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Characterization of Cannabinoid Receptor 2 Transcript Expression in B Cells

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by

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DEDICATION

I would like to dedicate this work to three important people that I lose during my time in the Ph.D. program here at the University of South Florida; my life partner William "Joe" Best, father Robert D. Sherwood, and grandmother Violet May Sherwood, may they all rest in peace. Thank you all for supporting me in all my life efforts. I would also like to dedicate this work to my mother Londa Sherwood for her never ending support and faith in me. Finally, I would like to thank my sister Deborah Parrott for without her love and support during the difficult times of losing my loved ones I would have never made it through this program.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Characterization of Cannabinoid Receptor 2 Transcript Expression in B Cells

Tracy Sherwood

ABSTRACT

Cannabinoids and cannabinoid receptors have been shown to play important roles in immune regulation particularly as modulators of anti-inflammatory cytokines and antibody production. The predominant cannabinoid receptor involved in this immune regulation is cannabinoid receptor 2 (CB_2), which is robustly expressed in B cells. Utilizing a combination of bioinformatics, 5' RACE, real time RT-qPCR, and reporter assays, we showed that human B cells from peripheral blood mononuclear cells (PBMC) expressed one CB_2 transcript while mouse B cells from spleen express three CB_2 transcripts. Alignment of the sequenced B cell RACE products to either the mouse or human genome, along with the GenBank mRNA sequences, revealed that the transcripts isolated in this study contained previously unidentified transcriptional start sites (TSSs). In addition, expression construct testing of the genomic region containing the TSSs of the mouse CB_2 exon 1 and 2 transcripts showed a significant increase of promoter

activity. Bioinformatics analysis for *cis*-sequences in the promoter regions identified DNA binding sites for NF-kB, STAT6, and Elk1 transcription factors activated by LPS, IL-4 and anti-CD40. Regarding variations in CB_2 transcript expression among the immune cell subtypes, RACE analysis showed that the exon 1b transcript is seen in B cells but not in T cells, dendritic cells or macrophages. Furthermore, RT-qPCR showed variations in transcript expression during B cell development as well as in resting versus LPS or IL-4/anti-CD40 stimulated B cells. The exon 1a transcript was predominant in pre-, immature and resting B cells whereas the exon 1b and 2 transcripts were enhanced in mature and activated B cells. These data showed for the first time that human B cells use one TSS for CB_2 expression while mouse B cells use multiple TSSs for the expression of three CB_2 transcripts, in which the expression of the individual transcript is related to immune cell type and/or cell activation state. Additionally, this is the first report in mouse B cells defining TSSs that are in genomic areas with promoter activity thus suggesting the location of two promoter regions. Defining the CB_2 transcript expression during various stages of B cell activation provide clues to therapeutic methods.

INTRODUCTION

Cannabinoids and Cannabinoid Receptors

The marijuana plant (*Cannabis sativa*) and preparations derived from it have been used for medicinal and recreational purposes for thousands of years. *Cannabis* produces ~60 unique compounds known as cannabinoids, of which Δ^9-tetrahydrocannabinol (THC) is considered the most important, owing to its abundance and psychoactive component (4, 29). Currently two cannabinoid receptors have been described. Cannabinoid receptor 1 (CB_1) the first to be identified (27), also known as the "central" cannabinoid receptor is found primarily in the brain and central nervous system (CNS) and is responsible for the psychoactive effect of THC. CB_1 is also found in cells of the male and female reproductive system, as well as in some peripheral organs, such as liver, fat and muscle cells. Cannabinoid receptor 2 (CB_2) (30), known as the "peripheral" cannabinoid receptor is almost exclusively found in the immune system, with highest expression observed in B cells. It is also found in the peripheral nervous system and in microglia. CB_2 has been shown to be the cannabinoid receptor

primarily responsible for the anti-inflammatory and possible immune therapeutic effects of cannabis (22) (38) (7).

The cannabinoid receptors belong to the G protein-coupled receptor (GPCR) superfamily, and are seven-transmembrane domain receptors, which act as a guanine nucleotide exchange factor (GEF) for associated G-proteins by exchanging bound GDP for GTP. The bound GTP activates the G-protein by causing the dissociation of the G-protein's α-subunit from the β and γ subunits. Two signal transduction pathways are affected by G-protein activation, the cAMP pathway and the phosphatidylinositol pathway. Cannabinoid receptors signal through a $G_{i/o}$-protein, in which dissociation of the α-subunit inhibits adenylate cyclase and thereby decreasing the production of cyclic AMP (19) (18) (2).

The cannabinoid receptors are activated by three general types of cannabinoids: 1. Phytocannabinoids, also known as the classical cannabinoids, which 66 have been isolated from *Cannabis*. 2. Endocannabinoids, produced endogenously by the body, in which two have been well characterized, arachidonolyethanolamine (Anandamide or AEA) and 2-arachidonynoyl glycerol (2-AG) endogenous. 3. Synthetic cannabinoids that are useful in experiments to determine receptor function. Numerous agonists have been design, some non-

selective, such as CP-55940, that bind CB_1 and CB_2 with similar affinity, as well as selective agonists, such as JWH-133, which binds only to CB_2. In addition, antagonists have been designed to either selectively block CB_1, SR141716, or CB_2, SR144528 (38) (24, 29, 31) (6).

B cells

B cells are antibody producing lymphocytes (white blood cells) that make up part of the adaptive immune system and responsible for the humoral immune response, the portion of immunity that is mediated by antibodies. On the surface of every B cell is a membrane-bound immunoglobulin (Ig) receptor, the B cell receptor (BCR), which binds to specific antigen. It is the BCR and the ability of the receptor to recognize antigen in its native form, as well as the maturation and production of large amounts of antibodies that distinguishes B cells from other types of lymphocytes. The "B" comes for the bursa of Fabricius, an organ in birds that is the site of hematopoiesis and B cell development and maturation. In mammals, the bone marrow is the site of hematopoiesis and where B cells are continually produced. Development occurs through several stages, each stage representing a change in the genome content at the Ig loci (Table 1). At the

immature stage B cells migrate to the spleen where the final stage of maturation occurs (3). Mature B cells express both surface IgM and IgD and are considered naïve until activated by antigen, which can occur either in a T cell-dependent or –independent manner(15).

The BCR and secreted antibody are comprised of four polypeptide chains, two identical heavy and two identical light chains with both constant (C) and variable (V) regions. In the heavy chain V region there are three segments; Variable (V), Diversity (D), and Joining (J), whereas light chain only contains V and J. Germ line DNA has up to 200 V genes, twelve D genes, and four J genes, which during B cell development randomly rearrange and recombine by a mechanism known as VDJ recombination to produce functional VDJ genes. The kappa (κ) and lambda (λ) chains of the Ig light chain loci rearrange similarly, except the light chain lacks a D gene. The first step of recombination for the light chain involves joining of V and J to give VJ before the addition C during transcription (14). Translation of the spliced mRNA for either K or λ results in formation of either the Igκ or Igλ light chain. At this point, the mature B cell is considered naïve until it is activated by antigen.

The five classes of antibody; IgA, IgD, IgE, IgG and IgM respectfully, are defined by the constant regions of the Ig heavy chain.

Upon exposure to antigen, naïve mature B cells are activated in the absence of T cell help (T cell-independent) by antigen cross-linking (48) of the IgM BCR and differentiate into functional IgM secreting plasma B cells (primary response). However, most antigens are T cell-dependent and require T cell help for maximum antibody production. Antigen primed T_H2 helper cells activate B cells through T cell Receptor (TCR) recognition of specific antigen presented on B cell MHC II, along with co-stimulation of CD40 on B cells by CD40 ligand on T cells (34). Cytokines are then secreted by T cells that bind to cytokine receptors on B cells and trigger B cell proliferation and differentiation into plasma and memory B cells. In addition, some of the functionally responsive IgM and IgD expressing mature B cells will undergo the process of Ig Class Switch Recombination (CSR) (46), in which rearrangement of the DNA places the recombined VDJ gene next to either IgG, IgE or IgA C gene, to produce Ig isotype specific secreting plasma and memory B cells (9). Each isotype has a distinct function, therefore the type of antigen and cytokines present in the B cell environment will determine which Ig isotype will be expressed in the differentiated plasma and memory B cells (Table 2). The memory B cells, formed from activated B cells, have BCRs that are antigen

specific so when exposed to the same antigen, a rapid and effective immune response occurs (secondary response) (45).

Table 1. B cell Developmental Stages.

Stage	**Heavy Chain**	**Light Chain**	**Ig**
Progenitor	Germline	germline	-
Early Pro	D-J rearrangement	germline	-
Late Pro	V-DJ rearrangement	germline	-
Large Pre	VDJ rearranged	germline	IgM in cytoplasm
Small Pre	VDJ rearranged	V-J rearrangement	IgM in cytoplasm
Immature	VDJ rearranged	VJ rearranged	Surface IgM
Mature	VDJ rearranged	VJ rearranged	Surface IgM & IgD

Table 2. Cytokines responsible for Ig class switching in mouse

T cell	Cytokine	Ig isotypes					
		IgG1	IgG2a	IgG2b	IgG3	IgA	IgE
T_H2	IL-4	+					+
	IL-5					+	
T_H1	IFNγ		+				
Treg	TGFβ			+		+	

+, indicates Ig that cytokine induces switching from IgM

Cannabinoid Effects on B cells

The molecular events of the peripheral cannabinoid receptor (CB_2) in immune cell function are still not fully understood. Nor are the mechanisms that regulate the CB_2 gene, *Cnr2*, during activation of immune cells. CB_2 message expression shows a pattern of mRNA expression that is most abundant in B cells followed by macrophages and then T cells (16, 25). Evidence also suggests that anti-CD40 (25), as well as IL-4 through activation of STAT6 (42), up regulate CB_2 message expression and that LPS (25) down regulates its message expression in B cells; however the mechanisms are still unclear. Furthermore, CB_2 has been implicated in B cell differentiation (8), migration (20, 39), proliferation (1, 13) and immunoglobulin isotype switching (1). These findings suggest a role of CB_2 as an immunomodulator. However, many gaps still exist in our understanding of this receptor's role in immune cell function, and in particular how is CB_2 receptor expression regulated in B cells.

Gene Regulation

The modern working definition of a gene is "a locatable region of genomic sequence, corresponding to a unit of inheritance, which is associated with regulatory regions, transcribed regions, and

or other functional sequence regions" (35, 36). A gene is basically a DNA sequence that contains the instructions for the production of a protein. The basic gene structure consists of a promoter regulatory region, and a transcribe region of exons (coding sequences) and introns (non-coding sequences) (40). Transcription is the first step leading to gene expression resulting in messenger RNA (mRNA), which is translated into protein (28). During transcription of a gene, the DNA sequence is targeted by RNA polymerase II (Pol II), which produces a complementary pre-mRNA strand, except that uracil (U) is used instead of thymine (T). The pre-mRNA strand is then spliced to form the mature mRNA strand that transports to the cytoplasm to be translated into protein. This process of DNA to RNA to protein is known as the "central dogma" of biology (Figure 1) (11).

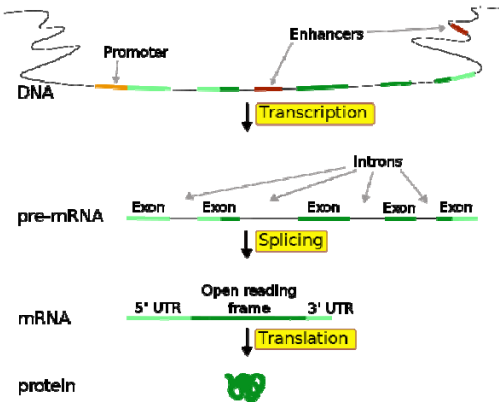


Figure 1. The "Central Dogma" of Biology. Several steps are involved in gene expression of a protein. Promoters and enhancers regulate what DNA sequence will be transcribed into pre-mRNA, which is then spliced into mRNA and translated into protein. This figure was obtained with permission from Wikimedia Commons.

The promoter is typically upstream adjacent to the gene and contains specific DNA *cis*-sequences, which provide binding sites for RNA Pol II and transcription factors (*trans*-factors). The promoter consists of two interacting components, the core promoter and the proximal promoter (51). The core promoter sequence is the minimal region of DNA required for Pol II to assemble with the basic *trans*-factors and form the pre-initiation complex for initiation of activator-independent (basal) transcription (17, 43). At the center of the core promoter sequence is the initiator (INR) sequence that contains the transcription start site (TSS), which is defined as the most 5' nucleotide of mRNA transcribed by Pol II (17, 41). The core promoter also contains the *cis*-sequences for the basic *trans*-factor elements, such as the TATA-box, the TFIIB recognition element (BRE) and the downstream promoter element (DPE), in which the TSS (+1) designates their position (Figure 2, provided by Wikimedia Commons) (43) (21). *Cis*-sequences upstream of the TSS are negative numbers counting back from -1, whereas downstream *cis*-sequences are positive numbers counting from the TSS (+1). The proximal promoter is the region generally upstream of the gene that contains regulatory elements involved in increasing transcription. These regulatory elements, also known as enhancers/repressors, can be several

kilobases (kb) away from the TSS. The large protein transcriptional complex, causes the DNA to bend back allowing for the placement of regulatory sequences far from the TSS (51).

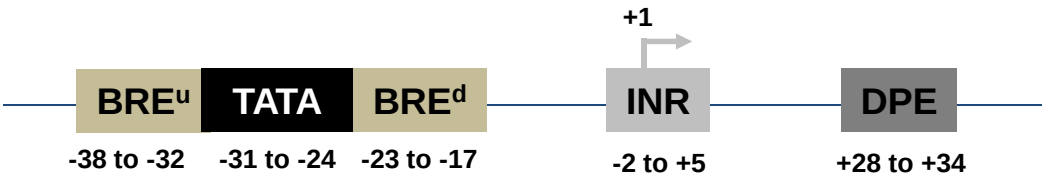


Figure 2. The Basic Core Promoter Elements. BRE, TFIIB recognition element, ^{u} upstream, ^{d} downstream TATA-box; DPE, downstream promoter element; INR, initiator; TATA, TATA-box

PROJECT SIGNIFICANCE

For centuries marijuana has been used recreationally and as a therapeutic for many ailments. However, little research was done investigating the beneficial medical effects of marijuana, until the discovery of the cannabinoid receptors and the endocannabinoid system. Since their discovery research in the field has grown exponentially. In which, recent developments have shown cannabinoids to be potent anti-inflammatory and immunosuppressive agents that mediate beneficial effects for many inflammatory diseases, including multiple sclerosis (10, 32, 33) rheumatoid arthritis, as well as allergy, an area of cannabinoid research in lab. Previous work done in our lab by Agudelo et al. 2008, showed that cannabinoids enhanced the IL-4/anti-CD40 mediated isotype switching from IgM to IgE in purified mouse B cells. In which, CB_2 was implicated as the main cannabinoid receptor mediating the observed enhanced increase of IgE in IL-4/anti-CD40 stimulated B cells.

The gene encoding for CB_2 is the *CNR2* gene, however the regulatory elements such as the promoter, transcriptional start site

(TSS), and *cis*/*trans*-factors have not been described in B cells. Therefore, research is needed to identify these gene regulatory elements responsible for the expression of CB_2 in B cells. The identification of the gene regulatory elements will lead to a better understanding of the pathways of CB_2 expression and the mechanisms of increased receptor expression with changes in B cell activity. Identification of CB_2 regulatory elements may also provide clues to how gene and protein expression can be therapeutically regulated in B cells. We propose to identify the location and sequence of the core promoter region(s) of the *CNR2* gene in mouse and human B cells as well as some of the associated proximal promoter elements. Because CB_2 is expressed highest in B cells, it is possible these cells contain unique features of the promoter contributing to enhanced CB_2 transcription.

OBJECTIVES

The focus of this project is to determine the *Cnr2* gene transcription start site (TSS) and associated promoter and *cis*-sequences involved in CB_2 mRNA expression in B cells as well as investigate transcript usage in resting and activated cells. Previous studies suggest a role of CB_2 in the immune regulation of B cells by demonstrating involvement in B cell differentiation, migration, proliferation, and immunoglobulin class switching to IgE. A preliminary computational analysis of the murine *Cnr2* gene and GenBank CB_2 mRNA clones revealed that two alternate transcripts containing different 5'UTR first exons (1 and 2) were reported. Three clones from immune tissues contained the exon 1 5'UTR, whereas clones originating from bone and liver contained the exon 2 5'UTR. This analysis indicated that more than one transcript is produced from the *Cnr2* gene, and that the transcripts may be related to cell type or function. From this, we **hypothesize that the *Cnr2* gene encodes multiple transcripts in B cells and other immune cells, which**

are varied following changes in cell function. In order to test this hypothesis, we propose the following aims:

Aim 1. To Determine the Extent of CB_2 Transcript Expression and TSSs in B cells

Evidence in the genomic databases suggested the occurrence of multiple CB_2 transcripts utilizing different first exons in various mouse tissues, as mentioned above. It is well known that the TSS is located either at the beginning of the first exon or upstream from it. Since 2 first exons (exon 1 and 2) have been reported, B cells possibly express multiple TSSs and CB_2 transcripts employing different first exons. Therefore, we will explore this possibility in resting B cells. To accomplish this aim, we will use the Switching Mechanism At 5' end of RNA Transcript – Rapid Amplification of cDNA Ends (SMART 5' RACE) to identify the number and location of the CB_2 TSSs in splenic and PBMC B cells.

Aim 2. To Characterize the *Cnr2* Gene Promoter in B Cells

It has been well established that accurate identification of the TSS leads to the location of the core promoter, which is usually -40 bp upstream to +40 bp downstream of the TSS. The basic elements that comprise the core promoter are the TATA-box, INR (Initiator), DPE

(downstream promoter element), and BRE (TFIIB recognition elements) (41). Identifying the promoter will lead to a better understanding of how the *Cnr2* gene is regulated in B cells, which in turn will lead to the elucidation of the mechanisms involved in the immunobiology of CB_2 and B cells. We will start with a bioinformatics analysis using web based analytical tools, such as Genomatix, to locate the putative promoter regions of the *Cnr2* gene. These will then be cloned into luciferase reporter gene expression vectors and transfected into purified splenic B cells to test for promoter activity. Truncations of the clones will be performed to identify core promoter and *cis*-regulatory sequences.

Aim 3. To Determine CB_2 Transcript Usage in Activated B cells as well as Other Immune Cell Subtypes

Since CB_2 is abundant in B cells and implicated in the involvement of various B cell functions, an understanding of transcript usage under varying conditions of B cell activation will be of value in designing future studies to regulate CB_2 expression. The GenBank data show that multiple CB_2 transcripts exists therefore, some of these could be unique to B cells and the various associated 5' UTR sequences could provide useful targets for selectively suppressing or enhancing receptor expression in B cells. In this aim, we will compare CB_2

transcript usage in resting and stimulated B cells. The literature shows that stimulation of B cells with anti-CD40 and/or IL-4, through STAT6 activation, increases CB_2 expression (8) (25) (1, 42), whereas, LPS stimulation suppresses expression (26). Therefore, to examine CB_2 transcript usage, we will stimulate purified B cells with stimuli reported to increase CB_2 expression, such as IL4 that activates STAT6, anti-CD40 that increases NFκB, and LPS, a known B cell mitogen, that binds to TLR4 and activates NFκB and/or IRF3. However, LPS has been shown to decrease CB_2 message, therefore results from these experiments may uncover possible repressor elements. To perform these experiments, B cells will be isolated and cultured alone or with the various stimuli, and analyzed by RT-qPCR at various time points following B cell stimulation.

Since we would like to know if any of the CB_2 transcripts are unique in B cells, we will also analyze other immune cell subtypes, such as T cells, dendritic cells and macrophages for the presence of CB_2 transcript variants.

MATERIALS AND METHODS

Bioinformatics Analysis

The bioinformatics programs used for this study include the Genetics Computer Group (GCG) SeqWeb v3.1 software package, Primer3, the Genomatix Suite, Consite, the Database of Transcriptional Start Sites (DBTSS), Ensembl and NCBI databases.

Mice

C57BL/6 mice, 8 to 10 wks old, and of mixed gender where obtained from NCI (Fredericksburg, MD) and housed and cared for in the University of South Florida Health Science Center animal facility, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Isolation of Mouse Splenocytes, T and B cells

Mice were euthanized by CO_2 asphyxiation, followed by removal of the spleens, which were placed in 12 ml of Hanks balanced buffer saline (HBBS) then pulverized with a Seward Stomacher® 80 (Lab

System, England) to release the splenocytes. The splenocytes where collected by centrifugation at 1100 rpm for 10 minutes at 10°C, and washed once with PBS. The T and B cells were then isolated by magnetic negative selection using the EasySep® mouse T or B cell enrichment Kits (StemCell Technologies, Canada) following the manufacturer's protocol. Total RNA was extracted from the lymphocytes immediately following isolation, except for B cells activated by LPS (5 μg/ml) for up to 8 hrs.

Human Subjects, Isolation of PBMCs and B cells

Human subjects recruited for this study were male and female laboratory workers at the University of South Florida, who gave informed consent. Venous blood (25 ml) was drawn into 4 K_3 EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ), then diluted 1:1 or 1:2 with RPMI 1640 medium (Sigma, St Louis, MO). The PBMCs were isolated from blood using Hisopaque®-1077 (Sigma Diagnostics, Inc.) following the manufacturer's protocol. B cell isolation was performed by magnetic negative selection using the EasySep® human B cell enrichment kit (StemCell Technologies, Canada).

Phenotypic Analysis of Immune Cell Populations

Mouse T and B cell subtypes were analyzed for enrichment by PCR and FACS analysis. RACE cDNA of the T and B cell samples was analyzed by PCR amplification using specific primers for the CD3ε chain of the T cell receptor and for the B cell marker CD19 (50). Enrichment of the B cell preparation is determined by the absence of CD3ε, while T cell enrichment is determined by the absence of CD19. PCR amplification was performed using 1 μl of RACE cDNA, 500 nM of each primer and Taq polymerase supplied with the SMART RACE cDNA Amplification kit (Clontech Inc., Madison, WI) in a final volume of 25 μl. Amplification was for 28 cycles using the MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA). β-actin was used as a loading control. FACS analysis of the purified mouse T and B cell populations was done by labeling 10^6 cells with fluorochrome-conjugated anti-mouse mAbs; CD19-PE, CD3-PerCP, NK-pan-FITC and F/480-APC (BD Pharmingen, San Jose, CA). The human B cell populations were analyzed for enrichment by labeling 10^5 cells with fluorochrome-conjugated anti-human mAbs; CD19-PE, CD3-FITC and CD14-APC (BD Pharmingen, San Jose, CA). All flow cytometric analysis was conducted using a FACS Caliber flow cytometer and Cell Quest software (Becton Dickinson, San Diego, CA, USA).

RNA Extraction

Total RNA was extracted from the cell populations by standard techniques using Tri-reagent (Sigma; 1 ml per 10^7 cells) and quantitated using the RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR). Just prior to cDNA synthesis, residual DNA was removed by treatment with Turbo DNA-*free*™ (Ambion Inc., Austin, TX) following manufacturer's protocol.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To synthesize the cDNA, 1.0 μg of the DNAse treated RNA was primed with 1 μl of random primers for 5 minutes at 70°C, then reverse transcribed (RT) at 37°C for 1hr using 15 U avian myeloblastosis virus (AMV), 40 Units RNasin (Promega, Corp., Madison, WI) and 1.25 mM mix of dNTPs (Promega Corp., Madison, WI) in a volume of 20 μl. The PCR reaction was carried out in 25 μl containing 1 μl cDNA, 500 nM of each primer (see Table 2), with 12.5 μl GoTaq Green Master Mix (Promega Corp., Madison, WI) and amplified using the MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA). The PCR amplification conditions were as follows; for the initial denaturation step, 95°C for 1 min, followed by 32 cycles at

95°C for 20 sec, 55°C for 30 sec, 72°C for 45 sec, with a final elongation at 72°C for 3 min.

SMART-5'-RACE

To identify the TSS we employed the technique; Switching Mechanism At 5' end of RNA Transcript Rapid Amplification of cDNA Ends (SMART™ RACE cDNA Amplification kit, Clontech Inc., Madison, WI) following manufacturer's protocol. Two reverse gene specific primers (GSP) were designed, for both mouse and human CB_2 using the GCG SeqWeb v3.1 software (see Table 3 for primer sequences). The mGSP1 (mCB_2-R301) binds within the ORF 301bp downstream of the ATG, while the hGSP1 (hCB_2-R298) binds 298bp downstream of the ATG. These were used with the universal primer mix (UPM) that anneals to the SMART sequence at the 5' end of the cDNA supplied by the kit for the initial PCR reaction. A second GSP2 (mCB_2-R217 and hCB_2-R163), located 84bp upstream of mGSP1, and 74bp upstream of hGSP1, was used with UPM in a nested PCR for CB_2 confirmation. RACE products were run on a 2% agarose gel, visualized with ethidium bromide and purified using the Perfectprep® Gel Cleanup kit (Eppendorf, North America) following manufacturer's protocol, and sent to the Moffitt/USF Molecule Biology Core lab for DNA sequencing.

The SeqWeb PileUp program was used to compare the RACE sequences with the GenBank m*Cnr2* and h*CNR2* sequences to confirm CB_2 identity, exon usage, and location of the TSSs (Figures 8, 9, and 10).

Table 3. SMART 5' RACE Primers Used to Identify the TSS.

Gene Specific Primers[a]	Sequence 5'-to-3'	5' binding site[b]
mGSP1-R301	CGACCCCGTGGAAGACGTGGAAGATGACAA	301 bp
mGSP2-R217	TGAACAGGTACGAGGGCTTTCT	217 bp
hGSP1-R298	GCCAGGAAGTCAGCCCCAGCCAAGCTGCCAA	298 bp
hGSP2-R163	GCACAGCCACGTTCTCCAGGGCACTTAGCA	163 bp

[a] GSP, gene specific primer; 1 denotes for the initial RACE PCR, 2 used for nested PCR.
[b] The number of base pairs from the start of translation in which the 5' end of the GSP binds for amplification of CB_2.

PCR and RT-PCR Primer Mapping

Genomic DNA was extracted from mouse splenic and human peripheral B cells using the Wizard® Genomic DNA Isolation System (Promega Corp., Madison, WI) following manufacturer's protocol. RNA was extracted, DNAse treated and reverse transcribed from mouse and human B cells as stated above. Using Primer3, forward primers were designed to flank the TSSs identified by 5'RACE (Figures 11 & 12) 2 to

10bp in either direction. Forward primers upstream the TSS should only amplify genomic DNA, whereas forward primers downstream of the TSS should amplify genomic DNA as well as cDNA derived from the CB_2 transcripts. The PCR reaction was carried out in 25 μl containing either 1 μl cDNA or 1 μl DNA, 500 nM of each primer (see Table 4), with 12.5 μl GoTaq Green Master Mix (Promega Corp.) and amplified using the MyCycler™ thermal cycler (Bio-Rad Laboratories, Inc). The PCR amplification conditions were as stated in section 2.6 with minor adjustments, the cycle number was increased to 35 and for the mouse samples elongation was increased to 1.5 minutes.

Table 4. Primers Used for Mapping the TSSs.

Primer Group[a]			Sequence 5'-to-3'[b]	Assay[c]	Size of amplicon
mE1b	DNA	A	ggaggaggcatgaggca	PCR	189 bp
	mRNA	B	ACACATAGCCTGGCACA	RT-PCR	171 bp
		C	GCGGTTGAATTCTCTCTTC		
		G	GACAAAGTTGCAGGCGAAGATCAC		755 bp
mE2	DNA	D	atacatcaaacacatccttg	PCR	224 bp
	mRNA	E	TTCTAGAAGGCACCCATGT	RT-PCR	189 bp
		F	CCTCTGCTCATTCAGGTACA		
		G	GACAAAGTTGCAGGCGAAGATCAC		567 bp
hE1	DNA	J	gcaagagaaagctggctt	PCR	99 bp
	mRNA	I	TCAACAGGTGCTCTGAGTG	RT-PCR	71 bp
		H	CTGAGGAGTCCCAGTTGTT		

[a] m, mouse; h, human; E1b, exon 1b; E2, exon 2; A, D, J, DNA forward primers; B, E, I, forward primers for amplification of mRNA derived cDNA; C, F, G, H, reverse primers.
[b] Primers designed to bind genomic DNA 5' of the TSSs are in lower case.
[c] RT-PCR, reverse transcription polymerase chain reaction.

Quantitative Real Time RT-PCR (RT-qPCR)

mCB_2 transcript exonal usage in resting, LPS (5μg/ml) and IL-4/anti-CD40 (3ng/ml, 0.5μg/ml) stimulated splenic B cells was measured by RT-qPCR, in which we employed a duplex Taqman PCR strategy; 4 mCB_2 exon specific primer sets and probes were designed, one each for the mCB_2 exons (1a, 1b, 2, and 3) and 1 primer and probe set for the endogenous β-actin control using Primer3 (see Table 5 for primer/probe sequences). The real-time PCR was carried out in 20 μl containing 1 μl cDNA, 300 nM β-actin and 500 nM CB_2 primers, 250 nM fluorescent probe (6-FAM for mCB_2 exon, ROX for β-actin), with 10 μl IQ™ Multiplex Powermix, and performed in the iCycler IQ™ Real-Time PCR detection system (Bio-Rad Laboratories, Inc). In brief, the reaction was performed in duplicate for each RT cDNA product (see above). Samples were heated for 10 min at 95 °C, followed by 50 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C.

Table 5. Primers and Taqman® Probes Used in This Study.

Primer pairs[a] and probe		Sequence 5'-to-3'[b]	Size of amplicon
mCB_2-E3	F	GCCGTGCTCTATATTATCCTGTCCTC	120 bp
	R	GACAAAGTTGCAGGCGAAGATCAC	
	P	6FAM-AGAAAGCCCTCGTACCTGTTCATCAGCA-BHQ1	
mCB_2-E1a	F	TCATCTGCGAAAGTGTGA	112 bp
	R	TTGTCCTGGCTATTCTGTATC	
	P	6FAM-CTGGAGCTGCAGCTCTTGGGAC-BHQ1	
mCB_2-E1b	F	ACACATAGCCTGGCACA	171 bp
	R	GCGGTTGAATTCTCTCTTC	
	P	6FAM-TCAAGTGAGTTGCAGGACAGCATAC-BHQ1	
mCB_2-E2	F	TTCTAGAAGGCACCCATGT	189 bp
	R	CCTCTGCTCATTCAGGTACA	
	P	6FAM-CTTCCTGTTGCTGTGTGCATCCT-BHQ1	
β-actin	F	GGGAATGGGTCAGAAGGACT	134 bp
	R	AGGTGTGGTGCCAGATCTTC	
	P	ROX-ATGTGGGTGACGAGGCCCAGAGCAA-BHQ2	

[a] E3, exon 3; E1a, exon 1a; E1b, exon 1b; E2, exon 2; F, forward primer; R, reverse primer; P, Taqman® probe.
[b] 6FAM, 6-carboxyfluorescein; BHQ1 or 2, Black Hole Quencher®-1 or 2.

Promoter Cloning

Using genomic DNA extracted from B cells (see above) and the pGL3-enhancer vector (Promega), clones were constructed to test for promoter activity surrounding the TSSs of the mouse exon 1 and 2 CB_2 transcript variants. The clones included the region from -359 bp to +205 bp of the TSS (+1) of exon 1a, whereas the exon 2 clones spanned the region from -189 bp to +205 bp of the exon 2 TSS (+1). The DNA regions were PCR amplified (see table 6 for primer sequences) and initially cloned into the pTOPO-Blue TA vector (Invitrogen) following manufacturer's protocol, then sub-cloned by standard methods, into the pGL3-enhancer vector via the Hind III restriction enzyme site.

Table 6. Promoter Clones and PCR Primers.

Promoter clone	Primers	Sequence 5'-to-3'	Promoter region cloned	Size (bp)
pGL3-E16	E1-352F E1+123R	GGCACATGTCACAGACAA GCGAAGAGTTAGGGAAGAGT	-270 bp to +205 bp exon 1a TSS(+1)	475
pGL3-E19	E1-14F E1+123R	CCTGCTGGGTCTCCAGAT GCGAAGAGTTAGGGAAGAGT	+68 bp to +205 bp exon 1a TSS(+1)	137
pGL3-E25	E1-441F E1-19R	GTTCAATTCCCAGCACCC CCCACGTAGGTCCCAAGAG	-359 bp to +63 bp exon 1a TSS(+1)	422
pGL3-P7	E2-F189 E2+R36	CTTGCCAGTTCCCAGTTTCA CAAGTCACATGGGTGCCTTCT	-189 bp to +36 bp exon 2 TSS(+1)	225
pGL3-P8	E2-F90 E2+R36	AGAAGAGGGACTTGCCCAAA CAAGTCACATGGGTGCCTTCT	-90 bp to +36 bp exon 2 TSS(+1)	126
pGL3-P10	E2+F13 E2+R205	TCTAGAAGGCACCCATGTGA CTGTGCCTCTGCTCATTCAG	+13 bp to +205 bp exon 2 TSS(+1)	192
pGL3-P11	E2-F189 E2+R101	CTTGCCAGTTCCCAGTTTCA AACAGGATGCACACAGCAAC	-189 bp to +101 bp exon 2 TSS(+1)	290
pGL3-P13	E2-F25 E2+R101	TCAAACACATCCTTGCCCTA AACAGGATGCACACAGCAAC	-25 bp to +101 bp exon 2 TSS(+1)	126

Transfection of B cells

Primary B cells were cultured for 24 to 48 hrs in RPMI medium containing 10% FCS, 10 ng/ml IL-4 and 500 ng/ml anti-CD40, then transfected (10^7cells/500μl RPMI in 0.4-cm cuvettes) with the pGL3-clones (10μg) by electroporation at 250 V and 800 μFarads using the Gene Pulser (BioRad). The transfected B cells were collected within 18 to 24 hrs after electroporation. For each cell sample a 50 μl aliquot was removed and mixed with an equal volume of Trypan Blue to obtain cell number and check viability. Cells were counted using a hemocytometer and compound light microscope.

Luciferase Reporter Assay

Cell lysates of the transfected cells were analyzed for luciferase activity using Promega's Luciferase Assay System, following manufacturer's protocol. In brief, cells were collected by centrifugation at 600 RCF for 10 minutes and washed 1X with PBS. Cells were lysed using 200 μl of CCLR, of which 20 μl was used for the luciferase assay and the remaining lysate was stored at -80°C. Each sample was in duplicate and luciferase activity was measured using the MLX luminometer (Dynex Technologies Inc., Chantilly, VA). A standard

curve was used to measure the amount of lucerifase protein in each sample.

Activation of B Cells

To activate B cells we used the B cell mitogen, LPS, and known inducers of immunoglobulin class switching IL-4 and anti-CD40. Purified primary splenic B cells were cultured in RPMI medium containing 10% FCS with either 5 μg/ml LPS or 3 ng/ml IL-4 and 0.5 μg/ml anti-CD40 for 1, 3, and 8 hrs. Total RNA was isolated at each time point and analyzed for transcript expression by RT-qPCR. Relative transcript expression was determined by the $2^{-\Delta\Delta Ct}$ method, in which β-actin was the endogenous control and time 0 (un-stimulated) was the calibrator.

RESULTS

Aim 1. To Determine the Extent of CB_2 Transcript Expression and TSSs in B cells.

Bioinformatics Analysis of the CB_2 Gene (Cnr2) and GenBank™ Clones.

Since the discovery of CB_2, several cDNA clones from various mouse and human tissues, as well as the complete gene sequence have been submitted to GenBank and available to researchers. We therefore took advantage of this resource to gain initial insight as to how the CB_2 receptor gene is expressed in B cells. Initially we explored genome databases, such as Ensembl and NCBI, to obtain the location and gene structure of mouse and human *CNR2*. The mouse *Cnr2* (m*Cnr2*) was reported to be located on chromosome 4, 24.7 kb in size, and produce at least two transcripts containing different 5' untranslated region (UTR) first exons (Figure 3A & 3B). Whereas human *CNR2* (h*CNR2*) was reported on chromosome 1, 39.4 kb in size, and express a single transcript (Figure 4A & 4B). A consensus was reported among the mouse and human clones in which the ORF,

encoding CB_2 protein, was within a single exon -- exon 3 for mouse and exon 2 for human (Figures 3 and 4). Further computational analysis using the GCG SeqWeb package to align the 5'UTR of the GenBank™ clones, revealed the mouse clones from various immune tissues share a similar 5'UTR first exon (exon 1) that differed in length at their most 5' nucleotide. Similarly, the clones reported from bone and liver share a second common 5'UTR first exon (exon 2) yet differ in length at the 5' nucleotide (Figure 3C). Analysis of human data (Figure 4C) showed only one full length human CB_2 clone containing a 5'UTR first exon (exon 1). This analysis suggested that m*Cnr2* utilizes multiple TSSs to produce at least two CB_2 transcript variants whereas the human gene utilizes only one. However, none of this existing data provided information as to the location of the TSS and CB_2 transcript variants utilized in B cells. Therefore, we began to investigate the TSS and CB_2 transcripts in B cells purified from mouse splenocytes and human PBMCs.

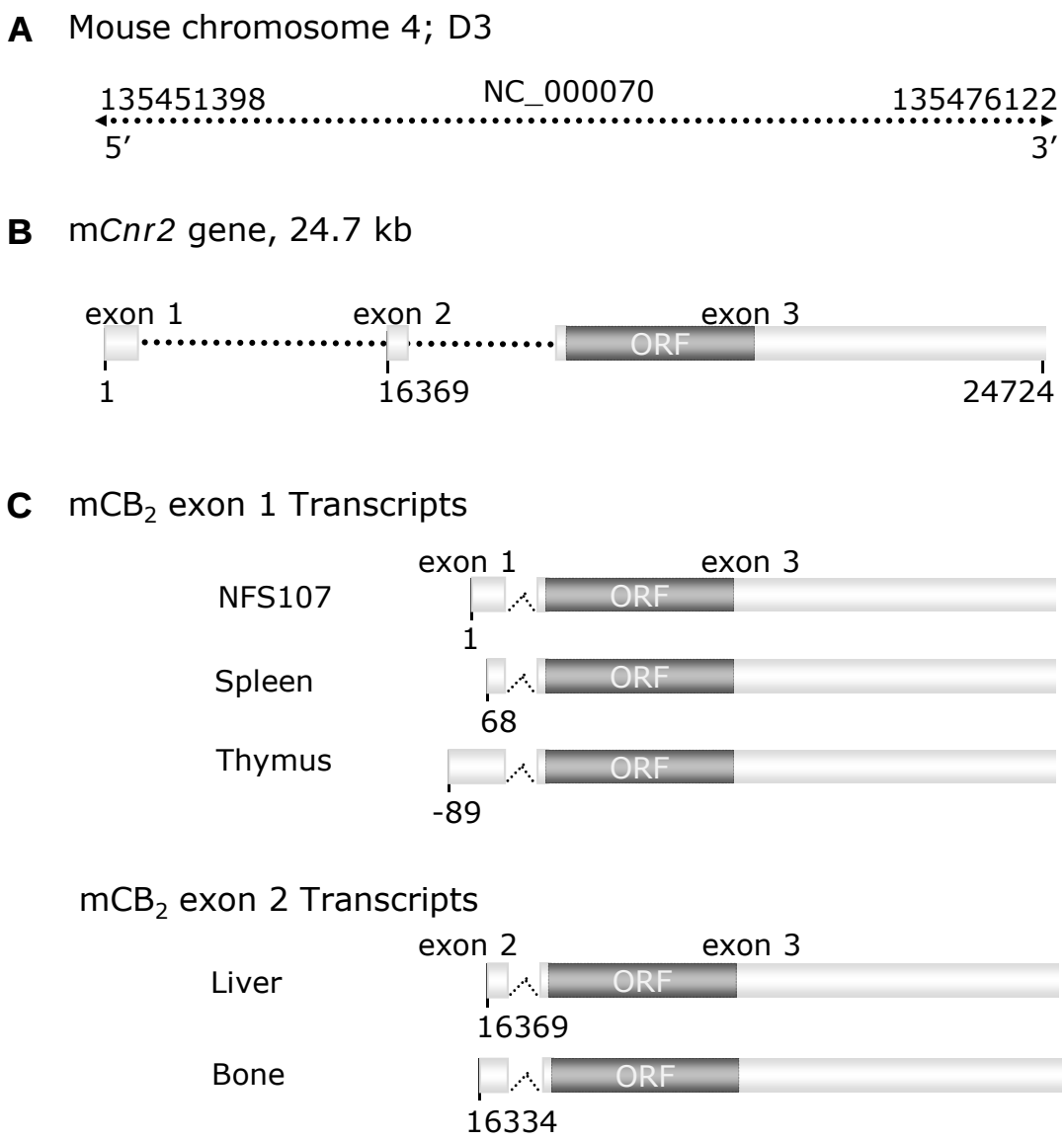


Figure 3. Computational Analysis of the Mouse *Cnr2* Gene.
A. Chromosome location of the m*Cnr2* (GenBank accession no. NC000070). **B**. The m*Cnr2* gene structure. Boxes represent exons, whereas white boxes are the untranslated region (UTR) and the dark grey shaded area is the protein coding region. ORF = open reading frame. Dotted lines are introns, which are spliced out to form mature mRNA. **C**. GenBank CB_2 mRNA transcripts; mCB_2 exon 1 transcripts are expressed in the murine leukemic cell line NFS107 (GenBank accession nos. X93168, NM009924), the spleen and thymus (GenBank accession nos. X86405, and AK037898), whereas mCB_2 exon 2 transcripts are expressed in liver and bone (GenBank accession nos. BC024052 and AK036658).

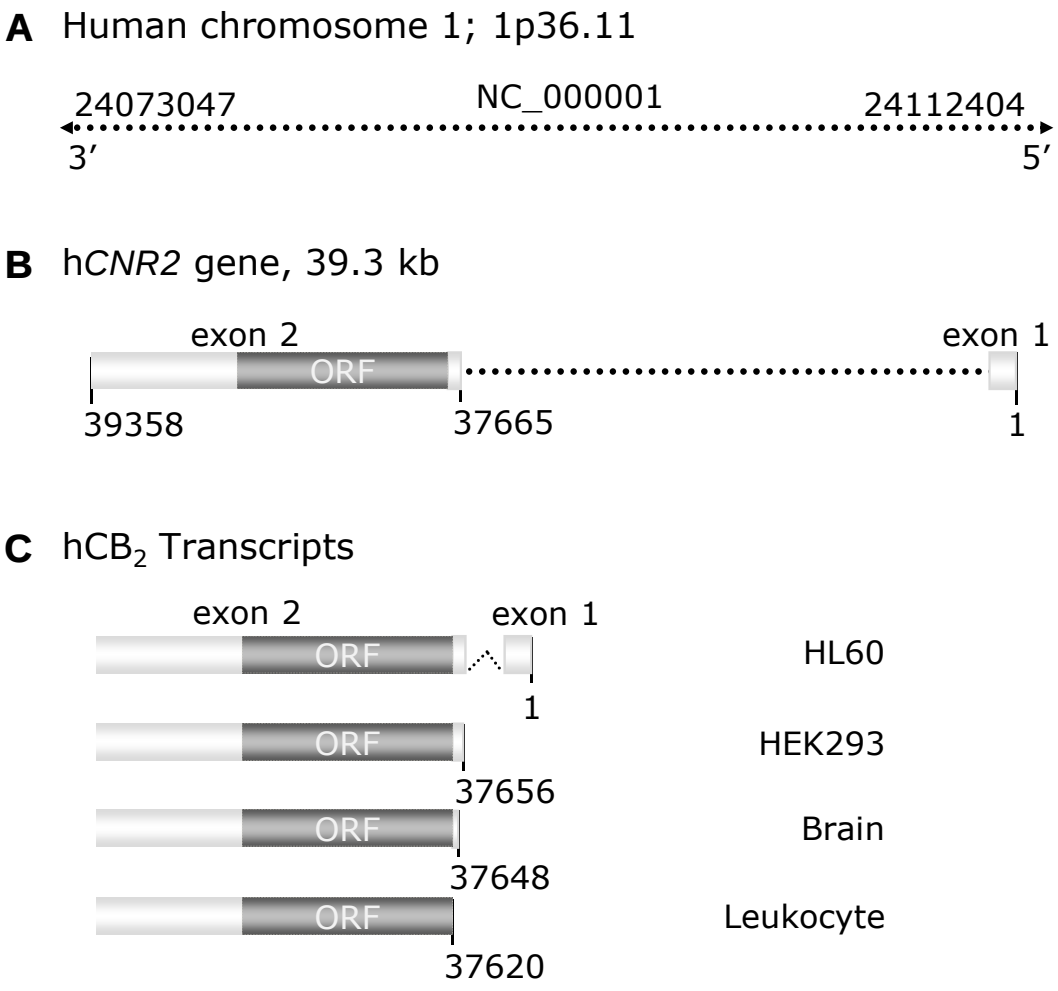


Figure 4. Computational Analysis of the Human *CNR2* Gene.
A. Chromosome location of the h*CNR2* (GenBank accession no. NC000001). **B**. The h*CNR2* gene structure. Boxes represent exons, whereas white boxes are the untranslated region (UTR) and the dark grey shaded area is the protein coding region. ORF = open reading frame. Dotted lines are introns, which are spliced out to form mature mRNA. **C**. GenBank hCB_2 mRNA transcripts are expressed in the human promyelocytic leukemic cell line HL60, human embryonic kidney cell line HEK293, brain, and leukocytes (GenBank accession nos. NM001841, AV430063, BC074767, and AM156854-6).

Phenotype of Lymphocyte Subtypes

Studies have shown that CB_2 mRNA is most abundant in mouse and human B cells (8, 16, 25) and the bioinformatic analysis performed above revealed that the m*Cnr2* produces at least two transcripts, whereas the h*CNR2* produces only one (Figures 5 and 6). However, from the database we could not find any information pertaining to the location of the TSS or CB_2 transcript usage in purified mouse and human B cells. Therefore, we began an analysis for CB_2 transcript initiation and usage in un-stimulated, resting purified B cells from mouse splenocytes and human PBMCs. T and B cells were purified using the EasySep® negative selection kits for mouse and human. Splenocytes from mice and blood mononuclear cells from humans were processed over antibody affinity columns to remove all lymphoid subtypes with the exception of B cells and T cells. We then employed RT-PCR and flow cytometry to determine enrichment of the lymphocyte subtypes. RT-PCR determined either the presence or absence of the T cell specific CD3ε message or the B cell specific message, CD19. Enrichment of the B cell preparation was determined by the presence of CD19 and absence of CD3ε, whereas T cell enrichment was determined by the presence of CD3ε and absence of CD19. PCR amplification was performed using CD3ε and CD19 specific

primers (50) for 28 cycles, in which weak to no visible CD19 bands were seen in the T cell populations and weak CD3ε bands were seen in the B cell population (Figure 5A). Because of the weak bands seen in the lymphocyte subtypes, we were unable to determine the percent purification of the lymphocyte subtypes. Therefore, to determine more precisely the purity of the subtypes, we performed flow cytometry analysis using CD19 and CD3 fluorescent labeled antibodies and demonstrated that the mouse B and T cell populations as well as the human B cell populations were enriched to greater than 95% (Figures 5 and 6). These results show that the purified lymphocyte subtypes were indeed highly enriched.

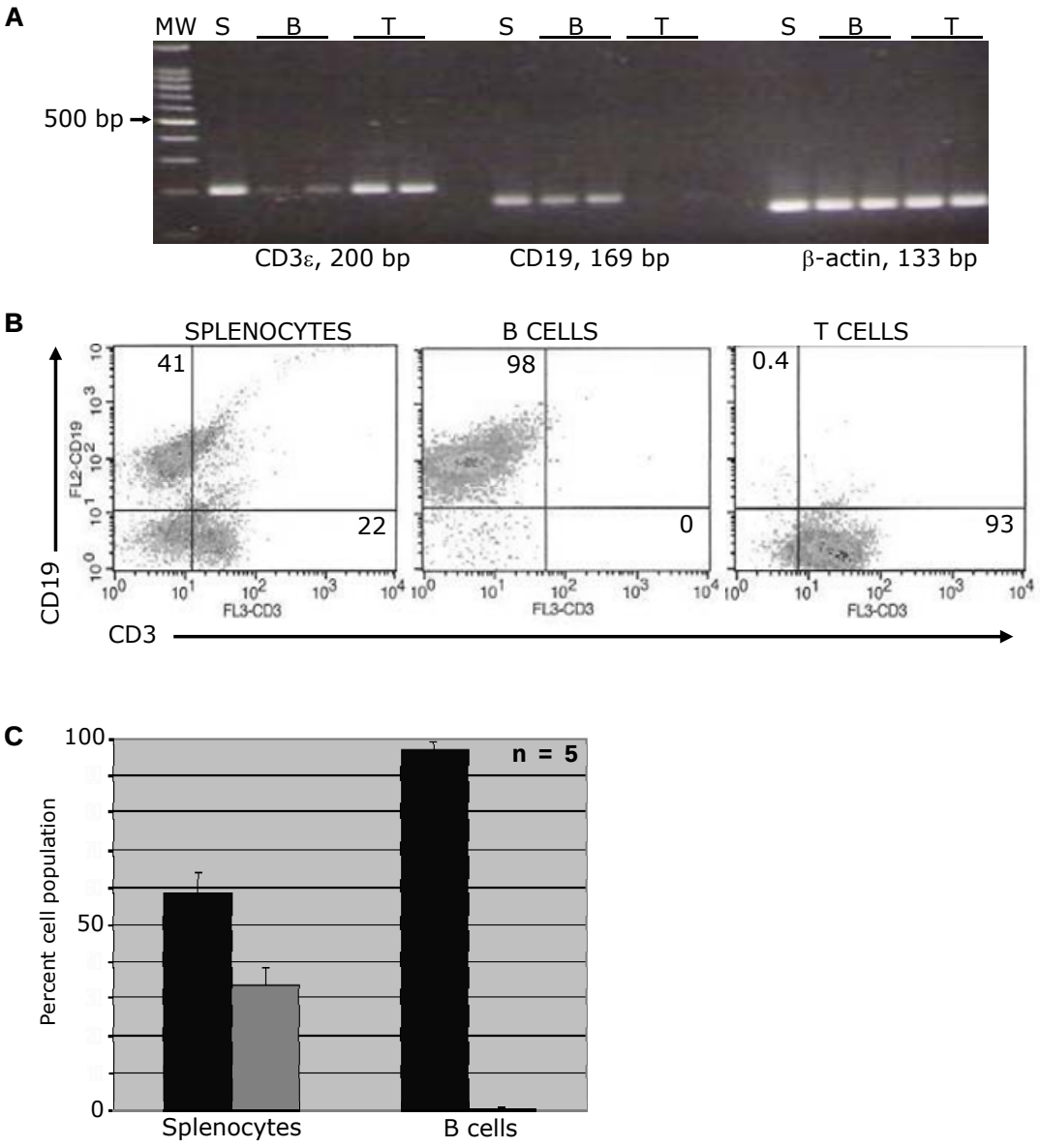


Figure 5. Phenotypic Analysis of Mouse Immune Cell Subtypes. Mouse B and T cells isolated from splenocytes by affinity purification (EasySep®) **A**. RT-PCR for the presence of the CD3ε message in T cells and for B cell specific marker CD19; β-actin used as loading control. **B**. Flow cytometry analysis of lymphocytes treated with CD19-PE, CD3-PerCP, NK-pan-FITC and F/480-APC anti-mouse mAbs (FITC and APC data not shown) to determine lymphocyte enrichment. **C**. Graph represents data of 5 independent B cell purification procedures (black bar). Grey bars are CD3+ cells.

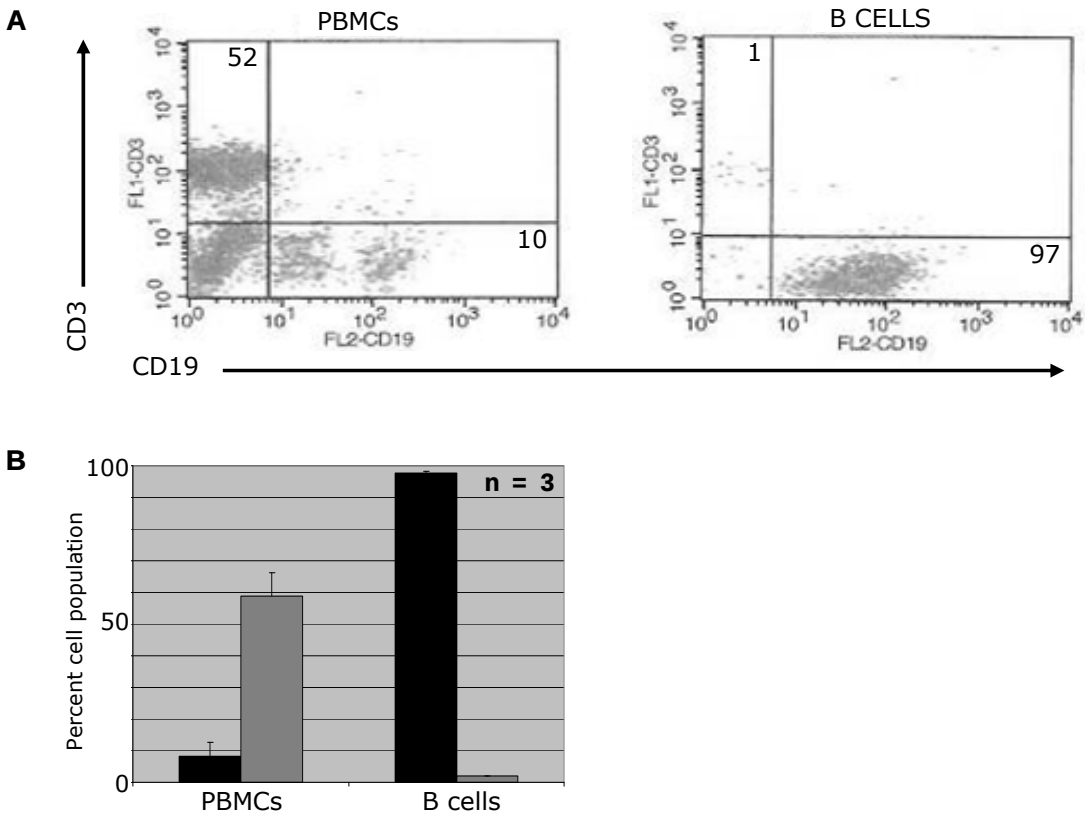


Figure 6. Phenotypic Analysis of Lymphocyte Subtypes Isolated from Human PBMCs.

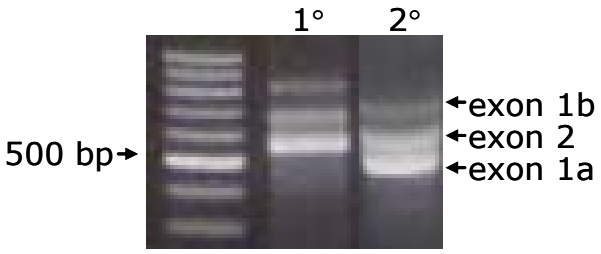
A. Human B cells isolated from PBMCs by affinity purification were analyzed with CD19-PE, CD3-FITC and CD14-APC anti-human mAbs (CD14 data not shown) to determine B cell enrichment. **B**. Graph represents data from 3 human donors. Black bars are B cells and grey bars are T cells.

Mouse and Human B cells Differ in the Number of CB_2 TSSs.

To determine the location of the m*Cnr2* TSS in purified B cells, we employed the SMART 5' RACE technique. Figure 7 shows RACE results of RNA isolated from mouse and human B cells. For mouse cells, we used the GSP1, mCB_2-R301, along with the UPM primer supplied with the kit. RACE PCR yielded three mCB_2 transcripts that were confirmed as CB_2 RACE products by nested PCR (Figure 7A). RACE was also performed on human B cell RNA using the hCB_2-R298 GSP1 with the UPM, followed by nested PCR using hCB_2-R163 GSP2, resulting in the demonstration of only one transcript (Figure 7B). In order to determine the relative gene location of the TSSs and 5'UTR structure of the CB_2 transcripts in B cells, the RACE products were isolated, sequenced and the nucleotides aligned for analysis. The location of the TSS was revealed by alignment of the 5' end of the RACE sequences with the UPM- SII oligo primer sequence and genomic DNA (Figure 8). Furthermore, alignment of the sequenced RACE products to either the mouse or human genome, along with the GenBank submitted mRNA sequences revealed several new aspects of CB_2 transcript expression in B cells. First the mouse transcripts were homologous to the *Cnr2* as well as the existing CB_2 mRNA data, with the exception that exons 1 and 2 in the transcripts we isolated were

longer by 14 to 294 nucleotides, respectively, indicating they contained previously unidentified TSSs. Mouse B cells also expressed and additional transcript, exon 1b, with three TSSs (Figure 9). Regarding transcript usage in human B cells, data obtained from three human subjects showed expression of only one first exon (Figure 10). To our knowledge, this is the first report identifying TSSs in B cells from mouse and human and these sequences have been submitted to GenBank (accession nos. FJ357033-6).

A. Mouse



B. Human

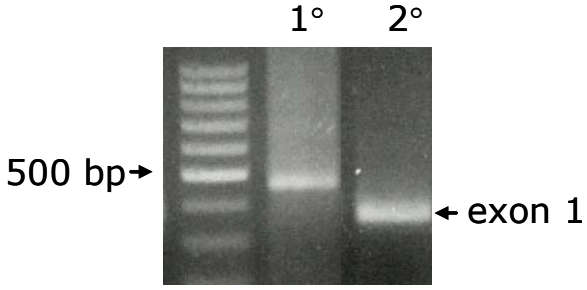
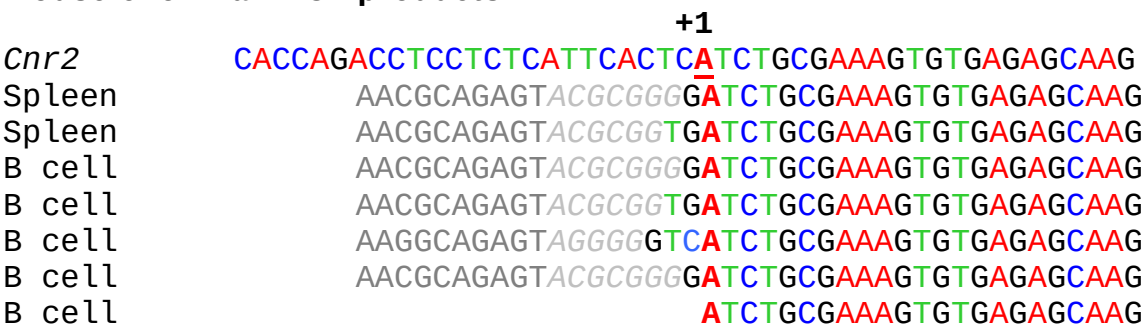
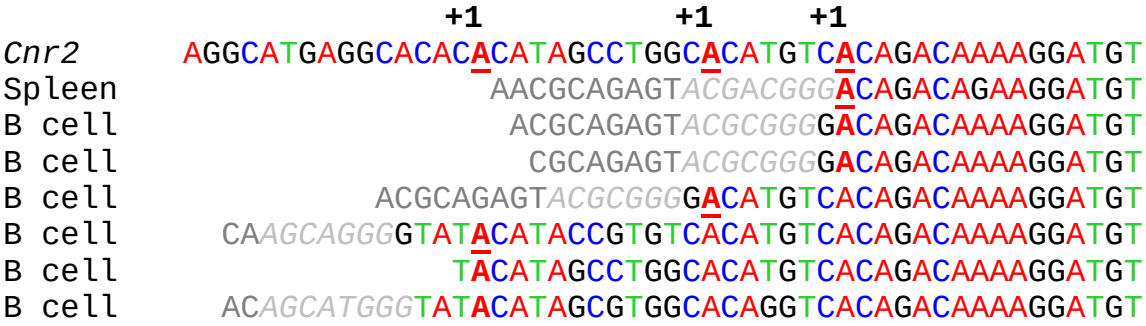


Figure 7. CB_2 Transcripts and TSSs Identified by 5' RACE.
Gel electrophoresis of the 5' RACE products visualized on a 2% agarose gel stained with ethidium bromide. primary PCR (1°), nested PCR (2°). **A**. Mouse 5' RACE products, 1° product length; exon 1b, 778-788 bp; exon 2, 614 bp; exon 1a, 543 bp. 2° size; exon 1b, 697-707 bp; exon 2, 533 bp; exon 1a, 459 bp **B**. Human 5' RACE product, 1°, 455 bp; 2°, 381 bp.

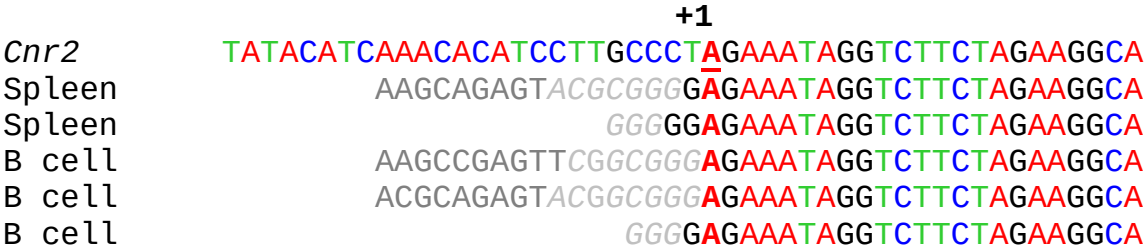
Mouse exon 1a RACE products



Mouse exon 1b RACE products



Mouse exon 2 RACE products



Human RACE products

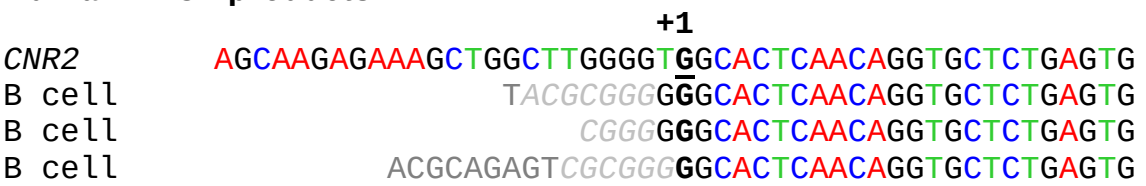
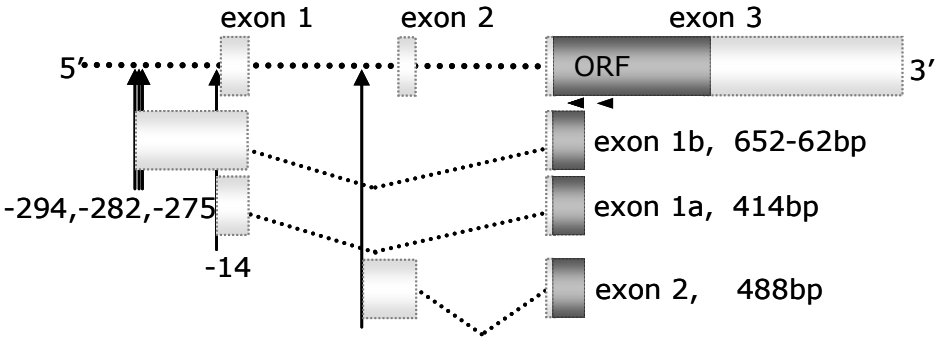


Figure 8. 5' RACE Products Reveal Location of the TSSs. To determine the TSS, the GCG SeqWeb PileUp program was used to align the 5' end of the RACE products with the *CNR2* gene and the UPM (AACGCAGAGT)-SII Oligo (*ACGCGGG*) sequences supplied with the RACE kit. TSSs are bold underlined and marked as +1. Shaded grey indicates the kit primers.



Exon 1a: 151 bp

-14

ATCTGCGAAAGTGTGAGAGCAAGAAACCCCAGGCTGGAGCTGCAGCTCTTGGGACCTACG
TGGGGGTCCCTGCTGGGTCTCCAGATCTGGATACAGAATAGCCAGGACAAGGCTCCACAA
GACCCTGGGGCCCAGCGGCTGACAAATGACA

Exon 1b: 412, 419, & 431 bp

-294 -282 -275

ACATAGCGTGGCACATGTCACAGACAAAAGGATGTAAACTTTACAGAGGTCAAGTGAGTT
GCAGGACAGCATACACCCGGGGCCAGATTAGAACCCAAGTTTCTGGAGTCTAAGGTCTAT
GCCTATGCCCTCCCCTGGCCAGAGTTCCTAGGAAGAGAGAATTCAACCGCAGGGCAAGAA
CACTGTGGCACTGAGGACCCAGAGGGGAAGTGGTAACCGGTACGGAAGGCCAGATCTCCT
CTCACTCACTTATCTGCACCAGACCTCCTCTCATTCACTCATTTGCGAAAGTGTGAGAGC
AAGAAACCCCAGGCTGGAGCTGCAGCTCTTGGGACCTACGTGGGGGTCCCTGCTGGGTCT
CCAGATCTGGATACAGAATAGCCAGGACAAGGCTCCACAAGACCCTGGGGCCCAGCGGCT
GACAAATGACA

Exon 2: 253 bp

-172

AGAAATAGGTCTTCTAGAAGGCACCCATGTGACTTGCAGAGGGTATCTCTATCTTCGTGG
AGACAGGGAGCCGGGCTTCCTGTTGCTGTGTGCATCCTGTTGTTCTCTTGTTAGGATGTC
CATCAAATGCATGCATTTCCTTTCCTAACTCTGGACAGTAACAGTCGTCTGCGGCCAAGC
TGTGCCTGAATGAGCAGAGGCACAGGCACCAGCCGTGGCCACCCAGCAAACATCTCTGCT
GACTCAGACTGGG

Figure 9. The *Cnr2* Gene Location of the TSSs and 5'UTR Sequences Identified by 5' RACE. CB_2 transcripts are labeled with their corresponding exon along with the number of nucleotides sequenced for each RACE product. The upward arrows represent the *Cnr2* gene location of the TSSs (underlined nucleotide) relative to position 1 (bold underlined nucleotide) of the Genbank™ accession nos. NM009924 for exons 1a & 1b and BC024052 for exon 2. Small black arrows mark the relative location of the GSPs. The 5'UTR exon sequences of the mCB_2 transcripts submitted to GenBank (accession nos. FJ357033-5).

Exon 1: 117 bp

GGGTGTGTTGTGGGTGGCTGGGCACTGGGAGCTGCCGGGGGGTGAGGAGTCCC
AGTTGTTTTTTGTCCTCTCCCAGGACCT**G**GCCGTGGGTGCCACTCAGAGCACC
TGTTGAGTGCC
-35

Figure 10. Human CB_2 5'RACE Transcripts have a Single TSS and 5'UTR. The *CNR2* gene location of the TSS (upward arrow). Number below the arrow represents the location of the TSS (underlined nucleotide) relative to position 1 (bold underlined nucleotide) of Genbank™ accession no. NM001841. Small black arrows mark the relative location of the GSPs. The CB_2 5'UTR exon 1 sequence submitted to GenBank (accession no. FJ357036).

To verify the relative location of the TSSs, we designed specific forward primers for PCR of either genomic DNA or cDNA reverse transcribed from 1 μg of total RNA. The strategy for these experiments is illustrated in figures 11A and 12A. In brief, the forward primers were designed so that either the 3' or 5' end borders the TSS. Consequently, the forward primer in which the 3' end is adjacent to the TSS will only amplify genomic DNA and not the cDNA derived from the mRNA transcripts, whereas the forward primer that adjoins the TSS at the 5' end will amplify both genomic DNA and cDNA. There is some limitation with this approach in that it is not as sensitive as 5' RACE in determining the TSS, but it does help confirm the relative location of the TSS and approximate 5' end of the transcripts. Therefore, using this approach we were able to confirm the TSS location of mCB_2 exons 1b and 2 (Figure 11B), as well as the hCB_2 exon 1 transcript (Figure 12B). Another limitation with the assay was that mouse exons 1a and 1b share identical sequences with the exception that exon 1b is 280 nucleotides longer at the 5' end. Consequently, primers designed to adjoin the TSS of exon 1a would not be able to distinguish genomic DNA from cDNA derived from exon 1b and would amplify both. Therefore, we could not use this approach

to verify the location of the TSS for transcripts containing exon 1a in B cells.

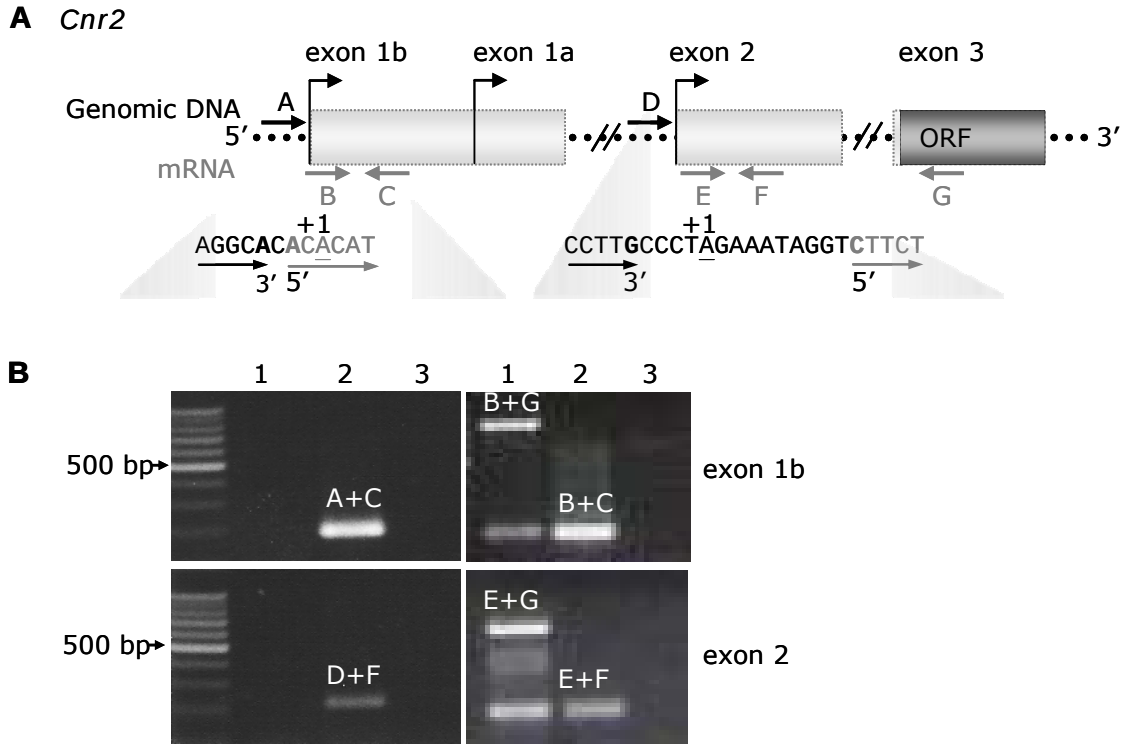


Figure 11. Primer Mapping of the mCB_2 TSSs. A. Illustration of the strategy for primer mapping of the mCB_2 TSSs. Forward primers A and D (black arrows) only amplify genomic DNA while forward primers B and E (grey arrows) amplify both genomic DNA and cDNA derived from CB_2 mRNA. The reverse primers C, F, and G (grey arrows) are shown. The blown out sequences illustrate where the forward primers bind in relation to the TSSs. The bold letters are the 3′ and 5′ end of the forward primers, and underlined nucleotides are the TSS **B**. Gel electrophoresis of the mapped CB_2 transcripts, where in lane 1 contains cDNA derived from 1 μg of total RNA from mouse splenic B cells, lane 2 is genomic DNA extracted from B cells, and lane 3 contains the no template control. The bands are labeled with the primer pair (white letters) used for PCR amplification. The panels are labeled with the exon TSS tested.

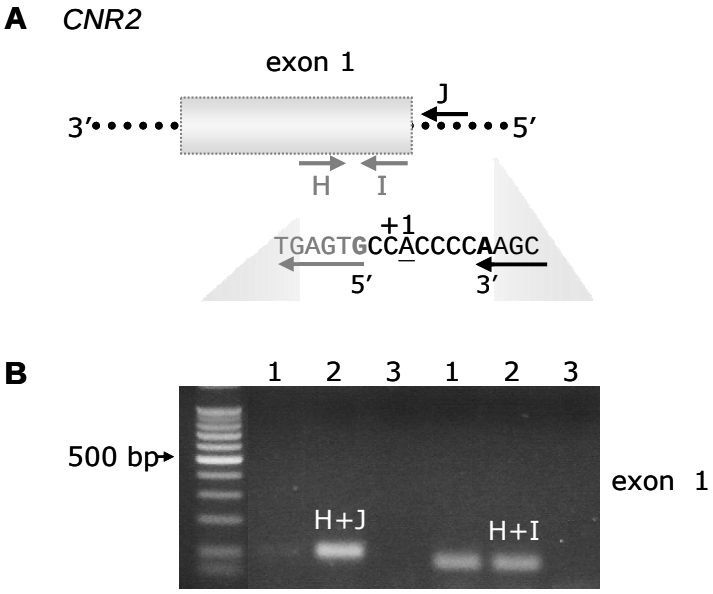


Figure 12. Primer Mapping of hCB_2 TSS. **A**. Forward primer J (black arrow) only amplifies genomic DNA and forward primer I (grey arrow) amplifies both genomic DNA and the cDNA derived from CB_2 mRNA. The reverse primer H (grey arrow) is shown. The blown out sequence illustrates where the forward primers bind in relation to the TSSs. The bold letters are the 3′ and 5′ end of the forward primers, and the underlined nucleotide is the TSS. **B**. Gel electrophoresis of the mapped hCB_2 transcript, where in lane 1 contains cDNA derived from 1 μg of total RNA from human PBMC B cells, lane 2 is genomic DNA extracted from B cells, and lane 3 contains the no template control. The primers used for each PCR are labeled in white above the bands.

Preferential Usage of the CB_2 Exon 1a Transcript Variant in Resting Splenic B Cells

The 5' RACE data revealed that resting splenic B cells expressed several CB_2 transcripts. Therefore, in order to determine which transcript was most abundant we used RT-PCR to quantify the transcripts. Using exon specific primers, semi-quantitative RT-PCR showed exon 3-containing transcripts and those containing variants of exon 1 rather than exon 2 predominated in resting B cells (Figure 13A). To better define these results we used quantitative real time RT-PCR (RT-qPCR). The primers and CB_2 exon specific taqman sequences, listed in table 5 and located as black boxes in the diagram of figure 13, were used in conjunction with β-actin primers and taqman probe in a duplex RT-qPCR. Since all three transcripts include exon 3, primers designed for this exon will amplify all the transcripts regardless of the first exon. In addition primers designed for exon 1a should amplify all transcripts containing exon 1, whereas primers for exon 1b and 2 were designed to specifically amplify transcripts containing only these exons. Our results show that the exon 1a transcript variant was the most abundantly expressed transcript in un-stimulated resting splenic B cells (Figure 13B).

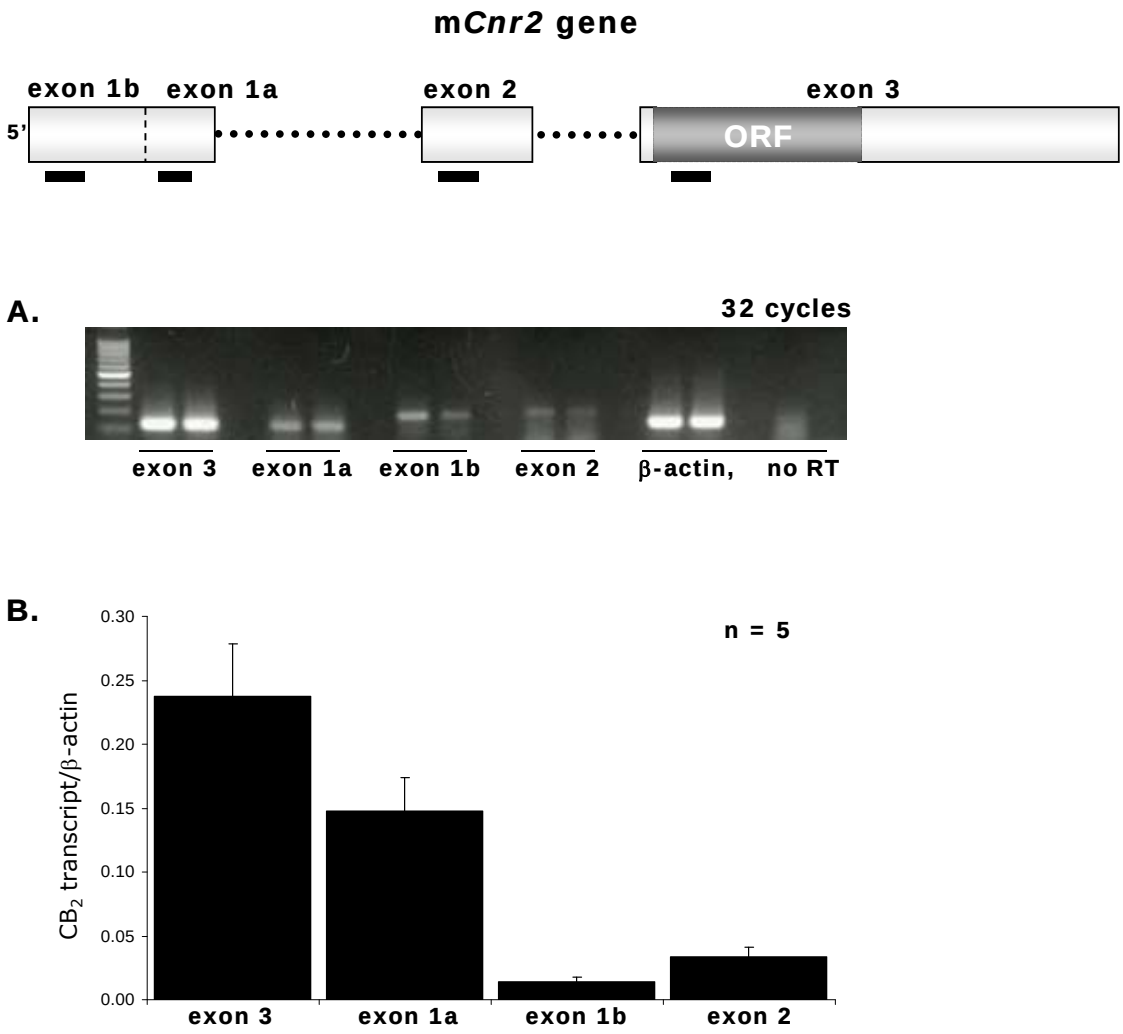


Figure 13. Quantitative real time RT-PCR (RT-qPCR) for mCB$_2$ mRNA Expression in Resting Splenic B cells. **A**. Semi-quantitative RT-PCR of the mCB$_2$ transcripts using exon specific primers and 2 separate mouse B cell samples. The samples were collected after 32 cycles of amplification and run on a 2% agarose gel visualized with ethidium bromide. **B**. Using taqman probes; RT-qPCR was performed to determine the major CB$_2$ transcript utilized in mouse B cells at basal transcription. Results were normalized with β-actin and expressed as a ratio of CB$_2$ transcript/β-actin. Data are means ± S.E.M. of five independent experiments.

Aim 2. Characterize the *Cnr2* Promoter in B Cells

Bioinformatics Analysis for Core Promoter Elements Near the TSSs

It has been well established that identification of the TSS will lead to the location of the core promoter, which is usually -40 bp upstream to +40 bp downstream of the TSS. The basic elements that comprise the core promoter are the TATA-box, INR (Initiator), DPE (downstream promoter element), and BRE (TFIIB recognition elements) (41). Therefore, we performed a bioinformatics analysis for the presence of consensus sequence of the core promoter elements in the vicinity of our RACE TSSs to tentatively identify the structure and location of the *Cnr2* core promoter. Using GCG SeqWeb, we aligned the 5' ends of the RACE sequences with that of previously described CB_2 mRNA sequences (GenBank accession nos. NM009924 for mouse, and NM001841 for human) as well as the *Cnr2* genomic region spanning -45 bp to +50 bp of the RACE TSSs (positions +1, Figure 13), followed by *in silico* analysis for core element consensus sequences. For exon 1a, we identified an INR sequence spanning the area -2 bp to +5 bp surrounding the TSS (+1), a TATA-like sequence at position -25 bp as well as multiple DPEs and DCEs at positions +10,18,31,36,38, and +44 bp. The RACE results for the exon 1b

transcript identified 3 TSSs, which from this analysis appear to have INR-like sequences. In addition, a GC/GAGA-box spanning 24 bp is present that is -24, -25, -26 bp as well as multiple DPE sites at +30,33,29, and +35 bp from its respective TSS. The exon 2 RACE transcript has an INR-like sequence, a TATA-box at -21 bp as well as DCE at +11 bp and a DPE at +25 bp (Figure 14A). The RACE transcripts from human also contain an INR-like sequence, as well as a TATA-box at -31 bp, a BRE[d] -24 bp and a DPE at +35 bp (Figure 14B). From this analysis it appears that the consensus sequences for core promoter elements are in the vicinity of the TSSs identified by the 5' RACE experiments. Furthermore, the analysis suggested that m*Cnr2* has three potential core promoters for the pre-initiation complex to assemble for transcription initiation, whereas h*CNR2* has a single putative core promoter.

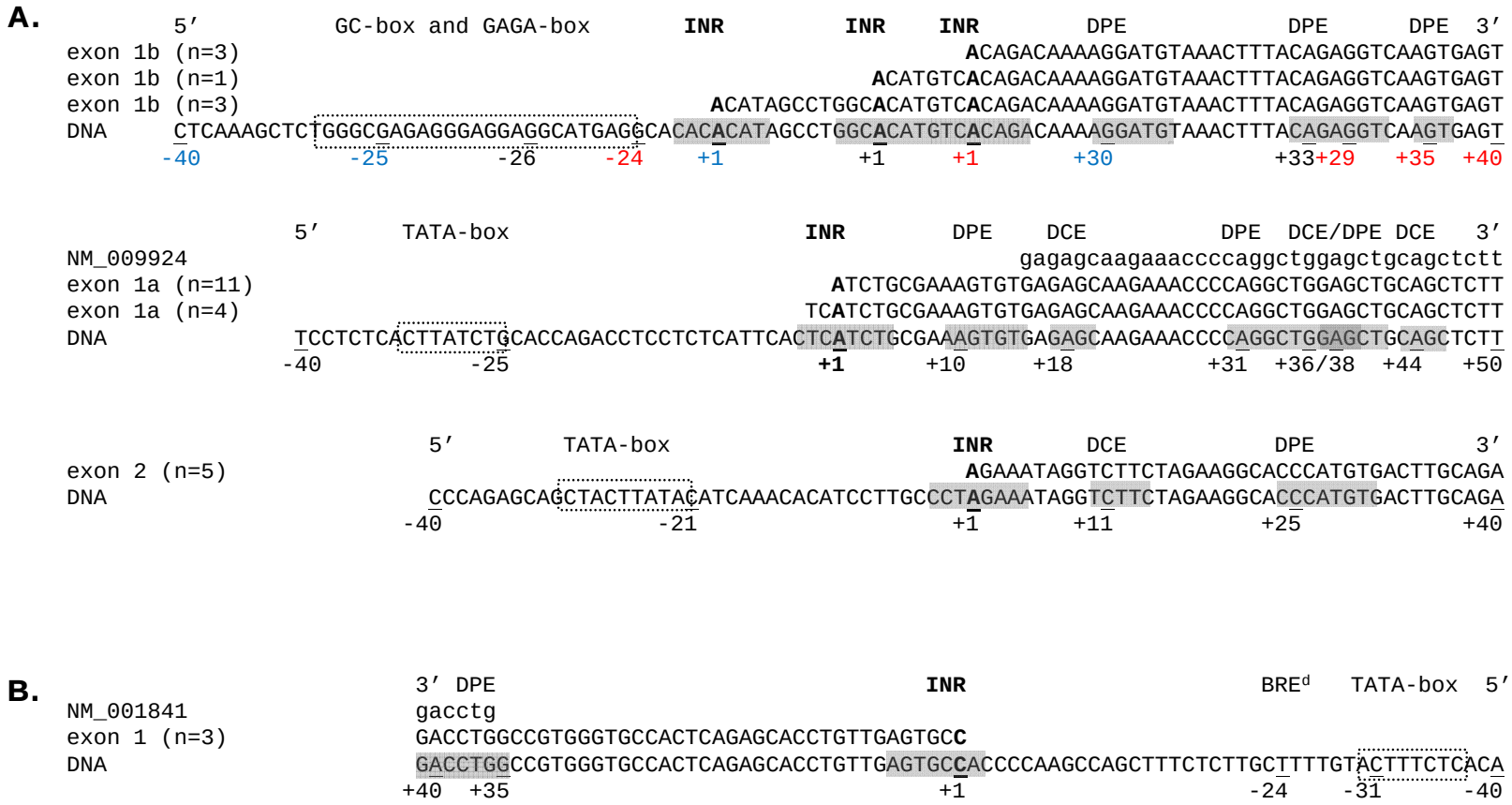


Figure 14. Putative Core Promoter Elements Near the TSSs. The gene region spanning approximately -40 bp to +40 bp of the TSSs was analyzed for core promoter elements. **A**. The putative core promoters of the three mCB_2 transcripts. For exon 1b the numbers for the TSSs (+1) are color coordinated with their respective core promoter element. **B**. The h*CNR2* gene putative core promoter. INR, Initiator (consensus sequence YYANWYY). DPE, Downstream promoter element (consensus sequence RGWCGTG) BRE^d, TFIIB recognition element downstream the TATA-box (consensus sequence RTDKKKK).

Bioinformatics Analysis to Identify Putative Promoters and cis-Sequences

It is well accepted that the promoter consists of two interacting components; the core promoter, in which the basic transcription factors join with Pol II at the TSS to form the pre-initiation complex and the regulatory elements that are involved in activated transcription (51). These regulatory elements consist of proximal elements, next to the core promoter, and enhancers/repressors elements, which can be located several kbs upstream or downstream the TSS. These regulatory elements, known as *cis*-sequences are the DNA binding sites for transcription factors. Identifying the *Cnr2* promoter and *cis*-sequences will provide insight into how this gene is regulated in B cells during different states of activation.

Bioinformatics has become a useful tool in identifying sequences that may be involved in regulating gene transcription that can then be experimentally tested. Therefore, to increase our understanding of the functional regulatory regions that control CB_2 transcription we utilized several web-base programs to tentatively locate the *Cnr2* promoter and *cis*-sequences. Functional regulatory regions tend to be close to the TSS, therefore our first analysis was to locate putative promoters near our 5' RACE TSSs. To accomplish this we used the Genomatix suite to analyze 1 kb of the *Cnr2* genomic sequence (obtain from

GenBank) surrounding the TSSs for exons 1 and 2. The analysis yielded two predicted promoter regions for the m*Cnr2* and one for the h*CNR2.* The first m*Cnr2* predicted promoter is 690 bp and spans the region -574 bp to +115 bp from the mCB_2 exon 1a TSS (+1). The second mCnr2 promoter is 601 bp and spans -362 bp to +238 bp from the mCB_2 exon 2 TSS (+1). The single h*CNR2* predicted promoter is 601 bp spanning -406 bp to +135 bp of the TSS (+1).

To identify putative *cis*-sequences, we used the same 1 kb genomic regions as above and the MatInspector program of the Genomatix suite, which this analysis yielded numerous *cis*-sequences that hampered us to decipher the true positives from the false. Therefore, we used the process of phylogenetic footprinting, in which ClustalW was used to align the entire *Cnr2* gene of mouse and human to find conserved regulatory regions*. A*lignment of the orthologous genes paralleled the Genomatix promoter prediction for the exon 1 region (Figure 15A). In addition, the orthologous alignment of the genomic region near the mouse exon 2 showed high conservation between mouse and human suggesting the possibility of an exon 2 for human *CNR2*, which the Genomatix analysis did not reveal (Figure 15B). Exon 1 and 2 regions were further analyzed using the Consite web-base program for conserved *cis*-sequence regions. The exon 1

alignment revealed conserved *cis*-sequences for Elk-1 and c-REL, as well as DNA binding sites for STAT6 and NF-kBp50 for mouse, GATA and STAT for human. Similar results were obtained for the exon 2 alignment indicating a conserved NF-kB, p65, c-REL *cis*-sequence and single STAT6 DNA binding sites for both mouse and human (Figure 15). The results obtained from these analyses exemplified the usefulness of employing bioinformatics as a tool to direct research in locating candidate *Cnr2* gene regulatory regions that can then be experimentally tested for functionality, as well as guide future research in understanding the interplay between the *cis*-sequences and *trans*-factors that regulate *Cnr2* in B cells.

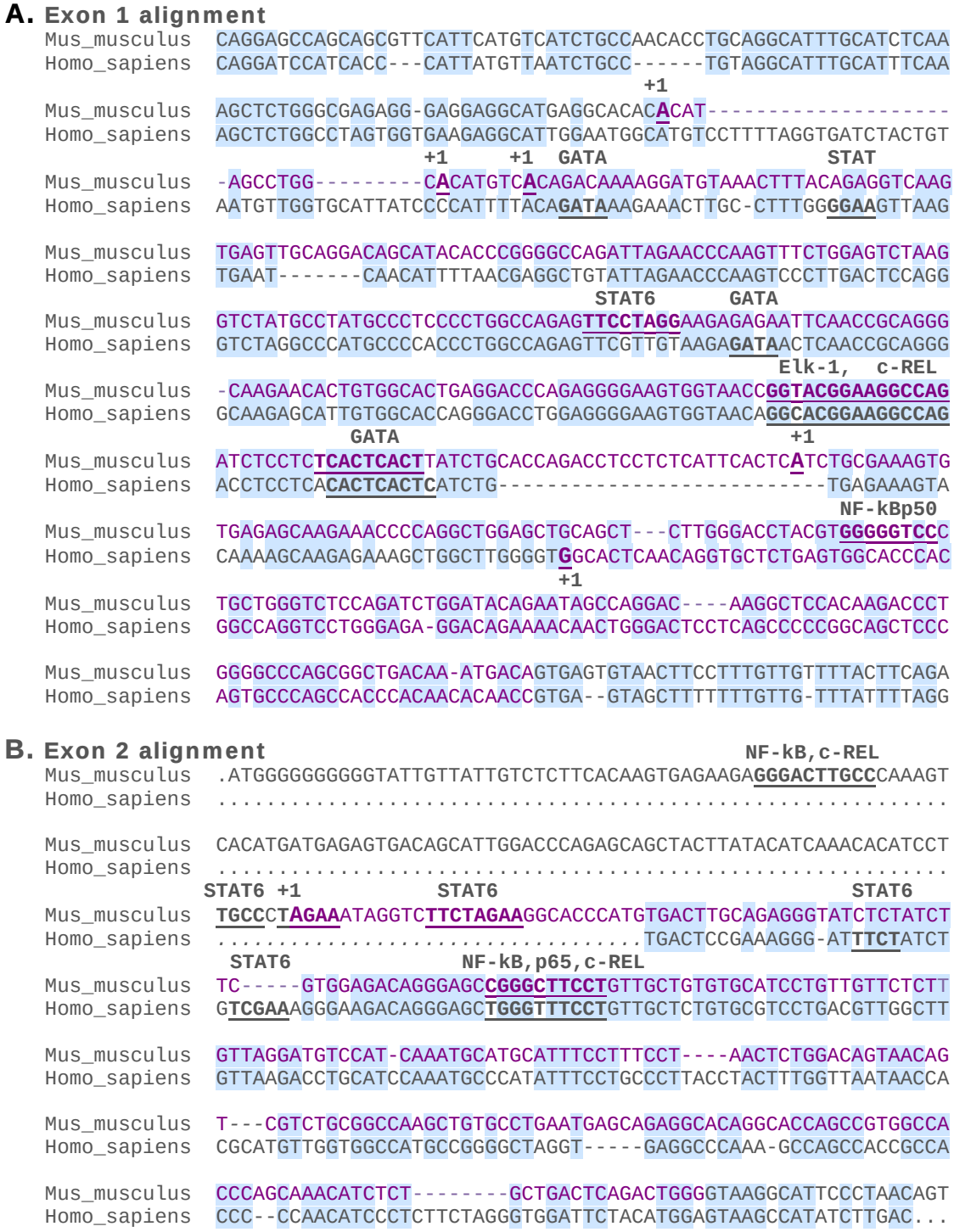


Figure 15. ClustalW Alignment of the Mouse and Human Putative Promoters. Nucleotides highlighted in blue represent the conserved regions of m*Cnr2* and h*CNR2* enabled prediction of the promoter region and *cis*-sequences (labeled and underlined). The 5' RACE TSSs (+1) are bold and purple nucleotides represent the 5' UTR exons.

Cloning of the Putative Cnr2 Promoters

From the bioinformatics analysis, the regions surrounding the TSSs for mouse exons 1 and 2 appeared to have core promoter elements, as well as enhancer elements, therefore we wanted to evaluate these regions for promoter activity. To test for promoter activity, we used genomic DNA from purified B cells to PCR amplify select regions of exon 1 (Figure 16A) spanning -359 bp to +205 bp of exon 1a TSS (+1) as well as the region from -189 bp to +205 bp of exon 2 TSS (+1). During PCR amplification, the Taq polymerase adds an adenosine nucleotide at the end of elongation thereby creating an A-tail that can be easily cloned into a TA-cloning vector, such as the TOPO-blue vector. Therefore, the PCR amplified DNA fragments were gel purified and initially TA-cloned into the TOPO-blue vector then sub-cloned into the pGL3-enhancer vector via the Hind III site by standard methods (Figure 16B). PCR screening was performed to determine insertion of the *Cnr2* clones into the pGL3-enhancer vector (Figure 16C). In total, three exon 1; pGL3-E16(-270,+205), E19(+68,+205), and E25(-359,+63) as well as five exon 2; pGL3-P7(-189,+36), P8(-90,+36), P10(+13,+205), P11(-189,+101), and P13(-25,+101) experimental *Cnr2* clones were chosen for evaluation of promoter activity.

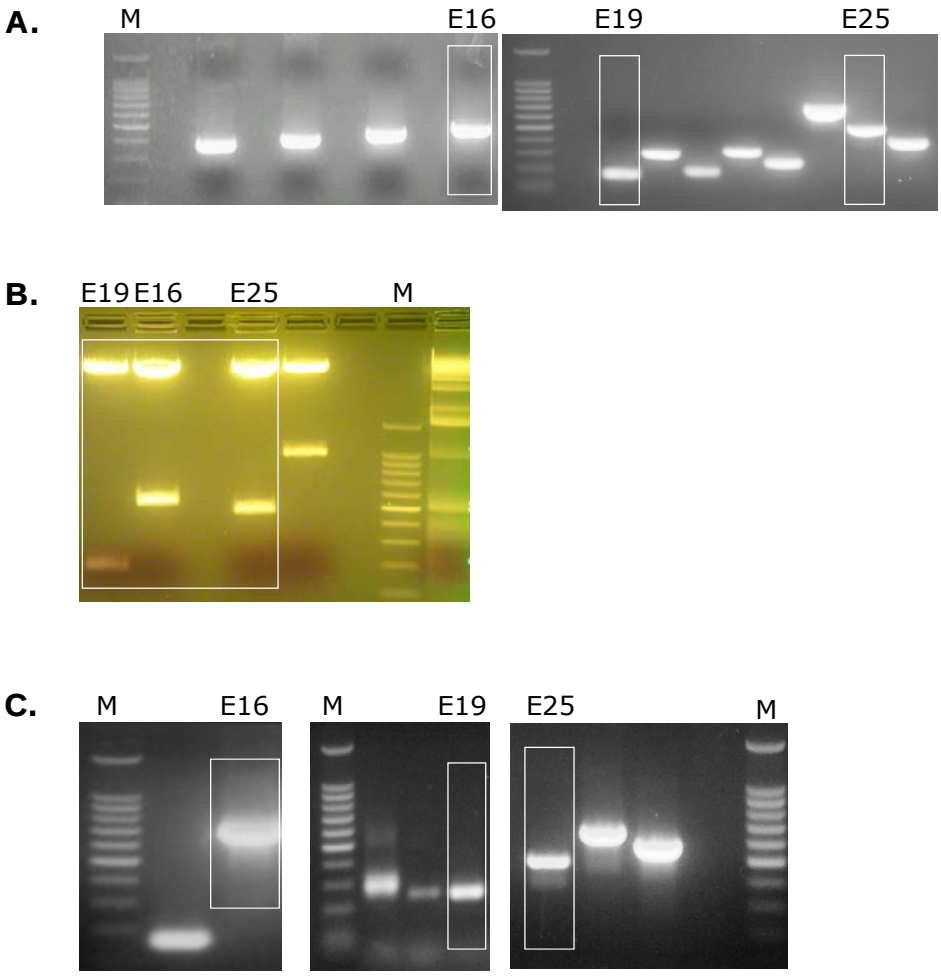


Figure 16. Cloning of the Exon 1 Promoter. A. PCR amplification of *Cnr2* promoter regions TA-cloned into the TOPO-blue vector. B. Hind III digest of TOPO- *Cnr2* clones for sub-cloned into the pGL3-enhancer vector. C. PCR amplification of *Cnr2* clones in the pGL3-enhancer vector.

Determination of Cnr2 Promoter Activity in B cells

The pGL3- *Cnr2* constructs were transfected by electroporation into IL-4/anti-CD40 stimulated primary B cells and 24 hrs later luciferase activity was determined for each construct. For exon 1 five constructs were analyzed, two control and three experimental vectors. The pGL3-enhancer vector does not have a promoter and contains only the SV40 enhancer downstream of the luciferase gene and therefore served as baseline activity. The pGL3-control vector contains both the SV40 promoter and enhancer and therefore exhibits full promoter activity. The pGL3-E25 experimental vector spans the region -359 bp to +63 bp (exon 1a, TSS +1) and contains all the TSSs and core promoter elements for exons 1a and 1b. The pGL3-E16 experimental vector spanning -270 bp to +205 bp contains the core promoter of exon 1a and a portion of the exon 1b core promoter. The GAGA-box and 1 TSS at -280 bp were excluded. The pGL3-E19 experimental vector spans from +68 bp to +205 bp and therefore did not contain either the exon 1a or exon 1b TSSs and core promoter *cis*-elements. Luciferase activation analysis for the exon 1 putative promoter demonstrated significant promoter activity for the pGL3-E25(-359,+63), pGL3-E16(-270,+205) and pGL3-control vectors, but not for pGL3-E19(+68, +205), indicating that the RACE TSSs we identified

were in *Cnr2* genomic regions that exhibited characteristics of a gene promoter (Figure 17A). In addition, pGL3-E25 had greater promoter activity then pGL3-E16 indicating that the GAGA-box and possibly the TSS at -280 bp are important for full promoter activity under these conditions.

The five exon 2 experimental clones used to evaluate promoter activity were pGL3-P11, which spans the genomic region -189 bp to +101 bp (exon 2, TSS +1) and contained the core and enhancer *cis*-elements. The truncated pGL3-P7 spanned -189 bp to +36 bp, was designed to exclude the 3' NF-kBp65, cREL *cis*-elements yet contained the core and 5' enhancer *cis*-elements. The pGL3-P8 was further truncated at the 5'end to span -90 bp to +36 bp and contained only core promoter *cis*-elements along with the 5' cREL enhancer *cis*-element. The pGL3-P13(-25,+101) truncated at the 5' end to include a third of the TATA-box and 3' core and enhancer *cis*-elements. The pGL3-P10(+13,+205) clone truncated at the 5' end to exclude the TATA-box and INR core cis-elements, but extended at the 3' end to include a portion of the GenBank designated exon 2 sequence. The reporter assay for the exon 2 clones demonstrated that the core promoter vector pGL3-P8 was sufficient to produce significant promoter activity. However, increased promoter activity was seen with

the pGL3-P7 clone, which contained the 5’ Sp1 *cis*-element as well as the pGL3-P11 clone that contained the 5’ Sp1 and the 3’ NF-kBp65, cREL *cis*-elements. No significant promoter activity was observed for either the pGL3-P10 or -P13 clones demonstrating that the TATA-box as well as the INR are needed for full promoter activity (Figure 17B). In all, these results demonstrated that the *Cnr2* genomic region containing the TSSs as well as core promoter *cis*-elements for exons 1a, 1b and 2 contained strong promoter activity as judged by these luciferase expression studies.

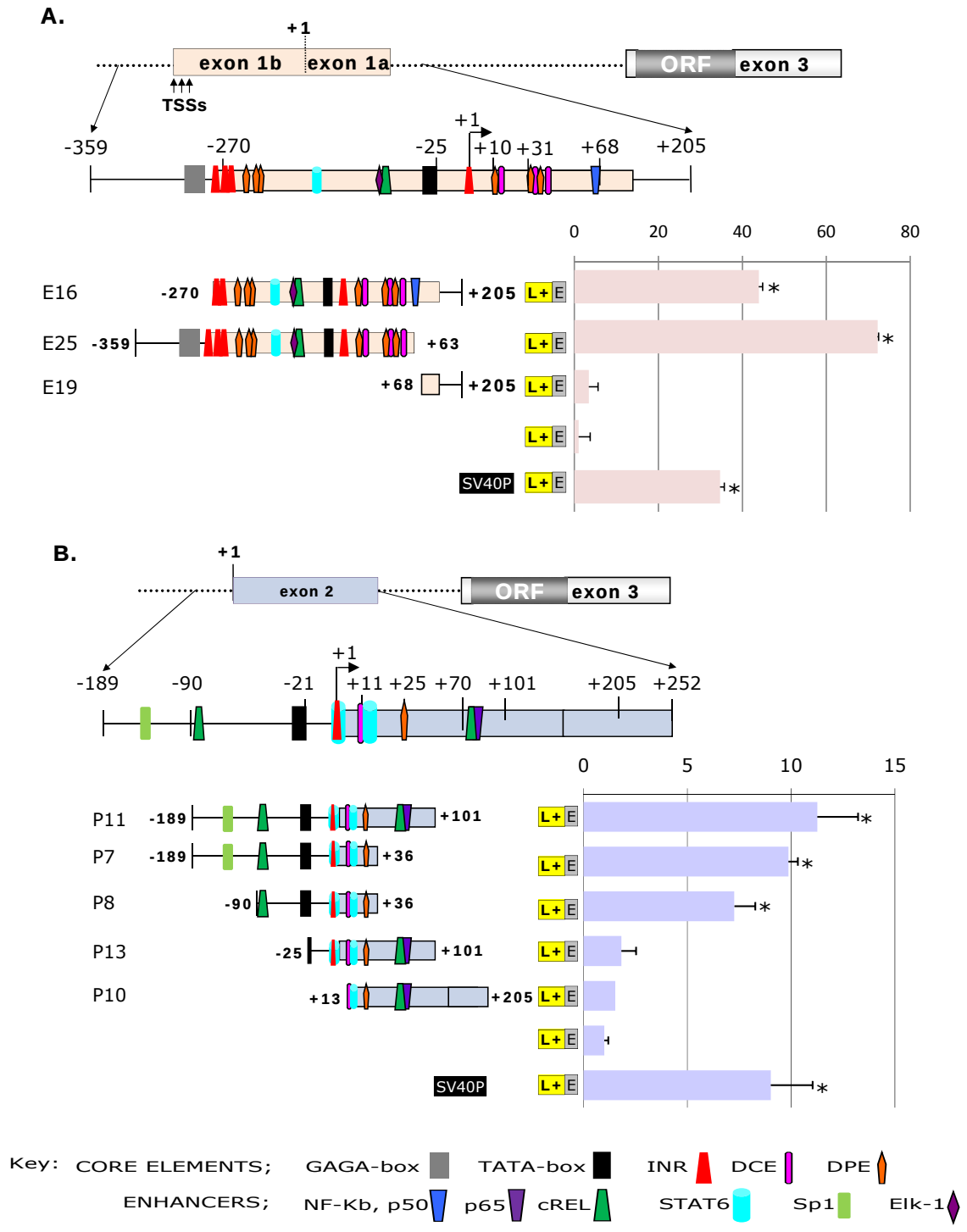


Figure 17. pGL3-*Cnr2* Luciferase Activity in IL-4/anti-CD40 Activated B Cells. A. Exon 1 putative *Cnr2* promoter pGL3-clones. **B.** Exon 2 putative *Cnr2* promoter pGL3-clones.

Aim 3. To Determine CB_2 Transcript Usage in Activated B Cells as well as Other Immune Cell Subtypes

CB_2 Transcript Expression in Mouse Activated B cells

Since CB_2 is abundant in B cells and implicated in the involvement of various B cell functions, an understanding of transcript usage under varying conditions of B cell activation is of interest. The literature shows that stimulation of B cells with anti-CD40 and/or IL-4, through STAT6 (*cis*-sequence found in both CB_2 putative promoter regions) activation, increases CB_2 expression (8) (25) (42), whereas, LPS stimulation suppresses expression (26). LPS through TLR4 triggers an intracellular signaling cascade, similar to anti-CD40/CD40 binding, that activates the *trans*-regulatory factors Elk1 and NF-kB (found in the CB_2 putative promoters). Both IL4/anti-CD40 and LPS promote B cell maturation and isotype switching.

Therefore, to gain a better understanding of CB_2 transcipt usage in activated B cells, we stimulated primary B cells with either LPS ornd the co-stimulatory molecule anti-CD40. To determine the relative expression of the CB_2 transcript variants, total RNA was collected at 1, 3, and 8 hrs following stimulation for RT-qPCR analysis. We looked at the expression of the CB_2 coding exon (exon 3, Figure 18A) and observed a steady increase over time following LPS stimulation.

Furthermore, using exon-specific primers and taqman probes revealed that LPS induced significant expression of the non-coding exon 1b and 2 transcripts, whereas, the exon 1a transcript remained at baseline (Figure 18B).

B cells activated by IL-4 and anti-CD40 undergo class switch recombination (CSR) changing the C region of the H chain to switch from IgM to IgE. Our lab has previously shown that IL4/anti-CD40 stimulation increased CB_2 expression in B cells at the message (25) and protein level (1). In addition to this, our lab has also shown that co-treatment of the IL-4/anti-CD40 stimulated B cells with the CBR agonist CP55940 increased immunoglobulin class switching to IgE.

To determine the effective dose of IL-4, we initially looked at CB_2 the coding exon 3 expression using two concentrations of IL-4 (3 and 10 ng/ml) with anti-CD40 (0.5 μg/ml). Exon 3 expression was significantly higher in B cells stimulated with 3 ng/ml of IL-4 compared to B cells stimulated with 10 ng/ml (Figure 19A). Therefore, we used the 3 ng/ml concentration of IL-4 with anti-CD40 to investigate CB_2 transcript variant expression. Following stimulation exon 3 increased within the first hour and maintained a steady state of expression thereafter (Figure 19A). On the other hand, non-coding exon expression increased increased 1 hr following stimulation with exon 2

continuing to increase over time, while the exons 1a and 1b returned to baseline by three hrs post stimulation (figure 19B). These results demonstrated for the first time that CB_2 transcript usage differs in B cells depending upon the state of activation of the cell with exon 1a predominating under basal conditions and exons 1b and 2 under varying conditions of activation.

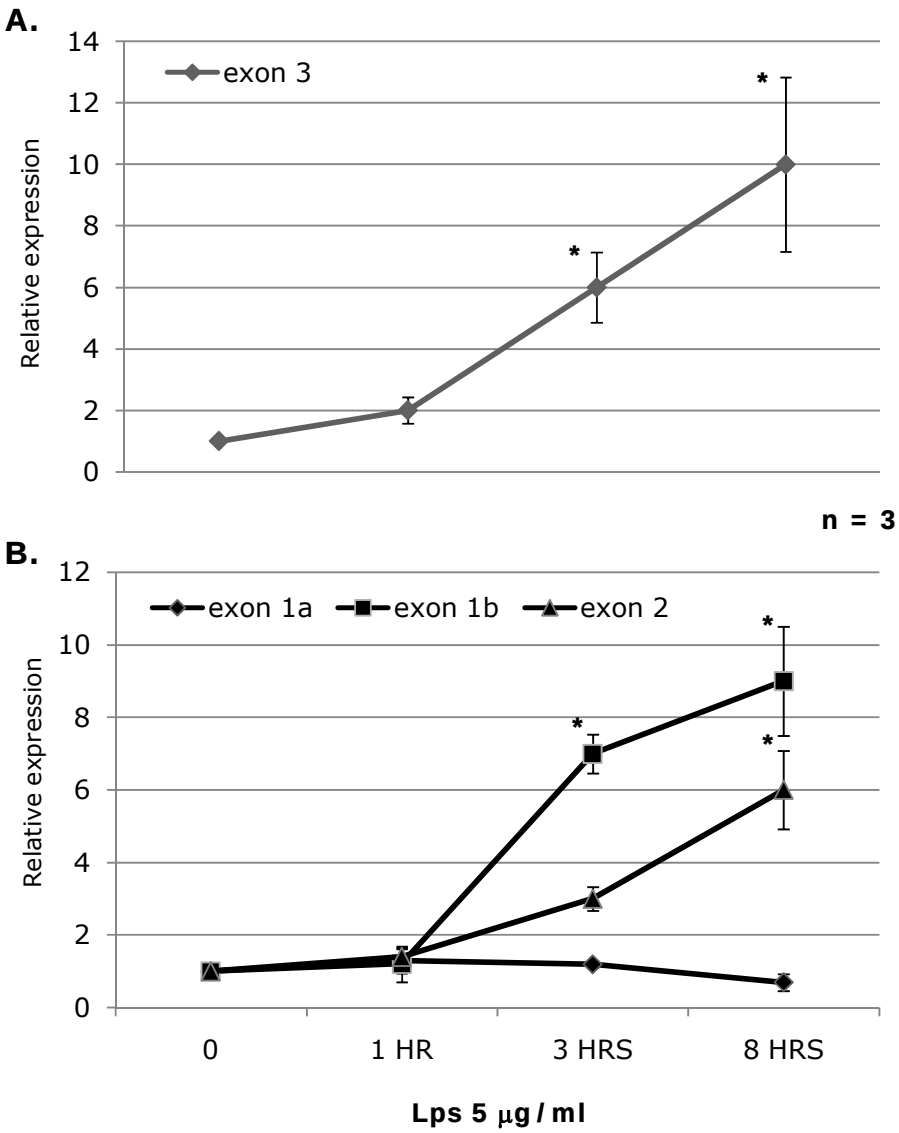


Figure 18. LPS Induces the Expression of the CB_2 Exon 1b and 2 Transcripts in Primary B cells. Primary B cells were cultured for 1, 3, and 8 hrs in RPMI medium containing 5 μg/ml LPS. Total RNA was isolated and 1 μg was used for RT-qPCR **A**. Total CB_2 message expression (exon 3) increases over time with LPS stimulation. **B**. Exon 1b and 2 transcripts expression is significantly increased overtime whereas the exon 1a transcript remains at baseline. Results were obtained by the $2^{-\Delta\Delta CT}$ method in which β-actin is the endogenous control and un-stimulated B cells (time 0) as the calibrator. Data are means ± S.E.M. of three independent experiments. * Significance at P = 0.05

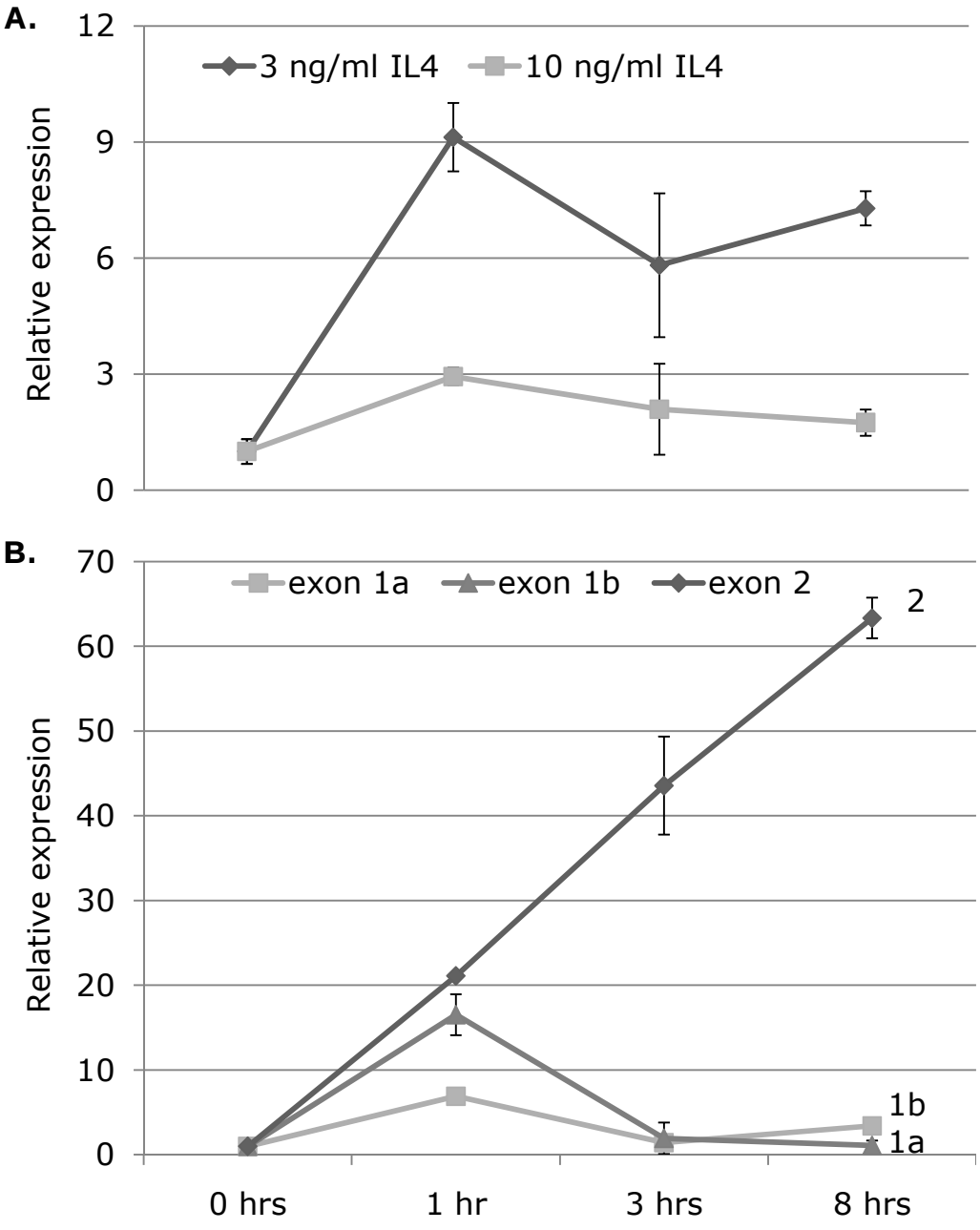


Figure 19. Primary B cells Stimulated with IL4 and anti-CD40. Primary B cells were cultured for 1, 3, and 8 hrs in RPMI medium containing IL-4 and 500 ng/ml anti-CD40. Total RNA was isolated and 1 μg was used for RT-qPCR **A**. Total CB_2 expression (exon 3) significantly increases with 3 ng/ml compared to 10 ng/ml IL-4 and 500 ng/ml anti-CD40. The exon 2 transcript steadily increases with stimulation of 3 ng/ml IL-4.

CB_2 Transcript Expression in Immune Cell Subtypes

The bioinformatics analysis performed in Aim 1 of the GenBank CB_2 clones suggested that expression of the first 5'UTR exon (1 and 2) variants could be related to tissue or cell type, the clones of immune tissue expressed exon 1, and other tissue types expressed exon 2. In spite of this, 5' RACE showed that B cells expressed CB_2 transcripts containing two variants of exon 1 and a single exon 2 variant. Since the previously described GenBank clones were obtained from immune tissues, and provided no information on transcript expression in immune cell subtypes, we wanted to investigate CB_2 expression in other immune cells other than B cells. Total RNA was isolated from purified un-stimulated T cells, dendritic cells, and macrophages for 5' RACE analysis. The results showed that CB_2 transcript expression was unique to immune cell subtypes. For example, T cells expressed only the exon 1a variant, whereas dendritic cells and macrophages expressed two transcript variants (exon 1a and 2). Most interesting was that the exon 1b variant was only observed in B cells (Figure 20). To confirm the expression of the transcript variants in the immune cell subtypes, RT-qPCR using exon specific primers and taqman probes was done (Figure 21). Coding exon 3 transcript expression was highest in B cells, followed by dendritic cells, then macrophages and T cells

expressing the least (Figure 21A). The exon 1a variant was the major CB_2 transcript seen in T cells. Dendritic cells and macrophages expressed both the exon 1a and 2 variants, however the relative expression of exon 2 was much higher in the dendritic cells (Figure 21B). The expression of the exon 1b variant was exclusive to B cells, though a negligible amount was observed in dendritic cells, which could be from amplification of residual genomic DNA. These results showed for the first time that the CB_2 transcript expression profile is different among the immune cell subtypes and that variant expression could be related to cell type and/or cell function. In addition, among these immune cell subtypes, the exon 1b variant was observed only in B cells and therefore could potentially be a cell specific target for CB_2 expression in this cell type.

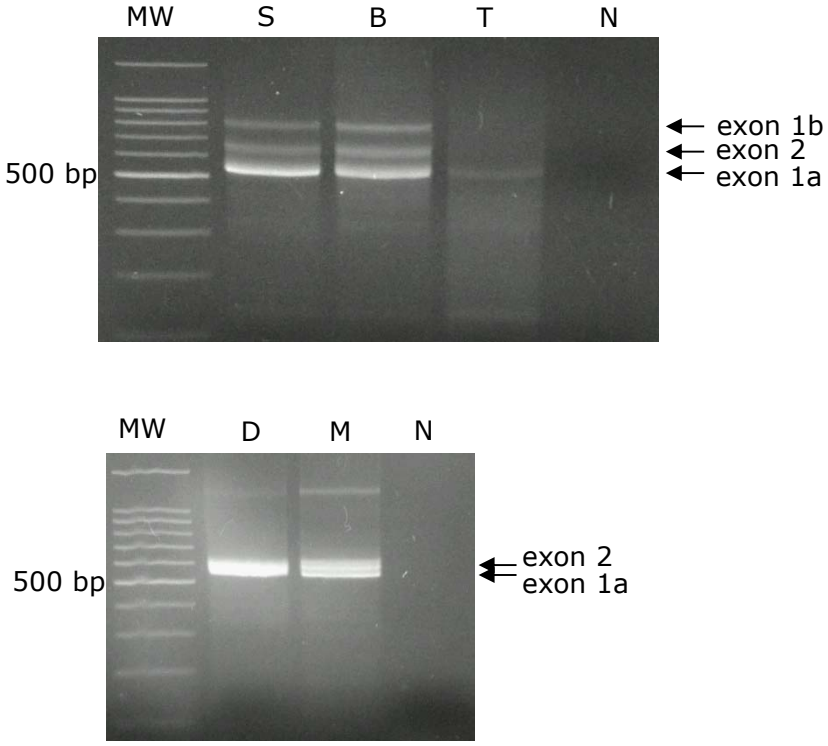


Figure 20. Immune Cell Subtypes 5' RACE CB_2 Transcripts. Gel electrophoresis of the RACE products isolated from Splenocytes, B cells , T cells (top panel) dendritic cells and macrophages (bottom panel).

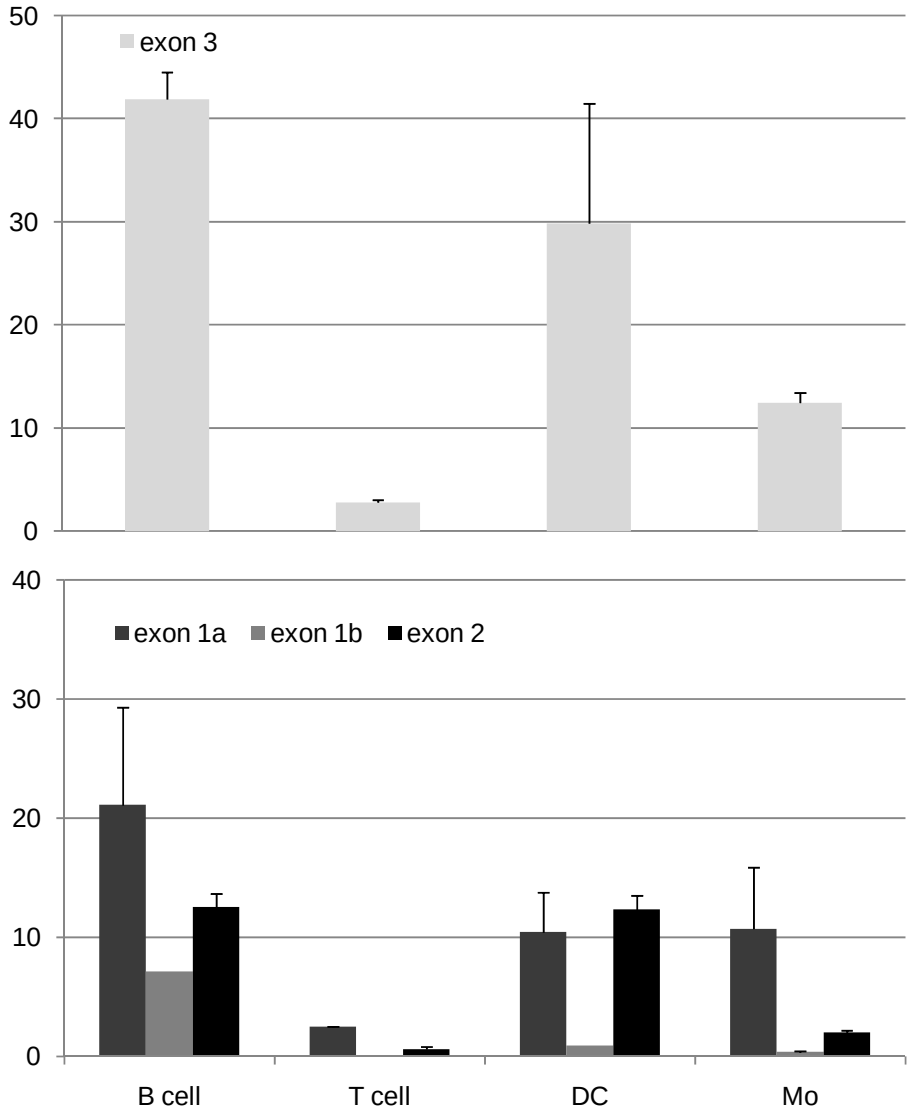


Figure 21. Quantitative RT-qPCR of the CB_2 Transcripts in Immune Cell Subtypes. Using 1 μg of total RNA isolated from un-stimulated B cells, T cells, Dendritic cells (DC) and macrophages (Mo) CB_2 transcript usage was determined. **A**. Total CB_2 expression (exon 3) in the immune cell subtypes. **B**. CB_2 transcript expression in the immune cell subtypes.

CB_2 Transcript Expression in Development of B cells

In A recent study investigating CB_2 mediation of immature B cell retention in bone marrow sinusoids (37) showed a two-fold higher expression of CB_2 in immature B cells compared to pre-B cells and that CB_2 deficiency led to a lower frequency of the Ig light -chain (λ^+) immature and mature B cells in the blood and spleen, thus suggesting a role for CB_2 in the formation of the B cell repertoire. In addition the results above have shown that expression of the CB_2 transcript variants can be related to immune cell subtype as well as the activation state of B cells. Therefore, we investigated CB_2 transcript expression in three different B cell lines representing B cell development from the pre-B stage to the mature stage.

The three mouse B cell lines used were; 18.81, an Abelson virus-transformed pre-B cell line that synthesizes only H chain, no light chain is produced. WEHI-231, an immature B lymphoma cell line that lacks Fc receptors and expresses only surface IgM and not IgD. K46μ, a mature B lymphoma cell line that expresses surface IgM and IgD. The 5' RACE of the different cell lines showed that the pre-B cell line expressed the exon 1a transcript and that the immature B cell line WEHI-231 and the mature B cell Line K46μ express all three transcripts but at different levels (Figure 22A). Therefore, we

performed RT-qPCR to determine the predominant CB_2 transcript expressed in these B cell lines. CB_2 coding exon 3 was observed to be the highest in WEHI-231 (immature) and lower in 18.81 and K46μ. The predominant CB_2 transcript observed in 18.81 and WEHI-231 was the exon 1a transcript, whereas the exon 1b transcript was predominant in the mature K46μ B cell line (Figure 22B). The data suggested that expression of the CB_2 transcript variants could be related to the developmental stage of B cells. In addition these results further support that CB_2 transcript usage varies in response to changes in B cell biology.

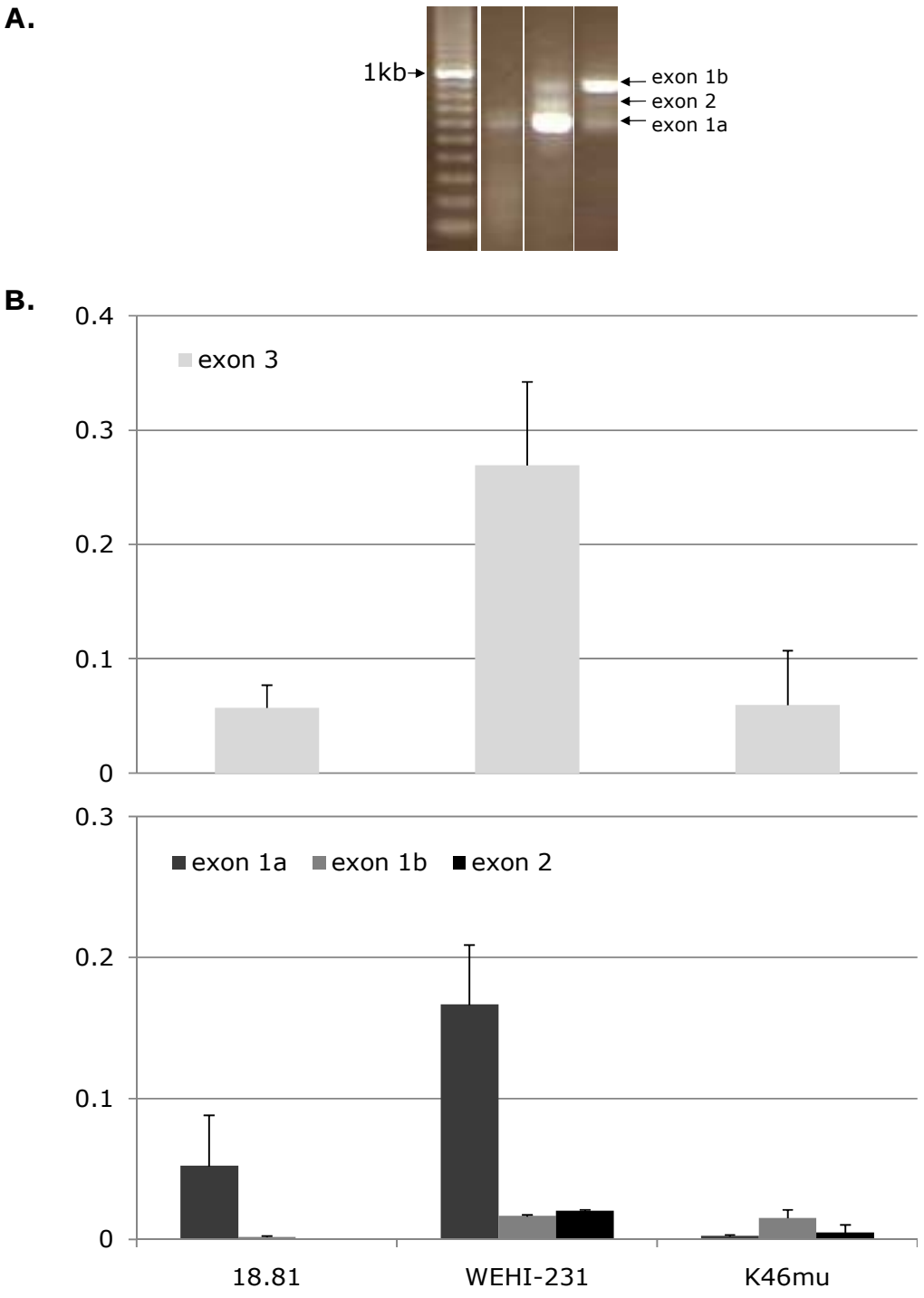


Figure 22. CB_2 Transcript Expression in B Cell Lines. A. Gel electrophoresis of the 5′ RACE products present in the B cell lines. **B**. RT-qPCR for CB_2 transcript expression in the B cell lines.

DISCUSSION

The relative robust expression of CB_2 in human and mouse B cells suggests that this receptor may have an important role in B cell biology. However, only a few reports have investigated the function of CB_2 in B cells. Furthermore, examination of the CB_2 transcript expression and *Cnr2* regulatory elements (i.e. promoter and TSS) in B cells had not been reported. Therefore, we investigated the genomic sequences involved in transcription of CB_2 by identifying the TSSs, mRNA transcripts and core promoter regions in purified resting and activated mouse B cells.

Our data provide the first evidence that resting splenic B cells in mice use multiple TSSs and express at least three CB_2 transcript variants. Based on present models of transcription initiation it is possible that two mechanisms of transcription could be involved in the generation of these variants: 1) alternative splicing of the 5'UTRs in the case of exons 1 or 2, and in fact donor-acceptor sites occur in these regions; and 2) alternative transcription initiation (dispersed initiation, see below) generating exon 1 variants that differ in the

length of their 5’ ends. The latter event may have occurred in the case of exon 1 in that we observed different lengths of the 5’ ends ranging over 295 bps and containing a cluster of four TSSs. Interestingly, a cluster of TSSs was predicted by the database, DBTSS, in the 5’ flanking regions of exons 1 and 2; furthermore, multiple TSSs were reported in GenBank CB_2 clones from various tissues in these same regions. Our RACE products from B cells identified new TSSs for exons 1 and 2 that were not only different than reported in other tissues but for the most part longer at the 5’ ends. Because of these many TSSs spread over hundreds of bps, we analyzed for core promoter sequences in these areas using an *in silico* approach. Interestingly, we found consensus core promoter sequences such as INR, DPE, DCE along with either TATA or GC boxes in abundance and in proximity to all of the TSSs expressed in mouse and the one TSS expressed in human B cells (see Figure 14). However, although present, these sequences were in different numbers and relative distances to the TSS position suggesting heterogeneity in core promoter activity under resting and activated conditions. Although the functional significance of multiple TSSs and core promoters is unknown, previous studies suggested this heterogeneity relates to cell type and/or cell activation state. This was observed in studies on the control of alternative first

exons of the glucocorticoid receptor (GR) which are under the control of specific transcription factors that control both tissue specific and cell activation state specific GR expression (49). This was also observed with adenosine A2A receptor (A2AR) 5'UTR splice variants wherein the long 5'UTR A2AR variants were observed in resting PMNs, whereas the short 5'UTRs were expressed to a greater extent in LPS- stimulated cells suggesting short 5'UTR variants were more efficiently translated (23) and suggesting the length of the 5'UTR can be a factor in determining tissue specificity and cell activation state.

In our mouse CB_2 studies, different TSSs and transcript expression were observed in different cell types. For example, resting T cells expressed only the exon 1a variant (Figures 20 & 21) and variants of this have been reported in thymocytes, splenocytes, and the macrophage like cell line, NFS107 (GenBank accession nos. AK037898, X86405, and NM009924). Whereas, bone and liver tissue (GenBank accession nos. BC024052 and AK036658) expressed the exon 2 variant though shorter at the 5'end than what was observed in B cells. Besides T cells, variation of CB_2 transcript expression was observed in other immune cell subtypes. For example, purified dendritic cells and macrophages expressed the exon 1a and 2 variants(Figure 20), though in dendritic cells the expression of the two

variants was more or less equal, whereas in macrophages the exon 1a variant was expressed five-fold higher than the exon 2 variant (Figure 21). Furthermore, resting splenic B cells expressed 3 CB_2 transcripts with an expression rank order of exon 1a > exon 2 > exon 1b, and of interest expression of the exon 1b variant was only observed in B cells (Figures 13 and 21). This variation in transcript expression among the various subtypes may be accounted for by variations in core promoter activity surrounding the different TSSs.

In contrast to the multiple TSSs and transcript variants we saw in mouse cells, human peripheral B cells collected from three different donors expressed a single CB_2 transcript and TSS (Figure 7). Interestingly, our observations in mouse and human are in line with those showing that two different strategies are employed by Pol II for transcription initiation. The h*CNR2* appears to utilize the more common strategy termed "focused initiation" in which a single TSS and the core promoter contains a TATA-box, BRE^d, INR, and DPE. On the other hand, the m*Cnr2* is more like the second strategy that involves multiple weak TSSs dispersed over DNA regions of approximately 50 to 150 bps, thereby dubbed "dispersed initiation" (21). The mechanisms of dispersed initiation are not clear but probably involve selective

usage of multiple upstream and downstream recognition and promoter elements similar to what we observed surrounding the mouse TSSs.

Different mCB_2 transcripts are not only associated with different cell types but also with different cell activation states. Using RT-qPCR, we showed that the mouse exon 1a transcript was predominantly expressed in resting splenic B cells (Figure 13) but that exons 1b and 2 were more pronounced in the LPS-activated B cells (Figure 18B), and that exon 2 increased in IL-4/anti-CD40 stimulated B cells (Figure 19B). A possible explanation of the observed variation in CB_2 transcript expression is the presence of proximal regulatory *cis*-sequences to the transcript TSS. Because in addition to core promoter activity, cell activation can lead to gene transcription through enhancer elements on the DNA either 5' or 3' to the core promoter region (5). Interestingly, the *in silico* analysis performed in Aim 2 identified proximal NF-kB (-82 and +72 bp) as well as STAT6 (-6 and +12 bp) *cis*-sequences 5' and 3' of the TSS(+1) for exon 2 (Figure 15B), which may account for the observed increase expression of the exon 2 variant in B cells activated by LPS or IL-4/anti-CD40 since it is well known that LPS, IL-4 and anti-CD40 activate B cells through an increase in NF-kB (12, 44, 47) and with IL-4 through activation of STAT6. In addition, pGL3-*Cnr2* reporter plasmid transfected mouse B

cells containing exon 2 genomic DNA constructs spanning -189 to +101 bp showed strong promoter activity when stimulated with IL-4 and anti-CD40 antibodies (Figure 17B); non-stimulated cells showed little luciferase activity (data not shown). Which the 5' NF-kB *cis*-sequence appears to be important for promoter activation, because constructs in which the 3' NF-kB site has been omitted still exhibit strong promoter activity (Figure 17B), in contrast to the much lower activity observed reporter constructs in which the 5' NF-kB site has been deleted (Figure 17B). Furthermore, it is In addition to NF-kB, STAT6 binding might also be involved in the significant increase of the exon 2 transcript observed in B cells stimulated with IL-4/anti-CD40, because two putative STAT6 sites are located at -6 and +12 bp of the TSS(+1) of exon 2 (Figure 15B). However, as reported previously (Thieu 2007), NF-kB may be required for binding of STAT6, supported by the minimal to no promoter activity observed in the exon 2 reporter constructs in which one or both STAT6 sites are present but the 5' NF-kB site has been deleted (Figure 17B). However, further analysis is required to determine the *Cnr2* regulatory relationship of these *cis*-sequences for the *trans*-factors under these conditions in B cells.

In addition to the variation of CB_2 transcript expression seen in activated B cells, differences in CB_2 transcript expression was also

observed in B cell development. RACE and RT-qPCR analysis of CB_2 transcript expression in three mouse B cell lines representing different stages of development showed that pre-B cells expressed only the exon 1a variant, whereas immature and mature B cells express all three variants, though at varying levels. Expression of the transcript variants was greatest in the immature B cells, notably the exon 1a variant, whereas mature B cells expressed mainly the exon 1b variant (Figure 22). Our results are in line with a recent report in which CB_2 transcript expression was higher in immature B cells located in bone marrow sinusoids when compared to other developmental stages and suggested a function for CB_2 in the formation of the B cell repertoire (Pereira 2009).

SUMMARY

In summary, we have characterized for the first time multiple TSSs that define alternative CB_2 transcripts in mouse splenic B cells as well as a single TSS and transcript in human PBMC B cells. We were able to confirm by RT-PCR primer mapping, the relative location of the TSS for mouse exons 1b and 2, as well as the human exon 1. These experimentally defined TSSs directed further *in silico* analysis and showed that these regions contain consensus sequences for multiple elements such as TATA-box, INR and DPE. These elements were found at the expected distances from the TSSs and by reporter assay experiments these segments contained significant promoter activity inferring that we correctly identified several of the TSSs in mouse B cells as well as identify the location of two promoters. In addition, we identified CB_2 transcript usage in resting B cells as well as other immune cell subtypes, in which the exon 1b transcript appears to be unique to B cells and therefore may serve as a therapeutic target in B cells. In addition, CB_2 transcript expression was different in the mouse B cell lines representing various maturation stages. Furthermore, we

identified CB_2 transcript expression in LPS and IL-4/anti-CD40 activated B cells, in which the exon 1b and exon 2 variants appear to be important.

The stimuli used in this study are known inducers of class switch recombination (CSR) and previous work done in our lab has suggested a role for CB_2 receptor activation in enhancing IL-4/anti-CD40 CSR from IgM to IgE, therefore it is possible from our work here, that exon 1b transcripts are unique to B cells and therefore provide a gene target for suppressing CB_2 expression in only B cells and not other immune cell subtypes. The identification of the CB_2 transcripts expressed during these conditions will guide future studies in regulating this receptor at the gene level, and provide possible gene targets(i.e. RNA silencing) for the therapeutic application in suppressing CB_2 and IgE production in Allergic diseases.

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