Transcriptional regulation of the Iγ2 promoter in Human B cells

Jessica A. Belser* and Lori R. Covey

Department of Cell Biology and Neuroscience,
Rutgers, The State University of New Jersey,
Nelson Laboratory, 604 Allison Rd, Piscataway NJ 08854

* Rutgers Undergraduate Research Fellow

Abstract

Human IgG2 (immunoglobulin G2) is the predominant IgG subclass that is expressed in response to bacterial capsular polysaccharides. An IgG2 deficiency can therefore result in compromised immunity to carbohydrate antigens. Expression of IgG antibodies require an intramolecular recombination event that is preceded by transcription from intragenic (I) promoters. Thus, generation of IgG2 antibodies requires the induction of the Iγ2 promoter. To elucidate the regulation of the Iγ2 promoter in response to T cell contact and cytokine signals, deletion constructs were made to test the role of sequences within the Iγ2 proximal promoter in response to CD40 activation and stimulation with IL-4. Upon transient transfection into Ramos 2G6 cells (an IgM^+ Burkitt's lymphoma cell line), we found that truncation of the distal 228 bp caused a considerable decrease in basal and induced expression in response to these signals. Site-specific mutagenesis of this region supported this finding. This data implicates a region containing an NF-κB site in the Iγ2 proximal promoter as a major regulator of transcriptional activity and provide a starting point to investigate further how this promoter is regulated.

Introduction

The IgG antibody is a major effector molecule of the humoral immune system constituting nearly 75% of plasma immunogloblulin (Ig) in humans. This glycoprotein has a high affinity for antigen and persists in circulation for sustained periods of time signifying its participation in a mature secondary antibody response [1]. In humans, IgG contains four distinct subclasses or isotypes, IgG1 to IgG4, each with specific functional properties conferred by the carboxy-terminal region of the heavy chain. Despite a significant homology in the amino acid sequences of the constant heavy region between all four isotypes and in the control regions for the specific genes, there is a strong bias of expression of IgG1 relative to the other IgG isotypes.

IgG2 is the major IgG isotype elicited in response to carbohydrate antigens at high concentrations and is more efficient than other IgG subclasses in complement-mediated cytolysis and activation of the alternate complement pathway [4]. IgG2 is also the only IgG subclass with the ability to form covalent polymers, which serves as an advantage against antigens that elicit only low affinity antibodies [5]. An IgG2 deficiency has been linked with increased susceptibility to bacterial capsular polysaccharides, especially those of streptococcus and pneumococcus. In particular, patients with a selective inability to mount an IgG2 antibody response experience a higher reoccurrence of infection by *streptococcus pneumoniae*, the cause of acute otitis media

(AOM) [6]. AOM, the most frequent bacterial infection in infants and young children, can be halted by an effective immune response that includes the production of antibodies such as IgG2 that target capsular polysaccharides. In addition, studies have found that children prone to recurrent AOM display low IgG2 anti-polysaccharide antibody levels [7]. Elucidating the mechanisms responsible for commitment to IgG2 during class switch recombination would provide valuable insight towards understanding how such a deficiency could occur.

Class switch recombination (CSR) is a DNA recombination event that elicits a change in effector function without altering an antibody's antigenic specificity. In brief, a new C_H region is juxtaposed to an already formed V_H region resulting in antibody molecules retaining antigen recognition but eliciting different effector functions. CSR requires participation of cytokines and contact-dependent signals from T cells [2]. In particular, the cognate ligand for CD40, known formally as CD154, which is expressed on activated CD4+ T cells, is absolutely required for the induction of CSR in B cells. Additionally, CSR requires expression of germline (GL) transcripts that originate from C_H intragenic (I) regions and include an I region exon spliced to the respective C_H exons [3].

The NF-κB/*c-rel* family of inducible transcription factors is essential for lymphocyte survival and activation, regulates both innate and adaptive immune responses, and interacts with CD40 responsive regions to contribute to promoter activity [8, 9]. Multiple putative NF-κB sites are located within the promoter regions of the different Iγ genes although it is not clear which sites contribute to functional responses. This study sought to identify regions within the proximal Igγ2 promoter that are responsible for regulating Iγ2 transcription and switch recombination. This was accomplished by parsing sequences in the Iγ2 promoter to determine which regions are necessary to constitute minimal promoter function. After identification of the minimal promoter, the sequence was analyzed for the presence of transcription factor binding sites and site-specific mutations were introduced to better identify the role a putative NF-κB site plays in the transcriptional regulation of the promoter. Promoter activity of all constructs was assayed by transient transfection into Ramos cells, a Burkitt's lymphoma line used as a model B cell system. These studies should provide valuable insight into the transcriptional regulation of the Iγ2 promoter and an extension to understand the specific regulation and expression of the IgG2 subclass of antibodies.

Materials and Methods

Cell lines and culture conditions

Ramos 2G6 cells, an IgM^+, non-EBV transformed Burkitt's lymphoma line previously isolated by Siegel and Mostowski [10], were used for transient transfections. The cells were cultured in RPMI 1640 media supplemented with 10% FBS, 1mM penicillin/streptomycin (Pen/Strep), and 1mM ^{L}-glutamine. 293 cells (American Type Culture Collection) were derived from adenovirus 5-transformed primary human embryonal kidney cells, and the 293/CD40L line was constructed by the stable transfection of pCT-BAM into 293 cells as previously described [11]. These cells were cultured in DMEM/F12 media with identical additives.

Transient transfections and co-culture conditions

Ramos 2G6 cells (0.5×10^7 per condition) were washed, resuspended in 0.4 mL RPMI with no additives and supplemented with 26 µg/mL DEAE-Dextran. Cells were incubated with 50 µg DNA and 1 µg pRLSV40 (*Renilla* luciferase-based control plasmid; Promega) for 10 min at 37°C and pulsed twice at 320 mV/960 µF (GenePulser apparatus; BioRad). Cells were incubated at room temperature for 10 min, washed, and plated 1mL/well in a 24-well plate that had been seeded 2-4 hrs earlier with either 5×10^5 293 or 293-CD154 cells per condition. Soluble IL-4 (200 U/mL) was added to appropriate wells. After 48 hours at 37°C, cells were lysed and assayed for luciferase activity using the Dual-Luciferase assay kit (Promega). Firefly and *Renilla* luciferase activity was measured using a luminometer in relative light units (RLU) and fold induction over basal expression was determined.

Deletion mutagenesis of the γ2 511 promoter using PCR

The Advantage - HF 2 PCR Kit (Clontech) was used to amplify templates of variable lengths. PCR reaction parameters were identical to those of the suggested protocol. To generate promoter deletions, primers were designed to flank both ends of the truncated promoter region, while creating a Hind III site 5' of the beginning of the new site to facilitate ligation into the pGL2E vector (Promega). All PCR products were subsequently digested with Hind III, and the Qiagen QIAquick PCR Purification Kit was used to remove impurities. Ligations were set up between each insert and the treated pGL2E vector. Ligation products were transformed with JM109 competent bacteria (Promega) (5 µl ligation product to 40 µl cells, 25 min incubation on ice, 45 s heat shock at 42°C, 2 min on ice, 1 hr incubation with 250 µl SOC media, incubated overnight on LB-AMP plates), screened for orientation, and prepared using the Qiagen Plasmid Maxi Kit.

Creation of site-specific NF-κB mutants by PCR

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to amplify templates containing several base-pair deletions to disrupt sequential regions within the 511 proximal promoter. PCR reaction parameters were 95 °C 30 sec for one cycle, then 95°C 30 sec, 65°C 1 min, 68°C 12 min (2 min/kb plasmid length) for 20 cycles. Primers were designed with 5-6 bp stretches of non-homology to interrupt the NF-κB site. PCR products were subsequently incubated with Dpn I to digest the parental DNA template and to select for mutation-containing synthesized DNA. Bacterial transformation (1 µl product used) was followed by colony selection, DNA miniprep, and confirmation of the mutation by sequencing.

Results

The γ2 proximal promoter is significantly weaker than the γ1 proximal promoter despite a high degree of sequence homology.

The ~500 bp proximal promoter regions of all four Iγ genes are highly conserved, yet previous work in our laboratory has shown that the Iγ1 promoter is significantly stronger than the Iγ3 promoter. To directly analyze the Iγ2 promoter and compare its strength relative to the Iγ1 promoter, both γ1-520 bp and γ2-511bp homologous proximal promoter sequences were cloned into the pGL2E luciferase expression vector and transiently transfected into Ramos B cells.

As seen in Fig. 1A, the Iγ1 basal promoter function was significantly stronger than the Iγ2 promoter with an approximately 4-fold higher level of activity. In contrast, both Iγ1 and Iγ2 promoters demonstrated similar patterns when induced by either CD154 or IL-4 and upregulated transcription in an additive manner when exposed to both signals (Fig. 1B). These findings suggest that despite the high degree of sequence homology between both proximal promoters, small discrepancies between these two sequences can contribute to highly biased basal expression towards Iγ1 activity.

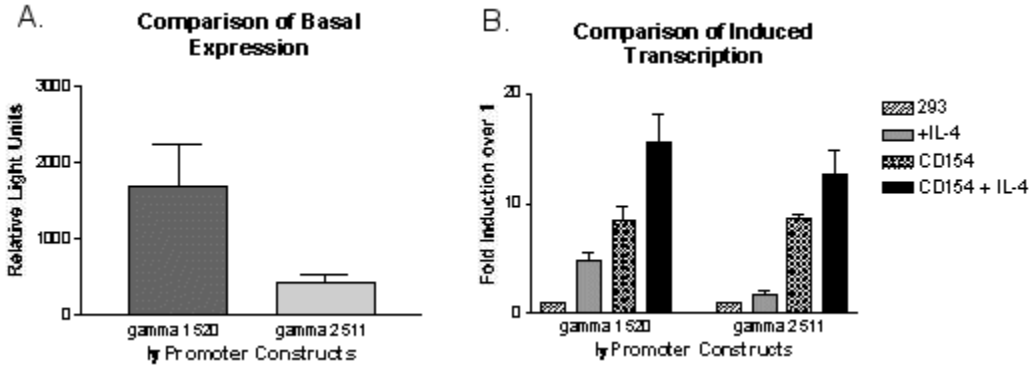

Figure 1. Iγ1 is a stronger promoter than Iγ2.
(A). The Iγ1-520 and Iγ2-511 proximal promoters were subcloned into the pGL2E vector and transiently transfected into Ramos 2G6 B cells. Analysis of transfection efficiency was accomplished by co-transfecting the pRLSV40 vector containing the firefly Renilla cDNA. Bars are representative of the average and SEM of five independent experiments.
(B). The Iγ1 and Iγ2 proximal promoter constructs were analyzed for inducible transcription in response to IL-4, CD154, and IL-4 plus CD154. Bars are representative of the average and SEM of five independent transfections.

The sequential truncation of the proximal promoter yields a marked decrease in transcriptional activity.

Sequence analysis of the proximal promoter region of the Iγ2 gene revealed a high level of identity with the Iγ1 region (Fig. 2). To better understand which regions were most essential for transcriptional regulation of the Iγ2 promoter, constructs were created within the Iγ2 proximal promoter that sequentially deleted regions of the promoter. Four constructs were created that contained deletions of sequences within the promoter and were named relative to the upstream Hind III site (bp #1): The γ2-21, γ2-138, γ2-228, and γ2-274 constructs contained 483bp (nt 21-511), 366 bp (nt 138-511), 276 bp, (nt 228-511), and 230 bp (nt 274-511), respectively (see Fig. 2). These constructs were introduced into Ramos B cells by electroporation and assayed for basal promoter activity and inducible transcription in response to signals from CD154 and IL-4 by measuring luciferase activity 48 hrs post transfection.

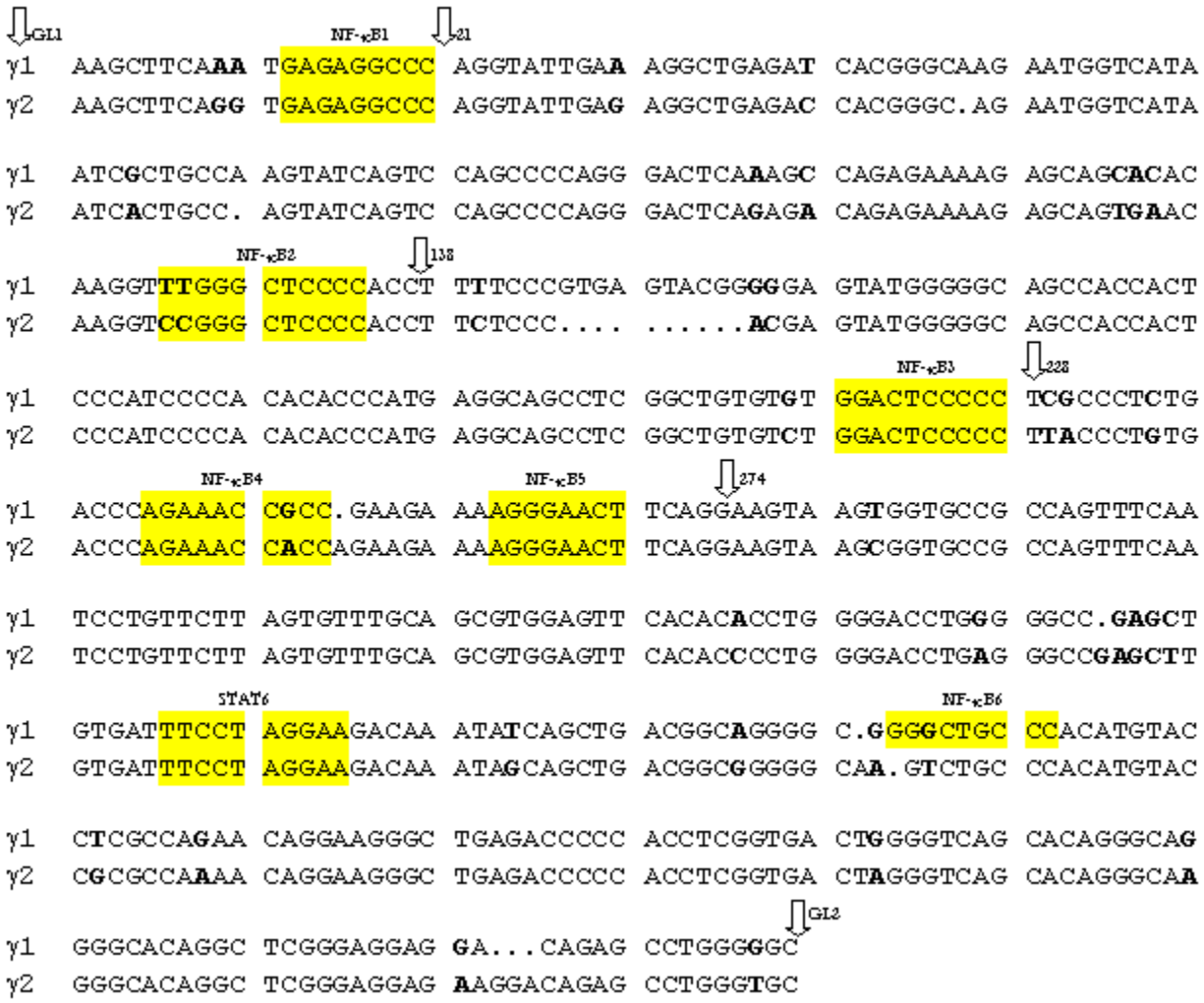

Figure 2. Sequence comparison of the proximal Iγ1 and Iγ2 promoter regions.
Shown is the sequence of the Iγ1 and Iγ2 promoter regions beginning at the upstream Hind III site (designated #1) and extending past the transcriptional start sites. Putative NF-κB and STAT6 sites are highlighted and the 5' end of each deletion construct is indicated by an arrow.

As seen in Fig. 3, the activities of the γ2-21 and γ2-138 constructs were very similar to the activity of the complete γ2-511 proximal promoter construct suggesting that sequences between 1 and 138 were dispensable with respect to full promoter activity. In contrast, a significant decline in both basal and induced promoter activity was observed when the γ2-228 and γ2-274 were introduced into B cells. These findings indicate that sequences between 138 and 228 play a crucial role in the transcriptional regulation of the Iγ2 proximal promoter, as their absence severely decreases the activity of the promoter.

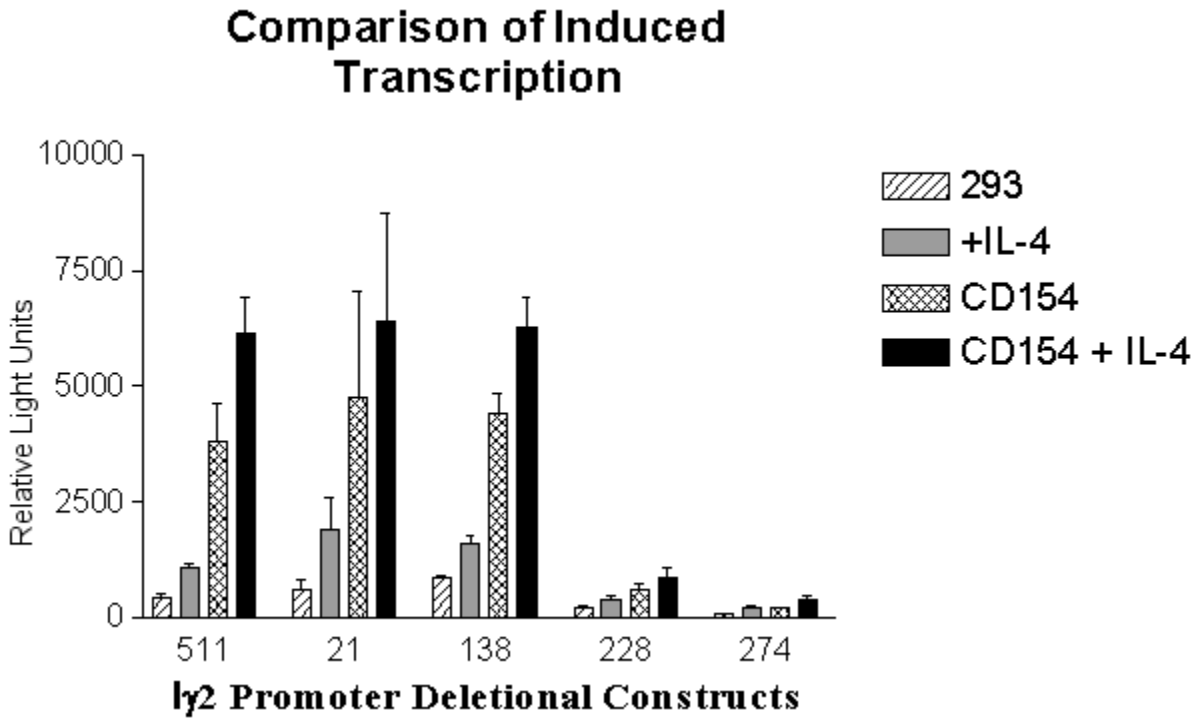

Figure 3: Basal and induced activity is disrupted in the Iγ2-228 and Iγ2-274 constructs.
Transient transfections using the different deletion constructs were carried out in Ramos 2G6 B cells. Upon transfection the cells were stimulated with either IL-4 and/or CD154. Transcriptional activation was measured by levels of luciferase activity in the cell extracts. Bars are representative of the average and SEM of five independent transfections.

Mutation of the third NF-κB site within the γ2 proximal promoter significantly reduces Iγ2 promoter activity.

Analysis of sequences between 138 and 228 revealed a single NF-κB site just upstream from nt 228 (designated NF- κB-3). Also, two putative NF-κB sites were identified between 228 and 274 (designated NF-κB-4 and NF-κB-5). To determine whether these sites were specifically responsible for the dramatic loss of promoter function in the γ2-228 and γ2-274 constructs, site-specific mutations were created within the third and fifth sites by PCR. The mutated fragments were cloned into pGL2E and these new constructs were termed mutNF-κB3 (mutation of the third binding site) and mutNF-κB5 (mutation of the fifth binding site). This approach was used to directly test whether the substantial decrease observed with the deletional mutants was due to the absence of NF- κB binding and not due to the possible removal of other transcription factor binding sites within the sequence. The mutated and the complete γ2-511 constructs were

electroporated separately into Ramos cells and assayed for luciferase activity 48 hours post-transfection.

Mutations in the third or fifth NF-κB sites did not appear to significantly affect the basal activity of the proximal promoter (data not shown), which may be due to the fact that the unmutated γ2-511 promoter already has a decreased intrinsic basal level of expression (Fig. 1). However, mutations of the NF-κB-5 site reduced the CD40- induced response by approximately 2-fold and mutations to the NF-κB-3 site dramatically reduced the CD154 and/or IL-4 responsiveness of the Iγ2 promoter to barely above background (Fig. 4). This response is similar to the γ2- 228 deletion construct that lacked the first three putative NF-κB sites (Fig. 3), suggesting that the third site is indeed responsible for the majority of induced transcriptional activity in response to CD40 signals.

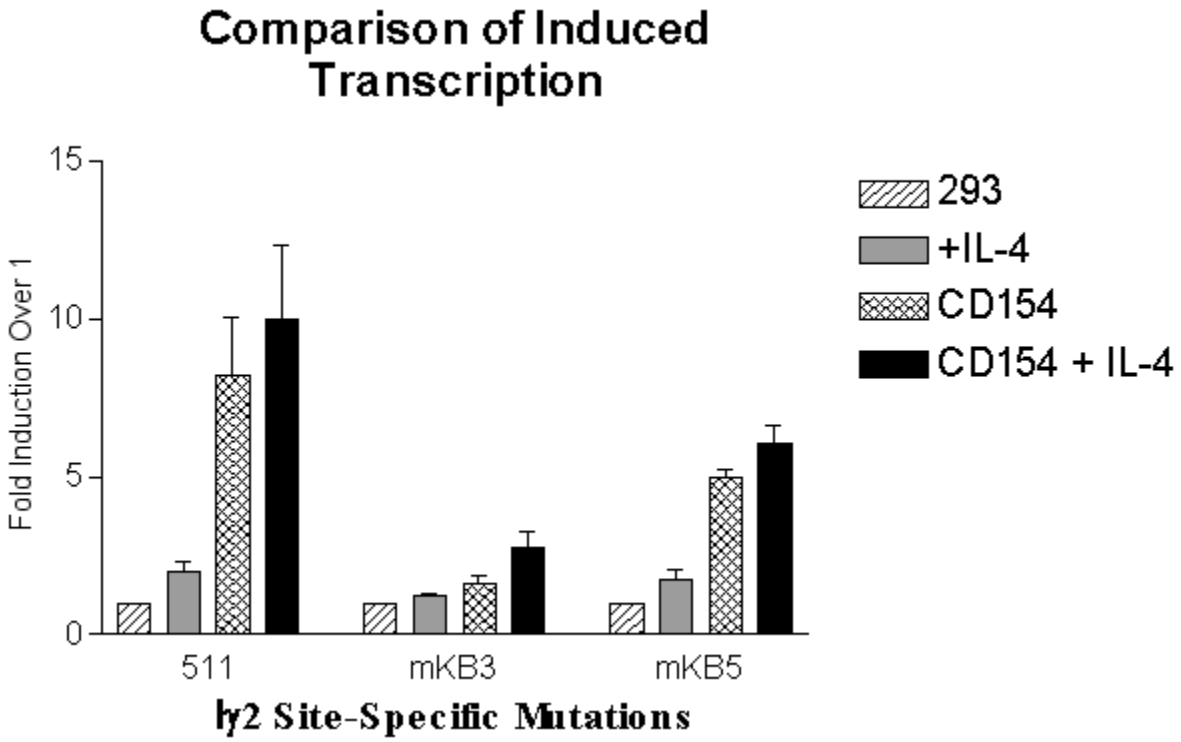

Figure 4: Mutations in the NF-κB-3 site affect inducible transcription.
Transient transfections using the mutNF-κB3 and mutNF-κB5 constructs were carried out in Ramos 2G6 B cells. Upon transfection the cells were stimulated with either IL-4 and/or CD154. Transcriptional activation was assessed by assaying levels of luciferase activity in cell extracts. Bars are representative of the average and SEM of five independent experiments.

Discussion

This study sought to elucidate the regulation of the Iγ2 promoter in response to T cell contact and cytokine signals. In particular, we sought to identify regions within the promoter that are important for regulating Iγ2 transcription. This research was conducted with the hope of more fully understanding the regulation of transcriptional activity of the Iγ2 promoter under basal and induced conditions. To achieve this, we analyzed four deletion mutants within the Iγ2 proximal promoter. Our results with the deletion constructs demonstrated that sequences between nt 138 and 228 are critical for basal and CD40 mediated activation.

To confirm this initial observation, we carried out site-specific mutagenesis to effectively "knock out" individual factor binding sites within the region. We chose to focus on the putative NF-κB site in the targeted region. By specifically mutating the third NF-κB site within the context of the complete proximal promoter, we demonstrated quite convincingly that the third NF-κB site was necessary for basal and induced expression of the Iγ2 proximal promoter.

Despite the high degree of sequence conservation between the Iγ2 and Iγ1 promoters there remains a significant disparity in their basal transcriptional activity which results in absolute higher levels of IL-4- and CD40- induced activities of the Iγ1 promoter. Previous research in our laboratory has identified a 36bp element 3' to the STAT site in the Iγ1 promoter that encodes multiple putative factor binding sites including NF-κB, CRE, SP-1 and ATF-2 which is believed to act as an "amplifier" of IL-4- and CD154-induced responses [13]. This motif is only partially conserved in the other Iγ subclass promoters. As the deletion or mutation of this element significantly reduces basal and indirectly, induced transcription, it is hypothesized that factor binding to sites within this element directly regulates Iγ1 transcription. The Iγ2 promoter contains a number of nucleotide changes within this 36bp element, disrupting every putative binding site identified in γ1. Limited conservation of this element on the Iγ2 proximal promoter could therefore account for the disparity found between γ1 and γ2 basal transcriptional responses.

An inability to mount an adequate IgG2 antibody response has been linked to persistent infection of such bacterial infections as acute otitis media and localized juvenile periodontitis [5, 14]. Achieving a broader knowledge of the molecular basis underlying the regulation of transcriptional activity of the Iγ2 promoter could prove useful in developing a more complete understanding of how this promoter works, ultimately contributing to an explanation of why differential switching occurs and how to counteract such an antibody deficiency.

Acknowledgments

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