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Mechanism of Transcriptional Regulation of C-Reactive Protein Gene Expression.

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Mechanism of Transcriptional Regulation of C-reactive Protein Gene Expression

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by

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ABSTRACT

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C-reactive protein (CRP) is an acute phase protein produced by hepatocytes whose serum concentration increases in inflammatory conditions including cardiovascular complications. Statins that are used in the treatment of cardiovascular diseases to reduce cholesterol also lower serum CRP levels. In human hepatoma Hep3B cells, CRP is induced in response to cytokines IL-6 and IL-1β. The objective of the study was to determine the mechanism of regulation of CRP gene expression in Hep3B cells in response to cytokines and to determine the effect of statins on CRP expression. Key findings of our research were: 1. IL-1β-activated NF-κB p50/p65 acted synergistically with IL-6-activated C/EBPβ in inducing CRP transactivation through the proximal CRP promoter. 2. A NF-κB site was localized in the proximal CRP promoter centered at position -69 overlapping the known OCT-1/HNF-1/HNF-3 sites. 3. The synergy between IL-6 and IL-1β in inducing CRP gene expression was partially mediated through the NF-κB site. 4. In the absence of C/EBPβ, a complex containing C/EBPζ and RBP-Jκ was formed at the C/EBP-p50-site. 5. Overexpressed C/EBPζ repressed both (IL-6+IL-1β)-induced and C/EBPβ-induced CRP expression. 6. OCT-1 repressed (IL-6+IL-1β)-induced CRP transactivation through the proximal CRP promoter. 7. Statins reduce cytokine-induced CRP gene expression at the transcriptional level. These findings led us to conclude that: 1. CRP transcription is determined by the relative levels of various transcription factors such as C/EBPβ, C/EBPζ, NF-κB and OCT-1 and their interaction with the proximal CRP promoter. 2. Inhibition of CRP transcription by statins is not due to an anti-inflammatory effect but due to the direct effect on CRP gene expression.

DEDICATION

This manuscript is dedicated to my father, Dr. Voleti Devarajulu Naidu and my mother, Dr. Moturu Subhadra Devi. My father, who forever stays in my heart, provides me the strength to aim high and accomplish. My mother inspires me to never stop from learning and to be courageous to seek the next big thing in life. I am forever indebted to her for the sacrifices she made, the encouragement she provided, her unwavering patience, and the support she gave throughout my life.

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ABBREVIATIONS

C/EBP	CCAAT/enhancer-binding protein
CREBH	cAMP response element-binding protein H
CRP	C-reactive protein
Dex	Dexamethasone
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
GADD153	Growth arrest and DNA damage inducible factor 153
gp130	Glycoprotein 130
HNF	Hepatocyte nuclear factor
IL-1	Interleukin-1
IL-6	Interleukin-6
JAK	Janus kinases
LDL	Low density lipoproteins
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
Mut	Mutant
NF-κB	Nuclear factor kappaB
NO	Nitric oxide
OCT	Octamer
Oligo	Oligonucleotide
PI3K	Phosphoinositide 3-kinase
RBP-Jκ	Recombination signal-binding protein Jκ,
SAA	Serum amyloid A

SLE	Systemic lupus erythematosus
SNP	Sodium nitroprusside
STAT3	Signal transducer and activator of transcription 3
TGFβ	Transforming growth factor
TNFα	Tumour necrosis factor
WT	Wild-type

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CHAPTER 1

INTRODUCTION

CRP is an evolutionarily conserved protein. In humans, serum CRP concentration increases in acute and chronic inflammatory conditions and in some non-inflammatory conditions such as stress and cell injury [1-3]. CRP belongs to the pentraxin family of proteins consisting of five identical subunits arranged in cyclic pentameric form. CRP binds in a calcium-dependent manner to phosphocholine-containing substances [4] such as the cell wall of *Streptococcus pneumonia,* modified low-density lipoproteins (LDL) and apoptotic cells [5-7]. The Calcium-binding site and the phosphocholine-binding site are located on the same side of the CRP pentraxin and are collectively referred to as the 'recognition face' of CRP [8, 9].

Functions Of CRP

CRP has been shown to be associated with several pathological states such as bacterial infections, atherosclerosis, rheumatoid arthritis, systemic lupus erythematosus (SLE), and cancer [7]. Binding of CRP to bacteria such as *Streptococcus pneumonia* has been shown [6]. CRP activates classical pathway of complement by binding to C1q and results in the clearance of bacteria [10]. CRP increases survival of mice injected with *Streptococcus pneumonia* [10, 11]. But CRP does not bind to some bacteria such as *Salmonella typhimurium* but increases survival of mice injected with these bacteria [12]. Recent research suggested that CRP activated lectin pathway of complement. Serum CRP levels also increase in chronic inflammatory conzditions such as atherosclerosis [13]. CRP binds to modified LDL and is found to be deposited in the atherosclerotic plaques [14]. Recent study has shown that CRP slows progression of atherosclerosis in mice with human like hypercholesterolemia [15]. CRP was shown to interact with auto-antigens. Studies have shown that injection of human CRP in mice prone to SLE results in prevention and reversal of nephritis [16]. Recent

research has shown that CRP enhances myeloma cell proliferation under stressed conditions and protects myeloma cells from chemotherapy-induced apoptosis [17]. The mechanism by which CRP exerts these functions is under investigation.

Significance Of Serum CRP Levels

Serum CRP concentrations in humans increase in both acute and chronic inflammatory conditions and in some non-inflammatory conditions [1, 2]. Serum CRP levels are used as an indicator of inflammation. There are variations even in the baseline serum CRP levels in normal human healthy population [13]. The American Heart Association recommended the use of baseline serum CRP levels as a predictor of future cardiovascular complications [13]. Serum amyloid A (SAA) is another major acute phase protein in humans [18]. In contrast to SAA, human CRP concentrations rise within 24h of an inflammatory event. Because CRP is involved in various pathological conditions, the unique regulation of CRP expression is highly significant to modulate serum CRP levels and to understand eukaryotic gene expression.

CRP Gene Expression

Human CRP is encoded by a single copy gene located on the short arm of chromosome 1. The CRP gene consists of two exons that encode the mature protein separated by an intron. CRP is primarily produced by hepatocytes [19]. Extrahepatic production of CRP was also reported, but hepatocytes primarily account for the serum concentration of CRP. Due to the limited availability of primary human hepatocytes, several cell lines such as HepG2, Hep3B, and HuH7 were used to study CRP gene expression. In these cell lines, several cytokines such as IL-6, IL-1β, TNFα, TGFβ, and IL-17 have been shown to influence CRP expression [18, 20-23]. Human hepatoma Hep3B cells are commonly used as a model to study CRP gene expression. In Hep3B cells, IL-6 and IL-1β are the two major cytokines that regulate CRP expression [24].

Effect Of Cytokines On CRP Gene Expression

In Hep3B cells, the CRP gene is regulated at the transcriptional level and is primarily produced in response to inflammatory cytokines, IL-6 and IL-1β [18, 20]. Dexamethasone (Dex) further enhances the cytokine-mediated CRP induction. IL-6 induces CRP expression modestly and IL-1β, which alone does not affect CRP expression, synergistically enhances the effects of IL-6. The mechanism of synergy between IL-6 and IL-1β is not known. CRP is produced by hepatocytes even in some non-inflammatory conditions such as stress, and the induction of CRP during stress requires transcription factor CREBH, which binds to a site located in the 5' untranslated region of the CRP gene [3]. The proximal CRP promoter (Fig. 1.1) contains binding sites for various transcription factors involved in CRP gene expression.

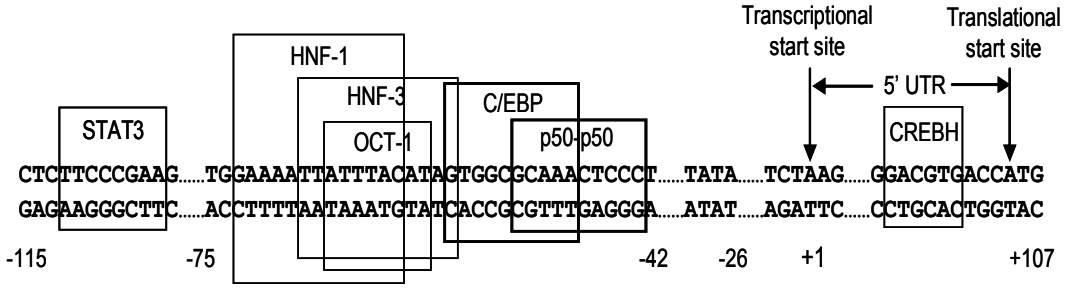

Figure 1.1. Proximal CRP promoter with the known transcription factor binding sites. +1 indicates the transcription start site. Sequences of the transcription factor binding sites are shown in boxes.

Constitutively Expressed Transcription Factors Involved In CRP Expression

Hepatocyte nuclear factor-1 (HNF-1), HNF-3 and OCT-1 are constitutive transcription factors that have overlapping binding sites in the proximal CRP promoter and are involved in maintaining the basal CRP transcription [24-26]. OCT family of transcription factors belong to structurally related POU domain factors found throughout the eukaryotes [27]. OCT-1 is a broadly expressed, versatile transcription factor involved in either activation or repression of genes [28-30]. In regulating gene expression, OCT-1 has been shown to

either bind to the regulatory regions and influence gene expression or directly interact with the transcription factors bound to the promoter sequences [30-32]. Even though OCT-1 binds to the proximal CRP promoter, the role of OCT-1 in CRP gene expression is not known. One of the objectives of this dissertation was to determine the role of OCT-1 in CRP gene expression. The overlapping multiple transcription factor binding sites (-59/-66) are critical for CRP expression. So the role of OCT-1 site in cytokine-induced and constitutive CRP expression will also be determined.

Mechanism Of Interleukin 6 (IL-6)-Induced CRP Expression

IL-6 activates several signaling pathways by binding to its receptor gp130. IL-6 binding to its receptor induces homodimerization of the gp130 IL-6 transducer leading to phosphorylation of Janus kinases (JAKs). Phosphorylation of JAKs results in activation of signal transducers and activators of transcription (STAT) factors at a single tyrosine residue [33-35]. Two other pathways are activated by IL-6 including the MAP kinase and PI3 kinase pathways [34]. IL-6 also activates the C/EBP family of transcription factors. There are six members in the C/EBP family of transcription factors that homodimerize or heterodimerize with each other and influence gene transcription [33].

IL-6 induces CRP expression by the activation of transcription factors C/EBPβ and STAT3 [36-39]. C/EBPβ binds to its site centered at -52 and -219 on the CRP promoter. STAT3 binds to its site centered at -108 [37, 38]. NF-κB (p50/p50) binds to a site centered at -46 overlapping the proximal C/EBP site [40, 41]. The overlapping C/EBP-p50 site is critical for IL-6-induced expression of CRP. IL-6-activated C/EBPβ and p50 homodimers occupy the C/EBP-p50 site under induced conditions, but a complex of unknown composition is formed at the C/EBP-p50 site when C/EBPβ is absent in the nuclear extracts [41]. This result suggested that the C/EBP-p50 site is not vacant when C/EBPβ is absent. One of the objectives of our study was to identify the composition of the complex formed at the C/EBP-

p50 site in the absence of C/EBPβ. The transcription factors that can potentially bind to the overlapping C/EBP-p50 site include other members of C/EBP and NF-κB family of transcription factors [42-44]. Another transcription factor RBP-Jκ has been shown bind NF-κB-sites [45, 46]. By using antibodies to the transcription factors described above, our aim was to determine the composition of the complex formed at the C/EBP-p50 site. This resulted in the identification of the basal transcription machinery associated with the CRP proximal promoter.

Mechanism Of Interleukin 1β (IL-1β)-Induced CRP Expression

IL-1β affects the expression of many inflammatory genes [43]. The mammalian Rel family of transcription factors include p50, p52, p65 (Rel A), Rel B, and c-Rel [42]. Classic NF-κB, the heterodimer of p50 and p65, is an activator of gene transcription while p50 homodimers have been shown to be involved in both activation and repression of genes [44]. IL-1β acts by the activation of NF-κB p50/p65. NF-κB p50/p65 is sequestered in the cytoplasm by binding to inhibitory κB (IκB) protein and is released by the phosphorylation and subsequent ubiquitination of IκB. Activated NF-κB p50/p65 then translocates to the nucleus and binds to the NF-κB binding sequences on the target genes called as κB-sites [44]. IL-6 induces CRP expression modestly whereas IL-1β, which alone does not have an effect on CRP expression, synergistically enhances the effects of IL-6. IL-1β activates NF-κB (p50/p65) in Hep3B cells. But the role of IL-1β in CRP expression was not known prior to this work.

Mechanism Of IL-6 And IL-1β Synergy

IL-6 induces CRP expression modestly, whereas IL-1β, which alone does not have an effect on CRP expression, synergistically enhances the effects of IL-6. Mechanism of synergy between IL-6 and IL-1β was not known. IL-1β activates NF-κB (p50/p65) in Hep3B cells.

Overexpressed NF-κB (p50/p65) induces CRP expression in Hep3B cells [47-49]. Hence it was proposed that an NF-κB response element that binds to NF-κB (p50/p65) must be present on the CRP promoter [47]. Because IL-6 activates C/EBPβ and IL-1β activates NF-κB p50/p65 in Hep3B cells, we hypothesized that the synergy between IL-6 and IL-1β was due to the synergy between C/EBPβ and NF-κB p50/p65. One of the objectives of this dissertation was to identify the NF-κB response element and to determine the functional role of NF-κB p50/p65 in (IL-6 and IL-1β)-induced CRP expression.

Effect Of Statins On CRP Expression

Serum CRP levels are elevated in chronic inflammatory conditions such as atherosclerosis [50]. Statins, which are cholesterol lowering drugs used in cardiovascular diseases, also lower serum CRP levels independently of their cholesterol lowering property [51, 52]. Statins produce nitric oxide (NO) and NO is known to regulate several inflammatory genes known to be involved in the pathogenesis of atherosclerosis [53-55]. Several NO donors such as SNP and SIN-1 have been used to study inflammatory gene responses [55, 56]. Prior to this work, it was not clearly known whether the effect of statins on serum CRP levels was due to a direct effect on CRP gene expression or due to decrease in inflammation. Another objective of our study was to determine the effect of statins on the production of CRP in Hep3B cells. Because statins also enhance NO production in hepatocyte cell lines, the effect of NO in CRP gene expression in Hep3B cells was also determined.

The dissertation research is primarily divided into 2 parts:

A. Determination of the mechanism of transcriptional regulation of basal and cytokine-induced CRP gene expression.
B. Elucidation of the role of statins in CRP gene expression.

Specific Aims

1. Determine the synergistic effect of NF-κB (p50/p65) with C/EBPβ and the functional role of NF-κB in the synergy between IL-6 and IL-1β in Hep3B cells.

   - We published the findings in Reference#58. The published findings are reproduced in Chapter 2.

2. Identify the complex formed on the C/EBP-p50 site in the absence of C/EBPβ in Hep3B cells.

   - We published the findings in Reference#57. The published findings are reproduced in Chapter 3.

3. Determine the role of OCT-1 in basal and cytokine-induced CRP gene expression.

   - We published some of the findings in Reference#58. Additional unpublished data are reported in Chapter 4 (Manuscript in preparation).

4. Determine the effect of statins and NO on CRP gene expression in Hep3B cells.

   - We published the findings in Reference#59. The published findings are reproduced in Chapter 5. Additional unpublished results are provided as supplemental data in Appendix 5A.

CHAPTER 2

Regulation of basal and induced expression of C-reactive protein through an overlapping element for OCT-1 and NF-κB on the proximal promoter

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Keywords: Acute phase reactants, Gene regulation, Transcription factors, Inflammation

Running Title: C-REACTIVE PROTEIN EXPRESSION

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(**Reference:** Voleti, B., Agrawal, A. 2005. J. Immunol. 175(5):3386-3390.)

Abstract

C-reactive protein (CRP) is an acute phase protein produced by hepatocytes. Minor elevation in the baseline levels of serum CRP is considered as an indicator of chronic inflammation. In hepatoma Hep3B cells, IL-6 induces CRP expression by activating transcription factors STAT3 and C/EBPβ. IL-1 synergistically enhances the effects of IL-6. The first 157 bp of the CRP promoter are sufficient for IL-1 synergy. Previously, NF-κB, a transcription factor activated by IL-1β in Hep3B cells, has been shown to increase endogenous CRP expression. The purpose of this study was to investigate the possible action of NF-κB on the 157 bp of the proximal promoter. Here, we show that NF-κB requires and acts synergistically with C/EBPβ on the CRP proximal promoter to regulate CRP expression. We located the regulatory element that consisted of overlapping binding sites for NF-κB (p50-p50 and p50-p65) and OCT-1. The κB-site was responsible for the synergy between NF-κB and C/EBPβ and was also necessary for the CRP transactivation by C/EBPβ through the C/EBP-site. Mutation of the κB-site decreased the synergistic effect of IL-1β on IL-6-induced CRP expression. Basal CRP expression increased dramatically when binding of both OCT-1 and NF-κB was abolished. Combined data from luciferase-transactivation assays and EMSA lead us to conclude that the binding of OCT-1 to the promoter, facilitated by p50-p50 in a novel way, represses while replacement of OCT-1 by p50-p65 induces CRP transcription in cooperation with C/EBPβ. This model for CRP expression favors the variation seen in the baseline serum CRP in normal healthy population.

Introduction

C-reactive protein (CRP)[2] is a multifunctional acute phase protein whose serum concentration increases in chronic and acute inflammation (1-4). CRP is primarily produced by hepatocytes (5, 6) and its synthesis is regulated at the transcriptional level (7-9). In human hepatoma Hep3B cells, IL-6 induces CRP expression modestly by activating transcription factors STAT3 and C/EBPβ (10-14). IL-1, which alone has no effect on CRP expression in Hep3B cells, synergistically enhances the effects of IL-6 (15). The first 157 bp of the CRP promoter are sufficient for synergistic induction of CRP expression by IL-6 and IL-1β (7, 12). Besides in Hep3B cells, STAT3 and C/EBPβ have been shown to act on CRP promoter in other hepatic cell lines too (16-20). On the CRP proximal promoter, within the first 157 bases, C/EBPβ binds to a site centered at -52 and STAT3 binds to a site at -108 (10-12). A second C/EBP-site is located at position -219 (11).

Three other transcription factors, HNF-1, HNF-3, and OCT-1, are involved in maintaining the constitutive expression of CRP (11, 21). OCT-1 is a broadly expressed versatile transcription factor of the POU family of homeo-domain proteins. OCT-1 performs many divergent roles in cellular transcriptional regulation partly owing to its flexibility in DNA binding and ability to associate with multiple and varied co-regulators. Although generally thought of as an activator of gene transcription, OCT-1 also represses transcription through a variety of mechanisms (22).

The mode of action of IL-1 in CRP expression is not defined. Because IL-1 activates NF-κB in Hep3B cells, it is hypothesized that IL-1 may be acting through activation of NF-κB (23). There are five NF-κB proteins: p50, p52, p65, Rel B, and c-Rel; they form homodimers or heterodimers with each other and bind to κB-sites on the promoter regions to modulate transcription (24). It has been shown previously that the NF-κB heterodimer p50-

p65 induces endogenous CRP expression in Hep3B cells (23); however, a binding site for p50-p65 has not been identified in the first 157 bp of the CRP promoter.

In the current study, we investigated the possible presence of a κB-site within the157 bp of the proximal promoter. We located a regulatory element, -74/-59, with overlapping binding sites for NF-κB and OCT-1. Our data indicate that the binding of OCT-1 to the promoter, facilitated by p50-p50 in a novel way, represses while replacement of OCT-1 by p50-p65 induces CRP transcription in cooperation with C/EBPβ.

Materials And Methods

Cell Culture, Cytokine Treatment, Transfection, And Luciferase Transactivation Assay

Hep3B cells were cultured in serum-free medium overnight for cytokine treatments as described previously (14). The confluency of cells was approximately 60% at the time of treatments. IL-6 and IL-1β (R & D systems) were used at concentrations of 10 ng/ml and 1 ng/ml, respectively, and the cells were treated for 24 h. For transient transfections, cells were plated into 6-well plates and transfected using FuGENE 6 reagent (Roche) as described previously (25). Luciferase reporter-CRP promoter constructs were used at 1μg plasmid per well. Cytokine treatments were started 16 h post-transfection. After 40 h of transfection, luciferase assays were performed as described previously (25). Luciferase activity was measured in a luminometer (Molecular Devices), which was programmed for the integration time of 10 s with no post-injection delay time.

Preparation Of Nuclear Extract And EMSA

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic kit (Pierce) and were used in EMSA as reported earlier (14). The oligonucleotide (oligo) GATCCGGGGACTTTCCATGGATGGGGACTTTCCATGG was used as the consensus κB site-containing probe. Oligos were obtained from Integrated DNA Technologies. The gel shift incubation buffer contained 40 mM KCl, 16 mM Hepes, pH 7.9, 1 mM EDTA, 2.5 mM DTT, 0.15% Nonidet P-40, 8% Ficoll, and 1 μg of poly dI-dC. The Ab to C/EBPβ (C19), p50 (H119), p65 (C20), HNF-1 (H205), and OCT-1 (C21) were purchased from Santa Cruz Biotechnologies. Unlabelled competitor oligos were used in 200-fold molar excess. DNA-protein complexes were resolved in native 4.5% polyacrylamide gels containing 2.5% glycerol. Gels were analyzed in a phosphorimager using ImageQuant software (GE Healthcare). Sequences of the top strand of the double-strand oligos, derived from the CRP

promoter and used in EMSA, were: Oligo 1: 19 bp long, ATGTTGGAAAATTATTTAC; Oligo 2: 25 bp long, CAATGTTGGAAAATTATTTACATAG; Oligo 3: oligo 2 with mutated κB-site, CAATGTTGG**TT**AAT**A**ATTTACATAG. The κB sites are underlined and the mutated bases are in bold.

Engineering Of CRP Promoter-Luciferase Reporter Constructs

The wild-type (WT) construct, Luc-157 WT CRP, has been described earlier (13). Luc-300 WT construct was prepared according to a previously published method (26). Briefly, genomic DNA (Promega) was used to PCR amplify a fragment corresponding to nucleotides -300/-1 of the CRP promoter, using the primers 5'- CCTAGATCTAGAGCTACCTCCTCCTGCCTGG and 5'-CCGACGCGTACCCAGATGGCCACTCGTTTAATATGTTACC. Primers were designed to contain the B*gl*II and M*lu*I restriction sites, respectively. PCR product was cloned into the luciferase reporter vector pGL2 basic (Promega) and the DNA sequence was confirmed. These two WT constructs were used as templates for mutagenesis. Constructs containing mutated κB and STAT3 site were generated using the QuickChange site-directed mutagenesis kit (Stratagene). The κB-site was mutated by substituting $^{-72}$AAAATT^{-67} with $^{-72}$TTAATA^{-67} using mutagenic primers: 5'-GCGCCACTATGTAAATTATTAACCAACATTGCTTGTTGGGGC and 5'-GCCCCAACAAGCAATGTTGGTTAATAATTTACATAGTGGCGC. The STAT3-site was mutated by substituting $^{-111}$TCCCGA^{-106} with $^{-111}$GATATC^{-106} using mutagenic primers 5'-GCTTCCCCTCTGATATCAGCTCTGACACCTG and 5'-CAGGTGTCAGAGCTGATATCAGAGGGGAAGC. Mutations were verified by sequencing. Plasmids were purified using maxiprep plasmid isolation kit (Eppendorf).

Results

NF-κB Acts Synergistically With C/EBPβ On The CRP Proximal Promoter

To determine whether NF-κB could induce CRP transactivation through the proximal promoter under any experimental condition, constructs of the WT promoter regions -300/-1 and -157/+3 linked to luciferase reporter (Luc-300 WT and Luc-157 WT) were transfected into Hep3B cells along with expression vectors for NF-κB (p50, p65) and C/EBPβ. NF-κB did induce CRP-promoter-driven luciferase expression, but only in the presence of C/EBPβ (Fig. 2.1). The amount of C/EBPβ was critical for inducing the effect of NF-κB on both -300/-1 and -157/+3 promoters. For 200 ng of p50 and p65 plasmids, > 20 ng of C/EBPβ plasmid was required to observe the synergistic induction. The data indicated that NF-κB required and acted synergistically with C/EBPβ bound to its proximal, but not the distal, site and that a κB-site must be located within the 157 bp on the CRP promoter.

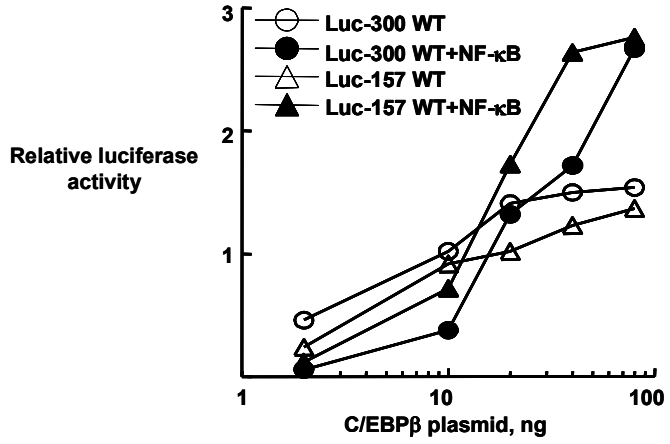

Figure 2.1. NF-κB acts on first 157 bp of the CRP promoter and synergizes with C/EBPβ to induce CRP-promoter (-157/+3 or -300/-1)-driven luciferase expression. A representative experiment is shown; 3 independent experiments exhibited similar patterns. Cells were transfected with CRP promoter-luciferase constructs (Luc-300 WT and Luc-157 WT) and plasmids encoding C/EBPβ (increasing doses) and NF-κB (p50 and p65, 200 ng each). CRP transactivation was represented as relative luciferase activity.

A κB-Site Is Located At Position -69 On The CRP Promoter

We read the DNA sequence of the CRP promoter and found a potential κB-site (-74/-63) overlapping the known binding sites for transcription factors HNF-1/OCT-1/HNF-3 (11, 21) (Fig. 2.2).

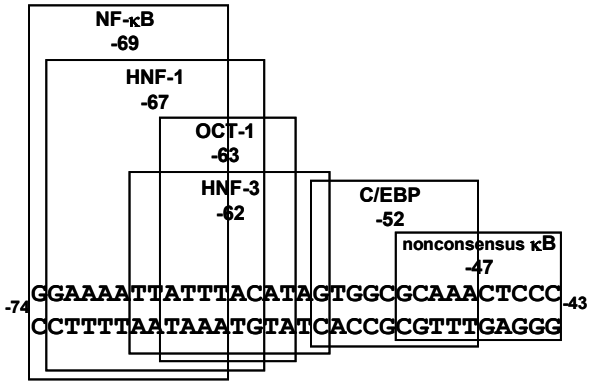

Figure 2.2. Localization of the κB-site on the CRP proximal promoter. The nucleotide sequence of the CRP promoter between positions -74 and -43, relative to the transcription start site, is shown. Sequence of the putative κB-site centered at -69, and known binding sites for other transcription factors are boxed.

Oscillation Between The Binding Of p50-p50/OCT-1 And p50-p65 On The Overlapping κB/OCT-1 Sites

Binding of NF-κB to the putative κB-site was determined by EMSA, using consensus κB-site probe and nuclear extracts from cells treated with IL-1β as the source of NF-κB (Fig. 2.3*A*). IL-1β-treatment induced formation of NF-κB p50-p65 complex (lanes 1-5). Oligos 1, 2, and 3 derived from CRP promoter and containing the κB-site were used as competitors. The 19 bp oligo 1 (-79/-61) did not compete efficiently with the probe for binding to NF-κB (lane 6); however, the 25 bp oligo 2 (-81/-57) competed (lane 7). To confirm that the competition was due to κB-site on oligo 2, the κB-site was mutated in oligo 3, and this oligo

did not compete for binding NF-κB (lane 8). Thus, the region -74/-63 on the CRP promoter is the κB-site and a certain flanking sequence is necessary for binding NF-κB.

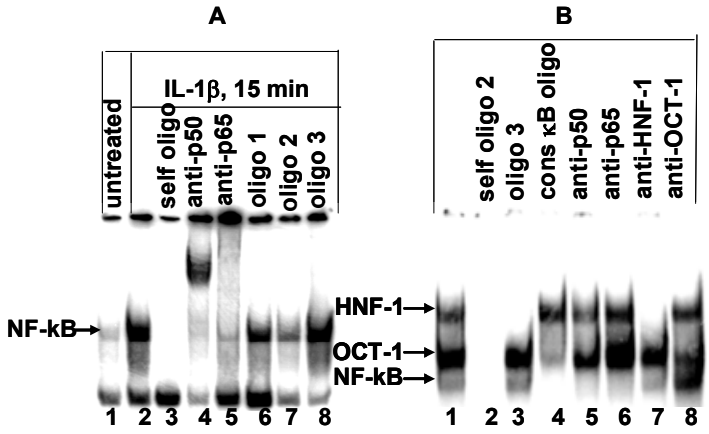

Figure 2.3. Binding of OCT-1 and NF-κB to the composite OCT-1/κB-site. *A*, CRP promoter's κB-site competes with the consensus κB-site for binding to NF-κB. EMSA using radiolabelled consensus κB-site probe and nuclear extract from IL-β-treated cells as the source of NF-κB; Competitor oligos 1-3, containing the putative κB-site, were derived from CRP promoter. Oligo 1: 19 bp containing the κB-site; Oligo 2: 23 bp containing the κB-site; Oligo 3: oligo 2 with mutated κB-site *B*, Direct binding of NF-κB to CRP promoter's κB-site. EMSA utilized oligo 2 as probe and nuclear extract from IL-β-treated cells. The competitors (200-fold excess of unlabeled oligos) and the Ab were added to the reaction mixtures before the addition of the probe. Results were analyzed by phosphorimager. The mobility of the free probe is not shown. A representative of 3 EMSA is shown.

EMSA, using oligo 2 as the probe, provided direct visualization of binding of NF-κB (Fig. 2.3*B*). Three specific complexes were formed (lanes 1, 2). The complex on the top was HNF-1 (lane 7), next complex was OCT-1 (lane 8), and the fastest migrating complex contained NF-κB p50-p65 (lanes 5, 6). Unexpected results were seen in lanes 3, 4 and 8. In lane 3, the mutated oligo 3 (with OCT-1-site intact) competed with HNF-1 complex only, showing that the mutation of κB-site abolished binding to OCT-1 also, in addition to NF-κB. This result indicated that the binding of OCT-1 to the probe required an intact κB-site. In lane 4, binding of OCT-1 to the probe, in addition to that of NF-κB, was drastically diminished in the presence of unlabelled consensus κB-site oligo, indicating dependence of OCT-1 on NF-

κB proteins for binding to its site. In lane 8, binding of p50-p65 to the probe was increased by the addition of anti-OCT-1 Ab, indicating that OCT-1 inhibited binding of p50-p65 to the probe. This result further indicated that the NF-κB proteins required for OCT-1-binding (lane 4) to the probe must be p50-p50, and explained the low intensity of the NF-κB complex seen in lane 1. We confirmed the capability of the κB-site to bind p50-p50 by using recombinant p50 (Fig. 2.4*A*). We conclude that the region -81/-57 on the CRP promoter binds either OCT-1 or p50-p65, and the binding of OCT-1 to its site requires prior transient binding of p50-p50 to the κB-site. Lastly, to confirm the finding that the binding of OCT-1 to its site was dependent on an intact κB-site as seen in lane 3 (Fig. 2.3*B*), an EMSA was performed using oligo 3 as the probe (Fig. 2.4*B*). Only one specific complex containing HNF-1 was formed.

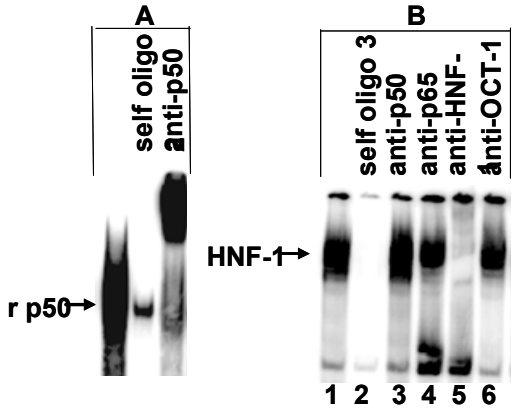

Figure 2.4. Binding of p50 and HNF-1 to oligo 2 and oligo 3 respectively. *A*, EMSA utilized oligo 2 as probe and recombinant p50 (4 gel shift units/lane). *B*, EMSA using oligo 3 as probe and nuclear extract from IL-β-treated cells. The competitors (200-fold excess of unlabeled oligos) and the Ab were added to the reaction mixtures before the addition of the probe. Results were analyzed by phosphorimager. The mobility of the free probe is not shown. A representative of 3 EMSA is shown.

The κB-Site Is Functional

To determine whether the κB-site mediated the synergistic effect of NF-κB on CRP transactivation by C/EBPβ, we conducted transactivation assays using Luc-300 m-κB and

Luc-157 m-κB constructs with the mutated κB-site (Fig. 2.5). In Luc-300 WT, NF-κB enhanced the inducing effect of C/EBPβ from 78-fold to 127-fold. In Luc-300 m-κB construct, NF-κB did not do so; instead, the effect of C/EBPβ alone was reduced by about 90% (from 78-fold to 8-fold) compared to WT construct, indicating that an intact κB-site was also necessary for maximum transactivation by C/EBPβ itself. Similar results were obtained with Luc-157 constructs. We conclude that the κB-site is responsible for synergy between NF-κB and C/EBPβ and is also necessary for the action of C/EBPβ through the C/EBP-site.

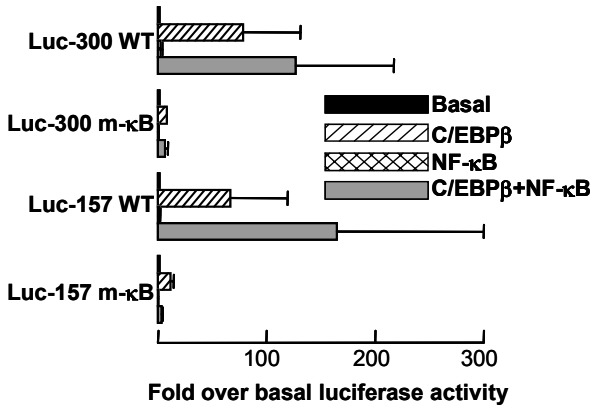

Figure 2.5. The κB-site and the C/EBP-site act together to regulate CRP expression. The basal luciferase activity for each construct is considered as 1 and the luciferase activity in response to C/EBPβ (80 ng) and NF-κB (p50 and p65, 200 ng each) is plotted as fold-induction over basal expression. The average ± SEM of 3 experiments is shown.

Participation Of The κB-Site In The Synergy Between IL-6 And IL-1β

To determine whether the κB-site mediated the synergistic effect of IL-1β on CRP transactivation by IL-6, we conducted transactivation assays using Luc-300 m-κB and Luc-157 m-κB constructs with the mutated κB-site (Fig. 2.6). In Luc-300 WT, IL-1β enhanced the inducing effect of IL-6 by 5.1-fold. In Luc-300 m-κB construct, the synergistic effect of IL-1β was reduced by 50% (from 5.1-fold to 2.6-fold) compared to WT construct. Similar results were obtained with Luc-157 constructs.

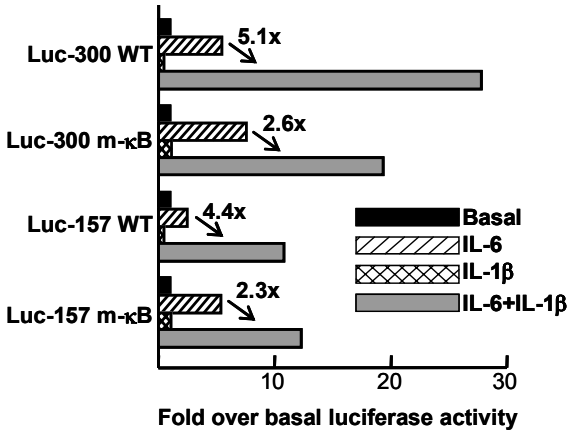

Figure 2.6. Synergistic effect of IL-1β on IL-6-induced CRP expression is only partially mediated by NF-κB. The basal luciferase activity for each construct is considered as 1 and the luciferase activity in response to IL-6 and IL-1β is plotted as fold-induction over basal expression. A representative experiment is shown.

The Overlapping κB And OCT-1 Sites Regulate Basal CRP Expression

Basal transactivation of CRP promoter Luc-300 m-κB and Luc-157 m-κB was up approximately 15-fold when compared to the basal transactivation of the corresponding WT promoters (Fig. 2.7).

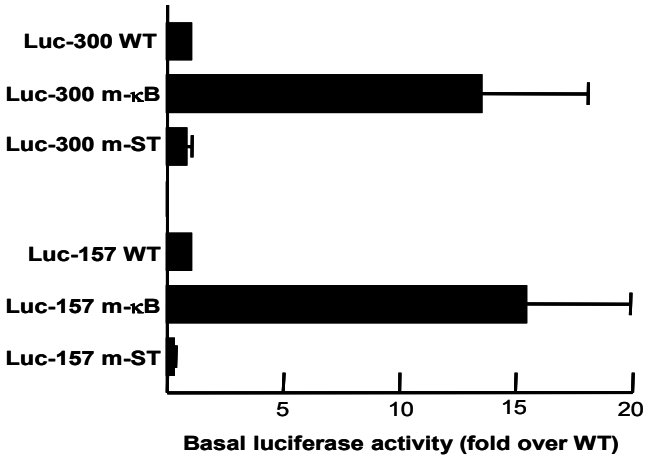

Figure 2.7. Basal level of CRP expression was elevated from the promoter with mutated κB-site. The basal luciferase activity of m-ST and m-κB constructs is plotted as fold-induction over that of WT construct whose basal activity was taken as 1. The average ± SEM of 5 experiments is shown.

As a control when the STAT3-site was mutated (m-ST), basal activity of the promoter did not increase. Since mutation of the κB-site abolishes binding of OCT-1 to the promoter (Figs. 2.3*B* and 2.4*B*), the transactivation results suggest that OCT-1 acts as a repressor of CRP expression.

Discussion

To explore further the mechanism of CRP gene expression, we evaluated participation of NF-κB in the induction of CRP gene expression through the proximal promoter. Our major findings were: 1. NF-κB p50-p65 acted synergistically with C/EBPβ to induce CRP transactivation through 157 bp of the promoter. 2. A minimum amount of C/EBPβ was critical for the NF-κB synergy. 3. A κB-site was located at position -69, overlapping the known OCT-1/HNF-1/HNF-3 sites. 4. The κB-site was required for the synergism between NF-κB and C/EBPβ. 5. The κB-site, in part, contributed to the synergism between IL-6 and IL-1β. 6. Basal CRP expression was increased dramatically when the binding of both OCT-1 and NF-κB to their cognate sites was abolished. 7. A novel interaction between OCT-1 and NF-κB dimers p50-p50 and p50-p65 was observed indicating that this binding site on the CRP promoter was a key element in regulating CRP gene expression under basal and inflammatory conditions.

A binding site for NF-κB p50-p65 within the proximal promoter of CRP gene was not identified in earlier attempts (13, 14, 23). The κB-site found earlier on the CRP promoter was located at position -2652 although a nonconsensus κB-site for binding p50-p50 was present in the proximal promoter at position -48. We found that p50-p65, in the presence of C/EBPβ, acted as an inducer of CRP expression. Moreover, the transactivation by C/EBPβ through the C/EBP-site located at position -52 also required the κB-site, strongly indicating a functional association between the two sites. The physical interaction *in vitro* and synergism in transcriptional activity between NF-κB and C/EBPβ acting through their adjacent sites is a general phenomenon and have been reported for a number of other gene promoters (27, 28). Previous data (13, 14) showed that p65-p65 inhibited the inducing effects of p50-p50 and also of C/EBPβ on CRP expression through the proximal promoter. In those transactivation

experiments utilizing overexpression approach, the amount of C/EBPβ was kept constant and the amount of p50 or p65 varying. In contrast, in this report, we utilized constant amounts of p50 and p65 with increasing amounts of C/EBPβ. It was not obvious whether the previously reported inhibitory effect of p65 was due to the sequestration of limited amount of C/EBPβ by p65 homodimers (13, 14).

The presence of a κB-site within the first 157 bp strongly indicated that this site could be the IL-1-response element on the CRP promoter. Results indicated that the activation of p50-p65, and the κB-site at -69, contributed only partially to the synergistic effect of IL-1 on IL-6-induced CRP gene expression. Our data support the notion that IL-1, besides activating NF-κB in Hep3B cells, participates in IL-6 synergy via other pathways as has been shown in the case of other IL-1-regulated genes (29). We present our data obtained from the mutational analysis of the CRP promoter and from the unique interaction of transcription factor on the OCT-1/κB-site in the form of a working-model (Fig. 2.8). This model would guide us to the next series of experiments to demonstrate the functions of OCT-1, p50-p50, and p50-p65 in regulating CRP expression. There are four features of this model: A. Absence of binding of any transcription factor to the OCT-1/κB-site enhances basal CRP expression. B. The binding of OCT-1 to the promoter requires prior transient binding of p50-p50 to the overlapping κB-site. C. OCT-1 represses basal CRP expression, consistent with the known role of OCT-1 as a repressor of gene expression (30). This model would guide us to the next series of experiments to demonstrate the functions of OCT-1, p50-p50, and p50-p65 in regulating CRP expression. There are four features of this model: A. Absence of binding of any transcription factor to the OCT-1/κB-site enhances basal CRP expression. B. The binding of OCT-1 to the promoter requires prior transient binding of p50-p50 to the overlapping κB-site. C. OCT-1 represses basal CRP expression, consistent with the known role of OCT-1 as a repressor of gene expression (30).

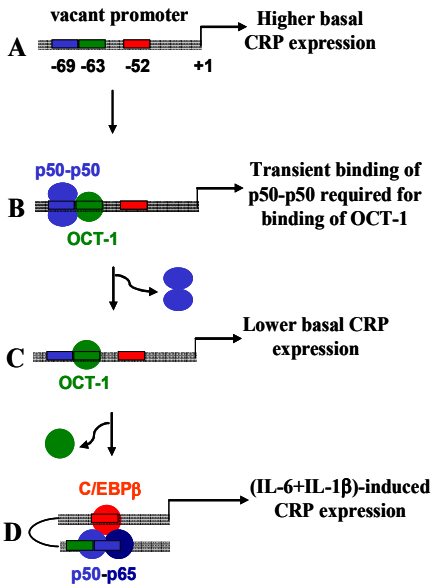

Figure 2.8. A model showing the role of the overlapping κB/OCT-1 sites functioning with the C/EBP-site in regulating basal and induced CRP expression. A. Vacant OCT-1, κB, and the C/EBP sites increase basal CRP expression. B. Binding of OCT-1 to its site requires prior transient binding of p50-p50 to the κB-site. Once OCT-1 is bound, p50-p50 leaves its site. C. The OCT-1-binding represses basal CRP expression. D. Cytokines such as IL-6 and IL-1β activate C/EBPβ and NF-κB p50-p65 respectively. A switch occurs between the repressor OCT-1 and p50-p65. Since the κB site is only 16 bp away from the C/EBP-site, a physical interaction between NF-κB and C/EBPβ is possible resulting in induced CRP expression.

Thus, basal CRP expression may vary depending upon the availability of free p50-p50 and OCT-1 in the hepatocyte nuclei. The importance of the ratio of various transcription factors in regulating gene expression has been documented earlier (31). D. Under inflammatory conditions, p50-p65 replaces OCT-1 to induce CRP transcription. Such oscillation between nucleoprotein complexes on the gene promoters has been described before (32). In addition, because the C/EBP-site is only 16 bp away from the κB-site, C/EBPβ and p50-p65 may form a stable ternary complex.

Acknowledgements

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CHAPTER 3

A novel RBP-Jκ-dependent switch from C/EBPβ to C/EBPζ at the C/EBP-binding site on the C-reactive protein promoter [1]

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Keywords: Inflammation, Acute phase reactants, Transcription factors

Running Title: RBP-Jκ AND C/EBPζ IN CRP TRANSCRIPTION

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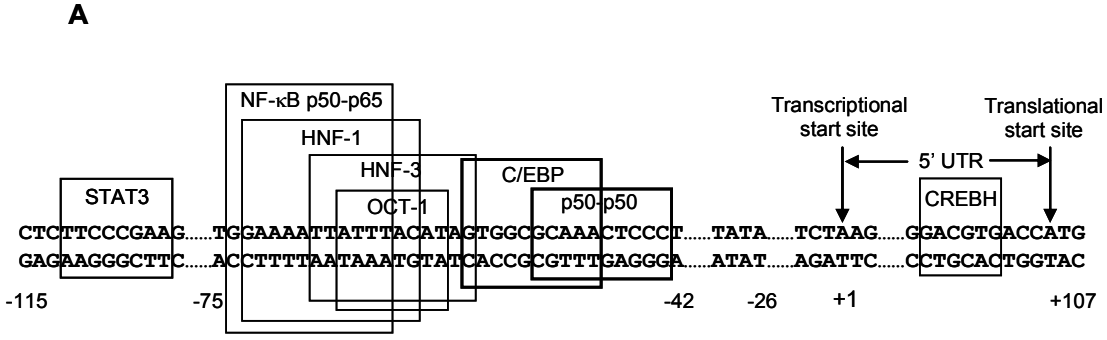
Abstract

The regulation of basal and (IL-6+IL-1β)-induced expression of C-reactive protein (CRP) in human hepatoma Hep3B cells occurs during transcription. A critical transcriptional regulatory element on the CRP promoter is a C/EBP-binding site overlapping a NF-κB p50-binding site. In response to IL-6, C/EBPβ and p50 occupy the C/EBP-p50-site on the CRP promoter. The aim of this study was to identify the transcription factors occupying the C/EBP-p50-site in the absence of C/EBPβ. Accordingly, we treated Hep3B nuclear extract with a C/EBP-binding consensus oligonucleotide to generate an extract lacking active C/EBPβ. Such treated nuclei contain only C/EBPζ (also known as CHOP10 and GADD153) because the C/EBP-binding consensus oligonucleotide binds to all C/EBP family proteins except C/EBPζ. EMSA using this extract revealed formation of a C/EBPζ-containing complex at the C/EBP-p50-site on the CRP promoter. This complex also contained RBP-Jκ, a transcription factor known to interact with κB sites. RBP-Jκ was required for the formation of C/EBPζ-containing complex. The RBP-Jκ-dependent C/EBPζ-containing complexes were formed at the C/EBP-p50-site on the CRP promoter in the nuclei of primary human hepatocytes also. In luciferase transactivation assays, overexpressed C/EBPζ abolished both C/EBPβ-induced and (IL-6+IL-1β)-induced CRP promoter-driven luciferase expression. These results indicate that under basal conditions, C/EBPζ occupies the C/EBP-site, an action that requires RBP-Jκ. Under induced conditions, C/EBPζ is replaced by C/EBPβ and p50. We conclude that the switch between C/EBPβ and C/EBPζ participates in regulating CRP transcription. This process utilizes a novel phenomenon, that is, the incorporation of RBP-Jκ into C/EBPζ-complexes solely to support the binding of C/EBPζ to C/EBP-site.

Introduction

C-reactive protein (CRP)[3] is a host-defense protein whose serum concentration increases in chronic and acute inflammatory and in some noninflammatory states (1-4). The synthesis of CRP in hepatocytes is regulated at the transcriptional level (5, 6). In hepatoma cells, IL-6 induces CRP expression by activating transcription factors STAT3 (7-10) and C/EBPβ (9, 11). IL-1β synergistically enhances the effects of IL-6, in part through the activation of NF-κB (12-14). The first 157 bp on the CRP proximal promoter is sufficient for the synergistic action of IL-6 and IL-1β in human hepatoma Hep3B cells (12). Three other transcription factors, HNF-1, HNF-3 and OCT-1, are involved in maintaining the basal expression of CRP (11, 14, 15). The hepatocytes produce CRP even when they are under stress and such induction of CRP expression requires transcription factor CREBH whose binding site is not in the promoter region but is located in the 5' untranslated region of the CRP gene (4). The binding sites of all known transcription factors involved in CRP expression through the proximal 157 bp of the promoter are shown in Fig. 3.1*A*. In this paper, we report the participation of two additional transcription factors, C/EBPζ and RBP-Jκ, in regulating CRP expression.



B

Oligo A from the CRP promoter	-78 TGTTGGAAAATTATTTACATAGTGGCGCAAACTCCCTTACT -38 GGTTACAACCTTTTAATAAATGTATCACCGCGTTTGAGGGA
Oligo A-m1: p50-p50 site mutated	TGTTGGAAAATTATTTACATAGTGGCGCAAA**G**T**GATA**TACT GGTTACAACCTTTTAATAAATGTATCACCGCGTTT**C**A**CTAT**
Oligo A-m2: p50-p65 site mutated	TGTTGG**TT**AAT**A**ATTTACATAGTGGCGCAAACTCCCTTACT GGTTACAACC**AA**TTA**T**TAAATGTATCACCGCGTTTGAGGGA
Oligo A-m3: Both p50 sites mutated	TGTTGG**TT**AAT**A**ATTTACATAGTGGCGCAAA**G**T**GATA**TACT GGTTACAACC**AA**TTA**T**TAAATGTATCACCGCGTTT**C**A**CTAT**
Oligo B from the CRP promoter	-65 TTTACATAGTGGCGCAAACTCCCT -42 GTATCACCGCGTTTGAGGGAATGA
Oligo C from the CRP promoter	-81 CAATGTTGGAAAATTATTTACATAG -57 GGTTACAACCTTTTAATAAATGTAT
C/EBP consensus oligo	TGCAGATTGCGCAATCTG CTAACGCGTTAGACGTTG
RBP-Jκ consensus oligo	TCTTCTAACGTGGGAAAATCCAGT AGAAGATTGCACCCTTTTAGGTCA
STAT3 consensus oligo	GCTTCATTTCCCGTAAAT GTAAAGGGCATTTAGCAG

Figure 3.1 A part of the CRP gene. *A.* The sequence of the CRP gene between positions -115 and +107, including -115/-1 region of the promoter, is shown. Sequences of the known binding sites for transcription factors on the promoter and 5' untranslated region (UTR) are boxed. The sequences under investigation in this manuscript, the overlapping C/EBP-binding and p50-binding nonconsensus κB sites, are in bold boxes. *B.* Sequences of the oligonucleotides used in EMSA. Mutated bases are in bold and underlined. Consensus sequences are underlined.

There are six members in the C/EBP family of transcription factors: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ. C/EBPζ is also known as C/EBP homologous protein 10 (CHOP10) and growth arrest and DNA damage-inducible gene 153 (GADD153). These C/EBP proteins can homodimerize or heterodimerize with each other. Except C/EBPζ-containing dimers, all other C/EBP dimers bind to the classical C/EBP-binding consensus sequence (16-20). C/EBPζ is ubiquitously expressed at low levels in proliferating cells including Hep3B and can be induced by cellular stresses (21). Within 157 bp of the CRP proximal promoter, a C/EBP-binding site is centered at position -52. A second C/EBP-site is located at -219 (11).

There are five members in the NF-κB family of transcription factors: p50, p52, p65, Rel B and c-Rel. These Rel proteins can also homodimerize or heterodimerize with each other (22, 23). Rel dimers bind to κB sites that are typically composed of five purines followed by five pyrimidines. The heterodimer of p50 and p65, the classical NF-κB, binds to a κB site centered at position -69 on the CRP promoter (14, 24). This κB site overlaps the binding sites for HNF-1, HNF-3 and OCT-1 (14). A second κB site on the CRP promoter is located at -2652 (25). The homodimers of p50 also bind to a nonconsensus κB site centered at position -47 overlapping the C/EBP-site (26-28). The overlapping C/EBP-site and p50-binding nonconsensus κB site (C/EBP-p50-site) on the CRP promoter is critical for the induction of CRP transcription (26, 28).

In addition to binding to NF-κB, some κB sites are also recognized by RBP-Jκ (also known as recombination signal-binding protein Jκ, CBF1 and CSL) (29-32). RBP-Jκ is a ubiquitously expressed transcription factor in mammals. The homologs of RBP-Jκ are also described: known as SuH in *D. melanogaster* and LAG-1 in the nematode *C. elegans* (33-36). The consensus sequence for the binding of RBP-Jκ is CGTGGGAAA although RBP-Jκ can bind to several variants of the consensus sequence (37-39). RBP-Jκ regulates transcription of genes in response to Notch signalling (36, 40). In the absence of signalling, RBP-Jκ binds to its target site and represses transcription by recruiting corepressors. In response to signalling the corepressors are replaced by coactivators (33, 36). Notch-independent functions of RBP-Jκ involve its recruitment into complexes containing other transcription factors, its interaction with components of transcription preinitiation complex (41, 42), and its competition with NF-κB for binding to κB sites (37, 43, 44).

The formation of a complex of unknown composition at the C/EBP-p50-site on the CRP promoter in Hep3B nuclei was reported earlier (26). This complex did not contain NF-κB proteins and was formed only when the nuclear extracts were pretreated with an oligonucleotide (oligo) containing the C/EBP-binding consensus sequence (26). In the current study, we identified the transcription factors present in this complex as C/EBPζ and RBP-Jκ. For the formation of C/EBPζ-containing complex at the C/EBP-p50-site on the promoter, the recruitment of RBP-Jκ into the complex was necessary. Our data suggest that a switch between C/EBPζ and C/EBPβ operates at the C/EBP-site on the CRP promoter to regulate CRP transcription. This uniqueness of the C/EBP-p50-site of the CRP promoter might be contributing to the acute phase nature of the induction of CRP gene expression.

Materials And Methods

Cell Culture, Cytokine-Treatment, Transfection, CRP Promoter-Luciferase (Luc) Reporter Constructs, And Luc Transactivation Assay

Hep3B cells (provided by Dr. G. J. Darlington, Baylor College of Medicine, Houston, TX) were grown as described previously (26). Cells were cultured in serum-free medium overnight for cytokine-treatments and transfections. The confluency of cells was approximately 60% at the time of treatments. IL-6 and IL-1β (R & D) were used at concentrations of 10 ng/ml and 1 ng/ml, respectively. The cells were treated with cytokines for 24 h for Luc transactivation assays. For transient transfections, Hep3B cells were plated into 6-well plates and transfected using FuGENE6 reagent (Roche) as described previously (27). CRP promoter-Luc reporter constructs were used at 1μg plasmid per well and the transcription factor expression vectors were used as mentioned in the figure legends. Total amount of plasmid DNA transfected was held constant using empty pCDNA3. The preparation of wild-type (WT) CRP promoter (-157/+3)-Luc reporter construct and the construct containing the mutated κB site has been described previously (14, 28). Expression vectors for C/EBPβ, C/EBPζ, and RBP-Jκ were obtained from Dr. P. F. Johnson (National Cancer Institute, Frederick, MD), Dr. N. J. Holbrook and Dr. J. L. Martindale (National Institute of Aging, Bethesda, MD), and Dr. L. D. Vales (University of Medicine and Dentistry, Piscataway, NJ), respectively. After 16 h of transfection, the transfected cells were either treated with cytokines for 24 h or left untreated. After 40 h of transfection, Luc transactivation assays were performed following the protocol supplied by the manufacturer (Promega), and the Luc activity was measured in a luminometer (Molecular Devices) as described previously (14).

Preparation Of Nuclear Extract And EMSA

Hep3B nuclear extracts were prepared using NE-PER nuclear and cytoplasmic kit (Pierce) as reported earlier (14). Primary human hepatocytes were purchased from Cambrex biosciences (catalogue number cc-2591). One ampoule of cryopreserved human hepatocytes (3-6 x 10^6 viable cells) was used to prepare 100 μl of nuclear extract using NE-PER nuclear and cytoplasmic kit. Four μl of hepatocyte nuclear extract was used for each reaction in EMSA. In the EMSA using purified recombinant human NF-κB p50 (Promega), 0.6 gel shift U of the protein was used for each reaction. EMSA on Hep3B nuclear extracts was carried out as described previously (13). Unless otherwise mentioned, the gel shift incubation buffer contained 16 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 2.5 mM DTT, 0.15% Nonidet P-40, 8% Histopaque, and 1 μg of poly(deoxyinosinic-deoxycytidylic acid). The sequences of the oligos derived from the CRP promoter, consensus oligos, and mutated oligos used in EMSA are shown in Fig. 3.1*B*. The C/EBP-binding (45), RBP-Jκ-binding (38), and STAT3-binding (46) consensus oligos were designed according to published sequences. Oligos were obtained from Integrated DNA Technologies. To prepare the probes, complementary oligos were annealed and labelled with either [α-^{32}P] CTP or [γ-^{32}P] ATP. In supershift experiments, Ab (2 μg) were added to the reaction mixture and incubated on ice for 15 min before addition of the probe. In competition experiments, 150 ng of unlabelled oligos were added to the binding reactions before addition of the Ab and probe. The Ab to C/EBPβ (C19), C/EBPζ (F168), HNF-1 (H205), HNF-3 (C20), and OCT-1 (C21) were purchased from Santa Cruz Biotechnologies. A rat monoclonal Ab to RBP-Jκ (clone K0043) was purchased from Institute of Immunology Co. Ltd., Tokyo, Japan. DNA-protein complexes were resolved in native 5% polyacrylamide gels containing 2.5% glycerol,

unless otherwise mentioned. Gels were analyzed in a phosphor imager using Image-Quant software (GE Healthcare).

Results

In The Absence Of C/EBPβ, A RBP-Jκ-Dependent C/EBPζ-Containing Complex Is Formed At The C/EBP-p50-Site On The CRP Promoter

To explore the composition of the complexes formed at the C/EBP-p50-site, we performed EMSA (Fig. 3.2) using Hep3B nuclear extracts and a 23 bp oligo derived from the CRP promoter as the probe (oligo B). Four DNA-protein complexes, three specific and one nonspecific, were formed (*lanes 1* and *2*). The specific complexes contained C/EBP proteins including C/EBPβ (*lanes 3* and *4*), indicating that some C/EBP proteins were constitutively active in Hep3B nuclei. Pretreatment (i.e. before the addition of radiolabelled probe) of nuclear extract with unlabelled C/EBP consensus oligo resulted in the appearance of a new intense complex comigrating with the nonspecific complex (*lane 4*), consistent with the previously reported observations (26). For clarity, we retain the use of the name "band D" for this new complex, as it was called earlier (26). Ab to RBP-Jκ diminished the intensity of band D, indicating that this complex contained RBP-Jκ (*lane 5*). Ab to C/EBPζ abolished band D, indicating that this complex contained C/EBPζ (*lanes 6* and *7*). When the nuclear extract was pretreated with RBP-Jκ consensus oligo to remove free active RBP-Jκ, the band D was not formed, confirming that the band D contained RBP-Jκ. In addition, this finding also suggested that RBP-Jκ was required for the formation of RBP-Jκ-C/EBPζ complexes at the C/EBP-p50-site (*lane 8*).

Two different patterns of complex formation were seen on this probe (oligo B) in various batches of nuclear extracts (pattern 1: *lanes 1-8*; pattern 2: *lanes 9-16*). In the second pattern, band D was formed even without pretreatment of nuclei with the C/EBP consensus oligo, indicating that this complex can also be formed in the presence of C/EBPβ (*lanes 9* and *10*). The

band D contained C/EBPβ (*lane 11*). Removal of active C/EBPβ from the nuclear extract by pretreatment with C/EBP consensus oligo resulted in the formation of much more intense band D (compare *lanes 9* and *12*). The anti-RBP-Jκ Ab did not affect the intensity of band D (*lane 13*). However, as in pattern 1 (*lane 8*), when the nuclear extract was treated with RBP-Jκ consensus oligo to remove free RBP-Jκ, the formation of band D was abolished (*lane 16*), indicating that RBP-Jκ was present in the complex and was required for the formation of this complex. Anti-C/EBPζ Ab abolished band D (*lanes 14* and *15*), indicating that the complex contained C/EBPζ. From these EMSA, we conclude that two types of C/EBPζ-containing complexes can be formed at the C/EBP-p50-site on the CRP promoter: C/EBPβ-C/EBPζ and RBP-Jκ-C/EBPζ. The formation of both types of complexes is dependent on the presence of RBP-Jκ and perhaps also on the binding of RBP-Jκ to the C/EBP-p50-site.

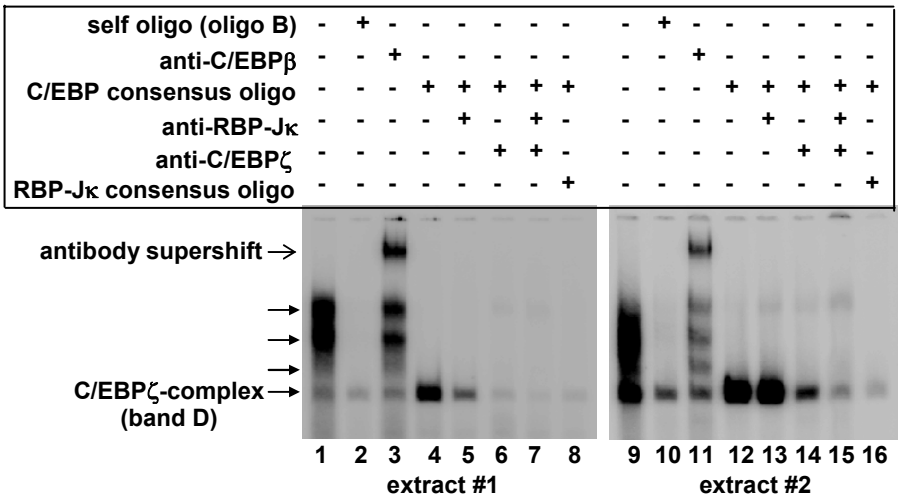


Figure 3.2. Formation of RBP-Jκ-dependent C/EBPζ-containing complex at the C/EBP-p50-site on the oligo derived from the CRP promoter. A representative EMSA using oligo B as the probe is shown. Two batches of Hep3B nuclear extracts were used. The sequences of the oligos used in this and subsequent EMSA are shown in Fig. 3.1*B*. Arrows point to the complexes formed on the probe. The mobility of the free probe in this and subsequent EMSA is not shown.

The Formation Of C/EBPζ-Containing Complex Is Independent Of The Binding Sites For OCT-1, HNF-1 And HNF-3 On The Promoter

To determine whether the binding sites for OCT-1, HNF-1, and HNF-3 on the CRP promoter influence the formation of C/EBPζ-containing complexes, we performed EMSA employing a 41 bp long oligo (oligo A), derived from the CRP promoter, as the probe (Fig. 3.3). Four specific complexes were formed (*lanes 1* and *2*). As was expected, three of the four complexes contained OCT-1 (*lanes 8* and *9*), HNF-1 (*lanes 8* and *10*), and HNF-3 (*lanes 8* and *11*). Pretreatment of nuclear extract with C/EBP consensus oligo did not affect the formation of complexes containing OCT-1, HNF-1 and HNF-3 (*lanes 3-7*); however, the intensity of the fastest migrating fourth complex (band D) was enhanced (compare *lanes 1* and *3*). Ab to RBP-Jκ alone did not affect the intensity of band D (*lane 4*). Ab to C/EBPζ substantially decreased the intensity of band D, indicating that this complex contained C/EBPζ (*lane 5*).

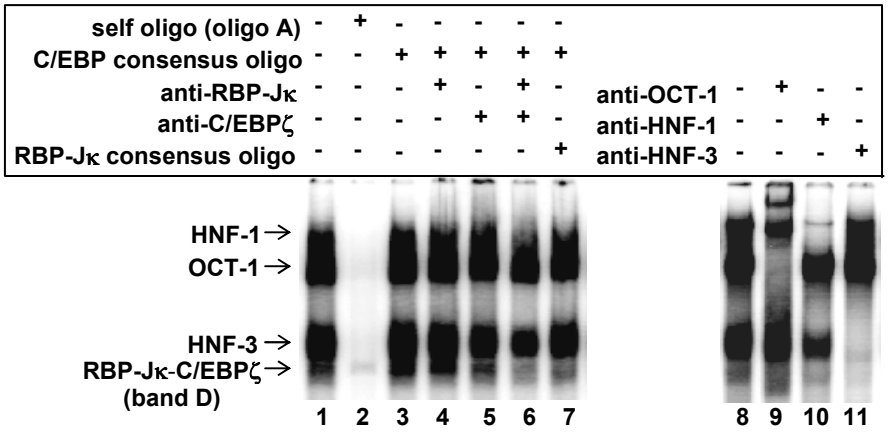


Figure 3.3. Effect of OCT-1, HNF-1 and HNF-3 sites on the formation of C/EBPζ-containing complex. A representative EMSA using oligo A as the probe and nuclear extract from Hep3B cells is shown.

The combination of the two Ab abolished the formation of band D, indicating the presence of both RBP-Jκ and C/EBPζ in the complex (*lane 6*). Importantly, the complex in band D was

competed by unlabelled RBP-Jκ consensus oligo, indicating that this complex contained RBP-Jκ and its formation required RBP-Jκ (*lane 7*), consistent with the data obtained from EMSA using oligo B. We conclude that the formation of C/EBPζ-containing complex is independent of OCT-1, HNF-1 and HNF-3 sites on the CRP promoter.

RBP-Jκ Associates With C/EBPζ, And Both Free RBP-Jκ And RBP-Jκ-C/EBPζ Bind To CRP Promoter

Next, we identified RBP-Jκ and RBP-Jκ-containing complexes in Hep3B nuclei by employing RBP-Jκ consensus oligo as the probe in EMSA. We also evaluated competition between RBP-Jκ consensus probe and the CRP promoter-derived oligos for binding to RBP-Jκ and RBP-Jκ-containing complexes (Fig. 3.4*A*). Specific complexes of two different compositions were formed on the RBP-Jκ consensus probe (*lanes 1* and *2*). Complex II, but not complex I, was supershifted by Ab to RBP-Jκ, indicating that complex II contained RBP-Jκ and it reacted with the anti-RBP-Jκ monoclonal Ab (*lanes 3* and *12*). Because the supershifted complex II comigrated with complex I (compare *lanes 1* and *3*), we could not determine whether the Ab to RBP-Jκ abolished the formation of complex I, if not supershifted. Because the anti-RBP-Jκ Ab is a monoclonal Ab, it is possible that this Ab recognizes free RBP-Jκ only and does not recognize RBP-Jκ when RBP-Jκ is complexed with other transcription factors. Because the probe in this EMSA was RBP-Jκ consensus oligo, complex I should contain RBP-Jκ. Complex I, but not complex II, was abolished by Ab to C/EBPζ, indicating that complex I also contained C/EBPζ (*lane 4*). Thus, in complex I, C/EBPζ was associated with RBP-Jκ. These data indicate the presence of both free RBP-Jκ and preformed RBP-Jκ-C/EBPζ complexes in the Hep3B nuclei.

Competitor oligos A, B, and C derived from the CRP promoter competed with the probe for binding to RBP-Jκ-C/EBPζ in complex I, indicating that this complex was capable of binding to CRP promoter (compare *lanes 6-8* with *lane 1*). The competitor oligos also competed with the probe for binding to RBP-Jκ in complex II, indicating that free RBP-Jκ was also capable of binding to CRP promoter (compare *lanes 15-17* with *lane 10*). Oligo B was a poor competitor compared to oligos A and C. Binding of RBP-Jκ to oligo C could be due to the presence of the κB site at position -69. Used as a control, the C/EBP consensus oligo competitor did not compete with any complex (*lane 9*), indicating that an RBP-Jκ-binding site was necessary for binding of C/EBPζ-containing complexes to the C/EBP-site.

To determine the role of the p50-site in the formation of RBP-Jκ-C/EBPζ complexes at the C/EBP-p50-site on oligo A, we mutated the p50-site. We mutated only those bases of the p50-site that do not overlap the C/EBP-site. We also mutated the κB site. The mutated oligos (shown in Fig. 3.1*B*) were used as competitors in an EMSA with RBP-Jκ consensus oligo as the probe (Fig. 3.4*B*). Similar to that shown in Fig. 3.4*A*, RBP-Jκ (complex II) and RBP-Jκ-C/EBPζ complexes (complex I) were formed on the probe (*lane 1*). Oligo A competed with the probe for binding to complex I (compare *lanes 1* and *3*) and complex II (*lanes 7* and *9*) indicating that both complexes were capable of binding to oligo A. Oligo A-m1 competed with the probe for binding to complex I (*lanes 1* and *4*) but competed less efficiently with the probe for binding to complex II (*lanes 7* and *10*), indicating that the mutation of the p50-site did not abolish the binding of RBP-Jκ-C/EBPζ to the C/EBP-p50-site. Oligo A-m2 competed with the probe for binding to complex II (*lanes 7* and *11*) but competed less efficiently with the probe for binding to complex I (*lanes 1* and *5*), indicating that the κB site also participates in the formation of complex I on oligo A.

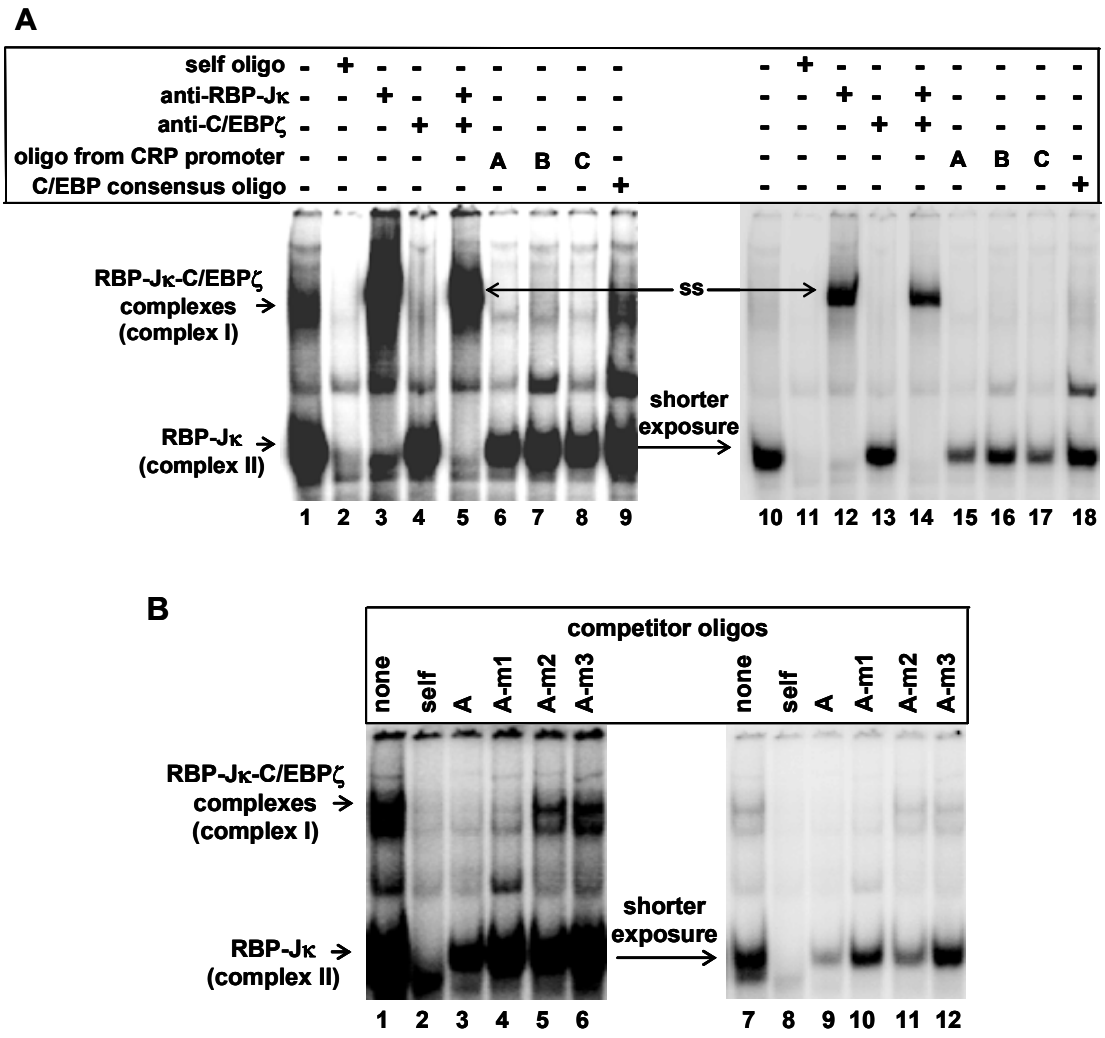


Figure 3.4. Association between RBP-Jκ and C/EBPζ. Two different exposures of the representative EMSA with RBP-Jκ consensus oligo as the probe and nuclear extract from Hep3B cells are shown. DNA-protein complexes were resolved in native 6% polyacrylamide gels. *A.* Hep3B nuclei contain RBP-Jκ-C/EBPζ complexes that bind to the oligos derived from the CRP promoter. The 'ss' represents Ab supershift. *B.* Effect of mutating the p50-sites on the binding of RBP-Jκ-C/EBPζ complexes to oligo A.

Oligo A-m3 did not compete with the probe for binding to complex I (*lanes 1* and *6*) and complex II (*lanes 7* and *12*) indicating that at least one of the two p50-sites was required for the formation of complex I on oligo A. The complex I often appeared in two forms as two bands. The additional component present in one of the two bands is not identified yet. Taken together,

these results suggest that a p50-site is necessary for the formation of RBP-Jκ-C/EBPζ complexes at the C/EBP-p50-site and that the κB site may be cooperating with the C/EBP-p50-site in regulating CRP transcription.

The RBP-Jκ-C/EBPζ Complex Was Also Formed On The Promoter When The Nuclear Extracts Were Pretreated With STAT3 Consensus Oligo

To determine whether the formation of RBP-Jκ-C/EBPζ complexes was stimulated in the nuclei pretreated only with the C/EBP consensus oligo that depletes the nuclei of C/EBPβ, we performed a control EMSA where the nuclear extracts were pretreated with STAT3 consensus oligo (Fig. 3.5*A*). The oligos A and B from the CRP promoter were used as probes. We expected that the band D would not be formed on these probes because the STAT3 consensus oligo does not bind C/EBP and, hence, will not deplete the nuclei of C/EBPβ. Surprisingly, the intensity of band D was enhanced when the nuclear extract was pretreated with the STAT3 consensus oligo (compare *lanes 1* and *3*). The complex in band D was competed by unlabelled RBP-Jκ consensus oligo, indicating that this complex contained RBP-Jκ and its formation required RBP-Jκ (*lane 4*), consistent with the results shown in Fig. 3.2. The intensity of the complex was reduced by individual Ab to C/EBPζ and RBP-Jκ and was abolished by the combination of both Ab, indicating that the complex contained both C/EBPζ and RBP-Jκ (*lanes 5-7*). Similar results were obtained with oligo A as the probe (*lanes 8-13*).

Because the STAT3 consensus oligo contains a polypyrimidine region, similar to that present in the p50-binding κB sites, we examined the ability of the STAT3 consensus oligo to bind to p50. As shown in the EMSA (Fig. 3.5*B*), purified p50 bound to STAT3 consensus oligo.

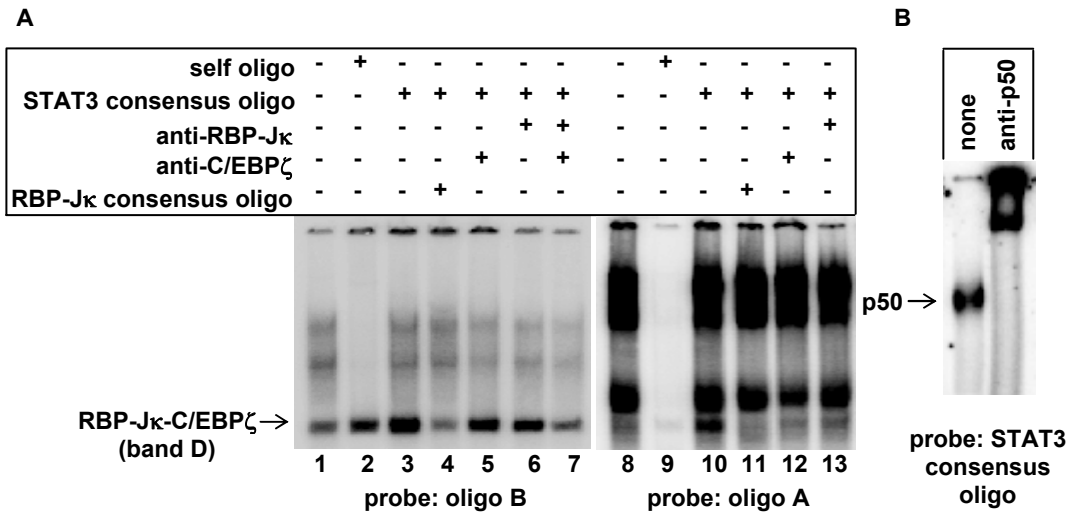


Figure 3.5. *A.* Formation of RBP-Jκ-C/EBPζ complexes at the C/EBP-p50-site in the presence of C/EBPβ. A representative EMSA using oligo B (*lanes 1-7*) and oligo A (*lanes 8-13*) as probes and nuclear extract from Hep3B cells is shown. *B.* Binding of p50 to STAT3 consensus oligo. A representative EMSA using purified p50 and the STAT3 consensus oligo as probe is shown. The gel shift incubation buffer contained 16 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 1 mM DTT and 8% Histopaque. DNA-protein complexes were resolved in native 6% polyacrylamide gels.

RBP-Jκ-Dependent C/EBPζ-Containing Complexes Are Formed At The C/EBP-p50-Site In The Nuclei Of Primary Human Hepatocytes

We next investigated the binding of RBP-Jκ-C/EBPζ complexes to the C/EBP-p50-site of the CRP promoter in the nuclei of primary human hepatocytes. We performed an EMSA using nuclear extracts from untreated hepatocytes and oligo B as the probe (Fig. 3.6).

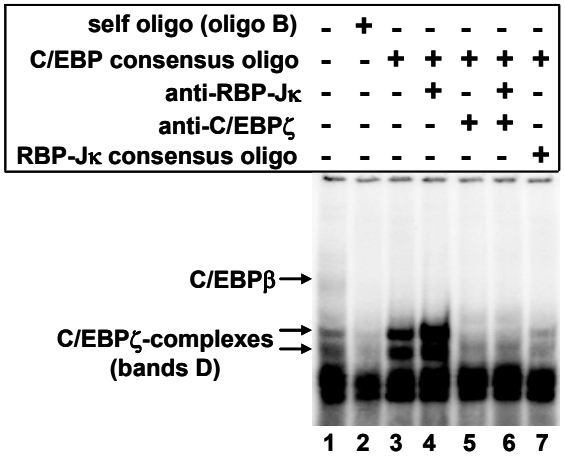


Figure 3.6. Formation of RBP-Jκ-dependent C/EBPζ-containing complexes at the C/EBP-p50-site on the CRP promoter in human hepatocytes. A representative EMSA using oligo B as the probe and nuclear extract from untreated primary human hepatocytes is shown.

Three specific complexes including a very faint complex were formed (*lanes 1* and *2*). The faint complex probably contained C/EBPβ because it was abolished by C/EBP consensus oligo (*lanes 3-7*), indicating negligible presence of C/EBPβ in the hepatocyte nuclei. Pretreatment of nuclear extract with the C/EBP consensus oligo resulted in enhancement of the intensity of the other two complexes (bands D; *lane 3*). Ab to RBP-Jκ did not diminish the intensity of bands D (*lane 4*), consistent with the results shown in *lane 13* of Fig. 2. Ab to C/EBPζ abolished bands D, indicating that both complexes contained C/EBPζ (*lanes 5* and *6*). When the nuclear extract was treated with RBP-Jκ consensus oligo to remove free active RBP-Jκ, the formation of bands D was abolished, indicating that RBP-Jκ was present in bands D and was required for the formation of these complexes (*lane 7*). The additional component in one of the two bands D is not identified yet. The difference between the effects of anti-RBP-Jκ Ab and RBP-Jκ consensus oligo might be due to the possible inability of anti-RBP-Jκ monoclonal Ab to recognize RBP-Jκ-C/EBPζ complexes. The RBP-Jκ consensus oligo, however, binds both RBP-Jκ and RBP-Jκ-

C/EBPζ. From these EMSA, we conclude that the RBP-Jκ-C/EBPζ complexes can be formed at the C/EBP-p50-site of the CRP promoter even in the nuclei of primary human hepatocytes.

Overexpressed C/EBPζ Inhibits Both (IL-6+IL-1β)-Induced And C/EBPβ-Induced CRP Expression

To evaluate the role of C/EBPζ in (IL-6+IL-1β)-induced CRP expression, we performed Luc transactivation assays. Overexpression of C/EBPζ inhibited (IL-6+IL-1β)-induced CRP promoter-driven Luc expression in a C/EBPζ-dose-dependent manner (Fig. 3.7A). Overexpression of RBP-Jκ with C/EBPζ was not required for the inhibitory effects of C/EBPζ on Luc expression (Fig. 3.7B). Thus, the role of C/EBPζ is to repress CRP expression.

To determine whether C/EBPζ inhibited the transactivating effect of C/EBPβ, Luc transactivation assays were performed by transfecting cells with both C/EBPβ and C/EBPζ. We first investigated the competition between C/EBPβ and C/EBPζ to regulate CRP expression based on their relative levels in the nuclei. We performed transactivation assays by trasfecting cells with increasing amounts of C/EBPβ in the presence of a constant amount of C/EBPζ (10 ng expression vector; Fig. 3.7*C*). Overexpression of C/EBPβ alone induced luciferase expression in a dose-dependent manner. At lower levels of C/EBPβ, overexpressed C/EBPζ inhibited C/EBPβ-induced Luc expression. At higher levels of C/EBPβ, overexpressed C/EBPζ did not inhibit C/EBPβ-induced Luc expression. These results indicate that relative levels of C/EBPβ and C/EBPζ are crucial to determine the extent of CRP expression. We then determined the inhibitory role of C/EBPζ by transfecting cells with 1 μg of C/EBPζ expression vector (Fig. 3.7*D*).

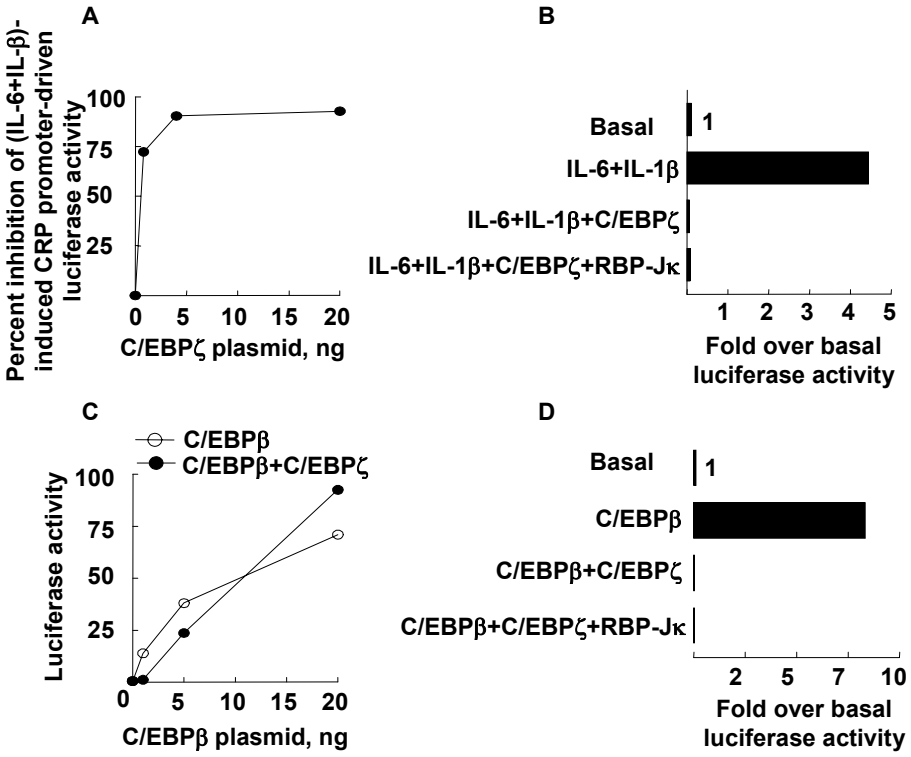


Figure 3.7. Loss of CRP promoter-driven Luc expression in cells overexpressing C/EBPζ. Representative Luc transactivation assays are shown; three independent experiments exhibited similar pattern. *A.* Cells were transfected with the CRP promoter-Luc construct and expression vector encoding C/EBPζ (increasing doses). After 16 h, cells were treated with cytokines. After another 24 h, transcription was measured as Luc activity. Percent inhibition of Luc activity in the presence of overexpressed C/EBPζ is plotted on the y-axis. *B.* As in *A*, except that 1 μg of each expression vector was used for transfection. Basal Luc activity is shown as 1 and the Luc activity in treated cells is plotted as fold-induction over basal activity. *C.* Cells were transfected with the CRP promoter-Luc construct and expression vector encoding C/EBPζ (10 ng) and C/EBPβ (increasing doses). After 40 h, transcription was measured and represented as relative Luc activity. *D.* As in *C*, except that 1 μg each of C/EBPζ and RBP-Jκ, and 50 ng of C/EBPβ plasmid were used for transfection. Basal Luc activity is shown as 1 and the Luc activity in treated cells is plotted as fold-induction over basal activity.

Overexpression of C/EBPβ alone induced Luc expression. Overexpressed C/EBPζ repressed C/EBPβ-induced Luc expression. The endogenous level of RBP-Jκ in the nuclei was found to be sufficient for C/EBPζ to repress C/EBPβ-induced Luc expression because overexpression of RBP-Jκ was ineffective.

Overexpressed RBP-Jκ Alone Has No Effect On CRP Expression

Overexpression of RBP-Jκ alone did not influence basal, IL-6-induced, (IL-6+IL-1β)-induced, or C/EBPβ-induced transactivation of the CRP promoter (Fig. 3.8*A*). We then investigated the effects of overexpressed RBP-Jκ on enhanced basal expression from the CRP promoter with the mutated κB site (Fig. 3.8*B*).

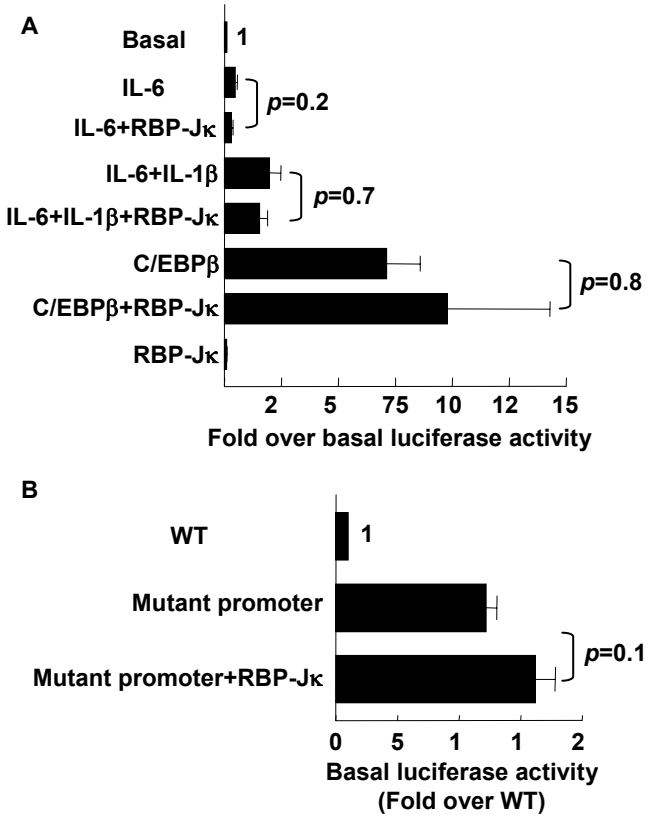


Figure 3.8. No change in the CRP promoter-driven Luc expression in cells overexpressing RBP-Jκ. Data shown represent mean + S.E.M. of five experiments. Mann-Whitney two-tailed test was used to determine *p* values. *A.* Cells were transfected with the CRP promoter-Luc construct and plasmids encoding C/EBPβ (50 ng) and RBP-Jκ (1 μg). Basal Luc activity is shown as 1 and the Luc activity in treated cells is plotted as fold-induction over basal activity. *B.* Two promoter constructs were used: WT promoter (-157/+3) and the mutant promoter (with the κB site at position -69 mutated). The Luc activity of the WT promoter is shown as 1 and the Luc activities of the mutant promoter in the presence and absence of RBP-Jκ are plotted as fold-induction over WT promoter activity.

Consistent with the previously published results (13), basal transactivation of mutated CRP promoter was increased approximately 10-fold when compared to the basal transactivation of the WT promoter. Overexpression of RBP-Jκ had no effect on the enhancement of basal expression from the mutated promoter. These results further suggest that the endogenous level of RBP-Jκ in the Hep3B nuclei was sufficient to participate in regulating CRP expression.

Discussion

We investigated transcription factors interacting with the overlapping C/EBP-binding and p50-binding sites on the CRP promoter and their role in regulating CRP expression. Our major findings were: 1. In the absence of C/EBPβ, a complex containing C/EBPζ and RBP-Jκ was formed at the C/EBP-p50-site in the nuclei of both Hep3B cells and primary human hepatocytes. 2. RBP-Jκ was required for the binding of C/EBPζ-containing dimers to the C/EBP-p50-site. 3. The formation of C/EBPζ-containing complexes was independent of the binding sites for OCT-1, HNF-1 and HNF-3 on the promoter. 4. Overexpressed C/EBPζ repressed both (IL-6+IL-1β)-induced and C/EBPβ-induced CRP expression. These findings suggest that the regulation of CRP transcription is partly determined by the relative levels of C/EBPβ and C/EBPζ in the nuclei. The C/EBPζ-mediated repression of CRP transcription involves a novel action of RBP-Jκ. RBP-Jκ associates with C/EBPζ-complexes to facilitate the binding of C/EBPζ to C/EBP-site on the CRP promoter.

Previously (26), EMSA was performed using an oligo containing the C/EBP-p50-site as the probe and Hep3B nuclear extracts pretreated with C/EBP consensus oligo as the source of transcription factors. This EMSA revealed the formation of a complex, called band D, on the probe. This complex did not appear to contain either C/EBP or NF-κB proteins (26). We, however, hypothesized that the complex might contain C/EBPζ because the C/EBP consensus oligo binds to and depletes the nuclear extract of all C/EBP proteins except C/EBPζ. Our alternate hypothesis was that the complex might contain RBP-Jκ because it has been shown that RBP-Jκ binds to certain κB sites (30, 32). On some gene promoters, RBP-Jκ competes with NF-κB proteins to repress NF-κB-induced transcription (37, 38, 43). Interestingly, we found that the complex present in band D contained both C/EBPζ and RBP-Jκ.

Unexpectedly, we observed formation of an intense RBP-Jκ-C/EBPζ complex at the C/EBP-p50-site in the nuclear extract pretreated with STAT3 consensus oligo, suggesting that the formation of band D could occur even in the presence of active C/EBPβ in the nuclei. This phenomenon can be explained by considering the sequence similarity between STAT3 consensus oligo and p50-binding nonconsensus κB site on the CRP promoter. Both sequences contain a polypyrimidine region (Fig. 3.1*B*). In addition to binding to STAT3, the STAT3 consensus oligo also binds p50. It was, therefore, likely that p50 was absorbed by the STAT3 consensus oligo in the pretreated nuclear extract and no p50 was available to bind to p50-site on the probe in EMSA. A vacant p50-site would then be occupied by RBP-Jκ facilitating the formation of band D. Together, these data indicate that C/EBPζ-containing complexes at the C/EBP-p50-site can be formed in two situations: when C/EBPβ is absent and when C/EBPβ is present but p50 is absent. RBP-Jκ is required in both situations. This interpretation also suggests that the binding of p50 to p50-site is a prerequisite for the binding of C/EBPβ to C/EBP-site.

In some EMSA, the Ab to RBP-Jκ did not affect the intensity of band D even if the formation of band D was invariably found to be dependent on the presence of RBP-Jκ. We interpret these results to indicate that the complex in such a band D contained RBP-Jκ-C/EBPζ dimers and that the monoclonal Ab to RBP-Jκ did not recognize these dimers. Our data show the existence of RBP-Jκ-C/EBPζ dimers in the nuclei and their capability to bind to RBP-Jκ consensus sequence, in addition to their binding to C/EBP-p50-site on the CRP promoter.

C/EBPζ inhibits expression of other C/EBP-inducible genes such as transferrin gene, in Hep3B cells (21). However, the mechanism of inhibitory action of C/EBPζ in transferrin gene expression involves a dominant regulatory effect. C/EBPζ dimerizes with other C/EBP proteins

to inhibit their binding to C/EBP-sites in the promoter (21, 47). Binding of C/EBPζ-containing dimers to a novel DNA target sequence, PuPuPuTGCAAT(A/C)CCC, has been reported earlier (48). However, the binding of C/EBPζ to this DNA sequence did not require either RBP-Jκ or an adjacent RBP-Jκ-binding site. On the CRP promoter, we found that the binding of C/EBPζ-containing dimers to the C/EBP-site required RBP-Jκ. This function of RBP-Jκ in facilitating the binding of C/EBPζ to the C/EBP-site is novel. Other mechanisms for the role of RBP-Jκ in gene expression have been described. For example, adjacent C/EBP-binding and RBP-Jκ-binding sites are present on the IL-6 promoter and it has been shown that RBP-Jκ suppresses activation of the IL-6 promoter by NF-κB and C/EBPβ (43, 49).

We observed two patterns of the formation of C/EBPζ-containing band D: either the complex was not formed until C/EBPβ was removed from the nuclear extract, or the complex was formed in the presence of C/EBPβ and its intensity was increased when C/EBPβ was removed. We also found that the inhibition of CRP expression by overexpressed C/EBPζ was related to the levels of C/EBPβ in the nuclei. These results suggest that the C/EBP-p50-site on the CRP promoter is a critical regulatory element and that the regulation occurs by C/EBP-dimers containing either C/EBPβ (activating) or C/EBPζ (inhibitory). The formation of a specific dimer causing activation or inhibition of CRP expression may depend upon the relative levels of C/EBPβ and C/EBPζ in the nuclei. The role of the relative levels of two transcription factors in the nuclei in regulating gene expression through a common site on the promoter has been described earlier (50). Because C/EBPβ induces synthesis of C/EBPζ (17), and C/EBPζ is also activated in acute phase response (51), our data suggest that C/EBPζ is a critical regulator of CRP expression.

In Fig. 3.9, we summarize our conclusions on the roles of C/EBPζ, C/EBPβ, RBP-Jκ and p50 in regulating CRP expression through the C/EBP-p50-site on the promoter.

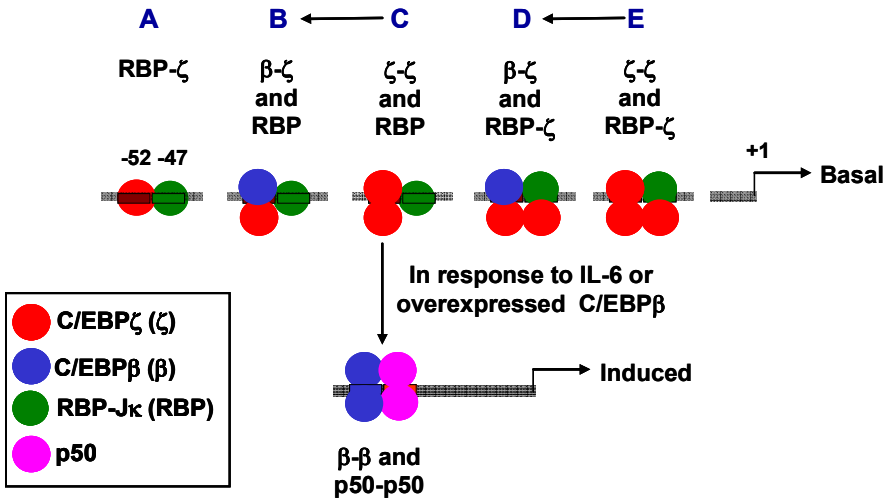


Figure 3.9. A model showing the switch between RBP-Jκ-C/EBPζ and p50-C/EBPβ at the C/EBP-p50-site on the CRP promoter. Under basal conditions, there are five possible arrangements (A-E) of transcription factors C/EBPζ and RBP-Jκ in the complex formed at the C/EBP-p50-site. Under induced conditions, C/EBPζ-containing dimers are replaced by C/EBPβ/β homodimers and this switch requires binding of p50 homodimers to p50-site. The formation of C/EBPβ/ζ heterodimers could be a transitory stage (C to B and E to D) between C/EBPζ/ζ homodimers and C/EBPβ/β homodimers.

Under basal conditions, there are five possible arrangements of transcription factors at the C/EBP-p50-site. 1. The RBP-Jκ-C/EBPζ dimer binds to C/EBP-p50-site (arrangement A). This is supported by the data that RBP-Jκ-C/EBPζ formed on the RBP-Jκ consensus oligo was competed by CRP promoter-derived oligos (compare complex I in *lanes 1* and *6-8* in Fig. 4). 2. RBP-Jκ binds to p50-site and supports binding of C/EBPβ/ζ heterodimer to C/EBP-site (arrangement B). This is supported by the data that, occasionally, RBP-Jκ, C/EBPβ and C/EBPζ were all constituents of band D (*lanes 11, 14,* and *16* in Fig. 2). 3. RBP-Jκ binds to p50-site and supports binding of C/EBPζ/ζ homodimer to C/EBP-site (arrangement C). This is supported by the data that the intensity of band D in most EMSA was diminished by individual Ab to RBP-Jκ

and C/EBPζ, and that the band D was abolished by the combination of both Ab. 4. RBP-Jκ-C/EBPζ dimer binds to p50-site and supports the binding of C/EBPβ/ζ heterodimer to C/EBP-site (arrangement D). This is a modified arrangement B, with RBP-Jκ being associated with C/EBPζ. 5. RBP-Jκ-C/EBPζ dimer binds to p50-site and supports the binding of C/EBPζ/ζ homodimer to C/EBP-site (arrangement E). This is a modified arrangement C, with RBP-Jκ being associated with C/EBPζ. In all five arrangements, RBP-Jκ was required because no complex was formed on the C/EBP-p50-site in the nuclei pretreated with RBP-Jκ consensus oligo. All these arrangements would cause repression of CRP expression and maintain basal expression. Under induced conditions, C/EBPζ-containing dimers are replaced by C/EBPβ/β homodimers and this switch requires binding of p50 homodimers to p50-site. The formation of C/EBPβ/ζ heterodimers (arrangements B and D under basal conditions) could be a transitory stage between C/EBPζ/ζ homodimers and C/EBPβ/β homodimers.

Footnotes

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[3] Abbreviations used in this paper: CRP, C-reactive protein; oligo, oligonucleotide; Luc, luciferase; WT, wild-type.

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CHAPTER 4

Effects of overexpressed transcription factor OCT-1 on C-reactive protein expression

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Key Words: C-reactive protein, inflammation, statin, nitric oxide, atherosclerosis

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Reference: Some of the findings were published in Reference#62. Additional unpublished data are reported in this chapter (Manuscript in preparation)

Abstract

Inflammation and cellular stress induce the expression of C-reactive protein gene in hepatocytes. In human hepatoma Hep3B cells, basal and (IL-6+IL-1β)-induced CRP expression is regulated at the transcriptional level. OCT-1 is a constitutively expressed transcription factor that binds to the proximal CRP promoter at -63 overlapping the binding sites for NF-κB, HNF-1, and HNF-3. A C/EBP site is centered at position -52 away from the OCT-1 site. In this study, we investigated the role of OCT-1 in CRP gene expression. In luciferase transactivation assays, overexpressed OCT-1 repressed (IL-6+IL-1β)-induced but not C/EBPβ-induced CRP promoter-driven luciferase activity. The amount of OCT-1 required to repress was less when the overlapping NF-κB site was mutated suggesting that the effect of OCT-1 is more pronounced when the overlapping NF-κB site is vacant. Deletion of the 8 bp OCT-1 site (-66/-59) overlapping the binding sites for HNF-1/3 and NF-κB resulted in the partial loss of cytokine-mediated induction of CRP indicating that the region is critical for IL-6 and IL-1β response. OC T-1 mediated inhibition was not completely lost when the OCT-1 site was deleted. These results led to the conclusion that OCT-1 represses CRP proximal promoter mediated CRP transcription and this repression may not be mediated through the OCT-1 site. The 8 bp region (-66/-59) is critical for (IL-6+IL-1β)-mediated induction of CRP expression. But more studies need to be done to determine the role of OCT-1 in CRP expression.

Introduction

C-reactive protein (CRP) is an acute phase protein whose serum concentration increases in acute and chronic inflammatory conditions and in some non inflammatory conditions such as stress and cell injury (1-4). CRP is primarily produced by hepatocytes (5). In human hepatoma Hep3B cells, the CRP gene is regulated at the transcriptional level. CRP is produced in response to cytokines IL-6 and IL-1β (6-8). In Hep3B cells, CRP production by cytokines is further enhanced by Dex. IL-6 induces CRP expression by activating transcription factors C/EBPβ and STAT3 (9, 10). C/EBPβ binds to the sites centered at -52 and -219 on the CRP promoter. STAT3 binds to a site centered at -108 (10). IL-1β, which alone does not induce CRP expression, synergistically enhances the effects of IL-6 partially by activating NF-κB p50/p65 that binds to a κB site centered at -69 (13-15). Another κB site on the CRP promoter is located at -2652. The proximal 157 bp of the CRP promoter is sufficient for the synergy between IL-6 and IL-1β in Hep3B cells. NF-κB p50/p50 bind to a site centered at -47 overlapping the proximal C/EBP site (11, 12). The overlapping C/EBP-p50 site is critical for the induction of CRP transcription by cytokines (12). Two additional transcription factors, C/EBPζ and RBP-jκ, have been shown to bind to the C/EBP-p50 site and regulate CRP expression (14). Hepatocyte nuclear factor-1 (HNF-1), HNF-3, and OCT-1 are constitutive transcription factors that have overlapping binding sites on the CRP promoter and are involved in maintaining the basal CRP transcription (16, 17).

OCT-1 belongs to the POU domain family of proteins (18). OCT proteins are a group of transcription factors that bind specifically to an octamer motif (ATGCAAAT) and related sequences (18). OCT-1 is expressed in all eukaryotic cells and regulates, either positively or negatively, the expression of a variety of genes (19, 20). In regulating basal promoter activity of some genes, OCT-1 has been shown to directly interact with several basal transcription factors

such as TATA binding protein (20, 22, 23). It has also been shown to interact with tissue specific transcription factors and cofactors mediating specific gene expression such as hormonal activation of MMTV (murine mammary tumor virus) and GnRH (gonadotropin-releasing hormone) promoters (24, 25). OCT-1 has also been shown to activate hormonally induced beta casein promoter activity by binding to the hormonal regulatory region of the beta casein gene promoter (25).

OCT-1 binds to the site centered at -63 overlapping the binding sites for HNF-1 and HNF-3 on the CRP proximal promoter. Previously, a binding site for NF-κB p50/p65 was identified overlapping the binding sites for OCT-1, HNF-1, and HNF-3 (15). This study showed that the region -74/-59 containing the overlapping binding site for NF-κB and OCT-1 is a critical regulatory region and suggested the requirement of NF-κB proteins for the binding of OCT-1 to its site. The current study further explores the role of OCT-1 in CRP gene expression. In this study, we performed northern blot and luciferase transactivation assays to determine the role of OCT-1 in CRP expression.

Materials And Methods

Cell Culture And Cytokine-Treatment And Transfection

Hep3B cells (provided by Dr. G. J. Darlington, Baylor College of Medicine, Houston, TX) were grown as described previously (19). Cells were cultured in serum-free medium overnight for cytokine-treatments and transfections. The confluency of cells was approximately 60% at the time of treatments. IL-6 and IL-1β (R & D) were used at concentrations of 10 ng/ml and 1 ng/ml, respectively. The cells were treated with cytokines for 24 h for Luc transactivation assays. For transient transfections, Hep3B cells were plated into 6-well plates and transfected using FuGENE6 reagent (Roche) as described previously (19).

Engineering Of CRP Promoter-Luciferase Reporter Constructs, And Luc Transactivation Assay

CRP promoter-Luc reporter constructs were used at 1μg plasmid per well and the transcription factor expression vectors were used as mentioned in the figure legends. After 16 h of transfection, the transfected cells were either treated with cytokines for 24 h or left untreated. After 40 h of transfection, Luc transactivation assays were performed following the protocol supplied by the manufacturer (Promega), and the Luc activity was measured in a luminometer (Molecular Devices) as described previously (19). The preparation of wild-type (WT) CRP promoter (-157/+3)-Luc reporter construct has been described previously. An expression vector for OCT-1 was obtained from Dr. Herr. The wild-type (WT) CRP promoter constructs, Luc-157 WT and Luc-300 WT, have been described earlier in Chapter 2. These two WT constructs were used as templates for mutagenesis. Engineering of constructs containing mutated κB site was described previously in Chapter 2. Constructs containing mutated OCT-1 site and mutated C/EBP site were prepared using the QuickChange site-directed mutagenesis kit (Stratagene) with

the WT constructs as the template. The OCT-1 site was mutated by deleting the 8 bp OCT-1 site by using mutagenic primers: 5'-GCAATGTTGGAAAATTAGTGGCGCAAACTCCCTTACTGC and 5'-GCAGTAAGGGAGTTTGCGCCACTAATTTTCCAACATTGC. The C/EBP site was mutated by substituting $^{-53}$CGCAA^{-49} with $^{-53}$ATATC^{-49} by using mutagenic primers: 5'-GGAAAATTATTTACATAGTGGATATCACTCCCTTACTGCTTTGG and 5'-CCAAAGCAGTAAGGGAGTGATATCCACTATGTAAATAATTTTCC. Mutations were verified by sequencing. Plasmids were purified using the maxiprep plasmid isolation kit (Eppendorf).

Preparation Of Nuclear Extract And EMSA

Hep3B nuclear extracts were prepared using NE-PER nuclear and cytoplasmic kit (Pierce) as reported earlier (19). EMSA on Hep3B nuclear extracts was carried out as described previously (19). Unless otherwise mentioned, the gel shift incubation buffer contained 16 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 2.5 mM DTT, 0.15% Nonidet P-40, 8% Histopaque, and 1 μg of poly(deoxyinosinic-deoxycytidylic acid). The sequences of the oligos derived from the CRP promoter and mutated oligos used in EMSA are as follows.

Oligo WT: 5'- TGTTGGAAAATTATTTACATAGTGGCGCAAACTCCCTTACT

Oligo mOCT: 5'- TGTTGGAAAATTAGTGGCGCAAACTCCCTTACT

Oligos were obtained from Integrated DNA Technologies. To prepare the probes, complementary oligos were annealed and labelled with either [α-^{32}P] CTP or [γ-^{32}P] ATP. In supershift experiments, Ab (2 μg) were added to the reaction mixture and incubated on ice for 15 min before addition of the probe. In competition experiments, 150 ng of unlabelled oligos were

added to the binding reactions before addition of the Ab and probe. The Abs to C/EBPβ (C19), p50 (H-119), p65 (H-286), HNF-1 (H205), HNF-3 (C20) and OCT-1 (C21) were purchased from Santa Cruz Biotechnologies. DNA-protein complexes were resolved in native 5% polyacrylamide gels containing 2.5% glycerol, unless otherwise mentioned. Gels were analyzed in a phosphor imager using Image-Quant software (GE Healthcare).

Results

OCT-1 Inhibits Cytokine-Induced Proximal 157 bp Promoter-Driven CRP Transcription: To clearly determine the role of OCT-1 in cytokine-induced CRP transcription, we performed luciferase transactivation assays. Construct of the wild-type (WT) promoter region -157/+3 linked to luciferase reporter gene (Luc 157-WT) was transfected into Hep3B cells along with increasing amounts of plasmid expressing OCT-1. In these assays, OCT-1 inhibited (IL-6 + IL-1β)-induced CRP transactivation in a dose-dependent manner (Fig.4.1*A*).

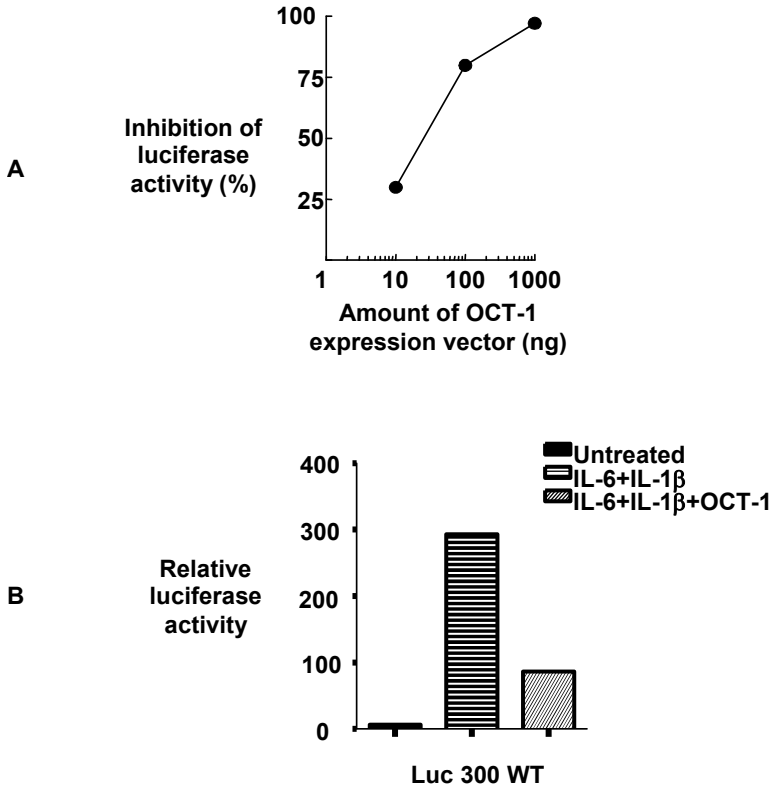
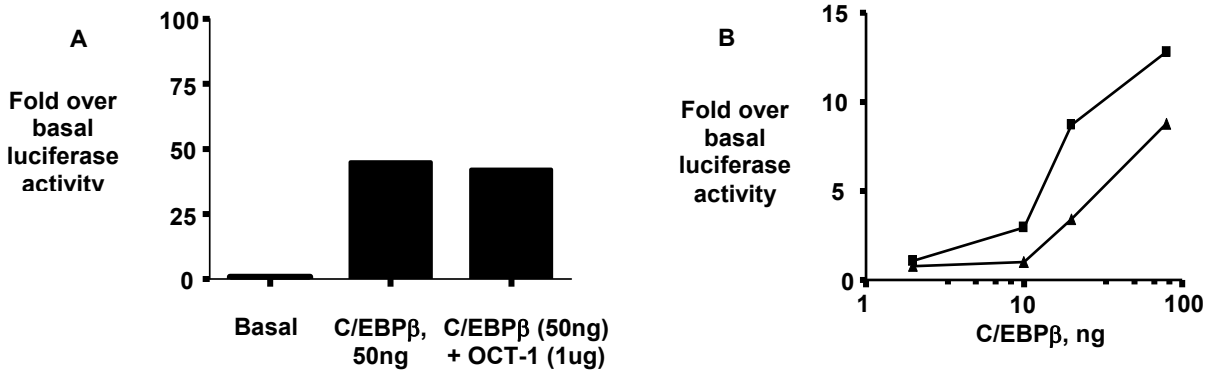


Figure 4.1. Effect of OCT-1 on cytokine-induced CRP promoter-driven luciferase activity. A. Cells were transfected with Luc 157 WT construct and the expression vector encoding OCT-1 (increasing doses). After 16h, cells were treated with cytokines. 24h after the cytokine treatment, CRP transcription was measured as luciferase activity. Percent inhibition of luciferase activity by OCT-1 is plotted on the y-axis. B. Same as in Panel A, but the Luc 300-WT promoter construct (-300/+1) was used. 1μg of OCT-1 was used in these experiments. Experiment was performed two times and a representative experiment was shown.

To determine if the presence of additional C/EBP site at -219 will have an effect on OCT-1 mediated inhibition, a construct of the wild-type (WT) promoter region -300/+1 linked to luciferase reporter gene (Luc 300-WT) was transfected into Hep3B cells along with 1μg of OCT-1. With Luc 300-WT also, OCT-1 inhibited (IL-6 + IL-1β)-induced CRP transactivation (Fig. 4.1*B*).

No Effect Of OCT-1 On C/EBPβ-Induced CRP Transactivation: To determine the mechanism of inhibition of OCT-1 on cytokine-induced transcription in luciferase transactivation assays, it is essential to understand the effect of OCT-1 on individual transcription factors activated by the cytokines IL-6 and IL-1β. IL-6 and IL-1β induce CRP expression by activating transcription factors STAT3, C/EBPβ and NF-κB p50/p65. To determine whether OCT-1 has an effect on C/EBPβ-induced CRP transactivation, luciferase transactivation assays were performed by transfecting Hep3B cells with Luc 300 WT (Fig. 4.2) along with plasmids expressing C/EBPβ and OCT-1. OCT-1 did not inhibit C/EBPβ-induced CRP transactivation. Different amounts of C/EBPβ and OCT-1 were used to determine the effect of OCT-1 and with all combinations of C/EBPβ and OCT-1, OCT-1 did not have an effect (Fig. 4.2*A*-*C*). These results indicate that OCT-1 inhibits cytokine-induced CRP transactivation by interacting with transcription factors, other than C/EBPβ, that are activated in response to cytokines.



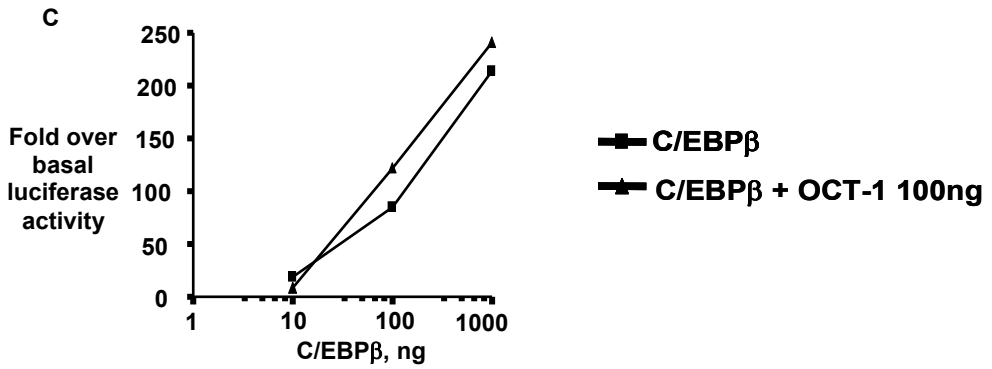


Figure 4.2. Effect of OCT-1 on C/EBPβ-induced CRP promoter-driven luciferase activity. A. Cells were transfected with Luc 300 WT construct and the expression vectors encoding C/EBPβ (50ng) and OCT-1 (1μg). 40h after transfection, CRP transcription was measured as luciferase activity. Basal luciferase activity is shown as 1 and the effect of C/EBPβ and OCT-1 is plotted as fold over basal luciferase activity on the y-axis. A representative experiment was shown. B. As in A, but increasing amounts of C/EBPβ (0-100 ng) was used and the effect of 100 ng of OCT-1 on C/EBPβ-induced transcription was measured. C. As in B, but different amounts of C/EBPβ (0-1000ng) was used.

OCT-1 Represses CRP Expression Better If The NF-κB Site Is Vacant And Is Not Occupied By NF-κB p50/p65: Previous study indicated that the binding of OCT-1 to its site was influenced by NF-κB proteins. To determine the role of NF-κB in OCT-1 mediated inhibition and to analyze the role of NF-κB binding on OCT-1 effect, we mutated the NF-κB binding site at -69. We mutated the NF-κB site at position -69 to determine the effect of OCT-1 on cytokine-induced CRP transcription in the absence of NF-κB binding. Constructs of the wild type (WT) promoter and the κB-site mutant promoter region -157/+3 linked to luciferase reporter gene (Luc 157 WT and Luc 157 mκB respectively) were transfected into Hep3B cells along with the plasmid expressing OCT-1. With both the constructs, there was a concentration-dependent inhibition of cytokine-induced CRP promoter-driven luciferase activity (Fig. 4.3), but the amount of OCT-1 required to repress was more in the presence than in the absence of NF-κB site. This result

indicates that OCT-1 is capable of repressing cytokine-induced CRP transactivation in a more efficient manner when the κB site is vacant and not occupied by NF-κB p50/p65.

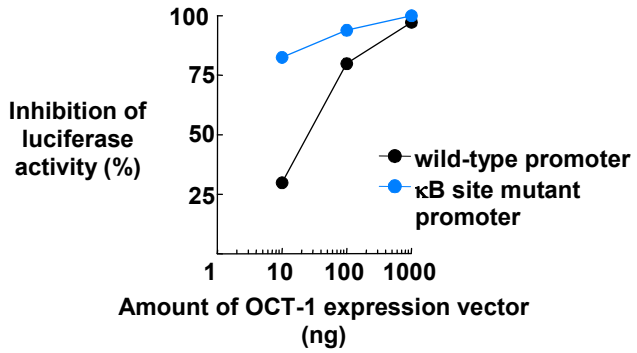


Figure 4.3. Effect of OCT-1 on cytokine-induced CRP promoter-driven luciferase activity (WT and κB-mutant promoters). Same as in Figure 2A, but two promoter constructs were used. WT promoter (-157/+3) and the mutant promoter with the κb-site at position -69 mutated.

Inhibition Of Cytokine-Induced CRP Transactivation By OCT-1 Is Not Mediated Through The OCT-1 Site: To determine whether the inhibition of cytokine-induced CRP transactivation by OCT-1 is mediated through the binding of OCT-1 to its site, luciferase transactivation assays were performed by mutating the OCT-1 site. Multiple mutations were carried out to selectively inhibit OCT-1 binding to its site, but due to the presence of overlapping binding sites for HNF-1, HNF-3, NF-κB p50-p65, and OCT-1, selective abolition of OCT-1 binding was not attained. With 2-4 bp mutations, OCT-1 remained bound (Data not shown). EMSA was performed by deletion of the entire 8bp OCT-1 site. Three specific complexes of HNF-1, OCT-1 and HNF-3 were formed on the wild type oligo WT (Fig. 4.4A, lanes 1-5), whereas deletion of the 8 bp region abolished the binding of OCT-1, HNF-1, and HNF-3 (Fig. 4.4A, lanes 6-10).

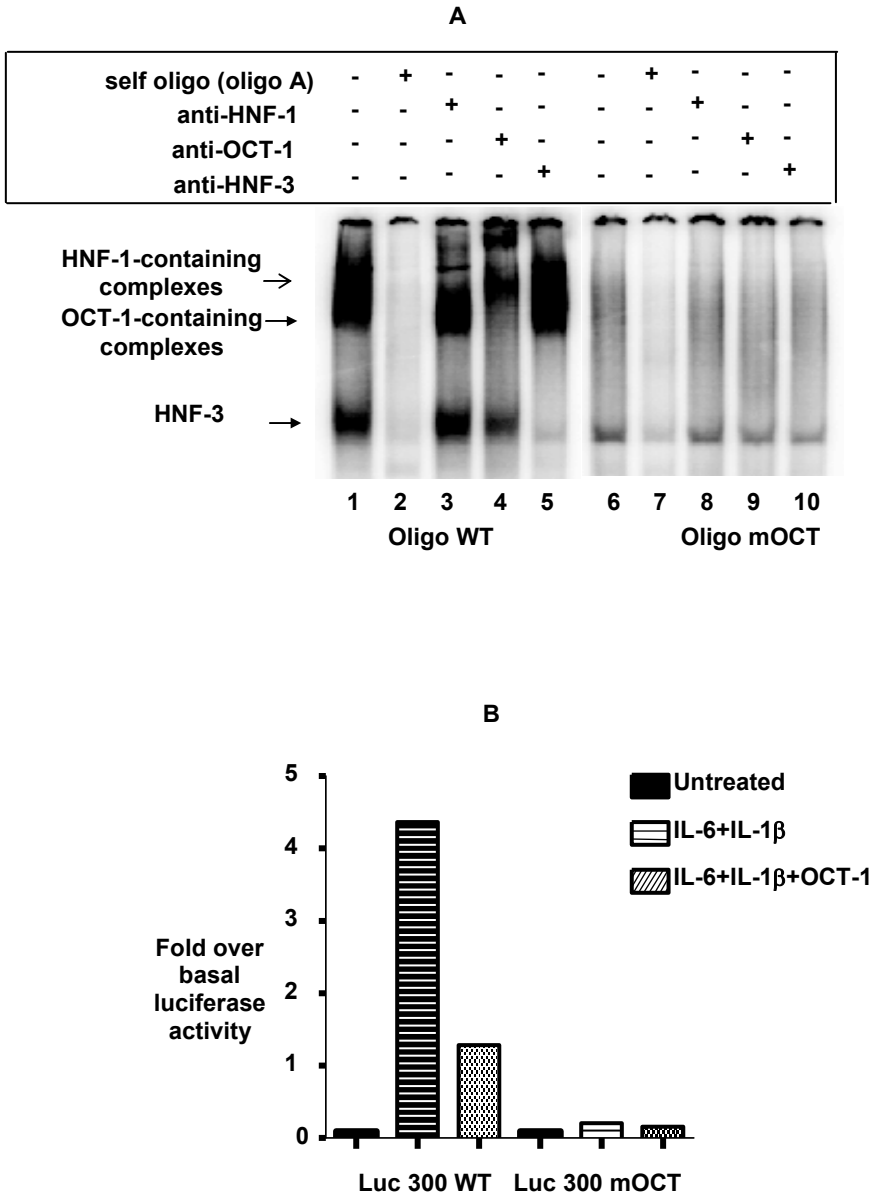


Figure 4.4. A. Deletion of the region -59/-66 (8 bp OCT-1 site) results in the loss of binding of transcription factors HNF-1, OCT-1 and HNF-3. EMSA using Oligo WT (lanes 1-5) and Oligo mOCT (Oligo with the 8 bp OCT-1 site deleted, lanes 6-10) as the probes and nuclear extracts from untreated Hep3B cells is shown. B. Inhibition of cytokine-induced CRP promoter-driven luciferase activity is not mediated through the OCT-1 site. Cells were transfected with Luc 300 WT and Luc 300 mOCT (8 bp OCT-1 site deleted by site-directed mutagenesis) along with the expression vector encoding OCT-1 as shown. After 16h, cells were treated with cytokines. Basal luciferase activity was expressed as 1 and the fold over basal luciferase activity is plotted on the y-axis. Experiment was performed two times and a representative experiment was shown.

8bp OCT-1 site was deleted from the Luc 300 WT and Luc 300 mOCT were transfected into Hep3B cells along with the plasmid expressing OCT-1. Overexpression of OCT-1 affected the cytokine-induced transactivation of Luc 300 mOCT similar to that of Luc 300 WT suggesting that the inhibitory effect of OCT-1 is not mediated through the OCT-1 site (Fig. 4.4*B*).

-59/-66 Region Of The CRP Promoter Is Required For The (IL-6+IL-1β)-Induced CRP Expression: To determine the role of the multiple transcription factor binding site (-59/-66) on the CRP promoter in cytokine-induced CRP expression, Luc 157 wt and Luc 157 mOCT were transfected into Hep3B cells and the effect of cytokines on CRP transactivation was determined. Absence of 8 bp region (-59/-66) containing the OCT-1 site and the overlapping binding sites for HNF-1 and HNF-3 in the CRP promoter resulted in partial loss of cytokine-induced CRP transactivation (Fig 4.5*A*) indicating that the -59/-66 region is required for the full cytokine response of CRP promoter. Similar results were obtained with Luc 300 WT and Luc 300 mOCT (Fig 4.5*B*) in which the proximal 300 bp of the CRP promoter was linked to the luciferase reporter gene. It was shown previously that C/EBP site on the CRP promoter is critical for IL-6 response. Mutation of C/EBP site (Luc 300mC/EBP) also resulted in the loss of cytokine-induced CRP transactivation (data not shown) indicating that both the -59/-66 region and the C/EBP site are critical for cytokine response.

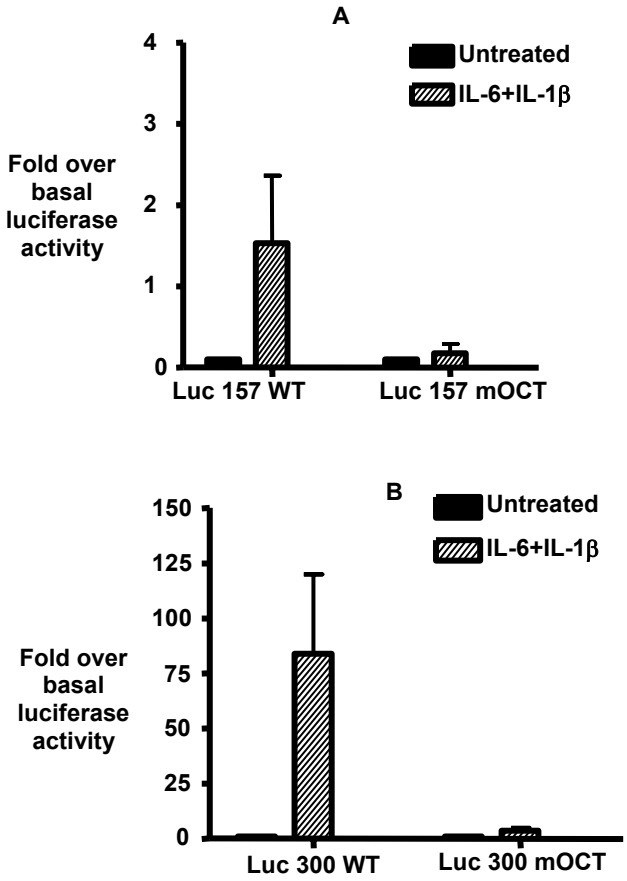


Figure 4.5. -59/-66 region of the CRP promoter is required for (IL-6+Il-1β)-induced CRP prmoter-driven luciferase activity. A. Cells were transfected with Luc 157 WT and Luc 157 mOCT (8 bp OCT-1 site deleted by site-directed mutagenesis) as shown. After 16h, cells were treated with cytokines. Basal luciferase activity is taken as 1 and the fold over basal luciferase activity is plotted on the y-axis. B. Same as in A, but promoter constructs, Luc 300 WT and Luc 300 mOCT (8 bp OCT-1 site deleted by site-directed mutagenesis) were used. Data shown represent mean + S.E.M of 3 experiments.

OCT-1 Binds To Its Site On The CRP Promoter Both In The Absence And Presence Of C/EBP Site On The CRP Promoter: We have previously shown that OCT-1 binds to the 23 bp oligonucleotide derived from the CRP promoter. To determine whether OCT-1 binds to its site in the presence of C/EBP site, EMSA was performed using a 45bp oligonucleotide (Oligo WT) derived from the CRP promoter and nuclear extracts derived from IL-1β-treated Hep3B cells. Four specific complexes were formed (Fig.4.6, Lanes 1, 2). The fastest migrating complex was

identified as NF-κB (p50-p65) by using antibodies to p50 and p65 (Lanes 3, 4 respectively). By using antibodies to HNF-1 and OCT-1, the top 2 complexes were shown to contain HNF-1 and OCT-1(Lanes 5, 6). Another specific complex containing HNF-3 was formed on the probe and was identified by using antibodies to HNF-3 (Not shown).

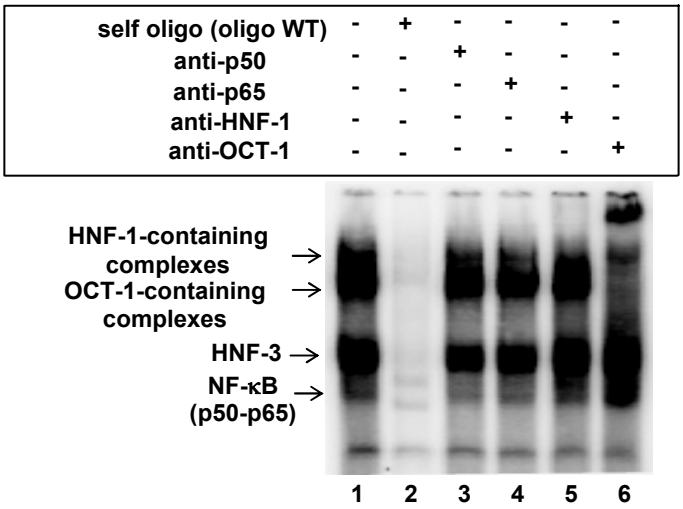


Figure 4.6. Formation of OCT-1 complex on the CRP promoter in the presence of C/EBP site. A representative EMSA using a 45 bp oligo WT as the radiolabeled probe is shown. Nuclear extract from IL-1β treated Hep3B cells was used. Arrows point to the complexes formed on this probe. Mobility of the free probe in this EMSA and the subsequent EMSA is not shown.

Influence Of HNF-1 On The Composition Of OCT-1 Containing Complex: The binding sites for HNF-1/-3 and OCT-1 are overlapping on the CRP promoter. To determine the influence of one transcription factor on the binding of another transcription factor, we performed EMSA using different amounts of nuclear extract (Fig. 4.7). In this EMSA, with the decrease in the amount of nuclear extract from 3μl to 1μl, there is a difference in the mobility of the complexes formed. The principle involved here is that as the amount of nuclear extract is decreased, one or more of the transcription factors (mainly HNF-1) in the nuclear extract become limited. This resulted in a change in the binding pattern indicating that the relative levels of transcription factors in the nuclei with respect to the radiolabelled probe determine the occupancy of the CRP promoter.

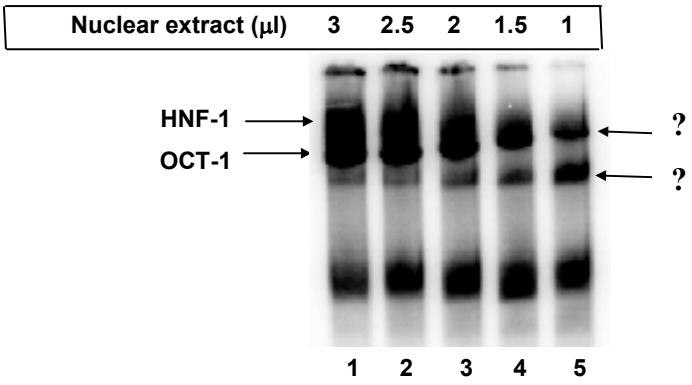


Figure 4.7. Influence of HNF-1 on the composition of OCT-1 containing complexes. A representative EMSA using the 45 bp oligo WT as the probe is shown. Nuclear extracts from untreated Hep3B cells were used.

Discussion

Previously, we have shown the binding of OCT-1 to the CRP promoter. To further elucidate the role of OCT-1 in CRP gene expression, we performed luciferase transactivation assays. Key findings from the study are:

1. OCT-1 binds to the CRP promoter both in the presence and absence of the adjacent C/EBP site.

2. OCT-1 inhibits (IL-6+IL-1β)-induced CRP transcription through the proximal 157 bp and 300 bp of the CRP promoter.

3. OCT-1 does not affect C/EBPβ-mediated induction of CRP transcription.

4. OCT-1 effect is more pronounced when the overlapping NF-κB site is vacant and not occupied by NF-κB p50/p65.

5. The -59/-66 region overlapping the binding sites for HNF-1/3 and NF-κB is critical for full (IL-6+IL-1β)-induced CRP transcription.

6. OCT-1 mediated inhibition was not completely lost when the OCT-1 site was deleted.

To determine the role of OCT-1, we performed luciferase transactivation assays using either proximal 157 bp or 300 bp of the CRP promoter that consists of binding sites for the known transcription factors that are involved in CRP transcription. OCT-1 inhibited (IL-6+IL-1β)-induced CRP transcription through the proximal 157 bp and 300 bp of the CRP promoter.

C/EBPβ is a major inducer of CRP transcription. To determine if OCT-1 inhibits CRP transactivation by interacting with C/EBPβ, the effect of OCT-1 on C/EBPβ-induced CRP transactivation was studied. OCT-1 did not have an effect on C/EBPβ-induced CRP transactivation. Previous studies have indicated that binding of OCT-1 to the CRP promoter requires p50/p50 homodimers and that NF-κB p50/p65 replaces OCT-1 and induces CRP

transcription (15). Our results with Luc 157mNF (Fig. 5.4) showed that OCT-1 has a more pronounced effect if the NF-κB site is vacant. Overexpressed NF-kB (p50/p65) induces CRP mRNA accumulation in Hep3B cells. Recently, OCT-1 has been shown to interact with p65 and repress NF-κB-dependent reporter gene expression of E-selectin and V-CAM-1 suggesting a possible effect of OCT-1 through the interaction with NF-κB p50/p65 on the CRP promoter (26). Since the effect of OCT-1 is influenced by the difference in NF-κB binding, effect of OCT-1 on NF-κB p50/p65-mediated accumulation of CRP mRNA will be studied. To determine the effect of OCT-1 on CRP mRNA accumulation, effect of OCT-1 on p50/p65 induced CRP transcription can be studied. Further studies need to be done to determine the mechanism of inhibition of proximal 157 bp and 300 bp-mediated CRP expression by OCT-1.

This study also showed that the cytokine effect on CRP expression requires the composite OCT-1/HNF-1/HNF-3/NF-κB site. C/EBPβ-mediated induction of CRP expression was also decreased in the absence of the region -59/-66 suggesting that the C/EBP site and the composite OCT-1/HNF-1/HNF-3/NF-κB site act together.

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CHAPTER 5

Statins and nitric oxide reduce C-reactive protein production while inflammatory conditions persist

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Key Words: C-reactive protein, inflammation, statin, nitric oxide, atherosclerosis

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Abstract

C-reactive protein (CRP) is made in liver and its serum concentration increases in inflammation. Measurement of serum CRP is recommended for use as an indicator of inflammation and predictor of atherosclerosis. Cholesterol-lowering drugs statins also lower CRP. To evaluate statin-mediated CRP reduction and to reassess clinical usefulness of CRP, we investigated regulation of CRP gene expression. Here, we show that pravastatin and simvastatin prevent the induction of CRP expression in human hepatoma Hep3B cells exposed to proinflammatory cytokines IL-6 and IL-1β. The nitric oxide (NO) donor, sodium nitroprusside, also prevented the induction of CRP expression while the CRP inducers IL-6 and IL-1β were present with the cells. The effect of NO on CRP expression was at the level of transcription. These findings suggest that the decrease in CRP level *in vivo* after statin-treatment does not necessarily reflect absence of inflammation, and that NO-releasing drugs have the potential to reduce serum CRP levels. Thus, the measurement of serum CRP levels alone in individuals on statin/NO-therapy is not as useful as was imagined.

Introduction

C-reactive protein (CRP) is an acute phase protein whose serum concentration increases even under chronic inflammatory conditions such as atherosclerosis (Agrawal, 2005; Black et al., 2004). CRP starts functioning *in vivo* probably after binding to ligands like phosphocholine-containing substances (Agrawal et al., 2002) such as modified low-density lipoproteins (Bhakdi et al., 2004; Chang et al., 2002), and extracellular matrix proteins such as fibronectin (Suresh et al., 2004). Any role of CRP in the pathogenesis of atherosclerosis is not certain, although elevated serum CRP is considered as a predictor of cardiovascular diseases (Libby and Ridker, 2004).

CRP is primarily produced by hepatocytes (Kushner and Feldmann, 1978) and can be experimentally induced in human hepatoma Hep3B cells by treatment with proinflammatory cytokines IL-6 and IL-1β (Ganapathi et al., 1991). In these cells, induction of CRP expression by (IL-6+IL-1β) is further enhanced by dexamethasone (Dex) (Ganapathi et al., 1991). Hep3B cells cultured in the presence of proinflammatory mediators represent an alternative to animal models of inflammation to investigate the mechanism of regulation of CRP gene expression.

In Hep3B cells, transcription factors C/EBPβ (Agrawal et al., 2001; Agrawal et al., 2003a; Cha-Molstad et al., 2000; Li and Goldman, 1996), STAT3 (Zhang et al., 1996), and NF-κB (Agrawal et al., 2003b) participate in the induction of CRP expression. On the CRP promoter, transcription factor C/EBPβ binds to a site at -52, STAT3 binds to a site at -108, and NF-κB binds to a site at -69 (Li and Goldman, 1996; Voleti and Agrawal, 2004; Zhang et al., 1996). A second C/EBP-site is located at position -219, however, the first 157 bp of the CRP promoter are sufficient for synergistic induction of CRP expression by IL-6 and IL-1β (Li and Goldman, 1996; Zhang et al., 1995).

Statins that lower cholesterol levels have also been shown to lower CRP levels in human blood (Nissen et al., 2005; Ridker et al., 2005). Statins enhance nitric oxide (NO) production from many cell types (Harris et al., 2004; Kaesemeyer et al., 1999), and since NO regulates expression of a number of genes in the hepatocytes (Bogdan, 2001; Davis et al., 2001), we explored the possible role of NO donors, and of statins, in CRP expression in Hep3B cells.

Materials And Methods

Cell Culture, ELISA, RNA Isolation, And Northern Blot

Hep3B cells were cultured in 100 mm dish containing 5 ml growth media and subjected to serum starvation overnight for cytokine, sodium nitroprusside (SNP), and statin treatments as described previously (Agrawal et al., 2001). The confluency of cells was approximately 60% at the time of treatments. IL-6 and IL-1β (R & D) were used at concentrations of 10 ng (100 U)/ml and 20 ng (200 U)/ml, respectively. Dex (Sigma) was used at 1μM. SNP (Fisher Scientific), pravastatin sodium salt (Wako Pure Chemical Industries Ltd.), and simvastatin sodium salt (Calbiochem) treatments were started 45 min prior to cytokine treatment. For CRP ELISA (Suresh et al., 2004), RNA isolation, and luciferase assay, the cells were treated with cytokines for 72 h, 40 h, and 24 h, respectively. Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen) and subjected to Northern blot exactly as described previously (Agrawal et al., 2003b). EcoRI-cut CRP cDNA clone (Agrawal et al., 2002) in the plasmid p91023 and GAPDH cDNA (Sigma) were used as probes in Northern blot.

Determination Of NO Production

NO production was determined using the Greiss reaction to monitor nitrite levels in cell culture media (Green et al., 1982). 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) was mixed with 50 μl of culture medium. OD_{550} was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} values of standard solutions of sodium nitrite (1-1000 nM).

Transfection And Luciferase Transactivation Assay

For transient transfections, cells were plated into 6-well plates and transfected using FuGENE 6 reagent (Roche) as described previously (Agrawal et al., 2003b). Luciferase reporter-

CRP promoter constructs were used at 1μg plasmid per well. SNP and cytokine treatments were started 16 h post-transfection. After 40 h of transfection, luciferase assays were performed as described previously (Agrawal et al., 2003b). Luciferase activity was measured in a luminometer (Molecular Devices).

Engineering Of CRP Promoter-Luciferase Reporter Constructs

The wild-type (WT) CRP promoter construct, Luc-157 WT, has been described earlier (Agrawal et al., 2001). Luc-300 WT construct was prepared according to a published method (Kleemann et al., 2003). Briefly, genomic DNA (Promega) was used to amplify a fragment corresponding to nucleotides -300/-1 of the CRP promoter, using the primers 5'-CCTAGATCTAGAGCTACCTCCTCCTGCCTGG and 5'-CCGACGCGTACCCAGATGGCCACTCGTTTAATATGTTACC. Primers were designed to contain the B*gl*II and M*lu*I restriction sites, respectively. The PCR product was cloned into the luciferase reporter vector pGL2 basic (Promega) and the DNA sequence was confirmed. These two WT constructs were used as templates for mutagenesis. Constructs containing mutated κB and STAT3 site were generated using the QuickChange site-directed mutagenesis kit (Stratagene). The κB-site was mutated by substituting $^{-72}$AAAATT^{-67} with $^{-72}$TTAATA^{-67} using mutagenic primers: 5'-GCGCCACTATGTAAATTATTAACCAACATTGCTTGTTGGGGC and 5'-GCCCCAACAAGCAATGTTGGTTAATAATTTACATAGTGGCGC. The STAT3-site was mutated by substituting $^{-111}$TCCCGA^{-106} with $^{-111}$GATATC^{-106} using mutagenic primers 5'-GCTTCCCCTCTGATATCAGCTCTGACACCTG and 5'-CAGGTGTCAGAGCTGATATCAGAGGGGAAGC. Mutations were verified by sequencing. Plasmids were purified using maxiprep plasmid isolation kit (Eppendorf).

Results

Pravastatin And Simvastatin Prevent (IL-6+IL-1β)-Induced CRP Production

Production of CRP by Hep3B cells in response to (IL-6+IL-1β) or (IL-6+IL-1β+Dex) was reduced when the cells were co-treated with pravastatin or simvastatin (Fig. 5.1). Requirement of higher concentrations of pravastatin for the observed inhibition of CRP expression indicated that the pravastatin we used here had poor accessibility to the cells and was not suitable for cell culture experiments. The inhibitory effect of statins was independent of the action of Dex.

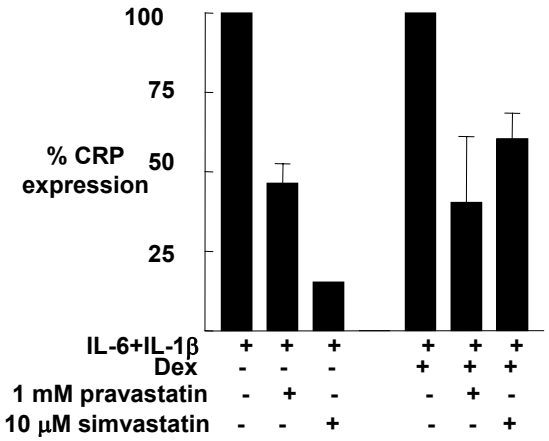

Figure 5.1. Statins prevent production of CRP by Hep3B cells while the cells are exposed to proinflammatory cytokines IL-6 and IL-1β. CRP expression in the absence of statins is plotted as 100%.

NO Prevents (IL-6+IL-1β)-Induced CRP Production

Treatment of Hep3B cells (Fig. 5.2) with the NO donor SNP (Yamamoto and Bing, 2000) alone did not affect CRP production. Pretreatment of cells with SNP prevented almost completely the induction of CRP production in response to (IL-6+IL-1β+Dex). The suppressing effect of NO on CRP expression was SNP-dose-dependent (Fig. 5.3A), as determined by ELISA. Increasing concentrations of SNP produced increasing concentrations of NO in the cell culture

medium (Fig. 5.3B). Another NO donor, S-nitroso-N-acetylpenicillamine (SNAP), also inhibited (IL-6+IL-1β+Dex)-induced CRP expression (data not shown).

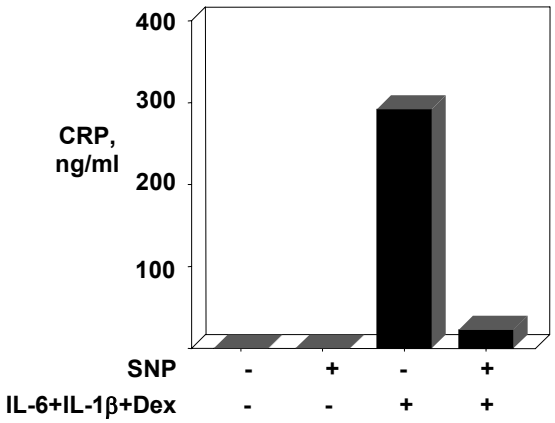

Figure 5.2. NO prevents production of CRP, as measured by ELISA, by Hep3B cells while the cells are exposed to proinflammatory cytokines. SNP was used at 200 μM. A representative experiment is presented.

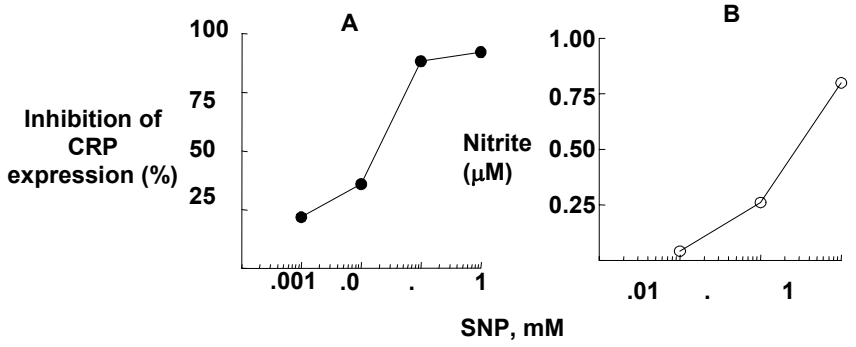

Figure 5.3. NO-dose-dependent suppression of CRP production. A representative experiment is presented. (A) CRP ELISA, Treatment with increasing concentration of SNP in the presence of a fixed concentration of IL-6+IL-1β+Dex. CRP expression without SNP-treatment was used to calculate percent inhibition. (B) Increased nitrite production in the culture media in response to increase in SNP.

Effect Of NO On CRP mRNA Accumulation

Employing Northern blot, the accumulation of CRP mRNA in Hep3B cells was measured in response to (IL-6+IL-1β+Dex)-treatment in the absence and presence of various concentrations of SNP. The inhibition of CRP expression by NO was at the level of transcription as indicated by the dose-dependent decrease in CRP mRNA accumulation (Fig. 5.4). A dose of 1mM SNP was not toxic to the cells as seen by GAPDH expression and trypan blue staining. As little as 1 μM SNP was sufficient to observe the inhibition of CRP expression, both at the protein level and at the mRNA level.

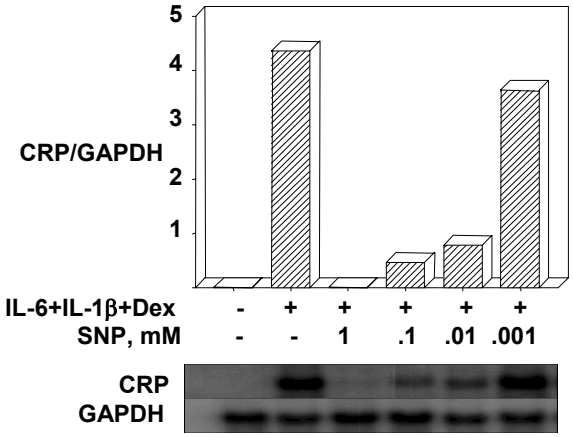

Figure 5.4. NO decreases CRP mRNA accumulation in Hep3B cells in the presence of IL-6 and IL-1β. Northern blot on total RNA was analyzed in a phosphorimager. Ratio of CRP to GAPDH mRNA accumulation is shown on the y-axis. A representative experiment is presented.

The STAT3-Site And The κB-Site Do Not Mediate The Inhibition Of CRP Expression By NO

Luciferase-transactivation assays were performed utilizing CRP promoter constructs with the mutated STAT3-site and mutated κB-site (Luc-300 m-κB, Luc-300 m-ST, Luc-157 m-κB, and Luc-157 m-ST) to evaluate their role in mediating the inhibitory effect of NO on CRP expression (Fig. 5.5). SNP-treatment of the cells inhibited (IL-6+IL-1±Dex)-induced

transactivation of all the mutated constructs, as SNP did on the WT promoters. Thus, the suppressing effect of NO on CRP expression was not through the STAT3 and κB sites. We could not employ mutagenesis to determine the role of the C/EBP-site because mutation of the C/EBP-site abolished all responses to cytokines (Agrawal et al., 2001; Cha-Molstad et al., 2000). By comparing 300 bp and 157 bp WT promoters, we ruled out involvement of the CEBP-site located at position -219 in mediating NO effects.

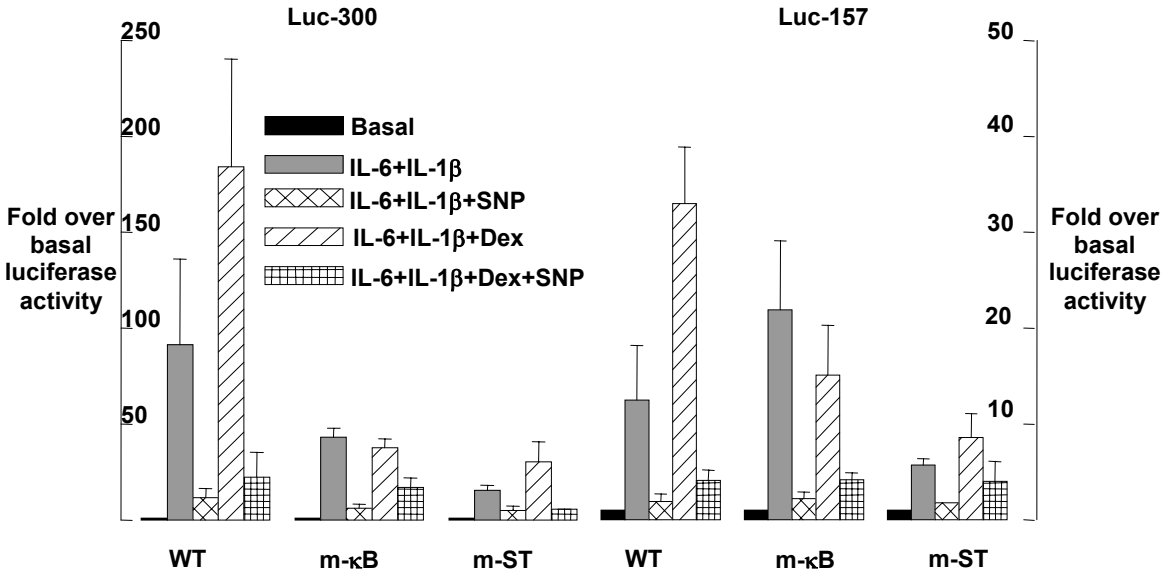

Figure 5.5. The STAT3-site and the κB-site do not mediate inhibition of CRP expression by NO. In all the promoter constructs, basal luciferase activity is shown as 1 and the luciferase activity in treated-cells is plotted as fold-induction over basal activity. The average ± SEM of 3 experiments is shown.

Discussion

Statins have been shown to lower CRP levels independently of their cholesterol-lowering activity (Nissen et al., 2005; Ridker et al., 2005). Since statins are known to generate NO and since NO modulates gene expression (Bogdan, 2001; Davis et al., 2001; Kaesemeyer et al., 1999; Harris et al., 2004), we investigated the effects of statins and NO on CRP expression in Hep3B cells. Our major findings were: 1. Pravastatin and simvastatin prevented induction of CRP expression in Hep3B cells exposed to proinflammatory molecules IL-6+IL-1β. We have not yet determined whether the statins' effect was mediated through NO generation. 2. NO donor SNP also prevented induction of CRP expression in response to IL-6+IL-1β. These findings suggest that the lowering of CRP by statins is not an indication of a decrease in the extent of proinflammatory cytokines and that the measurement of cytokines, but not of CRP, is required to assess anti-inflammatory outcome of statins.

In the presence of statins, the unresponsiveness of Hep3B cells to proinflammatory cytokines, resulting in the loss of CRP production, was not totally unexpected. It has been demonstrated previously that statins inhibit CRP production by IL-1-treated human CRP-transgenic mice, by IL-1-treated and IL-6-treated primary human hepatocytes, and by IL-1-treated human hepatoma cells (Arnaud et al., 2005; Kleemann et al., 2004; Verschuren et al., 2005). We interpret these data, generated from *in vitro* and *in vivo* models of inflammation, to conclude that even if inflammation persists, CRP is not produced due to the direct inhibitory action of statins on CRP-producing cells. We present this concept in a schematic diagram (Fig. 5.6) showing the reduction of CRP expression in response to statin/NO while the proinflammatory mediators may still be present with the hepatocytes.

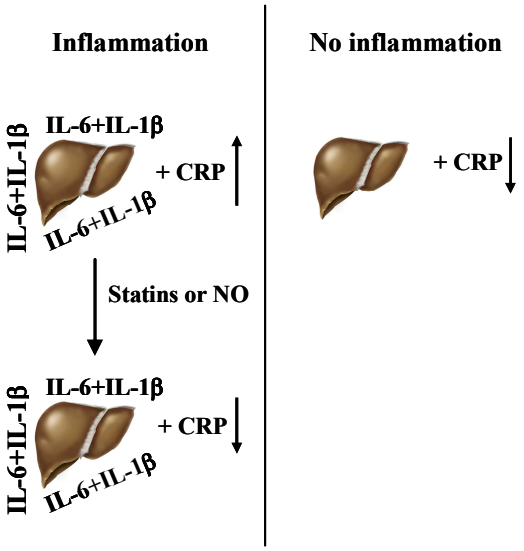

Figure 5.6. Schematic diagram of our *in vitro* experiment: Statins and NO reduce CRP production while the proinflammatory mediators are still present in the vicinity of CRP-producing hepatoma cells.

Earlier, it was proposed that the lowering of CRP production in response to statin-treatment might be due to the ability of statins to induce NO (Kaesemeyer and Caldwell, 2000). An inverse relationship between CRP and NO concentrations was shown in population-based studies (Braga et al., 1996; Cleland et al., 2000; Fichtlschrer et al., 2004). We have shown here the role of exogenously supplied NO in CRP expression by Hep3B cells exposed to cytokines. Hep3B cells, however, are capable of producing endogenous NO and under certain conditions e.g. hypoxia, IFNγ-treatment, the production of NO by Hep3B cells is enhanced (Imagawa et al., 2002; Yoshioka et al., 1997). Both NO and CRP are produced by liver in inflammation (Curran et al., 1989; Geller et al., 1993). Additionally, NO is produced by a variety of mammalian cells and is produced in numerous physiological and pathological conditions including during inflammation. Thus, it is likely that endogenous NO may also be participating in regulating CRP expression.

Our results support the hypotheses that NO might down-regulate CRP expression *in vivo* (Kaesemeyer and Caldwell, 2000; McCarty, 2004) and that the strategies to lower plasma CRP might be effective by improving NO bioavailability (Fischtlscherer et al., 2004). The contribution of CRP in the development of atherosclerosis has not been documented yet, but if a deleterious role of CRP is proposed, our findings raise the possibility of utilizing NO-releasing drugs to lower CRP expression. If the manipulation of NO is feasible, then the dose of statins sufficient to lower cholesterol levels need not be modified. NO-releasing aspirins have been developed for use to improve protection for the heart without the unwanted effects on the stomach (Napoli et al., 2002; Wallace et al., 2002). We suggest that, until a role of CRP in the pathogenesis of atherosclerosis is found, the NO-aspirin should be used with caution because it may lower CRP levels.

IL-6 and IL-1 are the main inducers of CRP expression (Castell et al., 1990; Ganapathi et al., 1991). Many other molecules such as IL-4, IL-11, TNF, TGFβ, and Dex have also been reported to participate in regulating CRP expression. IL-4 and TNF decrease IL-6-induced CRP expression (Gabay et al., 1999; Yap et al., 1991). IL-11 has been shown to increase CRP in a study with women participants (Gordon et al., 1996). TGFβ affects CRP expression at the post-translational level (Taylor et al., 1990). Our data indicate that NO is also a major player and is as important as IL-6 and IL-1 in regulating CRP expression.

One mechanism by which NO regulates gene expression is by directly influencing transcription factors. It is shown previously that p50-p50/C/EBPβ complex participates through a nonconsensus κB-site on the CRP promoter in inducing CRP expression (Agrawal et al., 2001), and the fenofibrate-mediated inhibition of IL-1-induced CRP expression is due to the decrease in the formation of p50-p50/C/EBPβ complexes (Kleemann et al., 2003). We

have not yet measured such complexes in SNP-treated cells. STAT3 was also shown to mediate the inhibitory effect of fenofibrates on CRP expression (Gervois et al., 2004). However, we found that neither the STAT3-site nor the κB-site was involved in mediating the NO effects. At present, we do not conclude about the mechanism of NO-mediated inhibition solely based on the luciferase assays.

The mechanism of statin/NO-mediated reduction in CRP mRNA levels is not clear, but the implications of our findings remain as significant for the evaluation of statin-mediated lowering of CRP in the presence of inflammation. We favor the advice that the CRP values to evaluate cardiovascular complications should be considered carefully (Kushner, 2002; Munford, 2001; Pepys, 2005).

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CHAPTER 6

SUMMARY

Major findings of our research were:

1. IL-1β-activated NF-κB p50-p65 acted synergistically with IL-6-activated C/EBPβ to induce CRP transactivation through 157 bp of the promoter.
2. A minimum amount of C/EBPβ was critical for the NF-κB synergy.
3. A κB-site was located at position -69, overlapping the known OCT-1/HNF-1/HNF-3 sites.
4. The κB-site was required for the synergism between NF-κB and C/EBPβ.
5. The κB-site, in part, contributed to the synergism between IL-6 and IL-1β.
6. A novel interaction between OCT-1 and NF-κB dimers p50-p50 and p50-p65 was observed indicating that this binding site on the CRP promoter was a key element in regulating CRP gene expression under basal and inflammatory conditions.
7. In the absence of C/EBPβ, a complex containing C/EBPζ and RBP-Jκ was formed at the C/EBP-p50-site in the nuclei of both Hep3B cells and primary human hepatocytes.
8. RBP-Jκ was required for the binding of C/EBPζ-containing dimers to the C/EBP-p50-site.
9. Overexpressed C/EBPζ repressed both (IL-6 and IL-1β)-induced and C/EBPβ-induced CRP expression.
10. Constitutive transcription factor, OCT-1 bound to the CRP promoter both in the absence and presence of C/EBP site.
11. OCT-1 either enhanced or did not have an effect on (IL-6 and IL-1β)-induced CRP mRNA accumulation.

12. But OCT-1 repressed (IL-6 and IL-1β)-induced CRP transactivation through the proximal 157 bp and 300 bp of the CRP promoter.
13. C/EBPβ is a major inducer of CRP transcription in Hep3B cells and OCT-1 did not repress C/EBPβ-induced CRP transactivation.
14. Effect of OCT-1 was more pronounced when the overlapping κB-site is vacant and not occupied by NF-κB p50/p65.
15. Selective abolition of OCT-1 binding to the CRP promoter could not be achieved due to the overlapping binding sites for transcription factors HNF-1, HNF-3 and NF-κB.
16. -59/-66 region of the CRP promoter containing the multiple transcription factor binding sites is required for the (IL-6 and IL-1β)-induced CRP expression.
17. Pravastatin and simvastatin prevented induction of CRP expression in Hep3B cells exposed to proinflammatory molecules IL-6 and IL-1β. We have not yet determined whether the effect of statins was mediated through NO generation.
18. NO donor SNP also prevented induction of CRP expression in response to IL-6 and IL-1β.
19. Effect of SNP was at the transcriptional level.
20. The STAT3 site and the NF-κB site located in the proximal CRP promoter did not mediate the effects of SNP.

These findings led to the identification of binding sites for 3 additional transcription factors, NF-κB p50/p65, RBP-Jκ and C/EBPζ. These three transcription factors were shown to be involved in CRP gene transcription. The following figure (Fig. 6.1) shows the modified CRP proximal promoter.

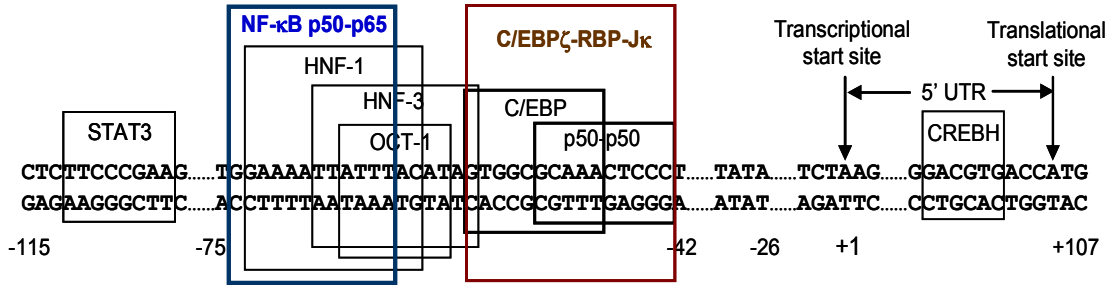

Figure 6.1. Proximal CRP promoter with the additional transcription factors binding sites. +1 indicates the transcription start site. Sequences of the transcription factor binding sites are shown in boxes. Colored boxes are used to show binding sites for the newly identified transcription factors involved in CRP transcription.

Major shortcoming of the study is that the findings are Hep3B cell line specific. The interactions of the transcription factors with the CRP promoter need to be confirmed in primary human hepatocytes and other hepatocyte cell lines such as HepG2 and Huh7. Another issue of concern is that we used naked DNA in the transcription factor binding studies, the findings may not be true at the chromatin level. In addition, the effect of each transcription factor on the endogenous CRP gene, instead of the proximal 300 bp or 157 bp of the CRP promoter, has to be shown to clearly elucidate the mechanism of CRP gene regulation.

We have identified two additional transcription factors, C/EBPζ and RBP-Jκ, that are involved in basal CRP expression [57]. C/EBPζ abolished both the cytokine-mediated and C/EBPβ-mediated CRP transactivation. These findings suggest that the regulation of CRP transcription is partly determined by the relative levels of C/EBPβ and C/EBPζ in the nuclei. It was shown that RBP-Jκ associates with C/EBPζ-complexes to facilitate the binding of C/EBPζ to the C/EBP-site on the CRP promoter, but the mechanism by which RBP-Jκ associates with C.EBPζ and the mechanism by which C/EBPζ represses CRP transcription are not known. Association of these transcription factors with the CRP promoter has to be validated at the chromatin level.

OCT-1 repressed (IL-6 and IL-1β)-induced CRP transactivation through the proximal 157 bp and 300 bp of the CRP promoter and the effect of OCT-1 varied with the mutation of NF-κB site. The results suggested that the relative levels of OCT-1 and NF-κB (p50/p65) and their binding to the proximal CRP promoter influence CRP transcription. Because overexpression of NF-κB (p50/p65) induces CRP expression, the effect of OCT-1 on NF-κB (p50/p65)-induced CRP transcription will provide the mechanism of OCT-1-mediated CRP transcription.

Inhibition of CRP gene expression by NO was at the transcriptional level. The STAT3 and NF-κB site did not mediate the inhibition of CRP mRNA accumulation by NO [59]. Effect of NO on CRP mRNA stability needs to be studied. Cycloheximide and Actinomycin D, which are commonly used agents to study mRNA stability, have differential effects on CRP mRNA stability [60]. CRP mRNA stability was enhanced by Actinomycin D and Cycloheximide does not have an effect on CRP mRNA accumulation suggesting complex regulatory processes [60]. Hence, these two agents cannot be used to study CRP mRNA stability but, instead, effect of NO donors on the rate of CRP transcription needs to be determined.

NF-κB site was identified in the proximal promoter and this site was shown to be involved only partially in the synergy between IL-6 and IL-1β. In many cells, NF-κB activation involves upstream protein kinase C (PKC) activation. The activation of PKC is also known to result in the phosphorylation of Ser 105 within the activation domain of C/EBPβ, thereby enhancing its transcriptional activity. The PKC pathway has been shown to be involved in the induction of CRP gene [61]. The PKC-dependent induction of CRP expression is only partially mediated by NF-κB and additional mechanisms such as C/EBP-dependent activation have been proposed [61]. In addition to the activation of transcription factors C/EBPβ and STAT3, IL-6

activates p38 and MAP kinase pathways. In Hep3B cells, recent reports have suggested the involvement of MAP kinase and PI3 kinase pathways in CRP gene expression.

In addition to IL-6 and IL-1β, other cytokines such as TNF-α and IL-17 have been shown to regulate CRP gene expression [23, 62]. IL-17 was reported to induce CRP gene expression in Hep3B cells and the induction is mediated by p38 MAPK and ERK1/2-dependent NF-κB and C/EBPβ activation [23]. The involvement of these pathways in the synergy between IL-6 and IL-1β in inducing CRP expression has to be further explored. Recent research showed the role of β-catenin in TNF-α-induced CRP transcription. An interaction of β-catenin, bound to the downstream regulatory region in the intron of CRP gene, with NF-κB p50/p50 was proposed in the study [62]. Further studies need to be done to determine the role of downstream regulatory regions in CRP gene transcription.

In addition to the cytokines, IL-6 and IL-1β, Dex enhances the cytokine-mediated induction of CRP gene. The mechanism by which Dex influences CRP gene expression is not known. In rat hepatocytes, Dex represses NF-κB DNA-binding activity in part through the upregulation of its inhibitor I-κBα [63]. Because NF-κB is involved in IL-1β-mediated CRP gene expression, effect of Dex on NF-κB activity in human hepatocytes needs to be studied. The effect of Dex on CRP gene expression was lost in both the constructs Luc 300m-κB and Luc 157 m-κB (Fig. 5.5) suggesting that the effect of Dex may be mediated through the NF-κB site located in the proximal CRP promoter. Dex did not enhance IL-6 and IL-1β mediated CRP gene expression in the construct Luc 300 mOCT, where the 8 bp region (-59/-66) in the proximal CRP promoter was deleted (Data not shown). The -59/-66 region partially overlaps the NF-κB binding

site suggesting the involvement of NF-κB site in Dex-mediated CRP induction. Further studies need to be carried out to fully determine the role of NF-κB site in Dex-mediated CRP induction.

During the past few years, the genetic bases for the variation in serum CRP levels have been proposed and these variations have been correlated with the predisposition of individuals to certain pathological states [64-66]. Several polymorphisms associated with the CRP gene have been shown to be responsible for the variation in basal serum CRP levels [67, 68]. In one of these correlation studies, a polymorphic $(GT)^n$ repeat was identified in the intron of CRP gene. At least nine different alleles were identified in a group of healthy European Caucasians and it was found that the different alleles affect baseline CRP levels [67]. Hence, the effect of relative levels of transcription factors in the hepatocytes on their binding to the proximal CRP promoter and the effect of polymorphisms associated with the CRP gene on the binding of transcription factors needs to be studied in detail to determine the mechanisms of regulation of CRP gene.

Future studies involve the use of more cell lines to validate the findings obtained with Hep3B cells. Chromatin immunoprecipitation assays have to be performed to determine the interaction of the transcription factors with the CRP promoter at the chromatin level. Involvement of additional signaling pathways in IL-6 and IL-1β mediated CRP induction and regulation of CRP gene by additional cytokines needs to be studied. Serum CRP levels in humans rise several thousand fold within 24h of an inflammatory event. The critical factor responsible for this unique regulation of CRP gene has to be identified. This dissertation work is a step ahead in elucidating the mechanism of CRP gene expression but much more needs to be done to understand the uniqueness of the CRP promoter.

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