FROM *IN VITRO* TO *IN VIVO*: CONTROL OF C-REACTIVE PROTEIN GENE 

EXPRESSION BY CYTOKINES 

by 

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Submitted in partial fulfillment of the requirements 
For the degree of Doctor of Philosophy 

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May 2008
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*We also certify that written approval has been obtained for any proprietary material contained therein.
This thesis is dedicated to my husband, Greg, and my daughter, Cora.
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ACKNOWLEDGEMENTS

I would like to thank my advisors Dr. David Samols and Dr. Irving Kushner for their training, guidance, and support. Dr. Samols taught me how to design and implement experiments and how to present scientific data in a clear and concise manner. Dr. Kushner taught me how to write logically, precisely, and clearly. Both Dr. Samols and Dr. Kushner helped me to become an independent scientist.

My other committee members, Dr. Hung-Ying Kao and Dr. Alan Levine were extremely helpful throughout my graduate career. Dr. Kao and his students helped me with a number of assays and allowed me to use a variety of equipment for my experiments. Dr. Levine provided excellent constructive criticism during all of my committee meetings and helped steer my project along its final path.

I thank Sui Bi Samols for teaching me how to perform a ChIP assay and for helping me optimize the ChIP assay. Hong Zhang taught me how to do real-time PCR and RT-PCR and was always available to discuss experimental ideas and conditions. Kris Stanya and Erin Reinecke both taught me how to perform several assays and were also available to discuss various experimental details and problems. I thank Dr. Richard Hanson for his kind recommendations and assistance with obtaining a postdoctoral position. I would also like to thank Dr. G.J. Darlington for providing Hep3B cells. This work was supported by National Institutes of Health grant #AG02467, the Metabolism Training Program grant #T32 DK-007319-28, and the Department of Biochemistry.
LIST OF ABBREVIATIONS

APP, acute phase protein
APR, acute phase response
CAT, chloramphenicolacetyl transferase
C/EBP, CAAT/enhancer binding protein
ChIP, chromatin immunoprecipitation
CREBH, cAMP responsive element binding protein H (a.k.a. CREB 3 like protein 3)
CRP, C-reactive protein
EMSA, Electrophoretic Mobility Shift Assay
Hep3B, hepatoma 3B
HNF, Hepatocyte Nuclear Factor
IFN γ, Interferon γ
IL, interleukin
K_{app}, apparent equilibrium binding constant
LAP, liver-enriched activating protein
LIP, liver-enriched inhibitory protein
NF-κB, Nuclear Factor κB
STAT, Signal Transducer and Activator of Transcription
TBP, TATAA Binding Protein
TGF β, Transforming Growth Factor β
TNF α, Tumor Necrosis Factor α
UPR, unfolded protein response
UPRE, unfolded protein response element
From *in vitro* to *in vivo*: Control of C-Reactive Protein Gene Expression by Cytokines

Abstract

by

DUPRANE PEDACI YOUNG

Expression of the acute phase protein C-reactive protein (CRP) is tightly regulated in hepatocytes. While very little CRP mRNA is transcribed normally, inflammatory stimuli are followed by a dramatic increase in mRNA synthesis and accumulation. Interleukins -6 and 1β (IL-6 and IL-1β) are believed to be the major cytokines responsible for induction of CRP and other acute phase proteins.

We previously demonstrated that *in vitro* c-Rel plays a novel regulatory role by forming a complex with C/EBPβ when C/EBPβ is bound to the CRP gene promoter following cytokine stimulation. c-Rel does not by itself bind to the DNA. In these studies we found that recombinant c-Rel(1-300) (lacks transactivation domain) increased the affinity of recombinant C/EBPβ for a CRP-derived C/EBP site (-53) at least 10 fold. C/EBPβ and c-Rel(1-300) were found to physically interact in solution, and overexpression of c-Rel in the presence of overexpressed C/EBPβ stimulated CRP transcription. We concluded that c-Rel(1-300) binding to C/EBPβ increased the affinity of C/EBPβ for the CRP-C/EBP(-53) site, and that the transactivation domain of c-Rel is not necessary for this effect, which depends on protein: protein contacts with C/EBPβ.

We also employed chromatin immunoprecipitation (ChIP) assays to determine the kinetics of transcription factor occupancy of these transcription factors on the
endogenous CRP promoter. C/EBPβ, STAT3, p50, and c-Rel were found bound to the endogenous CRP promoter in the absence of cytokines, and cytokine treatment markedly increased binding of only C/EBPβ. In addition, c-Rel and TBP appeared to occupy the promoter in parallel in the presence of cytokines. In the absence of cytokines, CRP mRNA accumulation was not measurable but began to increase by 3 h after exposure of cells to IL-1β + IL-6, peaking at 12 h with secondary peaks at 18 h and 24 h. The secondary peaks in mRNA expression paralleled the pattern of binding of c-Rel and TBP to the CRP promoter. We conclude that the CRP promoter has a low level of transcription factor occupancy in the absence of cytokines and induction occurs with binding of C/EBP, and that c-Rel and TBP are important for modulating CRP expression.
CHAPTER 1: INTRODUCTION

The Acute Phase Response (APR)

The acute phase response (APR) is a major systemic physiologic reaction to local inflammatory stimuli. Inflammation is a localized tissue response to infection, injury, or other trauma characterized by pain, heat, redness, and swelling. The inflammatory response includes both local and systemic changes, and consists of vasodilation, an increase in local capillary permeability, an influx of phagocytic cells and other immune cells, removal of foreign antigens, and restoration of the damaged tissue (Goldsby et al., 2000). The acute phase response is part of the systemic response to inflammation and involves physiologic alterations that are thought to aid in the defensive or adaptive capabilities of the host. Bacterial infection, trauma, myocardial infarction, surgical or other trauma, and strenuous exercise are some examples of inflammatory stimuli capable of invoking the acute phase response. The APR may dissipate with recovery or it may continue in chronic disease, resulting in a chronic acute phase response. The systemic changes that occur during the acute phase response perturbs homeostasis in various organs, including the brain, liver, bone, adrenal gland, muscle, and fat cells (Baumann and Gauldie, 1994; Kushner and Rzewnicki, 1997).

The acute phase response begins with local inflammatory stimuli such as an infection or injury. Phagocytic cells, natural killer cells, lymphocytes, and mast cells leave the blood stream and invade the local site of inflammation. These leukocytes release inflammation-associated cytokines such as transforming growth factor β (TGF-β), interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and
interleukin 6 (IL-6) into the circulation. When these cytokines reach the liver, they
influence the regulation of the acute phase proteins (APP) and the plasma proteins
produced in the liver (Gabay and Kushner, 1999).

There are about 50 human acute phase proteins classified as either positive or
negative (Gabay and Kushner, 1999; Kushner and Rzewnicki, 1997; Ruminy et al.,
2001). The serum concentration of positive acute phase proteins increases during an
acute phase response, while the concentration of negative acute phase proteins decreases.
C-reactive protein (CRP), serum amyloid A (SAA), several anti-proteases, complement
factors, and components of the clotting system are examples of positive acute phase
proteins. In humans, albumin and transferrin are negative acute phase proteins. The
range of fold-change in serum concentration varies from about a 50% increase for
ceruloplasmin and several components of complement to over 1,000-fold for CRP and
SAA (Gabay and Kushner, 1999; Kushner and Rzewnicki, 1997; Samols, 2002).

Although we often describe the acute phase response as a single event, it is really the sum
of the individual responses of the participating genes. Increased circulating levels of
some acute phase proteins, including CRP, can be detected within hours of the onset of
an inflammatory stimulus while others (fibrinogen and haptoglobin) increase more slowly
and display peak circulating levels several days later. Acute phase proteins are thought to
participate in a variety of inflammation associated activities. These activities include
killing or limiting the spread of a pathogen, protecting self from harmful elements of
inflammation (e.g., inhibition of proteases) and repair of tissue damage (Samols, 2002).

Although the acute phase proteins are highly conserved and are usually
comparable in different mammalian species, there are several important exceptions.
Under normal circumstances, CRP is present in only trace amounts in humans, rabbits, and mice. It is greatly induced by acute inflammatory stimuli in humans and rabbits, but not in mice. In rats, CRP is constitutively expressed at relatively high levels and only increases several-fold after an inflammatory stimulus. CRP homologs are present in all vertebrates and many invertebrates including mollusks, crustaceans, and arachnids. CRP is part of a family of proteins with five fold symmetry called the pentraxins. CRP’s closest relative is serum amyloid P, which is an acute phase protein in mice but not in humans (Gabay and Kushner, 1999; Ruminy et al., 2001; Samols, 2002).

**Regulation of Acute Phase Protein Gene Expression**

As stated earlier, the acute phase response begins with a local inflammatory stimulus that leads to systemic release of inflammation-associated cytokines. These cytokines act on the liver to influence the plasma levels of acute phase proteins as well as regulate proteins that remain in the liver such as transcription factors, intracellular enzymes, and surface receptors (Gabay and Kushner, 1999; Ruminy et al., 2001).

Cytokines are intercellular signaling peptides that are usually produced by activated cells. Most cytokines have multiple sources, targets, and functions. Cytokines that are produced during, and are active in, inflammatory processes are the main stimulators of gene expression of acute phase proteins (Ruminy et al., 2001). Inflammation-associated cytokines may be produced by a number of different cell types such as monocytes, macrophages, fibroblasts, endothelial cells, T lymphocytes, and epithelial cells (Gabay and Kushner, 1999). These cytokines contribute to the inflammatory process at local sites of injury and infection mostly by autocrine and
paracrine mechanisms, while the changes in acute phase protein concentrations are a result of blood-borne effects on hepatocytes (Gabay and Kushner, 1999; Gauldie et al., 1987; Ruminy et al., 2001).

The effects of inflammation-associated cytokines are regulated by circulating modulators of cytokine function, such as IL-1 receptor agonist (IL-1ra), soluble TNF-α receptors, and soluble IL-6 receptors (Samols, 2002). IL-1ra and soluble TNF-α receptors inhibit the effects of their associated cytokines, while soluble IL-6 receptors enhance the effects of IL-6. Other inflammation-associated cytokines such as IL-4 and IL-10, which generally have anti-inflammatory effects, inhibit the induction of some acute phase proteins. Circulating mediators such as hepatocyte growth factor (HGF), insulin, epidermal growth factor (EGF), colony stimulating factors (CSFs), and glucocorticoids may influence the response of APP to cytokines (Gabay and Kushner, 1999; Loyer et al., 1993; Samols, 2002).

Transcriptional regulatory mechanisms generally fall into two models. In the first model, transcription is regulated at the initiation step when the transcription factors bind to promoter DNA, assemble the pre-initiation complex, remodel chromatin, recruit RNA polymerase II and initiate transcription. In the second model transcription is regulated after the initiation step and involves transcript elongation or stability. Recent work by the Young lab demonstrated that 75% of all protein encoding gene proximal promoters had Pol II bound and had trimethylation of lysine 4 of histone 3 (H3K4me3), hallmarks of initiation, even when measurable mRNA was not being transcribed. They also found that most promoters in both embryonic stem cells and primary human liver cells had
methylation marks (demethylation of cytosine in CG dinucleotides) and Pol II bound (Guenther et al., 2007).

The general mechanisms that regulate gene expression elsewhere also apply to the acute phase protein genes. DNA regulatory elements in the promoter regions upstream of the RNA start sites are bound by transcription factors which associate with coactivators and corepressors to influence the chromatin structure, the general transcriptional machinery, and RNA polymerase II. Transcription factors are proteins with DNA binding domains that recognize and bind to DNA regulatory sequences in a gene promoter, and act as either activators or inhibitors. Coactivators and corepressors are proteins that bind to transcription factors to further increase or decrease gene expression beyond the transcription factor activity alone. These proteins are generally found in large complexes of proteins with multiple activities.

APP gene expression can be affected by translational, post-translational, transcriptional, or post-transcriptional mechanisms. Most acute phase protein changes, including CRP, are due to regulation at the transcriptional level. However, translational mechanisms of gene regulation occur with ferritin and transferrin, and post-transcriptional mechanisms have been shown to participate in the regulation of SAA, factor B, and several other acute phase proteins (Birch and Schreiber, 1986; Jiang et al., 1995a; Samols, 2002). Some cytokines, such as IL-1, have been shown to affect the stability of mRNAs in several systems (Holtmann et al., 2001; Winzen et al., 1999).

A number of transcription factors regulate the expression of positive acute phase proteins in response to IL-6, IL-1, and TNF-α. These transcription factors typically include liver enriched transcription factors such as CAAT/enhancer binding protein
(C/EBP) family members (β and δ), Signal Transducer and Activator of Transcription (STAT) (Figure 1b) proteins (STAT1 and STAT3), Hepatocyte Nuclear Factor (HNF - 1α, 3, 4, & 6), and general inflammation associated transcription factors such as the Rel family members (NF-κB) (Figure 2) (Ripperger et al., 1995; Samols, 2002; Wegenka et al., 1994). C/EBP family members produce multiple proteins from a single mRNA via several different mechanisms. C/EBPβ mRNA produces three isoforms, full-length, liver-enriched activating protein (LAP), and liver-enriched inhibitory protein (LIP) via alternative translation initiation. (Figure 1a). The inhibitory isoform, LIP, is a functional LAP antagonist and LIP/LAP ratios are tightly regulated (Alam et al., 1992; Luedde et al., 2004; Poli, 1998). STAT family members are activated by cytokine stimulation of the JAK/STAT pathway (Figure 1c) while activation of NF-κB family members are controlled by cytokine induced phosphorylation of IκB family members (Figure 3). IκB sequesters NF-κB proteins in the cytoplasm, and phosphorylation of IκB leads to its degradation and allows NF-κB proteins to translocate to the nucleus.
Figure 1: C/EBPβ and STAT3 protein domain maps and STAT3 signaling pathway.

a. Protein domain maps for all three isoforms of C/EBPβ are shown. LAP* is full length C/EBP. LAP is liver-enriched activating protein. LIP is liver-enriched inhibitory protein. The activation domains (AD) are shown in green. The negative regulatory domains (RD) are shown in blue. The basic region is shown in red and the leucine zipper (ZIP) is shown in yellow and black. b. Protein domain map for STAT3. Src Homology 2 (SH2) domain is shown. Tyrosine (Y) and serine (S) are phosphorylation sites. c. Cytokine signaling pathway for STAT3 activation via JAK phosphorylation.
Figure 1: C/EBPβ and STAT3 protein domain maps and STAT3 signaling pathway.

a. 

C/EBPβ, LAP*, LAP, LIP

<table>
<thead>
<tr>
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<tr>
<td>AD</td>
<td>AD</td>
<td>AD</td>
<td>RD</td>
<td>RD</td>
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<tr>
<td>Basic</td>
<td>ZIP</td>
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b. 

STAT protein domain

<table>
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<tr>
<td>N-terminal</td>
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<tr>
<td>coiled-coil</td>
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<tr>
<td>binding</td>
<td></td>
</tr>
<tr>
<td>linker</td>
<td></td>
</tr>
<tr>
<td>SH2</td>
<td></td>
</tr>
<tr>
<td>transactivation</td>
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YS

c. 

STAT3 signaling pathway

Cytokine → Receptor → Jak → Stat → Phosphorylation → Translocation to Nucleus
Figure 2: Domain maps for NF-κB and IκB families of proteins.

Protein domain maps for the NF-κB and IκB families of proteins are shown. The Rel homology domains (RHD) are shown in red and the ankyrin repeats are shown in green. The arrows point to endoproteolytic cleavage sites of p100/p52 and p105/p50. Transactivation domain (TD), leucine zipper (LZ), and glycine-rich region (GRR) are indicated.
Figure 2: Domain maps for NF-κB and IκB families of proteins.
Classical NF-κB is a p65/p50 heterodimer that acts as an activator. In our system, a p50 homodimer, which lacks a transactivation domain, acts as an activator.
Figure 3: Classical NF-κB activation pathway

- Cytokines and mitogens
  - Cytoplasm
    - IkB
    - p50p65
    - NF-κB
    - IKK complex
    - P
  - Nucleus
    - Transactivation
      - p50p65
      - mRNA
      - Basal Transcription Complex
  - IkB promoter elements
The major acute phase protein genes have been divided into two classes (Baumann and Gauldie, 1990; Samols, 2002). Class I genes are those that respond to IL-1 in addition to IL-6, while class II are responsive to IL-6 alone. Class I responsive genes include CRP, SAA, α1-acid glycoprotein (AGP), complement component C3, and rat haptoglobin. The promoters for all of these acute phase genes contain binding sites for C/EBP and Rel protein family members, often in close proximity (Poli, 1998). Although all members of the C/EBP family bind to similar sequences, the C/EBP β and δ are the isoforms that are usually activated during inflammatory events (Samols, 2002; Takiguchi, 1998).

Synergy is often observed between IL-1 and IL-6 in the Class I acute phase genes. Interaction between the Rel and C/EBP transcription factor families may account for part of this observed synergy (Agrawal et al., 2001a; Cha-Molstad et al., 2000; Xia et al., 1997). A well studied example of this is the SAA gene in human hepatoma, Hep3B, cells. Either IL-6 or IL-1 alone will only induce minimal gene expression, but together IL-1 and IL-6 greatly induce SAA gene expression. This observed synergy could be due to physical interactions between Rel A (p65 subunit of NFκB) and C/EBPβ given that the binding sites for these two transcription factors are in close proximity (Betts et al., 1993; Li and Liao, 1992; Samols, 2002; Xia et al., 1997).

In contrast to regulation of SAA gene expression in Hep3B, IL-1 alone has no effect on induction of CRP, but it greatly enhances CRP expression by IL-6. The synergistic effects of IL-1 are not observed unless the transcription factors induced by IL-6 (C/EBP and STAT3) are activated and the CRP-C/EBP(-53) binding site is present. Rel proteins also contribute in CRP induction in Hep3B cells, but in a novel way. There
appears to be an interaction between C/EBPβ and Rel p50 in a proximal region of the promoter where their binding sites overlap (Agrawal et al., 2001a; Cha-Molstad et al., 2000). c-Rel also appears to interact with C/EBPβ via protein-protein interactions and affects the binding affinity of C/EBPβ to the CRP promoter (Agrawal et al., 2003b; Cha-Molstad et al., 2007).

Class II acute phase genes are those that are responsive to IL-6 alone. A number of class II acute phase genes, especially fibrinogen, α-1-protease inhibitor, rat thiostatin, and rat α2M, are much more dependent on STAT3 than the class I acute phase genes. The major activator of STAT3 is IL-6, not IL-1. Thus, class II acute phase genes usually have multiple STAT binding sites that are necessary for their induction, and they may not have any C/EBP binding sites. In the fibrinogen genes, IL-1, which activates NF-κB, actually represses basal and IL-6 mediated transcription (Fuller and Zhang, 2001; Samols, 2002). This may be because many NF-κB binding sites (called κB sites) overlap STAT3 binding sites. Because the sites are overlapping, activated NF-κB may repress fibrinogen expression by displacing STAT3 on the promoter region. In addition to class II acute phase genes, STAT3 binding sites can also be found in the promoters of class I genes, such as CRP, where they regulate part of the IL-6 response. Thus, STAT3 is an important regulator of most acute phase proteins (Samols, 2002; Zhang et al., 1996b).

**C-Reactive Protein**

C-Reactive Protein is a serum protein and is a soluble pattern recognition molecule that acts as an opsonin. The human CRP gene is small, 2.3kb, contains a single 278 bp intron, and is located on chromosome 1q23.2 (Du Clos and Mold, 2004; Lei et al.,
In humans, the concentration of CRP in the plasma of most normal individuals is below 3mg/L, but its concentration may rise to more than 300 mg/L during the acute phase response (Du Clos and Mold, 2004; Kushner et al., 2006a). CRP plasma concentration rapidly drops to basal levels after the inflammatory stimulus is removed, thus the plasma level of CRP parallels the course of an inflammatory episode. CRP was discovered in 1930 in the plasma of patients suffering from pneumococcal pneumonia and was so named because it reacted with the C-fraction of the pneumococcus, which contained carbohydrates, specifically pneumococcal C-polysaccharide (PnC) (Tillet and Francis, 1930). Later it was found that CRP reacts with phosphocholine (PCh), a component of PnC and a constituent of many bacterial and fungal polysaccharides and of biological cell membranes (Gabay and Kushner, 1999; Volanakis, 2001).

CRP is part of the highly conserved pentraxin family of proteins; homologs are found in many invertebrates and all vertebrates. Pentraxins have five or ten identical non-covalently associated subunits arranged as a pentamer with radial symmetry, and display calcium dependent binding to a wide variety of ligands including phosphate esters, some polysaccharides, and electrolytes (Gewurz et al., 1995). Each subunit of CRP has 206 amino acid residues folded into a flattened jelly roll structure with fourteen anti-parallel β sheets arranged in two planes (Figure 4). The molecular weight of the entire pentamer is 115,135 Da (Du Clos and Mold, 2004; Mortensen, 2001; Shrive et al., 1996; Volanakis, 2001).
Figure 4: Co-crystal structure of CRP with phosphocholine.

The CRP-phosphocholine co-crystal structure was resolved at 3 Å and shows a ribbon diagram. CRP is a pentamer with five identical subunits. Each subunit contains a single α-helix (red) and 14 anti-parallel β-sheets (blue) arranged in two planes. Phosphocholine is shown in green and the Ca$^{++}$ ions are shown in yellow. White lines represent the loops.
Figure 4: Co-crystal structure of CRP with phosphocholine.
Phosphocholine binds the B-face (binding face) of each subunit of the pentamer in a calcium dependent manner; two calcium ions bind each protomer adjacent to a hydrophobic pocket. On the opposite side of each protomer, the A-face, is an α helix and a small cleft where the complement component C1q binds. Thus, the molecule has two faces, the binding face with five PCh binding sites and the effector face with C1q binding sites (Figure 4) (Agrawal et al., 2001b; Black et al., 2004; Du Clos and Mold, 2004; Mortensen, 2001; Volanakis, 2001). How the two faces communicate is unclear, as dramatic conformational changes with ligand binding have not been reported. Binding of C1q to CRP has been proposed to be a concentration dependent event rather than a conformation dependent event. If a sufficient number of CRP molecules bind ligands, the CRP molecules cluster, which permits C1q interaction with CRP. Phosphocholine is exposed on the surfaces of a number of bacterial species and is a component of sphingomyelin and phosphatidylcholine in eukaryotic membranes. In normal eukaryotic cells, the phosphocholine in these molecules are inaccessible to CRP; therefore, CRP can bind these molecules only in damaged or necrotic cells (Black et al., 2004; Du Clos and Mold, 2004; Volanakis, 2001).

CRP also binds to some nuclear constituents that do not contain PCh, such as small ribonucleoprotein particles, histones, chromatin, fibronectin, laminin, and polycations. Ligand-bound or aggregated CRP is recognized by the complement component C1q which subsequently activates the classical complement pathway in humans (Black et al., 2004; Mold et al., 2002b; Volanakis, 2001). CRP can interact with the immunoglobulin receptors FcγRI and FcγRIIb, which elicits a response from phagocytic cells. The FcγR binding site is adjacent to, or overlapping with, the C1q binding site on
the A-face of CRP. The ability to recognize pathogens via binding PCh and its ability to aid in their elimination by effecting phagocytic cells may make CRP an important part of the innate host defense system. CRP may also be involved in removal of damaged and necrotic cells by binding and aggregating on these cells, aiding in the restoration of normal structure and function of injured tissues (Black et al., 2004; Mold et al., 2002a).

In addition to its likely beneficial effects when circulating levels are high, some think CRP may be involved in the development of atherosclerosis when its plasma level is minimally elevated (3-10 mg/L). Many studies have established a correlation between minor CRP elevations and an increased risk of future cardiovascular events (Nissen et al., 2005; Paul et al., 2004; Pepys et al., 2006; Ridker et al., 2006; Ridker, 2005; Ridker, 2007; Ridker et al., 2001; Ridker et al., 2002; Verma et al., 2006). Known risk factors for cardiovascular events are smoking, high systolic blood pressure, high blood cholesterol (LDL), diabetes, being overweight or obese, physical inactivity, increased age, being male, and family history of cardiovascular disease. It is unclear if minor elevation of CRP improves prediction of future cardiovascular events beyond the nine risk factors listed above. All nine have been individually reported to be associated with minor CRP elevation. Based on these findings, the CDC, American Heart Association, and some researchers believe that CRP can be a clinically useful biomarker of cardiovascular disease risk and have recommended widespread screening of CRP levels. Because some studies have shown that CRP binds to oxidized LDL, alters nitric oxide levels, and activates complement, many researchers believe minor CRP elevation may be a causative agent in the development of cardiovascular disease (Chang et al., 2002; Munford, 2001; Patel et al., 2001).
However, the view that CRP is causative or a good predictor of future cardiovascular events is not universal. Many researchers believe that although CRP may be a reflector of disease, the evidence is weak that it is a causative agent of cardiovascular disease, and will not be useful either as a biomarker in detecting disease or as a target for treating or preventing disease (Kushner and Sehgal, 2002; Mortensen, 2001; Paul et al., 2004). CRP is too nonspecific and variable to be a useful marker. Minor elevations of CRP plasma levels are associated with a large number of conditions some of which have no apparent connection to inflammation, including socioeconomic and demographic factors, dietary and behavioral factors, and medical conditions, such as depression and sleep disorders. The wide variety of factors associated with minor CRP elevations may reflect a mild degree of tissue injury or stress, suggesting that the presence of distressed cells, rather than inflammation, as the common factor (Kushner et al., 2006a; Kushner and Sehgal, 2002; Mortensen, 2001).

*Regulation of C-Reactive Protein Gene Expression During Cellular Stress*

Our survey of conditions associated with minor elevation of CRP revealed that many of these are not obviously linked to inflammation as traditionally defined, but rather reflect cell stress, which may not require inter-cellular signaling by cytokines (Kushner et al., 2006b). A recent paper by Zhang et al (Zhang et al., 2006) describes such a cytokine-independent mechanism.

Our lab has always focused on the mechanism by which cytokines control CRP expression. However, recently the Kaufman group reported that the unfolded protein response (UPR) induces CREBH processing and SAP and CRP synthesis (Zhang et al.,
The UPR stimuli employed in the study only induced SAP and CRP, and it did not induce other acute phase proteins. Therefore, the UPR did not induce the full acute phase response and is the first cytokine-independent stimulus of CRP expression described.

The unfolded protein response is an intracellular stress related pathway induced by excess production of secretory proteins or protein misfolding in the lumen of the ER (Harding et al., 2002; Schroder and Kaufman, 2005). The unfolded protein response can eventually lead to apoptosis. Protein misfolding is involved in a large number of disease states, including several in which associations with minor levels of CRP have been noted. Among these are autoimmune diseases and diabetes. The lumen of the ER contains a number of chaperone and foldase proteins that aid in normal protein folding. Many potentially deleterious events such as protein overexpression, exposed hydrophobic regions in proteins, calcium misregulation, aberrant glycosylation and oxidized disulfides may lead to the UPR. The main sensor is the chaperone BiP, which binds to a large number of proteins in the lumen of the ER to prevent misfolding. If BiP remains sequestered by binding to misfolded proteins, the reduced level of BiP associated with ER transmembrane proteins leads to several cascades of events which include suppression of translation by PERK-mediated phosphorylation of eIF2α, IRE1-mediated endoribonuclease splicing of XBP1 mRNA and regulated intramembrane proteolysis of latent transcription factors ATF6 and CREBH. ATF6 is ubiquitously distributed in tissues while CREBH expression is restricted to hepatocytes. Following ER stress in hepatocytes, the bZIP-containing N-terminal 320 amino acids of CREBH (CREBH-N) is released from the full length transmembrane precursor (CREBH-F) and transported to the
nucleus where it dimerizes and binds to a specific cis-element called the unfolded protein response element (UPRE).

The murine SAP gene and the human CRP gene each contain a UPRE as do a variety of ER-associated chaperone and protein genes (Zhang et al., 2006). In the case of human CRP, the UPRE is located in the 5′UTR of exon 1. CREBH-N binds the CRP UPRE \textit{in vitro} and stimulates reporter activity in transfection studies. Compounds such as tunicamycin (blocks N-linked glycosylation), DTT (reduces disulfide bonds), Brefeldin A (interferes with ER:Golgi vesicle traffic) and thapsigargin (disrupts calcium homeostasis) all induce the UPR, CREBH-N processing, and SAP and CRP synthesis. However, some UPR stimuli do not induce expression of other acute phase proteins including SAA1/2, α1-acid glycoprotein or fibrinogen. Thus, the UPR does not induce the full acute phase response and is the first cytokine-independent stimulus of CRP expression described. UPR activation of CRP expression may reflect cellular stress and contribute to some of the minor CRP elevations noted above.

\textit{Regulation of C-Reactive Protein Gene Expression During the APR}

During an acute phase response, the serum concentration of CRP can rapidly increase up to 1000-fold after an acute inflammatory stimulus. Most of our understanding of the events regulating CRP gene expression comes from studies in cell lines derived from human hepatomas, such as Hep 3B, Hep G2, and NPLC. A few studies have confirmed these findings in primary human hepatocyte cultures. Because CRP regulation is species specific, rodent lines have only been minimally useful. In Hep 3B cells, nuclear run-on assays have shown that CRP gene expression is regulated
primarily at the transcriptional level (Kushner et al., 1995; Zhang et al., 1995). Although IL-6 is the principal inducer of CRP gene expression, IL-1, glucocorticoids, and other factors act synergistically with IL-6 to increase its effect (Ganapathi et al., 1991; Ganapathi et al., 1988; Ganter et al., 1989; Szalai et al., 2000; Toniatti et al., 1990a; Volanakis, 2001). A recent study has shown that IL-17 is also a potent inducer of CRP gene transcription (Patel et al., 2007).

The detailed picture of the CRP promoter is quite complicated and involves both transcriptional and non-transcriptional participants. The promoter has been analyzed for more than 20 years and early studies established the existence of two sites for C/EBP binding in the proximal promoter region with two HNF-1α binding sites and one STAT3 binding site located between the two C/EBP sites (Figure 5). In vitro studies showed that HNF-1α constitutively binds the two HNF-1α binding sites, and STAT3 and C/EBP bind their respective sites on the promoter in response to IL-6. The proximal C/EBPδ binding site located at -53 (CRP-C/EBP(-53)) appears to be the most critical for cytokine induced CRP expression (Arcone et al., 1988; Li and Goldman, 1996; Li et al., 1990; Majello et al., 1990; Ramji et al., 1993; Toniatti et al., 1990b; Zhang et al., 1996a).
Figure 5: Model of the proximal CRP promoter and relevant transcription factors.

The model of transcription factor assembly on the CRP promoter is based on EMSA and luciferase assay data. The relative positions of transcription factors C/EBPβ, STAT3, p50, c-Rel, and HNF-1α are shown.
Figure 5: Model of the proximal CRP promoter and relevant transcription factors.
In addition to the transcriptional regulation of CRP expression, post-transcriptional mechanisms also regulate CRP synthesis (Jiang et al., 1995b). In humans, the protein half-life in the circulation has been measured at \( \sim 19 \) h and the mRNA half-life in Hep3B cells has been measured at \( \sim 3 \) h (Kushner et al., 1995; Zhang et al., 1995). Under normal conditions, CRP is synthesized at low rates and the pentamer is rapidly assembled inside the endoplasmic reticulum (ER). Free subunits of CRP have never been detected. However, in primary rabbit hepatocytes most of the native pentamer is retained in the ER by two carboxylesterases and is very slowly secreted. During the acute phase response, secretion of CRP from the ER in primary rabbit hepatocytes becomes more efficient and the increased secretion rate is regulated independently of the rate of CRP synthesis (Hu et al., 1988; Macintyre et al., 1985). The increased rate of secretion (transit time) appears to be due to a decreased affinity of one of the carboxylesterases for CRP through post-translational modification and down regulation of the carboxylesterases (Yue et al., 1996). When fully induced, CRP is secreted at the diffusion rate. This is also true for albumin which, like CRP, is a rare non-glycosylated serum protein.

A potential OCT-1 site was identified at -63 on the CRP proximal promoter overlapping the CRP-HNF-1\(\alpha\)\((-67)\) site (Li and Goldman, 1996; Voleti and Agrawal, 2005). Using EMSA studies, Voleti and Agrawal found a non-consensus NF-\(\kappa B\) site at -69 that overlapped this OCT-1 site. Binding of OCT-1 to a 25-bp oligo (-81 to -57) derived from the CRP promoter required an intact \(\kappa B\) site at -69. OCT-1, NF-\(\kappa B\) p50-p65 heterodimers, and NF-\(\kappa B\) p50 homodimers could each independently bind this region of the CRP promoter. However, binding of OCT-1 inhibited binding of p65 to the -81 to
-57 region of the CRP promoter. Transient transfection and luciferase reporter assays using wild type and mutant CRP-κB(−69) CRP promoter constructs suggested that the CRP-κB(−69) site was functional. Overexpressed C/EBPβ transactivated the proximal CRP promoter luciferase reporter. Co-overexpression of C/EBPβ and p50-p65 enhanced induction of the CRP promoter compared to overexpression of C/EBPβ alone. Overexpression of p50-p65 alone did not transactivate the CRP promoter, and mutation of the CRP-κB(−69) site impaired luciferase expression. The authors theorized that binding of OCT-1 to the CRP promoter represses CRP transcription, while cytokine induced replacement of OCT-1 with NF-κB p50-p65 induces CRP transcription in the presence of C/EBP (Voleti and Agrawal, 2005).

Electrophoretic mobility shift assays (EMSA) studies and luciferase assays demonstrated that under non-induced conditions C/EBPζ occupies the CRP promoter at the CRP-C/EBPζ(−53) site. The presence of C/EBPζ inhibited cytokine (IL-6 and IL-1β) induction of the CRP promoter in luciferase reporter assays. The authors theorized that C/EBPζ participates in CRP gene regulation by acting as a repressor and that after cytokine induction C/EBPζ is replaced by C/EBPβ and p50 (Singh et al., 2007).

Our lab identified an IL-6 responsive STAT3 binding site that is necessary for optimal IL-6 induced transcription of CRP. STAT3 binds the human CRP promoter at position CRP-STAT3(−108) on the proximal promoter region. The site is specific to STAT3 because of the four base spacing between the flanking GG and CC, and neither STAT1 nor 5 has ever been detected at the position. The role of STAT3 in mediating IL-6 induction of CRP transcription was examined by transient transfection assays in human hepatoma 3B (Hep3B) cells (Zhang et al., 1996a). A series of 5′ deletions of human CRP
promoter chloramphenicolacetyl transferase (CRP-CAT) constructs were transiently transfected into Hep3B cells. After transfection, the cells were treated with IL-1β or IL-6 or both cytokines. IL-1 β had no effect on reporter expression without IL-6. Overexpression of a constitutively active form of STAT3 in the absence of cytokines was enough to increase CAT expression 2-3 fold. EMSA studies were used to demonstrate that recombinant STAT3 bound an oligomer of the CRP promoter in an IL-6 dependent manner. Northern analysis was used to measure the accumulation of endogenous CRP mRNA in cells stably transfected with pRc/CMV-STAT3 compared to un-transfected Hep3B cells. The presence of STAT3 greatly increased the amount of CRP mRNA detected in the Hep3B cells. Thus, we concluded that STAT3 participates in mediating IL-6 induced transcription of CRP (Zhang et al., 1996a).

Our lab studied the mechanism of the synergistic effect of IL-1β on IL-6 induction of CRP transcription. Since IL-1β activates the NF-κB system, we examined the effects of overexpressed Rel family members on CRP gene expression. These studies were done by transiently transfecting human Hep3B cells with CRP-luciferase reporter constructs and vectors containing Rel p50 or Rel p65 (Cha-Molstad et al., 2000). Unexpectedly, we found that overexpression of p50 induced CRP gene expression via a nonconsensus κB site overlapping the proximal C/EBP binding site on the CRP promoter. Traditional NF-κB is of a heterodimer of p65 and p50 and acts as a transcriptional activator mostly through the p65 subunit. Transactivation by p50 homodimers is unusual because p50 lacks a transcriptional activation domain and usually acts as a repressor when alone. Transient transfection assays using the -125 /+9 CRP promoter-luciferase reporter were done using constructs with mutations in the proximal CRP-C/EBP(-53) site.
and the overlapping p50 CRP-κB\(_{(-43)}\) site. These assays indicated that C/EBP\(\beta\) and p50 act together to induce CRP gene expression (Agrawal et al., 2001a).

We did additional studies with NF-κB. Overexpression of p65 was found to inhibit CRP gene expression if the amount of C/EBP in the system was low (Cha-Molstad et al., 2000). Mutation of the p50 binding site (CRP-κB\(_{(-43)}\)) inhibited the transcription activation effect of cytokines on CRP gene expression. Therefore, we concluded that the NF-κB system participates, in a non-classical way, in cytokine induced CRP gene expression (Agrawal et al., 2001a; Agrawal et al., 2003a; Cha-Molstad et al., 2000).

In Northern blot analyses, overexpression of either p50 or p65 in the absence of overexpressed C/EBP\(\beta\) or STAT3 had only modest effects on the production of CRP mRNA in Hep3B cells. However, overexpression of p50 with either overexpressed STAT3 or overexpressed C/EBP\(\beta\) acted synergistically to induce endogenous CRP mRNA production (Agrawal et al., 2003a). Agrawal et al. also found a consensus κB site centered at -2652 on the CRP promoter that was capable of binding p50/p65 heterodimers in EMSA studies.

Our lab expanded this approach and examined four Rel proteins, p50, p65, p52 and c-Rel, to determine whether Rel protein present in Hep3B nuclei aid in the binding of C/EBP\(\beta\) to the CRP-C/EBP\(_{(-53)}\) site on the proximal CRP promoter. Sequestration of all Rel proteins from Hep3B nuclei by incubation with a consensus κB oligonucleotide led to decreased formation of C/EBP\(\beta\)-DNA complexes on a wild-type CRP promoter oligomer and on an oligonucleotide that contained only the CRP-C/EBP\(_{(-53)}\) binding site but not the CRP-κB\(_{(-43)}\) binding site. This indicated that Rel proteins were involved in the binding of C/EBP\(\beta\) to its binding site by a mechanism independent of the CRP-κB\(_{(-43)}\)
site. EMSA studies also showed that c-Rel assisted in the formation of C/EBPβ-DNA complexes and that c-Rel binds to C/EBPβ itself but not to the CRP promoter DNA. Antibody depletion of c-Rel led to reduced C/EBPβ EMSA signals but did not alter the pattern of retarded bands or generate a supershifted band. Cotransfection of c-Rel enhanced the transactivation of the CRP-promoter-luciferase reporter construct in the presence of overexpressed C/EBPβ (Agrawal et al., 2003b). Thus, c-Rel improved C/EBPβ binding to the CRP-C/EBPβ(-53) site without binding to the DNA directly.

Despite the synergism found between C/EBPβ and Rel p50, we found that p50 and p52 did not affect the binding affinity of C/EBPβ to the CRP promoter and p50 could not account for the synergism observed with IL-1β and IL-6 treatment. In studies described in Chapter 2, using recombinant proteins in EMSA studies, we found that c-Rel(1-300) (truncated c-Rel protein missing the transactivation domain) increased the binding affinity of C/EBPβ for the CRP-C/EBPβ(-53) binding site on the CRP promoter at least 10 fold without detectable binding to the promoter DNA directly. This effect was independent of the p50 binding site (CRP-κB(-43)). C/EBPβ and c-Rel(1-300) were also found to physically interact in solution. Additionally, we examined the ability of overexpressed c-Rel(1-300) to transactivate the CRP promoter. Overexpressed c-Rel(1-300) alone did not transactivate the CRP promoter. However, overexpressing both c-Rel(1-300) and C/EBPβ enhanced the induction of the CRP promoter compared to overexpression of C/EBPβ alone (Cha-Molstad et al., 2007). We concluded that c-Rel(1-300) binding to C/EBPβ increased the affinity of C/EBPβ for the CRP-C/EBPβ(-53) binding site, and that the transactivation domain of c-Rel was not necessary for this effect, which depends on protein:protein contacts with C/EBPβ.
Together, these studies helped us create a model for the transcription factors involved in CRP gene expression (Figure 15, Chapter 3). In our model, transcription factors C/EBP β/δ, STAT3, Rel p50, and c-Rel participate in CRP gene expression following cytokine stimuli. C/EBP β and δ bind at two sites centered at -53 and -219, and STAT3 binds at -108. C/EBP binding the CRP-C/EBP_{-53} site is essential for CRP expression. Rel p50 binds the nonconsensus CRP-κB_{-43} site and both p50 and C/EBPβ are required for full induction of CRP expression by IL-6 and IL-1β. In our model c-Rel enhances CRP expression by facilitating binding of C/EBPβ to the CRP-C/EBP_{-53} site on the promoter, and c-Rel does not require its transcription activation domain for this effect. Our model also includes STAT3 binding to the CRP-STAT3_{-108} site. STAT3 is an IL-6 responsive transcription factor that can transactivate the CRP promoter and induce endogenous CRP gene transcription. Oct1, p65, and β-catenin are also involved in CRP regulation, although these roles have not been specifically associated with IL-1 or IL-6 responses and will therefore not be pursued in my studies.
CHAPTER 2: THE INTERACTION OF C-REL WITH C/EBPβ ENHANCES C/EBPβ BINDING TO THE C-REACTIVE PROTEIN GENE PROMOTER

This chapter has been published in Molecular Immunology by Cha-Molstad et. al. I am responsible for writing the published paper and for the data in figure 14.

INTRODUCTION

C-reactive protein (CRP) is a major human acute phase protein largely synthesized in hepatocytes following inflammatory stimuli. The serum concentration of CRP can rapidly increase 1000-fold after severe inflammatory stimuli (Black et al., 2004; Volanakis, 2001). In the human hepatoma cell line Hep3B, CRP gene expression is modestly induced by IL-6, while IL-1β alone has no effect. However, together IL-6 and IL-1β act synergistically to markedly induce CRP gene expression in these cells (Ganapathi et al., 1991). Cytokine induction of CRP gene expression occurs mainly at the transcriptional level (Ganter et al., 1989; Jiang et al., 1995a; Li et al., 1990; Zhang et al., 1995). Transcription factors C/EBPβ/δ, STAT3, and Rel p50 participate in CRP gene expression following cytokine stimuli. C/EBPβ and δ bind at two sites centered at -53 and -219, and STAT3 binds at -108. Rel p50 binds a non-consensus κB site centered at -43 that overlaps the proximal C/EBP site (Agrawal et al., 2001a; Cha-Molstad et al., 2000; Li and Goldman, 1996; Majello et al., 1990; Zhang et al., 1996a).

Interestingly, although the CRP-C/EBPβ(-53) binding site is indispensable for CRP expression, its sequence is far from the consensus sequence, raising the possibility that other transcription factors may be required to facilitate DNA binding. Overexpression of
C/EBPβ and p50 synergistically transactivated CRP expression through a complex cis element binding C/EBPβ (-53) and p50 (-43) (Agrawal et al., 2003b). In electrophoretic mobility shift assays (EMSA), antibodies to p50 and competition with NF-κB consensus oligos substantially reduced the binding activity of C/EBPβ on the CRP promoter while antibodies to p65 and competition with mutant NF-κB consensus oligos had no effect (Agrawal et al., 2001a).

Previous studies indicated that Rel dimers other than p50/p65 might play a role in facilitating and stabilizing C/EBPβ binding on the CRP promoter (Agrawal et al., 2001a; Agrawal et al., 2003b; Cha-Molstad, 2002; Cha-Molstad et al., 2000). In EMSA with IL-6 treated Hep 3B nuclear extracts using an oligo derived from the CRP promoter, we previously found that C/EBPβ-DNA complexes were supershifted by antibodies against c-Rel. Overexpressed full length c-Rel has been shown to transactivate CRP promoter luciferase constructs in the presence of overexpressed C/EBPβ. We concluded from these in vivo experiments that c-Rel binds to C/EBPβ on the CRP promoter and not to DNA (Agrawal et al., 2003b).

To further explore the role of c-Rel in C/EBPβ mediated upregulation of CRP expression, and to confirm the conclusion that the effect of c-Rel is exerted by binding to C/EBPβ rather than by binding to DNA, we employed recombinant transcription factors to reproduce the interactions in vitro. We used c-Rel(1-300) (truncated c-Rel protein missing the transactivation domain) to determine if the effects of c-Rel on C/EBP binding to the CRP-C/EBPβ(-53) site were independent of its transactivation domain. Surprisingly, c-Rel(1-300) facilitated C/EBP binding without being part of the final complex. These results suggested that c-Rel may contribute to CRP expression by enhancing binding of
C/EBPβ to its weak cognate site on the CRP promoter. We conclude that c-Rel(1-300) facilitates or stabilizes C/EBPβ binding on the CRP promoter without directly binding to the DNA itself and that the transactivation domain of c-Rel is not necessary for this effect.
MATERIALS AND METHODS

Materials

Prokaryotic expression vector, pET-11d and eukaryotic baculovirus expression vector, pVL1392, Sf9 cells, and TC-100 medium were generously provided by Dr. Cheng-Ming Chiang (Case Western Reserve University, Cleveland, OH). Baculovirus expression system (Pharmingen, San Diego, CA) and Cationic liposome solution (Invitrogen, San Diego, CA) were used according to manufacturer’s instructions. E.coli strain, BL21-Codon plus (DE3)-RP, was purchased from Stratagene (La Jolla, CA). Synthetic oligonucleotides were obtained from Integrated DNA Technology (Coralville, IA). Polyclonal antibodies to p50 (H119), c-Rel (N), C/EBPβ (C-19) proteins, and HRP-linked secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flag M2 affinity gel was purchased from Sigma (St. Louis, MO). Polynucleotide kinase (PNK), protein A-agarose, and T4 ligase were obtained from Roche (Indianapolis, IN). Precision protein standards, Acrylamide/Bis solutions, and PVDF membranes were purchased from Bio-Rad (Hercules, CA). ECL reagents were obtained from GE Bioscience (Piscataway, NJ). BCA protein assay reagent was purchased from Pierce (Rockford, IL). Luciferase activity was measured using the Promega luciferase system (Madison, WI), and the Lmax luminometer (Molecular Devices, Sunnyvale, CA). Hep3B cells were provided by Dr. G.J. Darlington (Baylor College of Medicine, Houston, TX). Expression vectors encoding c-Rel and C/EBPβ were provided by Dr. N. Rice (National Cancer Institute, Frederick, MD) and Dr. J.E. Darnell Jr. (Rockefeller University, New York, NY), respectively. Ligate-It ligation kit was purchased from USB (Cleveland, OH). TA Cloning kit and DH5α Maximum
Efficiency Competent cells were purchased from Invitrogen (Carlsbad, CA). Recombinant human IL-1β and IL-6 were purchased from R & D Systems (Minneapolis, MN).

*Plasmid Constructs*

The pF:C/EBPβ-11d and pF:p50-11d were expression constructs in pET11-d with an inserted N-terminal flag tag (Chiang and Roeder, 1995) containing cDNA encoding human C/EBPβ (amino acids 1-345) and human NF-κB p50 (amino acids 1-452). The full length C/EBPβ expression plasmid, pF:C/EBPβ-11d, was constructed by a three-way ligation between pET(flag)-11d vector digested with NdeI (5’) and HindIII (3’), a synthesized double stranded 71 bp oligo (5’TATGCAACGCCTGGTGCGCCTGGGACCCAGCATGTCTGCCGCTGCGCCGCCGCCGCCTGCTGCTTTAAATC 3’ and 5’CATGGATTTAAAGGCAGGCGGCGGCGGCAGCAGCCGACATGGCTGGGTCCCAGGCCAACCAGGCGTGGCA 3’) encoding the N-terminus of the full length C/EBPβ (amino acids 1-23) with overhanging enzyme sites of 5’ NdeI and 3’ NcoI, and a PCR product of LAP (amino acids 24-345) digested with NcoI (5’) and HindIII (3’). The primers used for PCR amplification of LAP were 5’ CAAGGATGACGATGACAAGCCATGGAAGTG 3’ and 5’

CAGCTTATCATCGATAAGCTTGCAGTG 3’. Rel p50 expression plasmid, pF:p50-11d, was generated by ligating pET (flag)-11d vector with PCR products of p50 using restriction enzyme sites, NdeI (5’) and HindIII (3’). The PCR primers were 5’
CATATGGCAGAAGATGATCCATATTTGGG 3’ and 5’
AAGCTTAGTCATCACTTTTGTCACAACC 3’ for p50.

To generate baculovirus expression constructs the pET-11d-based flag fusion protein expression plasmids were used as templates and PCR products were generated and ligated into pVL1392 using enzyme sites, NotI and SmaI. The upstream PCR primers used for subcloning were 5’ AGAATTCGCGGCCGCCATGGACTACAAAGACGA 3’ for C/EBPβ and p50. The downstream primers used were the same as those used to construct the pET-11d-based flag fusion protein expression plasmids. For ligation, the ends of PCR products were filled in with Klenow, cut with NotI, and cloned as NotI and blunt ended fragments. The baculovirus expression plasmids were designated as pVL(F:C/EBPβ)1392 for the full length flag tagged C/EBPβ and pVL(F:p50)1392 for the flag tagged p50.

The -256/+9 CRP promoter luciferase construct was created as previously described (Cha-Molstad et al., 2000). The c-Rel(1-300) insert was created by PCR. The PCR primers used were 5’ AAAAGCTTGGATCCATGGCCTCCGGTGCGTATAAC 3’ and 5’ GCCTCTAGAGTCGACTCAGAAAAGCAGAGTTGTCTTTTG 3’. The c-Rel(1-300) insert was TA cloned and the insert and RcCMV vector were digested with restriction enzymes BamH1 and XbaI, gel purified, and ligated.

**Protein Purification**

Flag tagged C/EBPβ and p50 were expressed in and purified from Sf9 insect cells infected with recombinant baculoviruses carrying the coding region of C/EBPβ and p50 following a published protocol (Wu et al., 1999). In brief, 1 μg of pVL1392 harboring
cDNA of C/EBPβ or p50 was mixed with 0.25 μg of BaculoGold linear DNA (PharMingen), 10 μl of cationic liposome solution (Invitrogen), and 0.5 ml of TC-100 medium. The reaction mixtures were vortexed vigorously, incubated at room temperature for 15 min, and then added to 60-mm plates to which ~ 2 to 3 × 10⁶ Sf9 cells were seeded. After incubating at room temperature with gentle rocking for 4 h, 1.5 ml of TC-100 medium was added to the plates. The plates were incubated in a 27 °C humidified chamber for 5 days. The supernatant was collected by spinning cells at 3,000 rpm for 5 min and designated as the P₀ virus stock. To generate a higher titer P₁ virus stock, 0.5 ml of P₀ was incubated with ~ 4 × 10⁵ Sf9 cells in a 60-mm plate containing 3 ml of TC-100 and the supernatant was collected after 5 days incubation. 0.5 ml of P₁ was used to infect 6 × 10⁶ Sf9 cells in a 150-mm plate containing 25 ml of TC-100. After 5 days incubation, 5 ml of the supernatant (P₂ virus stock) was added to infect Sf9 cells in 250 ml of suspension culture (~ 0.6 × 10⁶ cells/ml). The supernatant, virus stock P₃, was collected after 5 days of incubation and 25 ml of P₃ was used to infect 250 ml of Sf9 suspension culture (~ 0.6 × 10⁶ cells/ml) for protein expression. Following 44 h of protein expression, Sf9 cells were harvested by centrifugation at 3,000 rpm for 5 min, washed with cold phosphate-buffered saline, and resuspended in 15 ml of Bacterial Lysis Buffer (Chiang and Roeder, 1993). The cells were lysed using a French Pressure Cell at 1,500 Psi, centrifuged at 18,000 rpm for 20 min, and the supernatant was incubated with 0.1 ml of anti-FLAG M2-agarose (Sigma) at 4 °C overnight. The protein bound M2 agarose was washed with 10 ml of Bacterial Lysis Buffer plus 0.1 % Nonidet P-40 followed by four washes with 10 ml of BC300 (20 mM Tris, pH 7.9 at 4 °C, 20 % Glycerol, 0.2 mM EDTA, 300 mM KCl, 0.5 mM PMSF, and 1 mM DTT). Finally,
proteins were eluted with BC300 containing 0.2 mg/ml FLAG peptide and 0.1 % Nonidet P-40 (Chiang and Roeder, 1993). The protein concentration was determined using BCA protein assays (Pierce). Approximately 300 ng of each protein was loaded on and separated in an SDS-polyacrylamide gel (Fig. 1A, lanes 2 to 5). The purity of p50 was greater than 90 % as determined by coomassie staining while the purity of C/EBPβ was ~80 %. The purified C/EBPβ was visualized as four shadow bands with molecular weights ranging from 40 to 44 kDa, which probably resulted from post-translation modification, most likely phosphorylation. Human p52 (amino acids 1-447) and human c-Rel (amino acids 1-300) produced in E. coli were purchased from Santa Cruz. The full length p52 was produced in E. coli as an 80 kDa GST fusion protein. We did not perform additional procedures to ensure proper protein folding and activity was assessed by measuring binding to the Ig-κB oligo.

**EMSA**

The oligonucleotides (oligos) used in the EMSAs are listed in Fig. 1B. Complementary oligos were annealed and end-labeled with \[γ^{-32}P\]ATP using T4 polynucleotide kinase. EMSAs were carried out as published previously with some modifications (Levy et al., 1989). Purified recombinant proteins with concentrations indicated in the figure legends were incubated with 1 nM (binding assays) or 20 nM labeled double strand oligo probe in gel shift incubation buffer (50 mM NaCl, 20 mM Tris, pH 8.0, 1 mM MgCl₂, 1 mM DTT, 0.25 mg/ml BSA, 1 μg/ml of poly d(I-C), and 5% Glycerol) for 20 min at room temperature. In supershift experiments, antibodies (2 μg) were added to the reaction mixture and incubated at room temperature for 10 min.
before addition of the probe. In competition experiments, a 100 fold molar excess of unlabeled oligo was added to the binding reactions. The consensus oligos for C/EBP (Vinson et al., 1989), NF-κB (Muller and Harrison, 1995; Sen and Baltimore, 1986a; Sen and Baltimore, 1986b), and c-Rel (Butscher et al., 2001) were designed according to published sequences. DNA-protein complexes were resolved by 5 or 6 % native PAGE in 1X TAE (1X TAE= 0.04 M Tris-acetate and 1 mM EDTA) at 10 V/cm. The gels were dried and analyzed in a phosphorimager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Every experiment was repeated at least two times and representative EMSAs are shown.

**Data Analysis**

To measure apparent equilibrium binding constants ($K_{app}$), the fraction of DNA bound in each reaction was determined by dividing the intensity of the band of DNA bound by the total intensity of the bound and free DNA bands. The DNA bands were measured using a phosphorimager with ImageQuant software. Using Sigma Plot software, scattered plots were generated as fraction bound versus protein concentration.

In Sigma plot curve fitting and the Hill equation, $F=\frac{(L)^n/K^n}{[1+(L)^n/K^n]}$ (where $L = $ ligand concentration, $K = K_{app}$ apparent, $n = $ the Hill coefficient describing cooperativity, and $F = $ the fraction of ligand binding sites filled), were applied to determine $K_{app}$ and the binding cooperativity of p50 for Ig-κB and CRP-κB(-43) and C/EBPβ for C/EBP consensus oligo. This method is based upon studies showing cooperativity of the four oxygen molecules on hemoglobin.
**Immunoprecipitation and Western Analysis**

250 ng of C/EBPβ and c-Rel were mixed in 500 µl 1× EMSA binding buffer and incubated at room temperature for 15 min. 4 µg of polyclonal antiserum against C/EBP or c-Rel was added and incubated at 4 °C for 1.5 h. 20 µl of protein A-agarose (Roche) was added to each reaction and the incubation continued overnight. After the incubation, the protein A-agarose was pelleted by centrifugation at 1,000 × g for 30 s and washed with PBS three times. 40 µl of SDS-polyacrylamide gel sample buffer was added to release the immunoprecipitated proteins. The reactions were boiled for 3 min, centrifuged at 1,000 × g for 30 s, and the supernatant was transferred into a fresh microcentrifuge tube.

For Western blotting, 20 µl of the immunoprecipitate was loaded onto a 10 % SDS-polyacrylamide gel and separated by electrophoresis. After electrophoresis, the gel was equilibrated in 1× transfer buffer (39 mM glycine, 48 mM Tris base, 0.037 % SDS, and 20 % methanol) for 20 min. The separated proteins were electroeluted onto a PVDF membrane using a transfer apparatus (Idea Scientific) for 45 min. After transfer, the membrane was blocked in 5 % BSA in TBS (500mM NaCl and 20mM Tris) at 4 °C overnight, washed with 1× TBST (TBS + 0.05 % tween 20) three times, and incubated in polyclonal primary antibody (1:2000 dilution in 1x TBST) at room temperature for 1 h. The blot was washed three times in TBST and incubated with anti-rabbit IgG secondary antibody (1:5000 dilution in 1× TBST) conjugated with horse peroxidase at room temperature for 1 h followed by the three washes. The specific protein bands were detected using ECL reagent (Amersham Bioscience Corp) and imaged using VersaDoc Imaging system (Bio-Rad, Hercules, CA).
Cell Culture, Transient Transfection, and Luciferase Transactivation Assay

Cell culture, transient transfections, and transactivation assays were performed as previously described (Agrawal et al., 2003b). Briefly, human hepatoma 3B (Hep3B) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO₂. Hep3B cells were plated at 50% confluency in 6-well plates containing 2 mL culture media per well for transfection. Eighteen to twenty hours after plating, old media was replaced with fresh media with FBS, and cells were transiently transfected using FuGENE 6 reagent (Roche) following the manufacturer’s protocol. Diluted transfection solution (6 μL FuGENE 6 per μg DNA + serum-free RPMI 1640 medium to a final volume of 100 μL) was incubated for 5 minutes. Next the diluted transfection solution was mixed with one microgram of luciferase reporter-CRP promoter construct and varying amounts of the transcription factor expression vectors (5 ng C/EBPβ, 15 ng c-Rel1-300) and was incubated for 20 minutes at room temperature. The FuGENE 6-DNA mixture was applied to Hep3B cells and incubated for 28-30 hours at 37°C and 5% CO₂. After the incubation period, the media was changed to RPMI without FBS and cytokines IL-1β (1ng/mL) and IL-6 (10ng/mL) (R&D Systems, Minneapolis, MN) were added to the appropriate wells. Eighteen to twenty hours after cytokine treatment, the cells were washed in cold PBS two times and suspended in cold PBS. The cells were then pelleted by centrifugation at 13,000 rpm at 4°C in a tabletop centrifuge. Next, the cells were lysed with 60 μL 1x reporter lysis buffer (Promega, Madison, WI) and one freeze-thaw cycle. Twenty microliters of the cell extract supernatant was loaded into each well of an opaque, white 96-well microtiter plate. One hundred microliters of luciferase assay reagent (Promega,
Madison, WI) was added and luciferase activity was measured in a luminometer
(Molecular Devices, Sunnyvale, CA) that was programmed to read for 10 s with a 2 s
delay. Luciferase activity was normalized to the protein concentration of the extract
measured using the BCA protein assay kit from BioRad. Common internal transfection
standards such as pRSV-βgal were not used because they responded to overexpressed
transcription factors in this system. Assays were carried out in triplicate and the
experiments were repeated 3 times.
RESULTS

**C/EBPβ Binding to a Consensus Oligo and to the CRP-C/EBP_{(-53)} Oligo**

We performed EMSA assays to characterize the interactions of transcription factors on the CRP proximal promoter (-63 to -38). The preparations employed are shown in Figure 6a. We compared the binding affinity of baculovirus-expressed recombinant C/EBPβ for its proximal binding site on the CRP promoter (CRP-C/EBP_{(-53)}) to its affinity for an oligo containing a consensus C/EBP binding site (Figure 7). In this experiment, a fixed amount of oligo was incubated with increasing amounts of C/EBPβ (1.25 nM to 80 nM) (Figure 7a) and the fraction of the retarded specific protein-DNA complex over the total oligo calculated. C/EBPβ reached saturation on the consensus probe at a concentration of ~ 20 nM (Figure 7b).

Much higher concentrations of C/EBPβ (80nm to 740 nM) were required to measure the apparent equilibrium binding constant ($K_{app}$) for the CRP-C/EBP_{(-53)} site and a defined plateau could not be obtained. At a concentration of 80 nM, C/EBPβ displayed much stronger binding to the consensus oligo than to the CRP-C/EBP_{(-53)} oligo, and increased concentrations of C/EBPβ resulted in only modest increases in binding to the CRP oligo. The amount of free probe showed a minor decrease with increasing concentrations of C/EBPβ because free probe was distributed throughout the lanes as a result of the high protein concentration in the system. Therefore, the $K_{app}$ of C/EBPβ for the CRP-C/EBP_{(-53)} site could not be measured accurately. It was roughly estimated by calculating the fold difference of C/EBPβ concentration between the CRP-C/EBP_{(-53)} oligo and the C/EBP consensus oligo at a point where their values of the fraction of DNA bound were the same, and then multiplying the fold difference with the $K_{app}$ for the
C/EBP consensus oligo. Curve fitting (Figure 7b) using the Hill equation estimated a $K_{\text{app}}$ for C/EBP on the consensus oligo of $5.9 \pm 0.6$ nM with a cooperativity factor of $2.1 \pm 0.4$, indicating that C/EBP$\beta$ bound the consensus as a dimer. The $K_{\text{app}}$ for the CRP oligo was $>200$ nM and no cooperativity factor could be calculated. These results indicate that the CRP-C/EBP(-53) site is a relatively poor binding site for C/EBP$\beta$, exhibiting at least 30-fold weaker affinity for C/EBP$\beta$ than the C/EBP consensus oligo.
Figure 6: Protein preparations and sequences of oligonucleotides used in EMSAs.

a. ~300 ng of purified recombinant proteins were loaded onto a 10% SDS gel and stained with coomassie brilliant blue. The molecular weight marker is shown in lane 1. C/EBPβ and p50 expressed and purified from Sf9 cells are shown in lanes 2 and 3, respectively. p52 and c-Rel
(1-300) were purchased from Santa Cruz and shown in lanes 4 and 5, respectively.

b. The nucleotide sequence of oligonucleotides used in this study. Numbering of the CRP promoter oligo is relative to transcription site. The overlapping C/EBP and nonconsensus κB sites are boxed. Mutated bases in the κB sites of CRPm(κB) and CD28RE(mut) oligos are shown in bold italics. Consensus nucleotide sequences are underlined. Only the sequences of the sense strand are shown.
Figure 6: Protein preparations and sequences of oligonucleotides used in EMSAs.

a.

b.

CRP: 5' -TACATAGTGCGCAAACACTCCCTTACT- 3'

CRPm(κB): TACATAGTGCGCAAACGTGATTACT

Ig-κB: TCTGAGGGACTTTCCGTATC

C/EBP consensus: TGCAGATTGCGCAATCTGCAC

H-2Kb: CGGGCTGGGGATTCCCCATCTCGGTAC

CD28RE: GTTTAAAGAAATTCCAAAGAG

CD28RE(mut): GTTTAAAGACCATAGAAAGAG
Figure 7: C/EBPβ exhibits a 60 fold higher binding affinity for a C/EBP consensus oligo than for the CRP-C/EBP(-53) oligo.

a. EMSA using a fixed amount of DNA (1 nM) and increasing amounts of C/EBPβ. Either CRP-C/EBP(-53) or C/EBP consensus oligos were used as a radiolabeled probes. Arrows indicate the positions of the C/EBPβ-DNA complex and free double-stranded DNA. A representative of two experiments is shown.

b. A plot of C/EBPβ concentration versus fraction bound for the C/EBP consensus oligo. Two data sets were combined to generate the plot. One data set was derived from Figure 7a (open circles; o) and the other from an EMSA which is not shown (solid circles; •). As described in Methods, curve fitting (Hill equation) using sigma plot software is shown as a solid line.
Figure 7: C/EBPβ exhibits a 60 fold higher binding affinity for a C/EBP consensus oligo than for the CRP-C/EBP(-53) oligo.

(a) 

(b)
**Rel Family Members Binding to the CRP-κB(-43) Oligo**

In contrast, baculovirus-expressed recombinant p50 exhibited a $K_{\text{app}}$ for its binding site on the CRP oligo (CRP-κB(-43)), $K_{\text{app}} = 30 \pm 0.7 \text{ nM}$, that was close to its binding affinity for a consensus binding site (Ig-κB), $K_{\text{app}} = 11 \pm 0.8 \text{ nM}$, (Figure 8). After the fraction of DNA bound was measured (Figure 8a), a scatter plot was generated for both CRP promoter and Ig-κB oligos. Curve fitting to these plots exhibited a sigmoidal shape for the CRP-κB(-43) oligo, suggesting cooperative DNA binding of p50 (Figure 8b).

Binding of Rel family members to the CRP-κB(-43) site was further analyzed in EMSA experiments using 20 nM of the CRP oligo (Figure 9). As a control, an Ig-κB oligo was used as an NF-κB consensus site. p50 effectively bound the CRP-κB(-43) and Ig-κB oligos at a concentration of 50 nM and the identity of the retarded band was confirmed as being p50 by its complete supershift with an antibody against p50 (Figure 9, lanes 1 and 2). At a concentration of 192 nM p52 bound very poorly to the CRP-κB(-43) site (Figure 9, lanes 3 and 4) and only slightly better to the Ig-κB oligo. When p50 and p52 were combined, the results were no different from p50 alone (Figure 9, lanes 1 & 5). An antibody to p50 supershifted the entire p50-DNA complex band whereas an antibody to p52 showed no effect (Figure 9, lane 7). c-Rel$_{(1-300)}$ did not bind to the CRP-κB(-43) site or Ig-κB, even at a concentration of 300 nM (Figure 9, lanes 8-9). Concentrations of c-Rel$_{(1-300)}$ above 1.5 μM were required to observe a faint retarded band on the CRP oligo (data not shown). c-Rel$_{(1-300)}$ had little effect when mixed with p50 (Figure 9, lanes 10 – 12). We conclude that p50 dimers strongly bind the CRP oligo while p52 and c-Rel do...
not. As an additional control to demonstrate that the c-Rel\textsubscript{(1-300)} preparation was functional, an EMSA was performed with c-Rel\textsubscript{(1-300)} (300 nM) and a known c-Rel binding site (H-2K\textsuperscript{b} oligo) from the MHC class I promoter (Figure 9, lane 13). As shown in Figure 9, c-Rel bound to the H-2K\textsuperscript{b} oligo and the retarded band was supershifted with an anti c-Rel antiserum (Figure 9, lane 14).
Figure 8: p50 homodimer binding to the CRP-κB(-43) site has an affinity comparable to that for the Ig-κB site and exhibits cooperative binding to the CRP-κB(-43) site.

a. EMSA using an increasing concentration of p50 with a fixed amount of radiolabeled DNA probe (1 nM) containing the κB sites from either the promoter of CRP (CRP-κB(-43)) or the Immunoglobulin light chain κ (Ig-κB). Arrows indicate the positions of the p50-DNA complex and free double-stranded DNA.

b. A plot of p50 concentration (nM) versus fraction bound for both CRP-κB(-43) and Ig-κB. Two sets of data points were combined to generate each plot. One data set was derived from figure 8a (solid circles; •) and the other from an EMSA that is not shown (open circles; o). Curve fitting (Hill equation) using Sigma Plot Software is shown as a solid line.
Figure 8: p50 homodimer binding to the CRP-κB(-43) site has an affinity comparable to that for the Ig-κB site and exhibits cooperative binding to the CRP-κB(-43) site.

(a) Diagram showing p50/DNA complexes at different concentrations of p50 and Ig-κB.

(b) Graphs showing the fraction DNA bound as a function of [p50] (nM) for Ig-κB and CRP-κB.
Figure 9: Pattern of binding of Rel Family Members to CRP-κB(-43) and Ig-κB is similar.

EMSA using purified p50 (50 nM), p52 (150 nM), and c-Rel(1-300) (300 nM) with 20 nM of radiolabeled probe containing either the κB sites from the promoter of CRP (CRP-κB(-43)), the Immunoglobulin light chain κ (Ig-κB) or the MHC class I (H-2Kb) genes. Antibodies (2 μg) to p50, p52, and c-Rel were added as indicated in the figure before the addition of the labeled probe. DNA-protein complexes were separated by 5 % nondenaturing PAGE, and the results analyzed by phosphorimager. Radiolabeled probes used for each panel are indicated by an arrow. The mobility of free probe is not shown.
Figure 9: Pattern of binding of Rel Family Members to CRP-κB(-43) and Ig-κB is similar.

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c-Rel Enhances the Binding of C/EBPβ on the CRP-C/EBPβ(-53) Oligo

The effect of Rel proteins on C/EBPβ binding activity on the CRP promoter was examined by EMSA. C/EBPβ at 50 nM bound to the CRP oligo weakly (upper band, Figure 10a) and was completely supershifted by antibodies against C/EBPβ (Figure 10b, lanes 1 and 2). In the presence of a fixed amount of C/EBPβ, increasing amounts of p50 bound the CRP oligo as a faster migrating band separate from the C/EBPβ-DNA band. The intensity of the latter was unchanged in the presence of p50 (compare Figure 10a lane 1 with lanes 2-4). An antibody against p50 supershifted the faster migrating band (Figure 10b, lanes 3-5), establishing its identity. Addition of increasing amounts of p52 did not affect binding activity of C/EBPβ, and p52 containing complexes were not observed (Figure 10a, lanes 5-8). Interestingly, when increasing amounts of c-Rel(1-300) were added to a fixed amount of C/EBPβ, the intensity of the C/EBPβ-containing band markedly increased (Figure 10a, lanes 9-12). No separate c-Rel(1-300)-containing band was observed, consistent with results from Figure 9, indicating that c-Rel(1-300) does not bind the CRP oligo at the concentrations employed (Figure 10a, lanes 9-12). An antibody against c-Rel had no effect, indicating that the retarded band did not contain c-Rel(1-300) (Figure 10b, lanes 6-8). As a control, BSA did not affect C/EBPβ binding to the CRP oligo (Figure 10a, lanes 13-16). The enhancing effect of c-Rel(1-300) is presented graphically in Figure 10c. At a concentration of 1.5 μM of c-Rel(1-300), C/EBPβ with c-Rel(1-300) bound the CRP oligo five fold better than did C/EBPβ alone. The same results were observed with recombinant C/EBPβ purified after expression in E. coli (data not shown).
The effect of c-Rel(1-300) on C/EBPβ binding was further quantified by estimating the $K_{\text{app}}$ of C/EBPβ on the CRP oligo, in the presence and absence of 1.5 $\mu$M of c-Rel(1-300) (Figure 11). Between 5 and 200 nM C/EBPβ, c-Rel(1-300) shifted the $K_{\text{app}}$ of C/EBPβ from $> 200$ nM to $\sim 40$ nM, more than 5 fold. We conclude that c-Rel(1-300) facilitates or stabilizes C/EBPβ binding on the CRP promoter without directly binding to the DNA itself.
Figure 10: c-Rel_{1-300} but not p50 or p52 enhanced C/EBP\(\beta\) DNA binding on the CRP promoter.

a. EMSA using C/EBP\(\beta\), p50, p52, and c-Rel_{1-300} with 200 nM of the CRP promoter oligo as a probe. The C/EBP\(\beta\) concentration was fixed at 50 nM (lanes 1 – 16). Increasing amount of p50 (lanes 2 - 4), p52 (lanes 5 - 8), c-Rel_{1-300} (lanes 9 - 12), and BSA (lanes 13 - 16) were added as indicated in the figure. DNA-protein complexes were separated by 6 % nondenaturing PAGE, and the results analyzed by phosphorimager. The mobility of free probe is not shown.

b. EMSA was performed with the indicated Rel proteins with 2 μg of antibodies against C/EBP, p50, and c-Rel (lanes; 2, 4, 6, and 8) and without antibodies (lanes 1, 3, 5, and 7). Antibodies were added before addition of the labeled probes.

c. A graph showing the intensity of C/EBP\(\beta\)-DNA complex versus Rel protein concentration (●; p50, ■; p52, ▲; c-Rel, and x; BSA). The band intensity was measured using ImageQuant Software.
Figure 10: c-Rel(1-300) but not p50 or p52 enhanced C/EBPβ DNA binding on the CRP promoter.

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C/EBPβ +

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b.

C/EBPβ (50 nM) + + + + + + +
p50 (30 nM) + + +
c-Rel (1.5 μM) + + +
α-C/EBP (2 μg) + + +
α-p50 (2 μg) +
α-c-Rel (2 μg) +

C/EBPβ (50 nM) + + + + + + +
p50 (30 nM) + + +
c-Rel (1.5 μM) + + +
α-C/EBP (2 μg) + + +
α-p50 (2 μg) +
α-c-Rel (2 μg) +
Figure 11: c-Rel\textsubscript{(1-300)} increased $K_{app}$ of C/EBP\textbeta for CRP-C/EBP\textsubscript{(-53)} more than 10 fold.

EMSA using increasing amounts of C/EBP\textbeta alone or with c-Rel\textsubscript{(1-300)} (1.5\textmu M) with a fixed concentration (1nM) of the CRP promoter oligo as the radiolabeled probe. The C/EBP\textbeta-DNA complex and free double-stranded DNA are indicated with arrows. DNA-protein complexes were separated by 6 % non-denaturing PAGE, and the results were analyzed using ImageQuant software. $K_{app}$ was calculated using Sigma Plot (Hill equation) as described in the methods.
Figure 11: c-Rel(1-300) increased Kapp of C/EBPβ for CRP-C/EBP(-53) more than 10 fold.
Since c-Rel<sub>(1-300)</sub> was not detected in the C/EBPβ-DNA complexes by EMSA analysis, the interaction between c-Rel<sub>(1-300)</sub> and C/EBPβ was examined by immunoprecipitation followed by Western blotting (Figure 12). The inputs of c-Rel<sub>(1-300)</sub> and C/EBPβ (250ng each) yielded strong signals (Figure 12, lane 1). No signal was detected in reactions containing only c-Rel<sub>(1-300)</sub> or C/EBPβ with protein A agarose, indicating that neither protein interacts with protein A agarose (Figure 12, lanes 2 and 5). Antibodies to C/EBPβ did not bind to c-Rel<sub>(1-300)</sub>, and antibodies to c-Rel did not bind to C/EBPβ, indicating that antibodies employed were specific (Figure 12, lanes 3 and 6).

When C/EBPβ was immunoprecipitated by an anti-C/EBP antibody, c-Rel<sub>(1-300)</sub> was co-immunoprecipitated (Figure 12, lane 4) and when c-Rel<sub>(1-300)</sub> was immunoprecipitated by an anti-c-Rel antibody, C/EBPβ was coimmunoprecipitated (Figure 12, lane 7).

Next, we determined whether c-Rel<sub>(1-300)</sub> required the CRP-κB<sub>(43)</sub> site to exert its effect on C/EBPβ binding activity on the CRP promoter. We employed a wild type CRP promoter oligo (-63 to -38) and the same CRP oligo with a mutation in the κB site (CRP-κB<sub>m</sub>) (Figure 13). Increasing the amount of c-Rel<sub>(1-300)</sub> from 0.15 to 1.5 μM increased the binding of C/EBPβ on both oligos (Figure 13, lanes 1-4). An antibody to C/EBPβ completely supershifted the C/EBPβ band (Fig. 13, lane 5) while a c-Rel antibody had no effect on C/EBPβ DNA binding activity to either oligo (Figure 13, lane 6). Competition with 100 fold excess of an unlabeled oligo containing a consensus c-Rel binding site (CD28RE) dramatically abolished the enhanced C/EBPβ binding to the CRP oligo by c-Rel in both wild type and mutant CRP oligos (Figure 13, lane 7). In contrast, competition with a mutant CD28RE had only a modest effect (Figure 13, compare lane 3 and 8).
These results suggest that the CRP-κB(-43) sequence is not required for the enhancing effect of c-Rel on C/EBPβ binding to the CRP promoter.

Previously we demonstrated that overexpressed full length c-Rel (cRelFL) could transactivate the CRP promoter construct in the presence of overexpressed C/EBPβ (Agrawal et al., 2003b). Here we examined the ability of overexpressed c-Rel(1-300), which lacks the transcription activation domain, to similarly transactivate the CRP promoter (Figure 14). Overexpressed c-Rel(1-300) alone could not transactivate the CRP promoter (Figure 14, lane 3). However, overexpressing both c-Rel(1-300) and C/EBPβ enhanced the induction of the CRP promoter compared to overexpression of C/EBPβ alone (Figure 14, lanes 4 & 2). The reporter construct alone did not produce a signal (Figure 14, lane 1), and the reporter, as expected, responded to cytokines as a positive control (Figure 14, compare lane 1 with lane 5). We conclude that c-Rel does not require its transcription activation domain to enhance C/EBPβ induction of the CRP promoter.
**Figure 12: c-Rel<sub>(1-300)</sub> physically interacts with C/EBPβ in solution.**

Immunoprecipitation using 250 ng of c-Rel<sub>(1-300)</sub> and C/EBPβ. Proteins were precipitated using polyclonal antibodies against either C/EBPβ (lanes 3 and 4) or c-Rel (lanes 6 and 7) as described in *Materials and Methods*. As a control, C/EBPβ or c-Rel<sub>(1-300)</sub> was incubated with protein A-agarose (lanes 2 and 5). The precipitated proteins were resolved on a 10 % SDS PAGE and transferred to a PVDF membrane. The protein bands were detected using HRP-linked anti-rabbit antibodies and ECL and imaged using VersaDoc.
Figure 12: c-Rel(1-300) physically interacts with C/EBPβ in solution.

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</table>
Figure 13: The CRP-κB(-43) site is not required for the enhancing effect of c-Rel<sub>(1-300)</sub> on C/EBPβ binding to the CRP promoter.

EMSA using C/EBPβ, c-Rel<sub>(1-300)</sub> with the CRP promoter or CRPm(κB) as radiolabeled oligos. The C/EBP concentration was fixed at 50 nM (lanes 1 – 8) and the c-Rel concentration was increased as indicated. 2 μg of Abs (lanes 5 and 6) or competitor oligo (7 and 8) were incubated before the addition of radiolabeled oligos. DNA-protein complexes were separated by 6 % nondenaturing PAGE, and the results analyzed by phosphorimager. The mobility of free probe is not shown.
Figure 13: The CRP-κB(-43) site is not required for the enhancing effect of c-Rel(1-300) on C/EBPβ binding to the CRP promoter.
**Figure 14:** Overexpressed c-Rel(1-300) in combination with overexpressed C/EBPβ transactivates the CRP promoter.

Transient co-transfection of human Hep3B cells with c-Rel(1-300) (15 ng) and C/EBPβ (5 ng) and the -256/+9 CRP promoter luciferase reporter (1000ng). The cells were lysed and luciferase activity was measured using a luminometer. CRP promoter/luc reporter without any inducing factors (lane 1). C/EBPβ expression plasmid (5ng) alone (lane 2). c-Rel(1-300) expression plasmid (15ng) alone (lane 3). c-Rel(1-300) (15ng) and C/EBPβ (5ng) expression plasmids (lane 4). IL-1β (1ng/mL) + IL-6 (10ng/mL) alone (lane 5). This figure is representative of three experiments.
Figure 14: Overexpressed c-Rel(1-300) in combination with overexpressed C/EBPβ transactivates the CRP promoter.
DISCUSSION

Our major finding is that c-Rel(1-300), but not p50 or p52, enhanced C/EBPβ binding to a relatively weak C/EBP site on the CRP promoter (CRP-C/EBPβ(-53)), increasing the $K_{\text{app}}$ of C/EBPβ from $> 200$ nM to 40 nM. The c-Rel(1-300) effect did not require the CRP-κB(-43) site, and c-Rel(1-300) was not detected in the EMSA complexes observed on the CRP promoter oligo but could physically interact with C/EBPβ in solution. In addition, overexpressed c-Rel(1-300), despite lacking the transcription activation domain, was able to transactivate the CRP promoter, but only in the presence of C/EBPβ. Other Rel proteins, p50 homodimers, or p50/c-Rel heterodimers did not facilitate C/EBPβ DNA binding. These results are consistent with a model in which c-Rel upregulates CRP expression without binding directly to the CRP promoter, but rather by binding to C/EBPβ, and enhancing the binding of C/EBPβ to the CRP promoter. The transactivation domain of c-Rel is not necessary for this effect, which depends on protein:protein contacts with C/EBPβ.

Several reports have suggested that physical interactions between C/EBP and Rel proteins are responsible for transcriptional synergy of acute phase genes in response to both IL-6 and IL-1 (Agrawal et al., 2001a; Lee et al., 1996; Li and Liao, 1992; Li and Stashenko, 1992; Ray et al., 1995; Xia et al., 1997). Usually p65 has been involved, as shown for the serum amyloid A 1 promoter (Betts et al., 1993). However, the molecular mechanism by which these physical interactions exert transcriptional synergy has not been elucidated. Our and others’ previous studies have established that the CRP-C/EBPβ(-53) site is critical for IL-6 + IL-1 induction of CRP expression (Cha-Molstad et al., 2000; Li and Goldman, 1996). However, this CRP-C/EBPβ(-53) site binds C/EBPβ poorly,
raising the possibility that the ability of this site to bind C/EBPβ may need to be enhanced by the effect of other transcription factors. We propose that c-Rel, by facilitating or stabilizing C/EBP binding to the CRP promoter, plays such a role, and thus contributes to CRP expression.

Supporting this conclusion is our finding that antibodies against full length c-Rel supershifted C/EBPβ-containing complexes in nuclear extracts from cytokine treated Hep3B cells, even though we were unable to detect c-Rel(1-300) in EMSA complexes. In EMSA with IL-6 treated Hep 3B nuclear extracts using an oligo derived from the CRP promoter, we previously found that C/EBPβ-DNA complexes are supershifted by antibodies to c-Rel. This finding indicates that c-Rel binds to C/EBPβ on the CRP promoter (Agrawal et al., 2003b). Further support for this conclusion is our finding of physical interaction between c-Rel(1-300) and C/EBPβ in solution, detected by co-immunoprecipitation. These findings suggest that c-Rel(1-300) physically interacts with C/EBPβ, most likely through the Rel-bZIP domain (Schrem et al., 2004), but that the interaction requires full-length c-Rel or interactions with other proteins to be sufficiently stable to survive the electrophoresis conditions employed. Others have reported similar situations in which one transcription factor affects the affinity of DNA binding of another transcription factor. Banerjee et. al. reported a case in which the coactivator PC4 enhanced the binding of p53 to its cognate site in vitro (Banerjee et al., 2004). Chakravarty et. al. also reported a similar instance in which USA enhanced the binding of SREBP-1c for the PEPCK-C gene promoter (Chakravarty et al., 2004).

A mechanism has been proposed by which the DNA binding capacity of bZip proteins is enhanced by increased dimerization. Hepatitis B virus protein pX acts as a
chaperone, aiding in the dimerization of several bZIP proteins, with consequent increased binding of the these proteins for their DNA binding sites (Perini et al., 1999). pX did not alter DNA binding at high bZIP concentrations and its effect was sequence context dependent. In our system, c-Rel does not appear to be working through this mechanism. First, c-Rel was effective in increasing C/EBPβ binding to the CRP promoter even at high C/EBPβ concentrations. Second, recombinant C/EBPβ bound the consensus C/EBP oligo well in the absence of c-Rel even at low concentrations of C/EBPβ, indicating that it was already dimeric. Overall, we postulate that interaction between c-Rel(1-300) and C/EBPβ alters the conformation of C/EBPβ, resulting in enhanced binding to the weak CRP-C/EBP(-53) binding site.

Differences between the effect of c-Rel and of another Rel protein, p50, also shown to influence CRP expression, are of interest. In contrast to c-Rel(1-300), p50 bound to the CRP-κB(-43) site separately from C/EBPβ and showed no effect on C/EBPβ binding to DNA. No effect of p50 in C/EBPβ binding to the CRP promoter is somewhat surprising because antiserum against p50 has previously shown to diminish the C/EBPβ binding to the CRP promoter (Cha-Molstad et al., 2000). The CRP-κB(-43) site is a non-consensus κB sequence but is nonetheless a relatively good binding site for p50-containing dimers. Despite the relatively high affinity of recombinant p50 for the CRP-κB(-43) site, EMSAs using IL-6-induced Hep 3B nuclear extracts showed C/EBPβ-containing complexes but no separate bands containing Rel proteins bound directly to the CRP proximal promoter (Agrawal et al., 2001a). One possible explanation for the difference between results with nuclear extract and recombinant proteins is the position of the CRP-κB(-43) site, which is very close to the position occupied by TFIID. The latter
extends to around -45 (Gilmour et al., 1990; Sawadogo and Roeder, 1985; Van Dyke et al., 1988). p50 and p52 homodimers have also been shown to bind the κb site overlapping the initiator region of the HIV-1 promoter long term repeat and enhanced the binding of TFII-I to the initiation region (Montano et al., 1996). We postulate that p50 affects C/EBPβ indirectly, following binding to the CRP-κB(-43) site, by recruiting other transcription factors and TFIID or other general transcription factors, resulting in facilitation of C/EBPβ DNA binding or interaction between C/EBPβ and the general transcription factors. Alternatively, p50 may interact with C/EBPβ, changing its conformation, which would facilitate interactions between C/EBPβ and other factors.

c-Rel has been implicated in maturational and selection events during the T-cell double positive to single positive transition (Sen et al., 1995), B-cell survival from antigen receptor-mediated apoptosis (Owyang et al., 2001), transformation of a variety of hematopoietic cells (Gilmore et al., 2001), and regulation of IL-12 expression (Grumont et al., 2001). Our findings imply a novel role of c-Rel in CRP expression in facilitating DNA binding of C/EBPβ on the CRP promoter by increasing the affinity of the latter for a weak binding site. The effect of c-Rel(1-300) was most likely mediated by a Rel-bZip interaction (Schrem et al., 2004) and did not require c-Rel(1-300) DNA binding on the CRP promoter.
CHAPTER 3: BINDING OF C/EBPβ TO THE CRP PROMOTER IN HEP3B CELLS IS ASSOCIATED WITH TRANSCRIPTION OF CRP mRNA

INTRODUCTION

C-reactive protein (CRP) is a major human acute phase protein largely synthesized in hepatocytes following inflammatory stimuli. The serum concentration of CRP can rapidly increase up to 1000-fold after severe inflammatory stimuli (Black et al., 2004; Volanakis, 2001). In the human hepatoma cell line Hep3B, CRP gene expression is only modestly induced by IL-6, while IL-1β alone has no effect. Together these cytokines act synergistically to markedly induce CRP gene expression (Ganapathi et al., 1991). Cytokine induction of CRP gene expression occurs mainly at the transcriptional level. Transcription factors C/EBP β/δ, STAT3, Rel p50, and c-Rel participate in CRP gene expression following cytokine stimuli with C/EBP β/δ binding to two sites centered at -53 and -219 (Figure 15) (Ganter et al., 1989; Jiang et al., 1995a; Li et al., 1990; Zhang et al., 1995). HNF-1α also binds to two sites but this transcription factor is constitutively present and not activated by cytokines.

In Hep3B hepatoma cells the CRP-C/EBP(-53) site is essential for CRP expression (Cha-Molstad et al., 2000; Li and Goldman, 1996; Majello et al., 1990), although the sequence of this binding site differs from the consensus C/EBP binding sequence and, in vitro, C/EBPβ binds relatively poorly to this CRP-C/EBP(-53) site. This raises the possibility that other transcription factors may be required to facilitate C/EBP DNA binding. In fact, we have shown that c-Rel is such a protein. It does not bind DNA directly, but rather enhances CRP expression by binding to C/EBPβ, with consequent
enhancement of C/EBPβ binding to the CRP-C/EBPβ (-53) site on the promoter (Agrawal et al., 2003b; Cha-Molstad et al., 2007). In addition, p50 has been found to bind to a nonconsensus κB site (-43) and both p50 and C/EBPβ (-53) are required for full induction of CRP expression by IL-6 and IL-1β (Agrawal et al., 2001a; Agrawal et al., 2003a; Agrawal et al., 2003b; Cha-Molstad et al., 2000). Previous studies from our lab also showed that overexpressed STAT3 was able to transactivate the CRP promoter in response to IL-6 stimulation, that it activated the endogenous CRP gene in response to IL-6, and that STAT3 bound the CRP promoter in response to IL-6 (Zhang et al., 1996a).

Here, we employed chromatin immunoprecipitation (ChIP) assays in human Hepatoma 3B cells to confirm the involvement of the transcription factors identified in previous in vitro studies and to determine the kinetics of transcription factor occupancy on the endogenous CRP promoter. Of the implicated transcription factors, only binding of C/EBPβ to the endogenous CRP promoter markedly increased following cytokine exposure. C/EBPβ, STAT3, p50, c-Rel, and TBP were all found on the CRP promoter in both the absence and presence of cytokines, even though CRP mRNA accumulation was not measurable prior to cytokine treatment. CRP mRNA accumulation began to increase by 3 h after exposure of cells to IL-1β + IL-6, peaking at 12 h with secondary peaks at 18 h and 24 h. These results suggested that the CRP promoter has a low level of transcription factor occupancy in the absence of cytokine exposure, but that CRP mRNA is produced only after C/EBPβ binds the promoter. We also found that c-Rel and TBP appeared to occupy the promoter in parallel and may be responsible for modulating CRP expression, and that the pattern of CRP mRNA accumulation paralleled the pattern of binding of c-Rel and TBP to the CRP promoter.
Figure 15: Current model of the proximal CRP promoter and relevant transcription factors.

The model of transcription factor assembly on the CRP promoter is based on EMSA and luciferase assay data. The relative sites of binding of transcription factors C/EBPβ, STAT3, p50, c-Rel, and HNF-1α are shown.
Figure 15: Current model of the proximal CRP promoter and relevant transcription factors.
MATERIALS AND METHODS

Materials

Human recombinant cytokines IL-6 (206-IL) and IL-1β (201-LB) were purchased from R&D Systems (Minneapolis, MN). Synthetic oligonucleotides were obtained from Operon Biotechnologies, Inc. (Huntsville, AL). Rabbit polyclonal antibodies against C/EBPβ (sc-150), p50 (sc-7178), STAT3 (sc-482), c-Rel (sc-70), TFIID/TBP (sc-204), and TFIIE (sc-237) proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP conjugated anti-rabbit antibodies (12-348) were purchased from Millipore (Billerica, MA). SYBR Green (1988131) was purchased from Roche (Indianapolis, IN). Fluorescein (170-8780) and BioRad DC Protein Assay (Bradford method) reagents (500-0113, 500-0114, 500-0115) were purchased from Bio-Rad (Hercules, CA). Protein A agarose beads were purchased from Repligen (IPA-400HC) (Waltham, MA) and Upstate (16-157) (Lake Placid, NY). Salmon sperm DNA (D-7656) was purchased from Sigma (St. Louis, MO). BSA (10921), RNase A (70194Z), Proteinase K solution (76225), Aprotinin (11388), Leupeptin (18413), AEBSF (11118), RT-PCR Master Mix (78370), and HotStart-It Taq Master Mix (71196) were purchased from USB (Cleveland, OH). RNeasy Mini Kit (74104) was purchased from Qiagen, Inc. (Valencia, CA). Immobilon transfer PVDF membranes were purchased from Millipore (Billerica, MA). SuperSignal West Pico Chemiluminescent reagents (34080) were purchased from Pierce Biotechnology (Rockford, IL). HyBlot CL autoradiography film was purchased from Denville Scientific, Inc. (Metuchen, NJ).
**Cell Culture and Cytokine Treatment**

Human hepatoma 3B (Hep3B) cells were kindly provided by Dr. G.J. Darlington (Baylor College of Medicine, Houston, TX) and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37° C in a humidified atmosphere containing 5% CO₂. The media was changed to fresh RPMI 1640 + 10% FBS the day before cytokine treatment. Immediately prior to cytokine treatment the media was changed to RPMI 1640 without FBS and then treated with IL-6 (10 ng/mL) and IL-1β (1 ng/mL) for the indicated times.

**Chromatin Immunoprecipitation Assays**

A streamlined protocol for the ChIP assay developed at USB (Cleveland, OH) was used. Briefly, cells were cross-linked with 1% formaldehyde for 20 min at room temperature. The cross-linking reaction was stopped by the addition of glycine (0.125 M) for 5 min at room temperature. Prior to sonication, cell pellets were resuspended in ChIP lysis buffer (50 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-100, 0.1% DOC, 0.1% SDS) with protease inhibitors (Aprotinin 1mg/mL, Leupeptin 1 mg/mL, and AEBSF 100mM) to a volume of 1.0 ml – 1.5 mL containing 2x10⁴ cells/μL. Chromatin in the lysate was sonicated using a Fisher Scientific 550 Sonic Dismembrator for 8 cycles (Amplitude 4; 0.5 s on, 0.5 s off x 1 min) on ice with 2 min between cycles. DNA fragment sizes of 300-800 bp were confirmed by agarose gel electrophoresis.

Packed cell volume was used to estimate cell number and 2x10⁶ cells were used in each ChIP assay. Either 30 μL of a 50% slurry of Upstate protein A agarose or 50 μL 33% slurry of Repligen protein A agarose were used during the pre-clear and capture
steps. Upstate protein A agarose came as a 50% slurry pre-blocked with salmon sperm DNA and BSA. Repligen protein A agarose beads were washed two times in 1x PBS and two times in 1x TE, pH 8.0. Next, 5 mg BSA, 500 μg salmon sperm DNA, 0.075% sodium azide, and 835 1x TE, pH 8.0 were added to 500 μL packed protein A agarose for a final volume of 1.5 mL (33% slurry protein A agarose) and rotated overnight at 4°C.

The chromatin was pre-cleared for 1 h at 4°C, and 0-4 μg of antibody was added to the pre-cleared chromatin and rocked overnight at 4°C. Immune complexes were captured with protein A agarose for 1 h at 4°C, washed for 10 min at 4°C with 1 x 1 mL ChIP lysis buffer, high salt buffer (50 mM Tris pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton-100, 0.1% DOC, 0.1% SDS), and lithium salt buffer (20 mM Tris pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 0.5% DOC), and 2 x 1 mL 1x TE (pH 7.5). Immune complexes were eluted from the protein A agarose with 2 x 150 μL elution buffer (10 mM Tris pH 8.0, 5 mM EDTA, 1% SDS) at room temperature for 20 minutes. Cross-links were reversed overnight at 65°C in 0.2 M NaCl. RNA was digested with 10 μg RNase A at 37°C for 30 min and protein was digested with 50 μg Proteinase K at 45°C for 90 min. DNA was purified using phenol extraction and ethanol precipitation.

Purified DNA was re-suspended in 30 μL of 10 mM Tris-HCl pH 8.5. Total input DNA was diluted 1:10 for PCR. Each 25 μL PCR contained 2 μL DNA, 200 nM of each primer, and 12.5 μL of Hot Start-It (2x). Real-time PCR (25 μL) also contained 10 nM fluorescein and 0.2X SYBR green. The PCR protocol used was 95°C x 2 min followed by 30 cycles of 95°C x 30 s, 60°C x 30 s, and 72°C x 60 s; the final extension was 72°C for 5 min. Real-time PCR was performed using a Bio-Rad I-cycler. The real-time PCR protocol used was 95°C x 2 min followed by 40 cycles of 95°C x 30 s, 60°C x 30
s, and 72° C x 60 s; the final extension was 72° C for 5 min. Primers used to amplify the CRP proximal promoter region (-118 to +115) were 5’-CTCTTCCCAGCTCTGACACCT-3’ and 5’-AACAGCTTTCTCCATGGTCACGTC-3’.

**Data Analysis**

Each real-time PCR was performed in duplicate and cycle threshold (Ct) numbers were averaged. The background (mock, no antibody IP) Ct was subtracted from the ChIP Ct. After subtracting the background, the ChIP cycle thresholds were normalized to the Input cycle threshold. The resulting ΔCt represented the number of cycles above background for each ChIP. Each ΔCt represents a two fold change in signal; thus fold change = $2^{\Delta Ct}$. For graphic results the y-axis is expressed as fold change above background normalized to the input signal plotted against time of cytokine treatment.

**RNA Isolation and RT-PCR**

RNA was isolated using the Qiagen RNeasy kit following the manufacturer’s protocol. Each 25 μL RT-PCR contained 200 ng RNA, 200 nM of each primer, and 12.5 μL of RT-PCR Master Mix (2x). Primers used to amplify CRP cDNA for RT-PCR were 5’-TGGCCAGACAGACATGTCGAG-3’ and 5’-GGCTTCCCATCTACCCAGAAC-3’; the sense primer crosses the exon1/exon2 junction. Primers flanking the intron used to amplify β-Actin cDNA for RT-PCR were 5’-ACCCTGAAGTACCCCATCGAG-3’ and 5’-AGGCCGTACAGGGATAGCACAG-3’. The RT-PCR protocol used was 50° C x 30 min, 95° C x 2 min followed by 30 cycles of 95° C x 30 s, 60° C x 30 s, and 72° C x 60 s;
the final extension was 72°C for 5 min. The amplified cDNA was run on a 1.5% agarose gel and bands were quantified using ImageQuant software (GE Healthcare, Piscataway, NJ). CRP cDNA band intensities were normalized to β-actin cDNA band intensities.

Whole Cell Extraction, SDS-PAGE and ImmunoBlot

Whole cell extracts using Hep3B cells were made using RIPA buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.5% Triton X 100, 1X protease inhibitor cocktail added fresh). Cells were lysed with three freeze-thaw cycles and pipetting. Cell lysate protein concentration was measured using BioRad DC Protein Assay reagents following the manufacture’s protocol. Proteins were separated using an SDS-PAGE with a 4% stacking gel and a 6% separating gel. Each lane contained 17 μg of protein. Proteins were transferred to a PVDF membrane at 24 V, 1 A for 45 minutes at room temperature. Membranes were blocked in 5% milk in TTBS (0.1% v/v Tween 20 + TBS) at 4°C overnight. Primary antibodies were diluted 1:200 (C/EBPβ and c-Rel) or 1:1000 (TBP and TFIIE) in 5% milk + TTBS and blots were incubated at room temperature for 2 h. HRP conjugated donkey anti-rabbit antibody was diluted 1:2000 and blots were incubated at room temperature for 1 h. Chemiluminescence was generated using Pierce SuperSignal ECL reagents following the manufacture’s protocol. Proteins were imaged using HyBlot CL autoradiography film and Kodak M35A X-OMAT processor.
RESULTS

We performed chromatin immunoprecipitation (ChIP) assays and used antibodies against C/EBPβ, p50, STAT3, c-Rel, and TBP to follow their binding to the endogenous CRP promoter. A representative gel of the resulting PCR products (Figure 16) shows faint bands for C/EBPβ, p50, STAT3, c-Rel, and TBP at baseline, indicating that these transcription factors occupied the promoter in the absence of cytokines. All of these transcription factors increased their binding to the endogenous promoter following cytokine stimulation. The intensity of the PCR products in the C/EBPβ samples increased markedly after cytokine treatment, while modestly increased occupancy of STAT3, p50, c-Rel and TBP was also detected. These data demonstrate that the major transcription factors in our model of regulation of CRP gene expression occupy the CRP promoter in Hep3B cells, and that occupancy is enhance in response to cytokines.

To quantify transcription factor promoter occupancy, we performed real-time PCR on DNA isolated following ChIP assays from Hep3B cells treated with IL-6 + IL1β. Zero time values were determined in each case. Subsequently, three time courses were followed, ½ - 6 h, 8-16 h and 12-36 h. Increased C/EBPβ promoter occupancy was detected as early as 2 h after cytokine exposure and increased markedly through 10 h – 12 h before slowly falling through 36 h (Figure 17a-c). At its peak, the amount of CRP promoter DNA detected in the C/EBPβ immunoprecipitates was five-fold greater than at baseline. Rel p50 occupied the CRP promoter in the absence of cytokines and showed a modest elevation in promoter occupancy after 10 h – 12 h of cytokine exposure (Figure 18a-c). In contrast, cytokine exposure caused a slow two-fold increase in STAT3 occupancy through 10 h of cytokine exposure with a dip in occupancy after 14 – 18 h.

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(Figure 19a-c). These data suggest that C/EBPβ, p50, and STAT3 occupy the CRP promoter in the absence of cytokines and that C/EBPβ occupancy markedly increases, while p50 and STAT3 experience modest elevations after cytokine exposure.
**Figure 16: C/EBPβ, NF-κB p50, STAT3, c-Rel, and TBP bind the endogenous CRP promoter.**

Agarose gel of a ChIP assay performed on Hep3B cells treated with cytokines IL-1β & IL-6 for 0 h - 15 h, as described in Methods. Antibodies to C/EBPβ, NF-κB p50, STAT3, c-Rel, and TBP were used in the assays with primers flanking the CRP proximal promoter (-118 to +115). The Mock is a no antibody control. The Input is a 1:10 dilution of total chromatin after sonication and preclearing. C/EBPβ, NF-κB p50, and Input are shown in the first row. STAT3, c-Rel, and Mock are shown in the second row, and TBP is shown in the third row. This figure is representative of four experiments.
Figure 16: C/EBPβ, NF-κB p50, STAT3, c-Rel, and TBP bind the endogenous CRP promoter.
Figure 17: C/EBPβ binds the endogenous CRP promoter in response to cytokines.

Real-time PCR of ChIP assay performed on Hep3B cells treated with cytokines IL-1β & IL-6. Zero values were determined in each case. Subsequently three time courses were followed (a) ½ h – 6 h (n = 3) (b) 8 h – 16 h (n = 4) and (c) 12 h – 36 h (n = 4). The primer set was as described in figure 16. Graphs show C/EBPβ occupancy expressed as fold change after subtraction of Mock and normalization to Input signal (see Methods). Each graph is an average of 3-4 experiments, each done in duplicate. The error bars represent the standard deviation.
Figure 17: C/EBP\(\beta\) binds the endogenous CRP promoter in response to cytokines.

a. Average C/EBP\(\beta\) Occupancy on the CRP Promoter

b. Fold Change Over Mock

c. Time of Cytokine Treatment (h)
Figure 18: p50 occupancy of the CRP promoter remains nearly constant in the presence or absence of cytokines.

Real-time PCR of ChIP assay performed on Hep3B cells treated with cytokines IL-1β & IL-6. Zero values were determined in each case. Subsequently three time courses were followed (a) ½ h – 6 h (n = 3) (b) 8 h – 16 h (n = 4) and (c) 12 h – 36 h (n = 4). The primer set was as described in figure 16. Graphs show NFκB-p50 occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Methods). Each graph is an average of 3-4 experiments, each done in duplicate. The error bars represent the standard deviation.
Figure 18: p50 occupancy of the CRP promoter remains nearly constant in the presence or absence of cytokines.

a. Average p50 Occupancy on the CRP Promoter

b.

c.
Figure 19: STAT3 occupancy of the CRP promoter rises modestly in response to cytokines.

Real-time PCR of ChIP assay performed on Hep3B cells treated with cytokines IL-1β & IL-6. Zero values were determined in each case. Subsequently three time courses were followed (a) ½ h – 6 h (n = 3) (b) 8 h – 16 h (n = 4) and (c) 12 h – 36 h (n = 4). The primer set was as described in figure 16. Graphs show STAT3 occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Methods). Each graph is an average of 3-4 experiments, each done in duplicate. The error bars represent the standard deviation.
Figure 19: STAT3 occupancy of the CRP promoter rises modestly in response to cytokines.

a. Average STAT3 Occupancy on the CRP Promoter

b. 

c. 

Time of Cytokine Treatment (h) 

Fold Change Over Mock 

STAT3
CRP promoter occupancy by c-Rel and TBP changed in parallel, in a roughly biphasic pattern. In the two shorter time courses studied, CRP promoter occupancy by c-Rel and TBP peaked after 10 h of cytokine exposure, followed by a drop in occupancy at 12 h, and a second peak after 14 h (Figure 20a-b). Over a longer time course, occupancy of c-Rel and TBP peaked after 12 h of cytokine exposure, dropped from 18 h – 24 h, and peaked again after 30 h (Figure 20c). Although changes in c-Rel and TBP occupancy of the promoter occurred in parallel in every assay, the pattern of occupancy was not always the same from one experiment to the next, accounting for the large error bars in these panels. In one assay, c-Rel and TBP occupancy peaked 12 h – 18 h after exposure to cytokines, followed by a drop in occupancy through 36 h (Figure 20d). In yet another assay over the same time course, occupancy of both transcription factors dropped to a low through 24 h and peaked after 30 h of cytokine exposure (Figure 20e). Overall, these data suggest that cytokine exposure led to a biphasic occupancy pattern of c-Rel and TBP. We did not observe parallel changes in occupancy with any of the other transcription factor pairs used in these studies.

To determine the relationship between gene expression and transcription factor occupancy, we measured CRP mRNA accumulation by RT-PCR in Hep3B cells treated with cytokines (IL-1β + IL-6) over a 24 h period. CRP mRNA accumulation increased markedly from 3 h to 6 h after cytokine treatment (Figure 21a-b) and continued to increase through 12 h, before dipping transiently at 15 h and at 21 h. Since previous studies (Kushner et al., 1995; Zhang et al., 1995) determined the half-life of CRP mRNA in Hep3B to be ~3 h, the dips in mRNA accumulation are likely the result of dips in mRNA transcription. Overall, the mRNA accumulation pattern was similar to the pattern
of C/EBPβ occupancy on the CRP promoter, indicating that CRP mRNA accumulates only after binding of C/EBPβ to the CRP promoter. The peaks and valleys repeatedly seen in the mRNA accumulation after 12 h – 24 h of cytokine exposure are suggestive of the biphasic pattern observed for c-Rel and TBP occupancy of the CRP promoter.
Figure 20: c-Rel and TBP occupy the CRP promoter in parallel.

Real-time PCR of ChIP assay performed on Hep3B cells treated with cytokines IL-1β & IL-6. Zero values were determined in each case. Subsequently three time courses were followed (a) ½ h – 6 h (n = 3) (b) 8 h – 16 h (n = 4) and (c) 12 h – 36 h (n = 4). The primer set was as described in figure 16. Graphs show c-Rel (solid line, triangle data points) and TBP (dashed line, square data points) occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Methods). (a) – (c) Average of 3-4 experiments, each done in duplicate. The error bars represent the standard deviation. (d) – (e) Profiles from individual ChIP experiments of c-Rel and TBP promoter occupancy.
Figure 20 (a-c): c-Rel and TBP occupy the CRP promoter in parallel.
Figure 20 (d-e): c-Rel and TBP occupy the CRP promoter in parallel.

d. 

c-Rel and TBP Occupancy of the CRP Promoter

![Graph showing the fold change over mock for c-Rel and TBP over time.]

Time of Cytokine Treatment (h)  
0 6 12 18 24 30 36

Fold Change Over Mock  
0.0 2.0 4.0 6.0 8.0 10.0 12.0

c-Rel  
TBP

e. 

c-Rel and TBP Occupancy of the CRP Promoter

![Graph showing the fold change over mock for c-Rel and TBP over time.]

Time of Cytokine Treatment (h)  
0 6 12 18 24 30 36

Fold Change Over Mock  
0.0 5.0 10.0 15.0 20.0

c-Rel  
TBP

99
Figure 21: CRP mRNA accumulates in response to cytokines.

(a) Representative agarose gel of RT-PCR performed on Hep3B cells treated with cytokines IL-1β & IL-6 for the indicated times (hours). CRP mRNA levels are shown in the top row and β-Actin mRNA levels are shown in the bottom row. (b) Average quantification of band intensity measured using ImageQuant of CRP mRNA normalized to β-Actin mRNA (n = 4). Error bars represent the standard deviation.
Figure 21: CRP mRNA accumulates in response to cytokines.

(a)

CRP mRNA (0-24 hr)

(b)

Average Normalized Band Intensity vs. Time of Cytokine Treatment (h)

Average Normalized Band Intensity
We employed Western blotting to measure protein accumulation of C/EBPβ, c-Rel, and TBP in whole cell extracts of Hep3B cells treated with IL-1β + IL-6 over a 6 h period to determine whether new synthesis of these transcription factors occurs with cytokine induction. Three bands were present for C/EBPβ: full-length, liver-enriched activator protein (LAP), and liver-enriched inhibitory protein (LIP) (Figure 22a). Full-length and LAP protein levels were undetectable at 0 h, but LIP was present. Full-length and LAP protein levels increased substantially from 2 h through 6 h. A doublet was found for c-Rel, most likely representing phosphorylated and unphosphorylated forms (Figure 22b). Protein levels for c-Rel increased from 0 h to 2 h and then remained steady through 6 h. TBP protein levels remained constant from 0 h to 6 h of exposure to cytokines (Figure 22b). These data indicate that the amount of C/EBPβ and c-Rel protein increased in response to cytokine treatment.
Figure 22: C/EBPβ and c-Rel protein levels increase in response to cytokine treatment.

Immunoblot of Hep3B whole cell extracts following cytokine (IL-1β & IL-6) treatment for the indicated times (hours). 17 μg of whole cell extract was loaded into each lane. Antibodies to C/EBPβ, TFII3, c-Rel, and TBP were used in the immunoblots and detected as described in Methods. (a) C/EBPβ protein accumulation with an antibody against TFIIIE as a loading control. (b) c-Rel protein accumulation with an antibody against TBP/TFIID as a loading control. This figure is representative of three experiments.
Figure 22: C/EBPβ and c-Rel protein levels increase in response to cytokine treatment.

a. Time of Cytokine Treatment

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b. Time of Cytokine Treatment

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DISCUSSION

Our findings that IL-1β + IL-6 enhanced binding of C/EBPβ, STAT3, p50, c-Rel, and TBP to the CRP promoter in Hep3B cells confirms that these transcription factors participate in cytokine induced expression of CRP in its native context. Of the transcription factors we studied, only binding of C/EBPβ to the CRP promoter markedly increased following cytokine exposure, suggesting that its binding plays an important role in the overall pattern of CRP mRNA accumulation observed. C/EBPβ occupancy was also associated with increased C/EBPβ accumulation. In addition, the CRP mRNA accumulation had several peaks and valleys, similar to the biphasic, parallel patterns of CRP promoter occupancy observed with c-Rel and TBP. In previous studies we found that c-Rel enhanced the binding of C/EBPβ to the CRP-C/EBP(-53) site on the CRP promoter without binding the promoter itself (Cha-Molstad et al., 2007). Our findings in these studies indicate that C/EBPβ and c-Rel kinetics of binding to the proximal CRP promoter were not identical and suggest that the interaction between c-Rel and C/EBPβ is transient. We speculate that c-Rel binds C/EBPβ, helping it bind to the promoter, and then releasing C/EBPβ afterwards. We propose that c-Rel plays a role in modulating CRP expression, consistent with a model in which c-Rel enhances C/EBPβ binding.

Low levels of C/EBPβ, STAT3, p50, c-Rel, and TBP were found on the CRP promoter in the absence of cytokines, although CRP mRNA accumulation was not measurable before cytokine treatment. Transcriptional regulatory mechanisms generally fall into two models. In the first model, transcription is regulated at the transcription initiation step when the transcription factors bind the promoter and initiate transcription. In the second model transcription is regulated after the initiation step and involves...
transcript elongation or stability. Recent work by the Young lab demonstrated that 75% of all protein encoding gene proximal promoters had Pol II bound and had trimethylation of lysine 4 of histone 3 (H3K4me3), hallmarks of initiation, even when mRNA was not being transcribed. They also found that most promoters in both embryonic stem cells and primary human liver cells had methylation marks (demethylation of cytosine in CG dinucleotides) and Pol II bound (Guenther et al., 2007). Our finding that C/EBPβ, STAT3, p50, c-Rel, and TBP all bound the CRP promoter in the absence of cytokines suggests that CRP employs the second model of gene transcription regulation. Our data suggest that C/EBP is the necessary transcription factor for either completing transcription initiation or for elongation of the transcript. Low level transcription factor occupancy did not occur on every promoter; a control experiment in which we amplified the nephrin gene promoter in ChIP DNA isolated from Hep3B cells after cytokine exposure showed that the real-time PCR signal of several transcription factors was barely detectable above mock (data not shown).

We found that c-Rel and TBP occupied the promoter roughly in parallel and may be responsible for modulating CRP gene expression. c-Rel and TBP appeared to cycle on and off of the promoter throughout the time course studied with an apparent periodicity of 2-3 hours. The individual experimental variation in binding patterns for c-Rel and TBP resulted in the average occupancy to appear relatively flat and for the error bars to be large. We detected cycling without α-amanitin synchronization or instantaneous UV cross-linking prior to ChIP. Others have detected dynamic cycling of transcription factors using these methods (Hager et al., 2006; Nagaich et al., 2004; Reid et al., 2003); however, the cycling we detected appears to be in addition to the dynamic cycling
described by these groups. Cell cycle was not synchronized prior to cross-linking. However, because cells were ~90% confluent at the time of cross-linking most cells were no longer actively dividing. The lack of synchronization, cell cycle or promoter clearing, may explain why the periods of c-Rel and TBP occupancy varied from one ChIP experiment to the next. Despite the variations in the biphasic pattern observed for c-Rel and TBP occupancy between experiments, c-Rel and TBP occupancy repeatedly occurred in parallel and a biphasic pattern was always observed. We did not see parallel or repeated biphasic patterns of occupancy with any of the other transcription factors studied.

Our immunoblots showed that cytokines increased the amount of total cellular C/EBPβ and c-Rel. For C/EBPβ, only the inhibitory isoform LIP, a functional LAP antagonist (Alam et al., 1992; Luedde et al., 2004; Poli, 1998), was found in the absence of cytokines, while accumulation of all three isoforms increased within two hours of cytokine treatment. It is therefore likely that C/EBPβ occupancy of the CRP promoter detected in the absence of cytokines is the inhibitory form.

In the absence of cytokines, a doublet suggestive of phosphorylated and unphosphorylated c-Rel was detected, and the levels of both increased after cytokine exposure. Several studies have suggested that c-Rel is phosphorylated on its transactivation domain to increase transactivation activity (Sanchez-Valdepenas et al., 2006; Sanchez-Valdepenas et al., 2007; Yu et al., 2004). The finding that both phosphorylated and unphosphorylated c-Rel increased in response to cytokines may indicate that c-Rel has two functions in liver cells. Transcription of some genes requires that the transactivation domain be in its activated form (phosphorylated). For others,
such as CRP, c-Rel function does not require the transactivation domain. Our previous studies indicated that in vitro, the c-Rel transactivation domain was not required for it to enhance C/EBP binding to the CRP-C/EBP_{-53} element.

It should be noted that the C/EBPβ signal likely reflects occupancy at two sites at least, one centered at -219 and the other centered at -53 and that our ChIP experiments do not distinguish between the two. By transfection studies, it is the -53 site that is critical for CRP expression and is likely influenced by c-Rel (Agrawal et al., 2001a; Agrawal et al., 2003b; Cha-Molstad et al., 2007). Occupancy of p50 in the absence of cytokines may also occur at two p50 binding sites in the CRP promoter. Previous data indicated that p50 binds to a site overlapping C/EBPβ (CRP-κB_{-43}) in the presence of cytokines where it acts as an activator (Cha-Molstad et al., 2000). However, p50 may also bind a site overlapping the STAT3 site where it acts as a repressor in the absence of cytokines (A. Agrawal personal communication). Thus it was not unexpected to observe p50 occupancy in ChIP experiments in the absence of cytokines. Although STAT3 occupancy occurred later than generally observed in most systems, our ChIP data are in agreement with our previous studies that show prolonged activation of STAT3 following cytokine exposure of Hep3B cells (Zhang et al., 1996a).

We attempted to amplify the 3’UTR of the CRP gene approximately 2 kb downstream of the transcriptional start site to use as a negative control in our ChIP assays. However, we unexpectedly detected strong signals for C/EBPβ with these primers. Moreover, a recent study demonstrated that β-Catenin binds to a downstream element in the CRP gene and regulates CRP expression by looping of the downstream region of the gene to the proximal promoter (Choi et al., 2007). Looping of the
downstream region of the CRP gene into close proximity of the promoter would explain why we observed strong PCR signals when amplifying the 3’UTR of the CRP gene in ChIP assays.

In summary, our findings that IL-1β + IL-6 enhanced binding of C/EBPβ, STAT3, p50, and c-Rel, and TBP to the CRP promoter in Hep3B cells confirms that these transcription factors participate in cytokine induced expression of CRP in its native context. Of the transcription factors we studied, only binding of C/EBPβ to the CRP promoter markedly increased following cytokine exposure, indicating that its binding plays a critical role in CRP gene expression.
CHAPTER 4: SUMMARY

DISCUSSION

Our major finding from Chapter 2 is that c-Rel(1-300), but not p50 or p52, enhanced C/EBPβ binding to a relatively weak C/EBP site on the CRP promoter (CRP-C/EBP(-53)), increasing the $K_{\text{app}}$ of C/EBPβ from > 200 nM to 40 nM. The c-Rel(1-300) effect did not require the CRP-κB(-43) site, and c-Rel(1-300) was not detected in the EMSA complexes observed on the CRP promoter oligo but could physically interact with C/EBPβ in solution. In addition, overexpressed c-Rel(1-300), despite lacking the transcription activation domain, was able to transactivate the CRP promoter, but only in the presence of C/EBPβ. Other Rel proteins, p50 homodimers, or p50/c-Rel heterodimers did not facilitate C/EBPβ DNA binding. These results are consistent with a model in which c-Rel upregulates CRP expression without binding directly to the CRP promoter, but rather by binding to C/EBPβ, and enhancing the binding of C/EBPβ to the CRP promoter. The transactivation domain of c-Rel is not necessary for this effect, which depends on protein:protein contacts with C/EBPβ.

In Chapter 3, our findings that IL-1β + IL-6 enhanced binding of C/EBPβ, STAT3, p50, and c-Rel, and TBP to the CRP promoter in Hep3B cells adds support to the conclusion that these transcription factors participate in cytokine induced expression of CRP. Of the transcription factors we studied, only binding of C/EBPβ increased markedly following cytokine exposure, suggesting that its binding plays an important role in the overall pattern of CRP mRNA accumulation observed. C/EBPβ occupancy of the promoter was also associated with increased C/EBPβ protein accumulation. In addition,
CRP mRNA accumulation had several peaks and valleys, similar to the parallel patterns of CRP promoter occupancy observed with c-Rel and TBP. Our findings in these studies indicate that C/EBPβ and c-Rel kinetics of binding to the proximal CRP promoter do not match and suggest that the interaction between c-Rel and C/EBPβ is transient. We speculate that c-Rel binds C/EBPβ to help it bind to the promoter and releases C/EBPβ afterwards. We propose that c-Rel plays a role in modulating CRP expression, consistent with a model in which c-Rel enhances C/EBPβ binding.

A diagram of our proposed model is shown in Figure 23. In this model, all of the transcription factors are bound to the CRP promoter in the absence and presence of cytokines. However, LIP binds both C/EBP sites in the absence of cytokines while LAP binds the sites in the presence of cytokines. Thus, LIP inhibits CRP mRNA transcription in the absence of cytokines. We are also proposing that both STAT3 and p50 can bind the site at -108 in the absence of cytokines and that they may compete for this site. Our model also shows looping of the 3’ end of the CRP gene such that the 3’UTR is in close proximity to the proximal promoter region. Two models of transcription factor occupancy are shown after cytokine stimulation in which c-Rel and TBP are either bound to the promoter or free, reflecting the biphasic occupancy pattern observed in ChIP studies. The diagram also depicts c-Rel interacting with C/EBPβ or p50 or TBP after cytokine stimulation.
Figure 23: Theoretical model of kinetics of transcription factor occupancy on the CRP promoter before and after cytokine induction.

A diagram of the possible kinetics of transcription factor occupancy on the CRP promoter in the presence and absence of cytokines is shown. The CRP gene is shown as a blue box and possible looping of the 3’ region is shown. LIP (pale green, solid, oval); LAP (green, white dots, oval), p50 (dark green, white dots, round) STAT3 (yellow, solid, round), c-Rel (purple, solid, hexagon), HNF-1α (blue, solid, diamond), TBP/PIC (light blue, solid, octagon).
Figure 23: Theoretical model of kinetics of transcription factor occupancy on the CRP promoter before and after cytokine induction.
These studies have answered a number of questions regarding the role of c-Rel in CRP gene expression and the kinetics of C/EBPβ, STAT3, p50, and c-Rel occupancy on the endogenous CRP promoter. However, these studies have raised additional questions. Further examination of the mechanism by which c-Rel improves C/EBPβ binding affinity for the CRP-C/EBP(−53) site is warranted. In previous studies, we were able to detect binding of c-Rel and C/EBPβ in solution. Additionally, we need to verify if c-Rel is phosphorylated in Hep3B cells by performing Western blot assays with phospho-specific antibodies against phosphorylated c-Rel. We need to study the relationship between c-Rel and TBP to determine if they interact directly while binding the endogenous CRP promoter in parallel. In Chapter 3 we proposed that the 3’ end of the CRP gene loops such that it is in close proximity with the proximal promoter region. Thus, we also need to examine if looping occurs in our system. In addition to these studies, we need to further examine the roles of each promoter element in CRP gene expression in Hep3B cells by performing ChIP in Hep3B cells with stably transfected mutant promoter elements. Finally, we need to determine the effect of each transcription factor on mRNA production in endogenous CRP gene expression via knockdown assays.

To further examine the mechanism by which c-Rel improves C/EBPβ binding affinity for the CRP-C/EBP(−53) site we tried to detect the interaction between c-Rel and C/EBPβ in Hep3B cells using a mammalian two-hybrid system. We were unable to detect an interaction between C/EBPβ and c-Rel using this system. For these experiments, we created fusion proteins of VP16, a strong viral transcriptional activator, fused to the N-terminus of C/EBPβ. In another fusion protein the Gal 4 DNA binding
domain was fused to the N-terminus of the Rel homology domain of c-Rel (c-Rel1-300).
Thus c-Rel lacked a transactivation domain and could not activate the reporter system on its own. The VP16- C/EBPβ construct may have had folding problems preventing interaction between c-Rel and C/EBPβ. Repeating the mammalian two-hybrid assay with a C/EBPβ-VP16 fusion construct where VP16 is fused at the C-terminus of C/EBPβ may allow the protein to fold correctly and to interact with c-Rel. Alternatively, overexpression of C/EBPβ itself may stimulate transcription from the Gal4-c-Rel1-300 reporter. If so, tet regulated expression of C/EBPβ could be used to control the C/EBPβ:c-Rel interaction.

We also tried to detect the interaction between c-Rel and C/EBPβ in Hep3B cells using co-immunoprecipitation from whole cell lysates. Endogenous c-Rel and C/EBPβ in Hep3B cells are not abundant, thus the co-immunoprecipitation from whole cell extracts may not have been sensitive enough to detect the interaction. Co-immunoprecipitation on Hep3B whole cell lysates after overexpressing c-Rel and C/EBPβ would improve our chances of detecting an interaction between these two proteins. Additionally, the interaction may be transient; thus we may need to perform a more protracted study for the co-immunoprecipitation to be able to detect an interaction between C/EBPβ and c-Rel.

In another approach, we could use fluorescence anisotropy to confirm an interaction between C/EBPβ and c-Rel in solution and map the interaction domain. Measurements of fluorescent polarization (anisotropy) are made by observing the rotational motion of fluorescently labeled molecules in solution. Fluorescent molecules absorb and emit light in different geometrical planes; this intrinsic geometry results in the
polarization of fluorescently emitted light. The emitted light is depolarized (to varying
degrees) due to rotational motion of the molecule and fluorescence energy transfer
(extrinsic depolarization). Translational motion will not decrease the polarization of
fluorescence emission. Polarization and anisotropy are mathematically related, but the
expression for anisotropy is more easily applied and manipulated. The anisotropy of a
fluorescently tagged oligonucleotide (DNA) is measured, and values of bound and
unbound DNA are compared. Larger molecules (i.e. bound DNA) rotate less than
unbound molecules. As rotation decreases, the fluorescence anisotropy value increases
(Lundblad et al., 1996). This approach will allow us to detect and quantify the interaction
between C/EBPβ and c-Rel even if the interaction were transient. These same assays
could be used to detect an interaction between c-Rel and TBP in Hep3B cells.

In Chapter 3 we proposed that the downstream region of the CRP gene may loop
to the proximal promoter region. We would employ chromosome conformation capture
(3C) as described by the Jeong group (Choi et al., 2007) to test the looping theory.
Antibodies previously used in the ChIP assays would be used in the 3C experiment. In
3C, cells are cross-linked as in a normal ChIP assay. However, following cross-linking,
cells are lysed and nuclei are isolated. Next, enzyme digestion is performed using an
enzyme with known restriction sites in the CRP promoter and gene. After the digestion,
random ligation is done to ligate any DNA that is in close proximity to each other. Thus,
DNA that looped and is cross-linked together will now be ligated. Next, the cross-links
are reversed, and the proteins and RNA are digested away. To amplify the ligated DNA
products, primers are used that in a linear strand of DNA would face away from each
other, but in looped DNA ligated together now face one another. Therefore, we would be
able to detect what regions of the 3′UTR may be looping near the proximal promoter of the CRP gene. These experiments will likely demonstrate that the 3′UTR of the CRP gene interacts with C/EBPβ in the proximal promoter region of the CRP gene.

The role of c-Rel in the issue of IL-1 synergy also needs to be addressed. c-Rel is certainly a candidate as being a responsible party, but at this point the players involved in IL-1 synergy with IL-6 in regulating CRP gene expression are unknown. For c-Rel to be involved, we would have to demonstrate that IL-1 alone activates c-Rel but does not change its occupancy on the CRP promoter, and we would have to demonstrate that c-Rel ChIP profiles are not altered by IL-6 alone. These correlations would be a beginning. Demonstrating that overexpression of c-Rel could substitute for IL-1 or knockdown of c-Rel to prevent IL-1 synergy would add support.

To study the role of each promoter element in a native context we would stably transfect Hep3B cells with constructs containing mutant CRP proximal promoter elements and examine kinetics of transcription factor occupancy in response to cytokines via ChIP assays. These studies would allow us to confirm which transcription factors bind each promoter element in Hep3B cells in a native chromatin context. We propose that p50 binds the CRP-STAT3(-108) site in the absence of cytokines, while it binds the CRP-κB(-43) site in the presence of cytokines. In ChIP assays where either the CRP-κB(-43) site or the CRP-STAT3(-108) site, or both are mutated we would be able to determine if p50 binds the CRP-STAT3(-108) site in the absence of cytokines. This assay would also allow us to determine how each C/EBP binding site, -219 or -53, fits into the kinetics of C/EBPβ occupancy on the endogenous CRP promoter. I predict that C/EBPβ will bind the proximal site (-53) in response to cytokines and that C/EBPβ will bind the distal (-
219) site even in the absence of cytokines. In our ChIP assays, it is likely that the form of C/EBPβ that occupied the promoter in the absence of cytokines was the inhibitory isoform LIP, a functional LAP antagonist. Using antibodies that can distinguish between LIP and LAP would allow us to determine which form binds the promoter in the presence and absence of cytokines. Additionally, we would be able to confirm that c-Rel interacts with the CRP promoter through C/EBPβ at the CRP-C/EBP(-53) site. I expect ChIP assays on promoter constructs with mutations in the CRP-C/EBP(-53) site would eliminate C/EBPβ binding in response to cytokines and would eliminate detection of c-Rel in the system. However, if c-Rel also interacts with TBP, we would see a reduced c-Rel signal rather than an absence of signal.

The effect of each transcription factor on mRNA production and other transcription factor binding in endogenous CRP gene expression can be studied via siRNA to knockdown C/EBPβ, STAT3, p50, and c-Rel expression (individually) in Hep3B cells. ChIP assays on Hep3B cells after knocking down each transcription factor would allow us to analyze the effect of each transcription factor on the rest of the system. Measuring mRNA production in response to cytokines both individually and in combination in knockdown cells would allow us to determine the effect of each transcription factor on endogenous CRP gene expression. All of these studies are aimed at a global understanding of the interactions and interplay of the combination of factors that result in a transcriptional response to cytokines.

C-reactive protein gene regulation is a model of dramatic and rapid control of gene expression. We have studied the complex set of interactions on the CRP proximal promoter in detail using Northern blots and in vitro systems consisting of EMSA studies
and luciferase assays. The ChIP assays performed in Chapter 3 are the first studies done to examine the CRP promoter in a native chromatin context. These studies add to our understanding of how CRP gene expression is controlled.
REFERENCES


