Binding of C/EBPβ to the C-Reactive Protein (CRP) Promoter in Hep3B Cells Is Associated with Transcription of CRP mRNA

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Expression of the acute phase protein C-reactive protein (CRP) is tightly regulated in hepatocytes. Although very little CRP mRNA is transcribed normally, inflammatory stimuli are followed by a dramatic increase in mRNA synthesis and accumulation. IL-6 and IL-1β are believed to be the major cytokines responsible for induction of CRP and other acute phase proteins. Our previous studies, using transient transfection and EMSA experiments, implicated involvement of the transcription factors C/EBPβ, STAT3, Rel p50, and c-Rel in CRP induction. In the current study we used chromatin immunoprecipitation assays to determine the kinetics of transcription factor occupancy of these transcription factors on the endogenous CRP promoter. All of these transcription factors were found bound to the endogenous CRP promoter in the absence of cytokines, but cytokine treatment markedly increased binding of only C/EBPβ. In addition, c-Rel and TATA box-binding protein (TBP) appeared to occupy the promoter in parallel in the presence of cytokines. In the absence of cytokines, CRP mRNA accumulation was not measurable but began to increase by 3 h after exposure of cells to IL-1β plus IL-6, peaking at 12 h with secondary peaks at 18 and 24 h. The secondary peaks in mRNA expression paralleled the pattern of binding of c-Rel and TBP to the CRP promoter. We conclude that the CRP promoter has a low level of transcription factor occupancy in the absence of cytokines and induction occurs with binding of C/EBP, and that c-Rel and TBP are important for modulating CRP expression. The Journal of Immunology, 2008, 181: 2420–2427.

The C-reactive protein (CRP) is a major human acute phase protein largely synthesized in hepatocytes following inflammatory stimuli. The serum concentration of CRP can rapidly increase up to 1000-fold or more after severe inflammatory stimuli (1, 2). In the human hepatoma cell line Hep3B, CRP gene expression is only modestly induced by IL-6, whereas IL-1β alone has no effect. Together these cytokines act synergistically to markedly induce CRP gene expression (3). Cytokine induction of CRP gene expression occurs mainly at the transcriptional level. Transcription factors C/EBPβ, STAT3, Rel p50, and c-Rel participate in CRP gene expression following cytokine stimuli with C/EBPβ binding to two sites centered at −53 and −219 (see Fig. 1) (4–7). HNF-1α also binds to two sites but this transcription factor is constitutively present and not activated by cytokines.

In Hep3B cells the CRP-C/EBPβ (−53) site is essential for CRP expression (8–10) although the sequence of this binding site differs from the consensus C/EBP binding sequence, and in vitro C/EBPβ binds relatively poorly to this CRP-C/EBPβ (−53) site. This raises the possibility that other transcription factors may be required to facilitate C/EBP DNA binding. In fact, we have shown that c-Rel is such a protein. It does not bind DNA directly, but rather enhances CRP expression by binding to C/EBPβ, with consequent enhancement of C/EBPβ binding to the CRP-C/EBPβ (−53) site on the promoter (11, 12). In addition, p50 has been found to bind to a non-consensus kb site (−43) and both p50 and C/EBPβ (−53) are required for full induction of CRP expression by IL-6 and IL-1β (9, 11, 13, 14). Previous studies from our lab also showed that overexpressed STAT3 was able to transactivate the CRP promoter in response to IL-6 stimulation, that it activated the endogenous CRP gene in response to IL-6, and that STAT3 bound the CRP promoter in response to IL-6 (15).

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

Materials and Methods

Materials

Human recombinant cytokines IL-6 (206-IL) and IL-1β (201-LB) were purchased from R&D Systems. Synthetic oligonucleotides were obtained from Operon Biotechnologies. Rabbit polyclonal Abs against C/EBPβ (sc-150), p50 (sc-7178), STAT3 (sc-482), c-Rel (sc-70), TFIID/TBP (sc-204), and TFIIE (sc-237) proteins were purchased from Santa Cruz Biotechnology. HRP-conjugated anti-rabbit Abs (12–348) were purchased from Milipore. SYBR Green (1988131) was purchased from Roche. Fluorescein (170 – 8780) and Bio-Rad DC Protein Assay (Bradford method) reagents (500 – 0113, 500-0114, 500-0115) were purchased from Bio-Rad. Protein A-agarose beads were purchased from Repligen (IPA-400HC) (Waltham, MA) and Upstate (16 –157) (Lake Placid, NY). Salmon sperm DNA (D-7656) was purchased from Sigma-Aldrich. BSA (10921), RNase A (70194Z), proteinase K solution (76225), aprotinin (11388), leupeptin (18413), AEBSF (11118), RT-PCR Master Mix (78370), and HotStart-It Taq Master Mix (71196) were purchased from USB. RNeasy Mini kit (74104) was purchased from Qiagen. Immobilon transfer polyvinylidene difluoride membranes were purchased from Millipore. SuperSignal West Pico Chemiluminescent reagents (34080) were purchased from Pierce. HyBlot CL autoradiography film was purchased from Denville Scientific.

Cell culture and cytokine treatment

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

ChIP assays

A streamlined protocol for the ChIP assay developed at USB (Cleveland, OH) was used. Abs were used in the assays with primers flanking the CRP proximal promoter (−118 to +115), as described in Fig. 2. Data show C/EBPβ occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Materials and Methods). Results are an average of three to four experiments, each done in duplicate. Error bar represents SD. Statistical significance of each time point compared with basal levels was determined by a one-way ANOVA and is defined. *, p < 0.5.

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

FIGURE 2. C/EBPβ, NF-κB, p50, STAT3, c-Rel, and TBP bind the endogenous CRP promoter. Agarose gel of a ChIP assay performed on Hep3B cells treated with cytokines IL-1β and IL-6 for 0–15 h, as described in Materials and Methods. Abs to C/EBPβ, NF-κB, p50, STAT3, c-Rel, and TBP were used in the assays with primers flanking the CRP proximal promoter (−118 to +115). The mock is a no Ab control. Input is a 1/10 dilution of total chromatin after sonication and preclearing. C/EBPβ, NF-κB p50, and input are shown (top row). STAT3, c-Rel, and mock are shown in the middle, and TBP is shown in the bottom row. Results are representative of four experiments.

FIGURE 3. C/EBPβ binds the endogenous CRP promoter in response to cytokines. Real-time PCR of ChIP assays performed on Hep3B cells treated with cytokines IL-1β and IL-6. Zero time values were determined in each case. Subsequently three time courses were followed 30 min–6 h (n = 3 assays) (a), 8–16 h (n = 4) (b), and 12–36 h (n = 4) (c). Abs were used in the assays with primers flanking the CRP proximal promoter (−118 to +115), as described in Fig. 2. Data show C/EBPβ occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Materials and Methods). Results are an average of three to four experiments, each done in duplicate. Error bar represents SD. Statistical significance of each time point compared with basal levels was determined by a one-way ANOVA and is defined. *, p < 0.5.
were used in each ChIP assay. Either 30 μl of a 50% slurry of Upstate protein A-agarose or 50 μl of a 33% slurry of Repligen protein A-agarose were used during the preclear and capture steps. Upstate protein A-agarose complexes were captured with protein A-agarose for 1 h at 4°C, washed for 10 min at 4°C with 1× 1 ml ChIP lysis buffer, high-salt buffer (50 mM Tris (pH 8.0), 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), and lithium salt buffer (20 mM Tris (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and 2× 1 ml 1X TE buffer (pH 7.5). Immune complexes were eluted from the protein A-agarose with 2× 150 μl elution buffer (10 mM Tris (pH 8.0), 5 mM EDTA, 1% SDS) at room temperature for 20 min. Cross-links were reversed overnight at 65°C in 0.2 M NaCl. RNA was digested with 10 μg of RNase A at 37°C for 30 min and protein was digested with 50 μg of proteinase K at 45°C for 90 min. DNA was purified using phenol extraction and ethanol precipitation.

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

Fisher Scientific 550 Sonic Dismembrator for 8 cycles (amplitude 4; 0.5 s on, 0.5 s off for 1 min) on ice with 2 min between cycles. DNA fragment sizes of 300–800 bp were confirmed by agarose gel electrophoresis.

Packed cell volume was used to estimate cell number and 2× 10^6 cells were used in each ChIP assay. Either 30 μl of a 50% slurry of Upstate protein A-agarose or 50 μl of a 33% slurry of Repligen protein A-agarose were used during the preclear and capture steps. Upstate protein A-agarose beads were washed two times in 1X PBS and two times in 1X TE buffer (Tris-Cl (pH 8.0) and EDTA). Next, 5 μg of BSA, 500 μg of salmon sperm DNA, 0.075% sodium azide, and 835 1X TE buffer (pH 8.0) were added to 500 μl of packed protein A-agarose for a final volume of 1.5 ml (33% slurry) of protein A-agarose and rotated overnight at 4°C.

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

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

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**FIGURE 4.** p50 occupancy of the CRP promoter changes modestly in the presence of cytokines. Real-time PCR of ChIP assays performed on Hep3B cells treated with cytokines IL-1β and IL-6. Zero time values were determined in each case. Subsequently three time courses were followed 30 min–6 h (n = 3 assays) (a), 8–16 h (n = 4) (b), and 12–36 h (n = 4) (c). Abs were used in the assays with primers flanking the CRP proximal promoter (−118 to +115), as described in Fig. 2. Data show NF-κB p50 occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Materials and Methods). Results are an average of three to four experiments, each done in duplicate. Error bar represents the SD. Statistical significance of each time point compared with basal levels was determined by a one-way ANOVA at p < 0.5, but the experiment had insufficient statistical power to reliably calculate p values.

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**FIGURE 5.** STAT3 occupancy of the CRP promoter rises modestly in response to cytokines. Real-time PCR of ChIP assays performed on Hep3B cells treated with cytokines IL-1β and IL-6. Zero time values were determined in each case. Subsequently three time courses were followed 30 min–6 h (n = 3 assays) (a), 8–16 h (n = 4) (b), and 12–36 h (n = 4) (c). Abs were used in the assays with primers flanking the CRP proximal promoter (−118 to +115), as described in Fig. 2. Data show STAT3 occupancy expressed as fold change after subtraction of mock and normalization to input signal (see Materials and Methods). Results are an average of three to four experiments, each done in duplicate. Error bar represents the SD. Statistical significance of each time point compared with basal levels was determined by a one-way ANOVA at p < 0.5.
region (−118 to +115) were 5'-CTCTTCCCGAAGCTCTGACACCT-3' and 5'-AACAGCTTCTCCATGGTCACGTC-3'.

Data analysis

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

RNA isolation and RT-PCR

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

Whole cell extraction, SDS-PAGE, and immunoblot

Whole cell extracts using Hep3B cells were made using RIPA buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 1X protease inhibitor cocktail added fresh). Cells were lysed with three freeze-thaw cycles and pipetting. Cell lysate protein concentration was measured using Bio-Rad DC Protein Assay reagents following the manufacturer’s protocol. Proteins were separated using an SDS-PAGE with a 4% stacking gel and a 6% separating gel. Each lane contained 17 μg of protein. Proteins were transferred to a PVDF membrane at 24 V, 1 A for 45 min at room temperature. Membranes were blocked in 5% milk in TTBS (0.1% v/v Tween 20 plus TBS) at 4°C overnight. Primary Abs were diluted 1/200 (C/EBPβ and c-Rel) or 1/1000 (TFB and TFIIE) in 5% milk plus TTBS and blots
were incubated at room temperature for 2 h. HRP-conjugated donkey antirabbit Ab was diluted 1/2000, and blots were incubated at room temperature for 1 h. Chemiluminescence was generated using Pierce SuperSignal ECL reagents following the manufacturer’s protocol. Proteins were imaged using HyBlot CL autoradiography film and Kodak M53A X-OMAT processor.

**Statistical analysis**

One way ANOVA was used to determine statistically significant differences of each time point of transcription factor occupancy and mRNA accumulation with respect to baseline levels. Two-way ANOVA was used to compare the shapes of the c-Rel and TBP curves.

**Results**

We performed ChIP assays and used Abs against C/EBPβ, p50, STAT3, c-Rel, and TBP to follow their binding to the endogenous CRP promoter (Fig. 1). A representative gel of the resulting PCR products shows faint bands for C/EBPβ, p50, STAT3, c-Rel, and TBP at baseline (Fig. 2), indicating that these transcription factors occupied the promoter in the absence of cytokines. The interaction between c-Rel and the CRP promoter is indirect and occurs through C/EBPβ. All of these transcription factors increased their binding to the endogenous promoter following cytokine stimulation. The intensity of the PCR products in the C/EBPβ samples increased markedly after cytokine treatment, whereas modestly increased occupancy of STAT3, p50, c-Rel and TBP was also detected. These data demonstrate that the major transcription factors in our model of regulation of CRP gene expression occupy the CRP promoter in Hep3B cells, and that occupancy is enhanced in response to cytokines.

To quantify transcription factor promoter occupancy, we performed real-time PCR on DNA isolated following ChIP assays from Hep3B cells treated with IL-6 plus IL-1β. Zero time values were determined in each case. Subsequently, three time courses were followed: 30 min-6 h, 8–16 h, and 12–36 h. Increased C/EBPβ promoter occupancy was detected as early as 2 h after cytokine exposure and increased markedly through 10–12 h before slowly falling through 36 h (Fig. 3). At its peak, the amount of CRP promoter DNA detected in the C/EBPβ immunoprecipitates was 5-fold greater than at baseline. Rel p50 occupied the CRP promoter in the absence of cytokines and showed a modest elevation in promoter occupancy after 10–12 h of cytokine exposure (Fig. 4). In contrast, cytokine exposure caused a slow 2-fold increase in STAT3 occupancy through 10 h of cytokine exposure with a dip in occupancy after 14–18 h (Fig. 5). The changes in C/EBPβ occupancy are statistically significant as determined by one-way ANOVA. These data suggest that C/EBPβ, p50, and STAT3 occupy the CRP promoter in the absence of cytokines and that C/EBPβ occupancy markedly increases, whereas p50 and STAT3 experience modest elevations, after cytokine exposure.

CRP promoter occupancy by c-Rel and TBP changed in parallel, in a roughly biphasic pattern. In the two shorter time courses studied, CRP promoter occupancy by c-Rel and TBP peaked after 10 h of cytokine exposure, followed by a drop in occupancy at 12 h, and a second peak after 14 h (Fig. 6, a and b). Over a longer time course, occupancy of c-Rel and TBP peaked after 12 h of cytokine exposure, dropped from 18–24 h, and peaked again after 30 h (Fig. 6c). ANOVA analysis indicated that the two curves were parallel in all experiments as no two points in any of the curves were statistically different from one another. Although changes in c-Rel and TBP occupancy of the promoter generally occurred in parallel, the pattern of occupancy was not always the same from one experiment to the next, accounting for the large error margins in experiments. In one assay, c-Rel and TBP occupancy peaked 12–18 h after exposure to cytokines, followed by a drop in occupancy through 36 h (Fig. 6d). In yet another assay over the same time course, occupancy of both transcription factors dropped to a low through 24 h and peaked after 30 h of cytokine exposure (Fig. 6e). Overall, these data suggest that cytokine exposure led to a biphasic occupancy pattern of c-Rel and TBP. We did not observe parallel changes in occupancy with any of the other transcription factor pairs used in these studies.

To determine the relationship between gene expression and transcription factor occupancy, we measured CRP mRNA accumulation by RT-PCR in Hep3B cells treated with cytokines IL-1β and IL-6 for the indicated times (hours). CRP mRNA levels are shown at top and β-actin mRNA levels are shown at bottom. Average quantification of band intensity measured using ImageQuant of CRP mRNA normalized to β-actin mRNA (n = 4 measurements). Error bar represents SD.
cytokine induction. Three bands were present for C/EBPβ: full-length, liver-enriched activator protein (LAP) and liver-enriched inhibitory protein (LIP) (Fig. 9a). Full-length and LAP levels were undetectable at 0 h, but LIP was present. Full-length and LAP protein levels increased substantially from 2 through 6 h. Densitometric scanning of the image in Fig. 9 indicated that the accumulation of full-length C/EBPβ and LAP increased ~40% from 0 to 6 h, whereas LIP accumulation remained constant. A doublet was found for c-Rel, possibly representing phosphorylated and unphosphorylated forms (Fig. 9b). Protein levels for c-Rel increased from 0 to 2 h and then remained steady through 6 h. TBP protein levels remained constant from 0 to 6 h of exposure to cytokines (Fig. 9b). These data indicate that the amount of C/EBPβ and c-Rel protein increased in response to cytokine treatment.

Discussion

Our findings that IL-1β plus IL-6 enhanced binding of C/EBPβ, STAT3, p50, c-Rel, and TBP to the CRP promoter in Hep3B cells confirms that these transcription factors participate in cytokine-induced expression of CRP in its native context. Of the transcription factors we studied, only binding of C/EBPβ to the CRP promoter markedly increased following cytokine exposure, suggesting that its binding plays an important role in the overall pattern of CRP mRNA accumulation observed. C/EBPβ occupancy was also associated with increased C/EBPβ accumulation. In addition, the CRP mRNA accumulation had several modest peaks and valleys, similar to the biphasic, parallel patterns of CRP promoter occupancy observed with c-Rel and TBP. Our previous studies demonstrated that changes in CRP mRNA levels reflected changes in CRP plasma levels (7, 16). We have previously found that c-Rel enhanced the binding of C/EBPβ to the CRP/C/EBPβ−53 site on the CRP promoter without binding the promoter itself (12). Our findings in these studies indicate that C/EBPβ and c-Rel kinetics of binding to the proximal CRP promoter were not identical and suggest that the interaction between c-Rel and C/EBPβ is transient. We speculate that c-Rel binds C/EBPβ, helping it bind to the promoter, and then releases C/EBPβ afterward. We propose that c-Rel plays a role in modulating CRP expression, consistent with a model in which c-Rel enhances C/EBPβ binding.

Low levels of C/EBPβ, STAT3, p50, c-Rel, and TBP were found on the CRP promoter in the absence of cytokines, although CRP mRNA accumulation was not measurable before cytokine treatment. Transcriptional regulatory mechanisms generally fall into two models. In the first model, transcription is regulated at the transcription initiation step when the transcription factors bind the promoter and initiate transcription. In the second model transcription is regulated after the initiation step and involves transcript elongation or stability. Recent work by the Young lab demonstrated that 75% of all protein encoding gene proximal promoters had Pol II bound and had trimethylation of Lys4 of histone 3 (H3K4me3), hallmarks of initiation, even when mRNA was not detectable. They also found that most promoters in both embryonic stem cells and primary human liver cells had methylation marks...
Cycling we detected appears to be in addition to the dynamic cycling of transcription factors using these methods (18–20); however, the cross-linking before ChIP. Others have detected dynamic cycling of p50 may also bind a site overlapping the STAT3 site where it acts (21–23). Our previous studies indicated that C/EBP is the necessary transcription factor for either completing transcription initiation or for elongation of the transcript. Low level transcription factor occupancy did not occur on every promoter; a control experiment in which we amplified the nephrin gene promoter in ChIP DNA isolated from Hep3B cells after cytokine exposure showed that the real-time PCR signal of several transcription factors was barely detectable above mock (data not shown).

We found that c-Rel and TBP occupied the promoter roughly in parallel and may be responsible for modulating CRP gene expression. c-Rel and TBP appeared to cycle on and off of the promoter throughout the time course studied with an apparent periodicity of 2–3 h. The individual experimental variation in binding patterns for c-Rel and TBP resulted in the average occupancy to appear relatively flat and for the error bars to be large. We detected cycling without α-amanitin synchronization or instantaneous UV cross-linking before ChIP. Others have detected dynamic cycling of transcription factors using these methods (18–20); however, the cycling we detected appears to be in addition to the dynamic cycling described by these groups. The cell cycle was not synchronized before cross-linking. However, because cells were ~90% confluent at the time of cross-linking, most cells were no longer actively dividing. The lack of cell cycle synchronization or promoter clearing may explain why the periods of c-Rel and TBP occupancy varied from one ChIP experiment to the next. Despite the variations in the biphasic pattern observed for c-Rel and TBP occupancy repeatedly occurred in parallel and a biphasic pattern was always observed. ANOVA analysis indicated that the two curves were parallel in all experiments as no two points in any of the curves were statistically different from one another. We did not observe parallel or repeated biphasic patterns of occupancy with any of the other transcription factors studied.

Our immunoblots showed that cytokines increased the amount of total cellular C/EBPβ and c-Rel. For C/EBPβ, only the inhibitory isoform LIP, a functional LAP antagonist (21–23), was found in the absence of cytokines, whereas accumulation of all three isoforms increased within 2 h of cytokine treatment. It is therefore likely that C/EBPβ occupancy of the CRP promoter detected in the absence of cytokines is the inhibitory form. In the absence of cytokines, a doublet suggestive of phosphorylated and unphosphorylated c-Rel was detected, and the levels of both increased after cytokine exposure. Although we do not know the identity of the doublet, several studies have suggested that c-Rel is phosphorylated on its transactivation domain to increase transactivation activity (24–26). Our previous studies indicated that in vitro, the c-Rel transactivation domain was not required for it to enhance C/EBP binding to the CRP-C/EBPβ signal. We did not observe parallel or repeated biphasic patterns of occupancy with any of the other transcription factors studied.

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In the absence of cytokines, a doublet suggestive of phosphorylated and unphosphorylated c-Rel was detected, and the levels of both increased after cytokine exposure. Although we do not know the identity of the doublet, several studies have suggested that c-Rel is phosphorylated on its transactivation domain to increase transactivation activity (24–26). Our previous studies indicated that in vitro, the c-Rel transactivation domain was not required for it to enhance C/EBP binding to the CRP-C/EBPβ signal. We did not observe parallel or repeated biphasic patterns of occupancy with any of the other transcription factors studied.

It should be noted that the C/EBPβ signal likely reflects occupancy at two sites, one centered at −219 and the other centered at −53 and that our ChIP experiments do not distinguish between the two. By transfection studies, it is the −53 site that is critical for CRP expression and is likely influenced by c-Rel (11–13). Occupancy of p50 in the absence of cytokines may also occur at two p50 binding sites in the CRP promoter. Previous data indicated that p50 binds to a site overlapping C/EBPβ (CRP-ΔxB-43) in the presence of cytokines where it acts as an activator (9). However, p50 may also bind a site overlapping the STAT3 site where it acts as a repressor in the absence of cytokines (A. Agrawal, unpublished observation). Thus it was not unexpected to observe p50 occupancy in ChIP experiments in the absence of cytokines. Although STAT3 occupancy occurred later than generally observed in most systems, our ChIP data are in agreement with our previous studies that show prolonged activation of STAT3 following cytokine exposure of Hep3B cells (15).

We attempted to amplify the 3′ untranslated region of the CRP gene −2 kb downstream of the transcriptional start site to use as a negative control in our ChIP assays. However, we unexpectedly detected strong signals for C/EBPβ with these primers. Moreover, a recent study demonstrated that β-catenin binds to a downstream element in the CRP gene and regulates CRP expression by looping of the downstream region of the gene to the proximal promoter (27). Looping of the downstream region of the CRP gene into close proximity of the promoter would explain why we observed strong PCR signals when amplifying the 3′ untranslated region of the CRP gene in ChIP assays.

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

Our results have practical use. It has been suggested that CRP plays a significant role in disease pathogenesis (28–33). Other studies indicate that CRP exerts protective action against pneumococcal infection in mice (34–36). Should further investigations support this hypothesis, intimate knowledge of the molecular mechanisms leading to CRP induction could permit the development of clinically useful therapies.

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Disclosures
The authors have no financial conflict of interest.

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