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Molecular regulation of class switch recombination to IgE through ϵ germline transcription

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The role of germline (GL) transcription in class switch recombination

The ability of B cells to express immunoglobulins with identical antigen specificity but different effector functions results from their capacity to undergo class switch recombination (CSR). This process consists of an intrachromosomal DNA recombination event that involves repetitive switch (S) regions located 5' of each C_H gene, except C_δ, and results in the juxtaposition of different downstream C_H genes to the expressed VDJ gene (reviewed in Ref. 1). The S region involved in recombination is not targeted at random. Instead, CSR appears to be directed by T-helper-cell-derived cytokines in conjunction with the regulation of B-cell proliferation and differentiation. Thus, human B cells activated by CD40 cross-linking in the presence of IL-4 or IL-13 switch from IgM to IgE (2, 3).

Molecular analysis has shown that cytokine-dependent induction of CSR to a particular C_H gene almost

invariably correlates with the transcriptional activation of the same gene in germline (i.e., unrearranged) configuration. All GL transcripts share the same overall structure, and originate from TATAA-less promoters a few kilobases upstream of the S region, proceed through short noncoding exons (I_H exons), the S regions and C_H exons, and are polyadenylated near the normal poly(A) site for mature IgH mRNAs. The I_H exon is then spliced to the normal acceptor site in the first exon of the C_H gene. During CSR, the region containing the GL promoter and the I_H exon is deleted as part of a switch circle. GL transcripts are "sterile"; i.e., they do not appear to be translated into protein, because they contain stop codons in all reading frames (reviewed in Ref. 1).

Targeted mutagenesis experiments have indicated that integrity of the GL transcription units (promoters and/or exons), rather than transcription through the S regions, is essential for CSR (4–8). More recently, splicing of primary GL transcripts has been shown to constitute an essential step in CSR activation (8–10). This finding implies that a spliced-out RNA species may be an integral part of the CSR enzymatic machinery, and/or some of the factors responsible for RNA splicing may also be involved in DNA recombination (see below). Supporting the hypothesis of a functional connection between RNA splicing and CSR are the identification of proteins

Abbreviations. BSAP: B-cell-specific activator protein; C/EBP: CAAT enhancer binding protein; CSR: class switch recombination; GL: germline; IL-4R: IL-4 receptor; IL-4RE: IL-4-responsive element; NF- κ B: nuclear factor- κ B; S region: switch region; STAT: signal transducer and activator of transcription.

involved in RNA processing as components of S region DNA-binding complexes (11, 12) and the precedent of yeast factors involved in both splicing and meiotic recombination (13). Involvement of the splicing machinery is also suggested from studies on the distribution of CSR junctions in CH12F3-2 cells that undergo efficient cytokine-dependent CSR *in vitro* (14). CSR junctions were found not only within but also outside the S region. However, they were rarely found within the I exon, suggesting that breakpoints of CSR are restricted to the intron region of GL transcription. If processing of transcripts and recombination of DNA are really coupled as they appear to be (see below), CSR would be a very unique system with unprecedented character (15).

The full extent to which GL transcripts participate in CSR remains unclear, but their role is currently being reinterpreted. For a number of years, the invariable association between GL transcription and isotype switching was taken to support the accessibility model, according to which chromatin opening coupled with transcription allows the CSR recombinase to access the target DNA. However, this model could not account for isotype switching induced by stimuli (e.g., glucocorticoids) that do not affect GL transcription (16). In addition, it was convincingly argued that, since GL transcripts must be spliced together for CSR to occur, they cannot be simply involved in chromatin remodeling (17). Indeed, recent evidence suggests that the choice of antibody class in CSR may be regulated by GL transcription through the formation of RNA/DNA hybrids that select the sites of recombination. This view is supported by biochemical experiments demonstrating that transcripts of the S regions in immunoglobulin genes remain associated with the template DNA after transcription *in vitro* (18). Mouse and human S regions contain G-rich sequence repeats. The G-rich transcribed RNA appears to form a stable hybrid with the C-rich DNA template, leaving the other DNA strand (the nontemplate strand) on its own in a single-stranded state (19). Most importantly, after B cells from mouse spleen are induced to undergo CSR *in vivo*, RNA/DNA hybrids form within the S regions of the DNA. These RNA/DNA hybrids appear to be mechanistically important for CSR, because mice transgenic for ribonuclease H (an enzyme that specifically snips the RNA in the hybrids) showed a marked reduction in hybrid formation and, in parallel, lower IgG levels and inhibition of CSR in their splenic B cells (20).

What structural features of the S region are required for formation of the stable hybrid during transcription remains to be established. Although mouse and human S regions are G-rich, and runs of guanines are known to form unusual structures, this may not be a crucial feature because the S region upstream of the Ig C μ gene in *Xenopus* is A-T rich (21). It is possible that the stretches of purine-pyrimidine asymmetry, the repeated sequences, and the numerous palindromes (sequences that read

identically in both directions) in the S regions of mouse, man, and *Xenopus* are essential for CSR. How RNA/DNA hybrids regulate CSR is also unclear, but it is likely that they serve as the recognition target for a CSR machinery still uncharacterized. Splicing may be required to stabilize the hybrids by removing extra nucleotides incapable of participating in their formation (17).

The counterintuitive conclusion warranted by these data is that the intronic region spliced out of the primary GL transcript, rather than the processed I $_H$ -C $_H$ transcript itself, may represent what is required to target recombinase activity to the appropriate S region. The current model for the control of CSR to IgE through GL transcription is presented in Fig. 1. In the absence of cytokine stimulation and CD40 engagement, a naive B cell expresses IgM and has the IgH locus in GL (unrearranged) configuration. Even under these conditions, I μ -C μ GL transcripts are constitutively expressed (step 1). After stimulation with IL-4 or IL-13 (and possibly with the cooperation of CD40 cross-linking), the ϵ GL promoter is activated (see below), and transcription is initiated (step 2). Primary ϵ GL transcripts contain the I ϵ exon, S ϵ , and C ϵ (step 3), and share the overall structure, albeit not the sequence, of primary μ GL transcripts. Upon splicing of the primary GL transcripts (step 4), mature I $_H$ -C $_H$ transcripts are released, whereas the spliced intron corresponding to S region sequences remains associated with template DNA. The resulting stable RNA/DNA hybrid represents the target for a still unidentified endonuclease that might initiate CRS. Joining of the two S regions involved in CSR, followed by DNA repair mediated by a number of factors, including Ku proteins (22) and DNA mismatch repairing enzymes (23), results in the formation of a hybrid S μ /S ϵ region, bringing C ϵ in close proximity to the rearranged VDJ complex. The excised DNA forms a circle and is rapidly degraded (step 5).

Regulation of the ϵ GL promoter

The postulated mechanisms through which S region transcription and splicing may cooperate to target CSR reiterate the critical role of cytokine-dependent induction of GL transcription in determining the isotype specificity of switching. Thus, it is necessary to understand how individual cytokines activate specific GL promoters in order to understand how the isotype specificity of CSR is achieved.

Full activation of the human ϵ GL promoter in response to IL-4 and CD40 engagement requires a constellation of nuclear factors that are either constitutively expressed or recruited to the promoter by specific activating signals. In the last few years, our group has performed a systematic characterization of the proximal region of the human ϵ germline promoter by electrophoretic mobility shift assays (EMSA) and

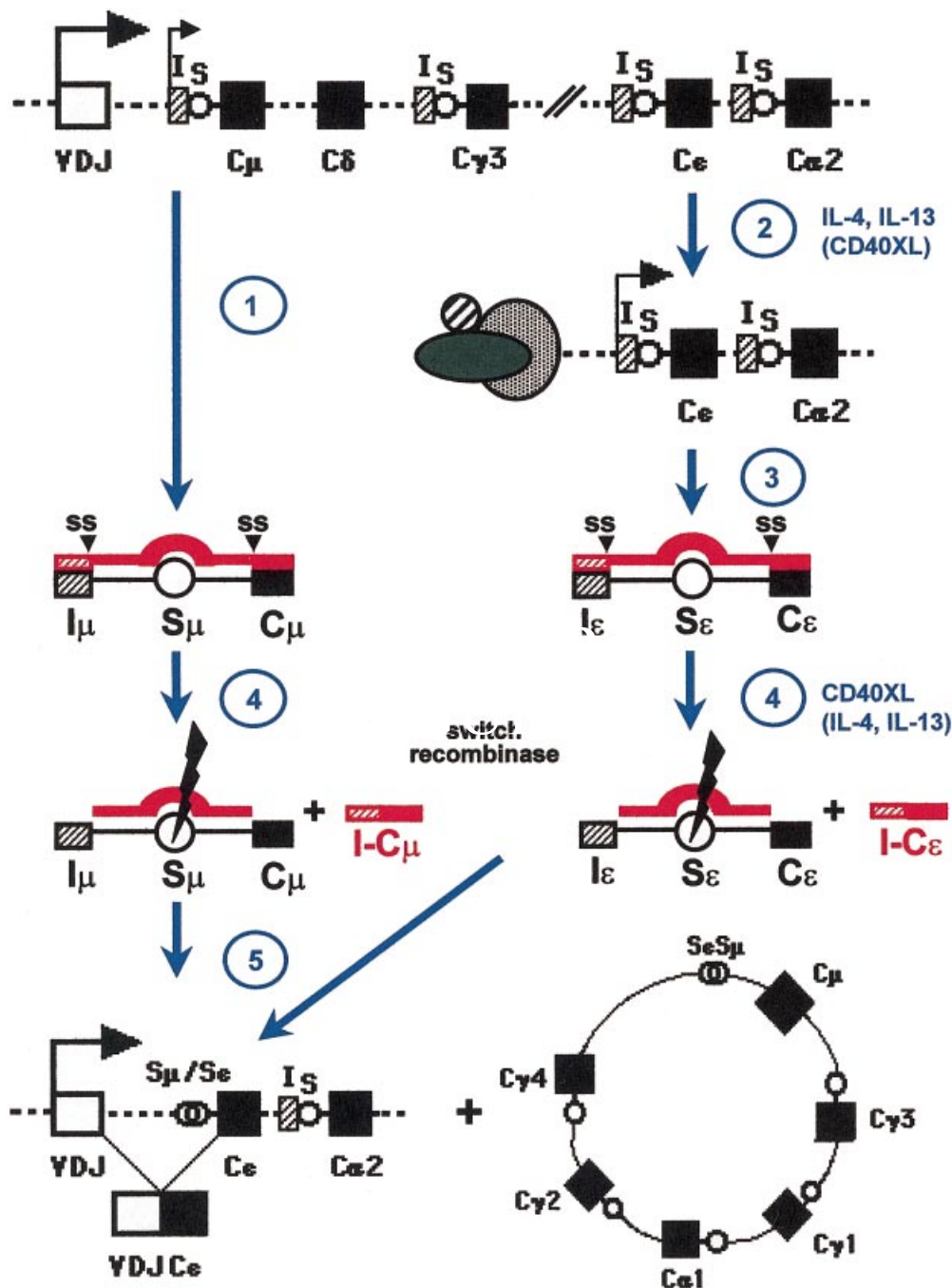


Figure 1. Induction of class switch recombination to IgE through GL transcription. The model is extensively discussed in the text. The different steps in CSR are labeled in blue. RNA is indicated in red. 1) Constitutive expression of primary μ GL transcripts; 2) cytokine (CD40)-dependent activation of the ϵ GL promoter; 3) cytokine-dependent expression of primary ϵ GL transcripts; 4) splicing of primary GL transcripts, release of mature I - C_H GL transcripts, and formation of stable hybrids between the S region and the intron of GL transcripts; the hybrids target the switch recombinaison to the appropriate S region; 5) S_μ/S_ϵ recombination and formation of circles containing the excised DNA. ss: splice site.

reporter analysis. EMSA analysis provides information on the number and affinity of specific binding sites for nuclear proteins in a DNA fragment. The specificity of protein–DNA interactions is assessed with oligonucleotide competitors to compare the affinities of different DNA sequences for the protein(s) of interest. Another useful feature is the ability to identify the protein(s)

involved in the formation of the complex of interest, using antibodies to specific transcription factors in an attempt to modify the mobility of the protein/DNA complex, or to prevent its formation. Once a protein binding to a given DNA motif has been identified by the pattern of oligonucleotide competition and antibody-mediated supershifting, the original probe may be tested

for its ability to bind the transcription factors of interest expressed in recombinant form. Reporter assays, on the other hand, allow the characterization of promoter sequences essential to drive transcription of a reporter construct with the same stimuli known to activate the endogenous gene. The functional role played by individual transcription factors in regulating promoter activity can be assessed with constructs that contain mutations shown to abrogate binding of specific nuclear proteins.

The IL-4-responsive element (IL-4RE), a region that binds STAT6 and Myb proteins

The human ϵ GL promoter is exquisitely IL-4/IL-13-inducible. Promoter response to Th2 cytokines appears to rely critically on a highly conserved sequence 5' to the major initiation sites for ϵ GL transcripts. This region (position -109/-80 in Fig. 2) represents an IL-4RE that binds STAT6 and recombinant CAAT enhancer-binding (C/EBP) proteins, and is sufficient to confer IL-4 inducibility on a heterologous promoter in transient transfection studies (24, 25).

STAT6, whose essential role in IL-4-dependent ϵ GL transcription (26) and IgE switching (27–30) is well established, belongs to the recently identified family of signal transducers and activators of transcription (STAT) (31). Binding of IL-4 to its receptor (IL-4R) leads to activation by tyrosine phosphorylation of two receptor-associated cytoplasmic tyrosine kinases, Janus kinase (JAK)3 and JAK1 (reviewed in Ref. 32). These kinases are believed to induce rapidly phosphorylation of critical tyrosine residues in the intracellular domain of the IL-4R α chain. These phosphorylated tyrosine residues provide docking sites for the latent cytosolic transcription factor STAT6. STAT6 binds to these docking sites via its SH2 domain, and is in turn tyrosine-phosphorylated by the receptor-associated JAKS. Phosphorylated STAT6 then homodimerizes, translocates to the nucleus, and binds to the promoter of a number of genes, contributing to the activation of

transcription (33). STAT6 preferentially binds dyad symmetric half-sites separated by four base pairs (TTC-N₄-GAA) (34). DNA binding specificity is localized to a region of 180 amino acids at the N terminal side of the SH3 domain (35). The discovery of JAKs and STATs provided an explanation for the apparent paradox that the IL-4R, as well as several other cytokine receptors, lacks kinase domains, and yet couples ligand binding to tyrosine phosphorylation.

While the -109/-80 region functions as an IL-4RE, IL-4 inducibility was surprisingly lost in constructs containing a slightly shorter sequence (position -105/-83), even though this region contained intact binding sites for both STAT6 and C/EBP (36). This discrepancy raised the possibility that deletions introduced at the two ends of the IL-4RE in the -105/-83 construct destroyed unidentified binding sites for nucleoprotein(s) important in the regulation of ϵ GL promoter activity. Indeed, we have recently been able to show that the IL-4RE contains at its ends two motifs that strongly resemble the consensus binding site for the Myb family of transcription factors (PyAAC^G/T_G) (37), and bind endogenously expressed Myb proteins. The myb1 site overlaps the 5' half of the putative C/EBP site, whereas myb2 overlaps the 3' half of the STAT6 element (Monticelli et al., submitted for publication) (Fig. 2).

The presence of binding sites for Myb proteins at the two ends of the human IL-4RE in the ϵ promoter may provide a mechanistic explanation for the long-sought link between germline transcription, switch recombination, and B-cell proliferation (38). Indeed, full expression of the transactivation potential of two myb family members, A- and B-Myb, requires unmasking of their transactivation domain through phosphorylation of the carboxy-terminal regulatory domain (39, 40). Phosphorylation appears to be mediated by cyclin A/cdk2 and cyclin E/cdk2 kinases, which are specifically expressed at the G1/S transition (40–42). Thus, the carboxy-termini of A- and B-Myb

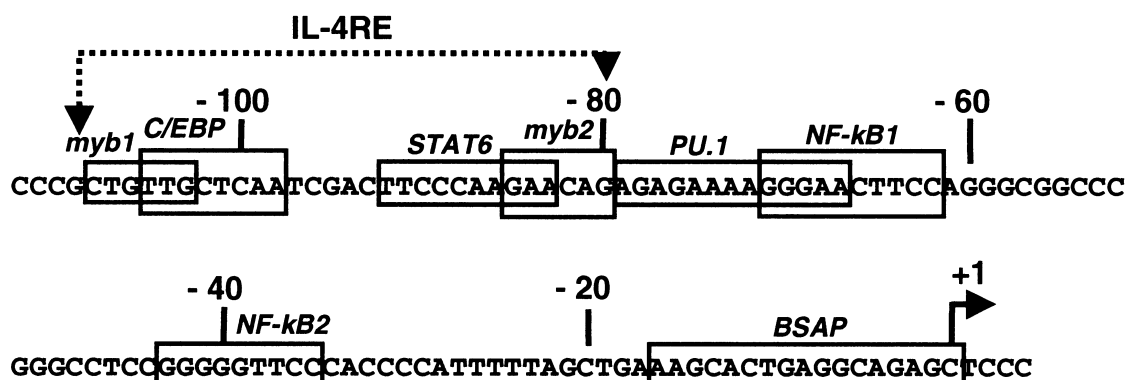


Figure 2. The proximal human ϵ GL promoter. Position +1 corresponds to the major ϵ RNA start site in BL-2 cells (65). Binding sites for transcription factors identified in the promoter are boxed and labeled. The IL-4RE and its boundaries are indicated by dashed line and arrowheads.

act as cell-cycle sensors. Furthermore, RNA for A- and B-*myb* is expressed in a cell cycle-dependent manner during late G1 and early S-phase (41, 43). This dual control mechanism ensures that maximal A- and B-Myb activity peaks in the G₁/S phase. Myb binding may also contribute to the early steps of ϵ GL gene activation. Indeed, recent data indicate that both Myb and STAT6 proteins interact with the coactivators p300 and CBP, thereby recruiting histone acetyltransferase activity to their cognate sites (44, 45). Thus, binding of STAT6 and Myb to adjacent sites in the IL-4RE may play a key role in chromatin remodeling, as well as in the interaction with the basal transcription machinery.

BCL-6

STAT6-binding sites share homology with the DNA recognition motif for BCL-6, a transcriptional repressor expressed at high levels in germinal center B cells (46). Indeed BCL-6, a POZ/zinc-finger protein, has been shown to bind the STAT6 site in the CD23b promoter and block IL-4-dependent transcription of this gene in transient transfection assays (47). Furthermore, it has been proposed that BCL-6-mediated repression through the STAT6 site may contribute to silencing the IL-4-inducible ϵ GL promoter under basal conditions (48). This hypothesis would be consistent with the finding that mutations of the STAT6 recognition element in the ϵ GL promoter may result in markedly increased levels of basal transcription (49, 50), suggesting a disruption in the recruitment of a repressor to these sites. We showed that BCL-6 is expressed in the BL-2 cell line used in our experiments. However, this factor appeared to play no role in the regulation of transcription from the human proximal ϵ GL promoter. Indeed, EMSA analysis failed to show BCL-6 binding to the STAT6 site; furthermore, mutations of the STAT6 element in the ϵ GL reporter construct abrogated STAT6 binding but had no effect on the basal activity of the reporter gene in transient transfection assays.

It is noteworthy that the recognition sites for BCL-6 and STAT6 are similar but not necessarily identical. Unlike the STAT6 elements in the proximal region of the ϵ promoter, an optimal BCL-6 site defined *in vitro* (46) and the one found in the CD23b promoter (51) contain three, not four, nucleotides between the two palindromic half-sites. It is possible that endogenous BCL-6 binds with high affinity only to elements that contain a 3-bp spacer, i.e., to sites recognized by most STAT proteins. In contrast, STAT6 is known to bind sites with 3-bp spacers, but, in addition, has a unique ability to interact with 4N sites (34, 35). This would imply that BCL-6 may interact with some but not all

STAT6-binding sites, but at the same time could modulate events triggered by other STAT proteins as well.

NF- κ B/Rel proteins and their association with STAT6

The region immediately downstream of the IL-4RE contains two sites that bind NF- κ B/Rel proteins with comparable affinity (Fig. 2) and appear to be essential for promoter function (see below). The upstream site (NF- κ B1) bound essentially p50/p65 heterodimers. c-Rel binding was also detectable in cells stimulated with IL-4 and/or anti-CD40 mAb. The downstream NF- κ B site (NF- κ B2) bound the same NF- κ B/Rel family members. In addition, we detected p50 homodimers in extracts from cells treated with anti-CD40 mAb, with or without IL-4, and p50/RelB heterodimers in untreated and IL-4-stimulated cells.

Perhaps the most interesting outcome of the NF- κ B studies was the demonstration that NF- κ B/Rel proteins directly associate with IL-4-inducible STAT6, and synergize with it in activating IL-4-dependent transcription (see below). Similar findings were reported for the murine ϵ and γ 1 GL promoters, in which a STAT6 element is located upstream of two or three NF- κ B binding sites (52–54). EMSA analysis with a probe (position –97/–57) that spans both the STAT6 and the NF- κ B1 elements revealed three major specific complexes in cells treated with IL-4, with or without anti-CD40 mAb (Fig. 3). Complex 2 was IL-4-inducible, and contained STAT6, because it was specifically competed by an oligonucleotide (STAT6, –97/–72) containing the STAT6 binding site, but not by an NF- κ B consensus sequence (κ BH2), nor by an unrelated oligonucleotide (COUP). Furthermore, complex 2 was completely supershifted by an anti-STAT6 antiserum. Complex 3 contained NF- κ B/Rel proteins, because it was specifically competed by κ BH2, but not by STAT6. Complex 3 was supershifted by an anti-NF- κ B p50 antiserum and inhibited by an antiserum to p65, but was unaffected by addition of anti-STAT6 antiserum. Most importantly, an additional band (complex 1) was detected. Complex 1 contained both STAT6 and NF- κ B nucleoproteins, because it was specifically competed by STAT6 and κ BH2 oligonucleotides, and supershifted or inhibited by antisera to STAT6 and NF- κ B family members (p65, p50, and c-Rel). These data show that, consistent with findings in the murine model (55), STAT6 and NF- κ B physically associate when binding to their adjacent cognate sites in the human ϵ GL promoter.

BSAP

The B-cell-specific activator protein (BSAP, Pax-5) is a homeodomain-class transcription factor specifically expressed in the B-cell lineage from pro-B to mature

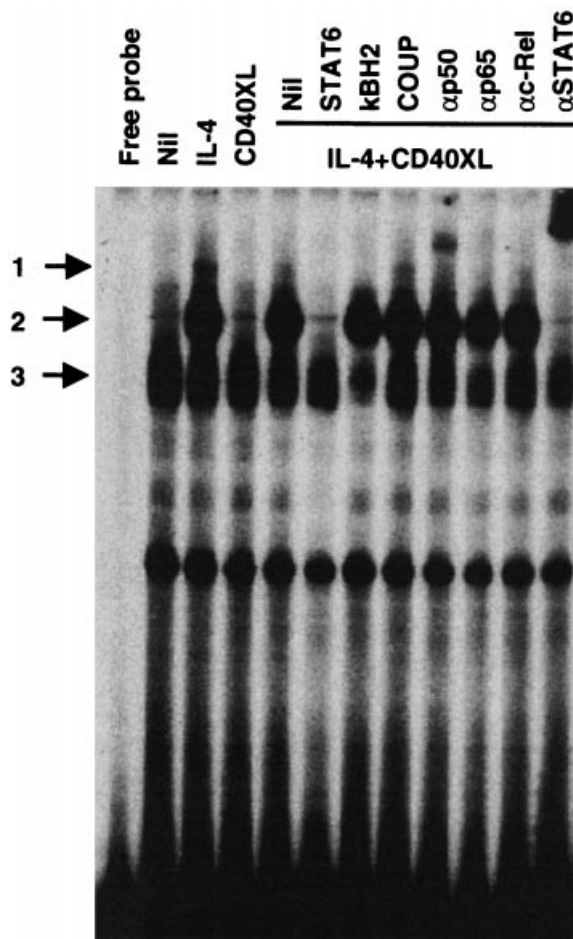


Figure 3. Association between NF- κ B/Rel and STAT6 proteins. Nuclear extracts (5 μ g) from serum-starved BL-2 cells incubated in the presence or absence of IL-4 (100 U/ml) and/or anti-CD40 mAb (5 μ g/ml) for 30 min were analyzed by EMSA, using as probe an oligonucleotide spanning both the STAT6 and NF- κ B1 sites in the ϵ GL promoter (position -97/-57). Extracts from BL-2 cells stimulated with IL-4+anti-CD40 mAb were preincubated with rabbit antibodies (2 μ g/reaction) specific for individual NF- κ B/Rel proteins or unrelated factors for 30 min on ice before adding the probe. Competitors were added at 100-fold molar excess. Specific complexes containing STAT6 and/or NF- κ B proteins are indicated by arrows.

B cells, but not in plasma cells (56). Binding sites for BSAP have been found in various promoters of B-cell-related genes (e.g., CD19, V_{preB1} , $\lambda 5$, and blk), including the Ig gene locus (reviewed in Ref. 57). BSAP binds to a highly conserved region immediately upstream of the major I ϵ transcription initiation site in both murine (58) and human (59) B cells (Fig. 2). Notably, among the factors that regulate ϵ GL transcription, BSAP is unique in that it is specifically expressed in B cells. GL transcription through the immunoglobulin locus is a B-cell-specific event as well. Thus, it is tempting to speculate that BSAP may be involved in the tissue-specific activation of immunoglobulin GL promoters, several of which contain BSAP-binding sites.

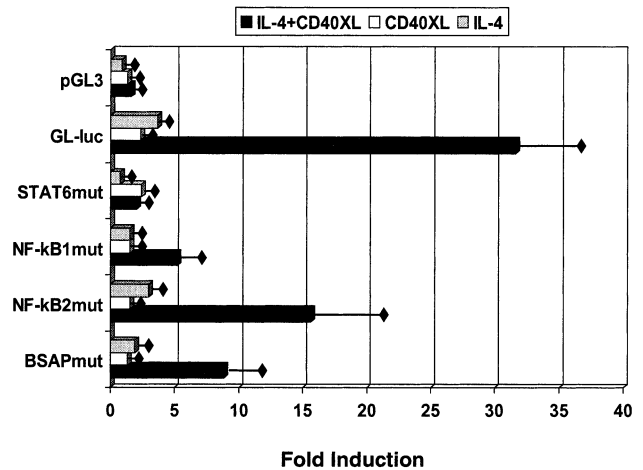


Figure 4. Reporter analysis of the proximal ϵ GL promoter. BL-2 cells were transiently cotransfected with the reporter constructs and an RSV- β -gal control plasmid using DEAE-dextran, and split into aliquots that were incubated in the presence of IL-4 (100 U/ml; gray bars), anti-CD40 mAb (5 μ g/ml; white bars), or a combination of the two stimuli (black bars). Luciferase activity was assessed 48 h later and normalized for transfection efficiency and total protein content. Values obtained in mock transfected cells were subtracted as background. Fold induction represents the ratio of relative luciferase activity (cpm/ μ g of protein) between treated and untreated cells. The results show the mean $\pm$ SE of at least three independent experiments, each performed in duplicate.

Functional analysis of the human ϵ GL promoter

Having identified a set of transcription factors that bind the human ϵ GL promoter, we then assessed the functional role played by these nucleoproteins in regulating promoter activity. For this purpose, luciferase reporter constructs containing the human ϵ GL promoter (either wild-type or mutated at specific binding sites) were transiently transfected into BL-2 cells. Reporter activity was measured in cells left untreated or stimulated with IL-4 and/or anti-CD40 mAb. As shown in Fig. 4, the wild-type promoter was activated 3.6-fold by IL-4, 2.3-fold by CD40 engagement, and 31.6-fold by a combination of the two signals. Thus, CD40 cross-linking enhanced IL-4-dependent transcription by 8.7-fold, pointing to a strong synergism between the two stimuli.

Mutations introduced in the reporter constructs had profound but different effects on the activation of the promoter in response to IL-4 and/or CD40 engagement. Mutations of the STAT6 and NF- κ B1 binding sites had dramatic effects on promoter activity; i.e., they abolished both the response to IL-4 and the CD40-dependent enhancement of ϵ transcription (80% and 70% inhibition, respectively). Mutation of the NF- κ B2-binding site impaired IL-4 responsiveness only minimally (19% inhibition), and decreased the synergism between IL-4 and CD40 signaling by only 40%, indicating that NF- κ B proteins bound more downstream may not be primarily involved in the

interaction with STAT6. Mutation of the BSAP-binding site strongly impaired both the IL-4-induced activation and the CD40-dependent enhancement of ϵ GL promoter activity (59). Simultaneous mutation of both NF- κ B sites, with or without the BSAP element, abrogated not only CD40 responsiveness but also IL-4-dependent activation, even though the STAT6 element was left intact (data not shown). The reporter construct containing mutations of the STAT6 and NF- κ B1 and/or NF- κ B2 sites was functionally indistinguishable from the empty pGL3 vector (data not shown).

Our results clearly indicate that regulation of human ϵ GL transcription involves complex interactions between factors recruited through different signaling pathways. NF- κ B/Rel proteins cooperate with STAT6 for the synergistic activation of IL-4-dependent human ϵ GL transcription. NF- κ B activity was critical not only for the CD40-dependent enhancement of IL-4-induced ϵ GL transcription, but also for IL-4 responsiveness in the absence of antibody-mediated CD40 cross-linking. Both effects are likely to reflect the physical association between NF- κ B/Rel proteins and STAT6. Upon interaction with NF- κ B, STAT6 appears to undergo critical quantitative and functional changes: DNA-binding affinity is substantially enhanced, and, most importantly, transactivating ability is induced (35). Thus, direct STAT6/NF- κ B interactions are necessary for the synergistic activation of transcription from promoters containing both cognate sites. Notably, activation of neither STAT6 nor NF- κ B requires *de novo* protein synthesis, allowing the rapid transmission of the IL-4/CD40 signal to the nucleus. More recently, it has been shown that the upstream NF- κ B site overlaps with a PU.1-binding motif. Abrogation of DNA binding of both PU.1 and NF- κ B resulted in loss of IL-4 inducibility of IgE GL promoter constructs (60). This result suggested that PU.1, as well as NF- κ B factors, may cooperate functionally with STAT6 to mediate IL-4 induction of the ϵ GL promoter.

BSAP may contribute to IgE switching not only through direct activation of the ϵ GL promoter, but also through differential regulation of GL transcription. BSAP inhibited α GL transcription and switching to IgA in sIgM-positive murine I.29 μ cells, but enhanced ϵ GL transcription and switching to IgE (61). The

opposite effects of BSAP on ϵ and α GL transcription exemplify the ability of this factor to activate or inhibit transcription, depending on the protein(s) with which it interacts at a particular binding site (62, 63), and the affinity for its different binding motifs. During a B-cell immune response, BSAP maintains its activator functions but is relieved of its repressor functions. This selective targeting of BSAP activities was shown to be regulated by a concentration-dependent mechanism whereby activator motifs for BSAP had a 20-fold higher binding affinity than repressor motifs. An exchange of activator and repressor motifs, however, showed that the context of the motif, rather than the affinity, determined whether BSAP operated as an activator or repressor (63).

The role of C/EBP proteins in regulating human ϵ GL transcription through the putative site in the IL-4RE remains unclear. Because sites for C/EBP and STAT6 proteins are often adjacent or overlapping in the promoters of IL-4-regulated genes, this arrangement was considered to serve a functional purpose (64). Although both factors can bind DNA independently, C/EBP β would stabilize STAT6 binding, thereby synergistically activating IL-4-dependent transcription (25). Indeed, the 5' portion of the IL-4RE binds recombinant C/EBP β . However, we found no evidence for interactions with endogenous members of this family in BL-2 cells. Of note, production of IgE upon stimulation with IL-4 and a trimeric CD40 ligand was normal in C/EBP β -deficient mice (27). Ig ϵ GL transcription in this model was not specifically assessed; however, these findings imply that C/EBP β may not be necessary for IL-4-induced IgE switching *in vivo*.

In conclusion, multiple factors contribute to the regulation of ϵ GL transcription upon binding the proximal region of the promoter. It will be interesting to characterize additional regulatory elements in the genomic region 5' to the 110 bp analyzed in the present study, as well as long-range interactions involving the 3' α 2 enhancer. Furthermore, it will be important to compare nucleoprotein binding and functional properties of the ϵ and γ 4 GL promoters, since they both respond to IL-4 and CD40 cross-linking, but show distinct patterns of activation *in vivo*.

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