4

TKCAT γ1[-177,-14]TKCAT γ1[-148,-14]TKCAT γ1[-177,-78]TKCAT γ1[-148,-78]TKCAT γ1[-177,-91]TKCAT
DISCUSSION

Structure of Germ-Line γ1 RNA

The facts that each of the unrearranged CH genes (except Δ) have been shown to be transcribed under conditions that promote specific class switching (Stavnezer-Nordgren and Sirlin, 1986; Lutzker et al., 1988; Severinon et al., 1990), that where examined, these transcripts have an I exon located 5' to the S region, and that transcription proceeds through the S region in the sense direction (Lutzker and Alt, 1988; Stavnezer et al., 1988; Berton et al., 1989; Radcliffe et al., 1990; Rothman et al., 1990a, 1990b; Gerondakis, 1990; Sideras, 1009) suggest that this transcription has a function in class switching.

In order to clarify how transcription of the unrearranged Cγ1 gene is regulated, it was first imperative for me to determine the structure of germ-line γ1 transcripts. As shown in the results, like other germ-line RNAs, the germ-line γ1 RNA also has an I exon. The structure of germ-line γ1 RNA is similar to other germ-line RNAs. The Iγ1 exon has an unique 3' splice site which uses consensus splice donor and acceptor sequences, exon-ag/gtaag-intron-cag/exon (Shapiro and Senapathy, 1987). Compared with other germ-line RNAs, Iγ2b uses the same splicing consensus sequences (Lutzker and Alt, 1988), whereas germ-line α and ε RNAs do not use the splicing consensus sequences (Radcliffe et al., 1990; Lebman et al 1990; Gaff
and Gerondakis, 1990). Germ-line $\alpha$ RNA has multiple 3' splice sites. It is not clear whether these multiple splice sites have a specific function in class switching.

As true for all other germ-line RNAs, germ-line $\gamma_1$ RNA also has multiple initiation sites and no TATA and CAAT boxes located 5' of their initiation sites (except germ-line $\alpha$ RNA has an inverted CAAT box). Some of the initiation sites of $\alpha$, $\gamma_2b$, $\mu$, and $\gamma_1$ RNA have similar nucleotide sequences (Fig 2D), suggesting that they may use a common transcription factor. However, these sequences differ from the initiator sequences that have been defined for other genes that lack TATA and CAAT boxes (Sehgal et al., 1988; Smale and Baltimore, 1989). The promoters which lack obvious TATA and CAAT boxes have been divided into two classes (Smale and Baltimore, 1989). The first class has GC-rich (GGGCGG) promoters, found primarily in some housekeeping genes (Sehgal et al., 1988). The promoters belonging to this class usually have multiple start sites distributed over a fairly large region and contribute to the constitutive expression of these genes. The second class, which lack the GC-rich motif, usually have one or a few tightly clustered RNA initiation sites and many of these promoters are regulated during cell differentiation (Smale and Baltimore, 1989). However, the promoter for germ-line $\gamma_1$ RNA, as well as promoters for other germ-line RNAs, do not have GC-rich motifs but have multiple initiation sites spread over a region of about 100 nucleotides, suggesting this type of promoter may represent yet another class of promoters lacking the TATA or CAAT
box. The initiation sites of germ-line γ1 RNA are essential for the transcription of germ-line γ1 RNA as shown by the fact that deletion of all the initiation sites abolishes luciferase expression regardless of the presence of 5' flanking sequences, suggesting that the initiation sites themselves may be involved in the regulation of transcription of the germ-line γ1 gene.

The germ-line γ1 and α RNAs differ from the germ-line γ2b and μ RNAs in that the γ1 and α transcripts have small ORFs with Met initiation codons in contexts that should allow relatively efficient translation, whereas the germ-line γ2b and μ RNAs have small ORFs with Met codons in poor contexts for translation according to Kozak (1986, 1987). The major germ-line α RNA in I.29μ cells, which has an ORF encoding a 43 amino acid polypeptide (that differs from the sequence encoded by the γ1 ORF), appears to be located on small polysomes in I.29μ B lymphoma cells and this association is enhanced by LPS treatment (Radcliffe et al., 1990), suggesting that germ-line α RNA might be translated. Thus, it is interesting to speculate that germ-line α and γ1 RNAs encode polypeptides which may function in class switching. This is probably not the only function of these transcripts, since it appears likely that not all germ-line transcripts are translated. The germ-line transcripts may simply be an indicator that CH genes are in a transcriptionally active state or participate directly in regulation of class switching.

The determination of the structure of germ-line γ1 RNA allowed me to begin studies to define the DNA regions necessary for
regulated expression of the germ-line γ1 RNA. The definition of these DNA sequences is important for understanding how switching to IgG1 is regulated.

γ1 Promoter Is Expressed and Induced in L10A6.2 and A20.3 Cells
Germ-line γ1 RNA can be induced in spleen cells by treatment with IL-4 or LPS plus IL-4 (Stavnezer et al., 1988; Berton et al., 1989; Esser and Radbruch, 1989; Severinson et al., 1990). However, I was unable to induce expression of the germ-line γ1 promoter-luciferase plasmids transfected into LPS activated spleen cells with IL-4 in the presence of LPS or PMA, although I found that the spleen cells transfected with these plasmids do express luciferase (data not shown). Therefore, I tested a number of B cell lines for their ability to express the -1491LUC plasmid. I found two B cell lines, L10A6.2 and A20.3, that demonstrated inducible expression of the germ-line γ1 promoter-luciferase plasmids. Furthermore, endogenous germ-line γ1 RNA can be induced in A20.3 cells by PMA plus IL-4. The regulation of the germ-line γ1 RNA differs in these two lines from that in spleen cells in that IL-4 alone induces germ-line γ1 RNA in mouse spleen B cells with or without LPS stimulation but not in L10A6.2 and A20.3 cells. IL-4 is able to synergize with anti-Ig or with PMA to induce transcription directed by the germ-line γ1 promoter in these two cell lines.
Expression of the 5'Sγ1-LUC plasmids differs in these two cell lines. In L10A6.2, an IgM+ cell line, the 150 bp 5' to the first RNA initiation site promotes almost the same activity as the most active 5' flanking segment I tried, whereas in A20.3, an IgG2a+ cell line, this 150-bp promotes only one fourth the basal activity and less than one half the IL-4 inducibility as the 1491 bp flanking segment. Thus, an element, or elements, 5' to the -150 bp region contributes to activity in A20.3 cells but appears to be inactive in L10A6.2 cells. In addition, the 5'Sγ1-LUC plasmids responded somewhat differently in these two lines to certain inducers. It is possible that the germ-line γ1 promoter may be induced in B cells of different stages of differentiation by different stimuli and this induction may be mediated by different transcription factors.

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 hydrolysis of phosphatidyl inositol, resulting in formation of diacylglycerol (DAG) and in a calcium flux, and also induces protein tyrosine kinase activity (Coggeshall and Cambier, 1984; Campbell and Sefton, 1990; Gold et al., 1990). DAG activates protein kinase C (PKC), the subsequent effects of which are unknown. Treatment with the phorbol ester PMA also activates PKC. I found that treatment with anti-Ig or PMA induces expression of the 5'Sγ1-LUC plasmids and IL-4 acts synergistically with these inducers. The finding that anti-Ig
and/or PMA treatment alone induces the germ-line γ1 promoter in the two cell lines is surprising in that these reagents have not been shown to augment class switching by splenic B cells, although anti-idiotype antibody has been found to augment LPS-induced switching to IgA in the I.29μ lymphoma cell line (Stavnezer et al., 1985). However, it has been shown that injection of anti-IgD antibody into mice greatly increases the serum level of IgG1 and IgE (Finkelman et al., 1986, 1988). The increase of IgG1 cannot solely be explained by the effect of IL-4, which is also 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 IgG1 production. Furthermore, co-injection of IFNγ profoundly inhibits the IgE production but only moderately inhibits IgG1 production. I hypothesize that anti-IgD activates the PKC pathway which induces transcription of germ-line γ1 genes and thereby directs switching to IgG1 in these mice.

The two cytokines which inhibit induction of the γ1 promoter have previously been shown to down-regulate endogenous germ-line γ1 transcripts and/or switching to IgG1 by splenic B cells. IFNγ inhibits the induction of germ-line γ1 RNA by IL-4 (Berton et al., 1989; Severinson et al., 1990) and inhibits IL-4 induced switching to IgG1 (Snapper and Paul, 1987). I found that IFNγ inhibits the IL-4 synergy with PMA in both B cell lines and reduces PMA induction of the γ1 promoter in A20.3 cells. TGFβ1 has been reported to induce germ-line α RNA in spleen cells and to induce switching to IgA but to
reduce expression of all other isotypes (Coffman et al., 1989; Kim and Kagnoff, 1990; Lebman et al., 1990a, 1990b). I found that TGFβ inhibits induction of the γ1 promoter by PMA and synergy with IL-4 in both cell lines. Therefore, the inhibitory effects of IFNγ and TGFβ on induction of IgG1 expression appear to be achieved via inhibition of expression of the γ1 promoter. Thus, it is highly likely that these cytokines actually inhibit the switch recombination event by reducing transcription of the unrearranged Sγ1-Cγ1 gene segment, thereby reducing its accessibility. I also tested the effect of several other cytokines which had not been shown to have a specific effect on expression of IgG1, and these cytokines had no effect on the germ-line γ1 promoter.

Molecular Analysis of Germ-Line γ1 Promoter

Within 150 bp upstream of the first initiation site of germ-line γ1 RNA are located sufficient sequence elements for inducibility by PMA and synergy with IL-4 in L10A6.2 cells. The palindromic AP-1 sequence element, TGACTCA, which has been shown to mediate transcriptional activation in response to the phorbol ester-activated, PKC-dependent signal transduction pathway (Angel et al., 1987) is not present within this 150 bp segment, although there is an element that resembles an AP-1 site, at -160/-154 (TGAGTCT). This element does not appear to contribute to PMA inducibility conferred by segments of the γ1 promoter ligated to a minimal TK promoter since
its deletion had no effect. I decided to make linker-scanning mutations to across this region to find the elements responsible for PMA and PMA plus IL-4 inducibility.

Linker-scanning analyses indicated that multiple elements are required for γ1 promoter activity and for inducibility by PMA and synergy with IL-4. I noted several sequence elements that matched known transcription factor binding sites in the 150 nucleotide region 5' to the first initiation site. A CACCC box is repeated 4 times in this region (Fig. 11A). Each of these repeats (except the one located at -26/-18) contributes to basal level expression and is essential for PMA and PMA plus IL-4 inducibility except the CACCC box located at -89/-80 which is less important for PMA inducibility in A20.3 cells. Another CACCC box at -26/-18 also appears necessary for PMA inducibility in A20.3 cells. A CACCC box is required for transcription of the mouse β-globin gene (Cowie and Myers, 1988; Dierks et al., 1983). An inverted CACCC box has also been found in the germ-line α promoter (Y.-C.A. Lin and J.S., submitted). The putative CACCC transcription factor has not been identified.

A TIE-like element may also be essential for the basal level expression, PMA, and PMA plus IL-4 inducibility of the γ1 promoter in L10A6.2 cells and for basal level expression and IL-4 synergy in A20.3 cells. TIEs are involved in TGFβ inhibition of several genes and bind a protein complex containing c-Fos (Kerr et al., 1990).

The sequences located between -120/-104 match perfectly a C/EBP binding site whose core sequence, TT/GNNGNAAT/G, is present
in many viral enhancers and mamalian promoters, including the Ig heavy chain enhancer and Ig heavy chain variable region promoters (Akira et al., 1990; Peterson et al., 1988). 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) have characterized a new member of the C/EBP family, Ig/EBP, which is expressed in all cell lines and all tissues examined but is most abundant in immature 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). Mutation of the C/EBP binding site in the γ1 promoter abolishes the induction by PMA and synergy with IL-4. Whether Ig/EBP or C/EBP or another member of this family contributes to the PMA inducibility and IL-4 synergy is unknown. The C/EBP site overlaps an element homologous to two binding sites for an IL-4 inducible protein in the promoter for the class II Aαk gene (Boothby et al., 1988). This IL-4 RE shares homology with consensus sequences of the C/EBP binding site (Fig. 12) and it seems possible that the IL-4 RE specific DNA-binding protein also belongs to the C/EBP family.

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 (Pettersson and Schaffner, 1987; Karim et al., 1990). The PU box is recognized by murine Ets-1, a product of the proto-oncogene, ets-1 (Gunther et al., 1990) and by a macrophage and B cell specific transcription factor PU.1 (Klemsz et al., 1990) whose amino acid sequence in the binding domain has considerable homology with proteins belonging to the ets oncogene family. Mutation of the PU box reduces basal level expression and decreases the inducibility by PMA and synergy with IL-4. The PU box is also found 5' to the initiation sites for germ-line γ2b, γ3, and α RNAs (at -16/-6 relative to the first initiation site of germ-line γ2b RNA, at two sites located at -155/-145 and at -38/-28 relative to the 5' end of germ-line γ3 cDNA, and at -69/-59 5' to the first start site of germ-line α RNA) (Fig. 12) (Lutzker et al. 1988; Rothman et al., 1990; Y.-C.A. Lin and J.S., submitted). I did not find any other of the putative γ1 promoter elements in the published sequences of the γ3 and γ2b 5' flanking regions. Since factor PU.1 is not expressed in EL-4 cells and the germ-line γ1 promoter is also not active in EL-4 cells, it is interesting to speculate that the PU box is one of the determinants responsible for cell-specific expression of the germ-line γ1 promoter.

The sequences located between -41/-31 match with two elements: one is a perfect αβ-interferon response element (αβ 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 γ1 promoter.

The DNA elements located within 150-bp region function interdependently to contribute to the basal level expression and inducibility of the γ1 promoter-luciferase fusion constructs. I made several 5'Sγ1TKCAT heterologous constructs (Fig. 14) to test if the elements in 150-bp region can confer PMA and PMA plus IL-4 inducibility to minimal TK promote. The fact that 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 γ1 RNA initiation sites) and that the -177/-78 and -148/-78 segments confer PMA inducibility only and that the -177/-91 fragment does not confer any inducibility indicate that sequences within -148/-78 are sufficient for PMA inducibility but sequences located within -78/14 are also required to obtain IL-4 synergy. These results, combined with my other analyses, demonstrated that synergy with IL-4 is mediated by multiple interdependent elements.

Previously, it has been shown that either an AP-1 or AP-2 element (Mitchell et al., 1987; Imagawa 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. A serum response element (SRE) located in the c-Fos promoter is also a target for PKC-dependent and -independent signals (Gilman et al., 1986; Sheng et al., 1988). The PKC-dependent signal is mediated by
a protein called p62TCF (for ternary complex factor) (Shaw et al., 1989; Graham and Gilman, 1991). None of these sequence elements (except an imperfectly matched AP-3 site which makes only a minor contribution to γ1 promoter activity) are located in the 150 bp region that confers PMA inducibility to the γ1 promoter. PKC appears to be a main target for PMA stimulation (Nishizuka et al., 1984), although PMA has multiple biological effect on different cells. It is likely that PMA is inducing transcription of other genes whose products induce the γ1 promoter since the earliest time I have demonstrated PMA inducibility is at 3 hours and since the response is not maximal until 12 hours after addition of PMA. I have not examined earlier times. Other alternatives are possible but appear less likely. The mechanism underlying the IL-4 synergy with PMA is completely unknown.

**Biological Significance**

Since I find that cytokines which have been shown to regulate switching to IgG1 regulate the germ-line γ1 promoter, The results presented here begin to provide a description of the mechanism of regulation of accessibility of the Sγ1-Cγ1 gene segment. IL-4, which induces switching to IgG1 and also induces germ-line γ1 RNA, induces expression of the germ-line γ1 promoter in the presence of PMA. IFNγ and TGFβ, which down-regulate switching to IgG1, inhibit the IL-4 activation of the germ-line γ1 promoter.
My results suggest that cross-linking of antigen-receptor on B cell may be involved in inducing accessibility of the Cγ1 gene and thereby, inducing switching to IgG1. The signal provoked by cross-linking the antigen receptor and mimicked by PMA can induce the γ1 promoter. Sequences located within -148/-78 5' to the first initiation site of germ-line γ1 RNA are sufficient to transfer this inducibility to another promoter. This is the first case of which I am aware in which multiple functionally interdependent elements are required to respond to PMA.
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