Regulation of Human C-Reactive Protein Gene Expression by Two Synergistic IL-6 Responsive Elements

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ABSTRACT: To study the mechanism of interleukin-6 (IL-6) induction of human C-reactive protein (CRP) gene expression, we have utilized a human hepatoma (PLC/PRF/5) cell culture system to analyze the *trans*-acting factors which bind to the 300 bp 5′-flanking region of human CRP gene. *In vitro* gel mobility shift analyses and methylation interference assays demonstrated that NFIL-6α interacted with two IL-6 responsive elements, and HNF-1α and HNF-3/Octamer-like factors interacted with the downstream IL-6 responsive element in the human CRP promoter. *In vivo* functional analysis by transient transfection of plasmid constructs containing site-specific mutations in one or two IL-6 responsive elements in the CRP promoter fused to a reporter gene, chloramphenicol acetyl transferase (CAT), demonstrated that the binding of NFIL-6α to two IL-6 responsive elements resulted in synergistic induction of the gene. When HNF-1α or HNF-3/Octamer-like factors were independently bound to their corresponding sites, they had either a positive or negative effect, respectively, on IL-6 inducible transcriptional activity.

The body's initial response to infection, inflammation, or tissue injury is the acute phase response. The major site of synthesis of most of the acute phase proteins is in the parenchymal cell of the liver, the hepatocyte. Human C-reactive protein (CRP)[1] is the prototype acute phase reactant (Hurlimann & Hochwald, 1966; Kushner & Feldmann, 1978) which increases 1000-fold or more in the serum (Kushner, 1982).

The precise *in vivo* function of CRP has been elusive. Nevertheless, CRP has a number of recognition and activation capabilities. *In vitro*, it has been found to bind phosphorylcholine, chromatin, fibronectin, laminin, 70 kDa polypeptide of U1 snRNP, galactose-containing polysaccharides, and polycations. *In vivo*, it has been observed to bind the complement component, C1q, resulting in the activation of the classical complement pathway; to act as an opsonin for bacteria, fungi, and parasites; and to bind to neutrophils and monocytes/macrophages. In addition, CRP has been reported to stimulate IL-1 and TNF production by macrophages, to induce platelet-mediated cytotoxicity of schistosomula, and to inhibit malaria liver stage (sporozoite) development (Kilpatrick & Volanakis, 1991; Ballou & Kushner, 1992).

It was found that a cytokine (Hirano et al., 1986), later referred to as interleukin-6 (IL-6), could induce a variety of acute phase reactant genes in liver hepatocytes and in cultured hepatoma cells (Gauldie et al., 1987). Thus the single-copy gene for human CRP represented a good model for studying cytokine-inducible liver-specific gene expression. We previously established an *in vitro* human hepatoma PLC/PRF/5 cell culture system (Goldman & Liu, 1983) in which CRP transcription and translation could be monitored after stimulation by the cytokine, IL-6 (Goldman & Liu, 1987). We functionally identified two IL-6 responsive elements which were located proximal to the site of initiation of transcription. In addition to these two IL-6 responsive elements, several positive and negative *cis*-acting DNA elements were also identified (Li et al., 1990). Recently, it was reported that in the human hepatoma cells, Hep3B, IL-1 could dramatically boost IL-6 inducible CRP gene transcription (Zhang et al., 1995).

Several liver-enriched DNA-binding proteins have been characterized and cloned which belong to the C/EBP, HNF-1, HNF-3, and HNF-4 families (Lai & Darnell, 1991; Simone & Cortese, 1992; Tronche & Yaniv, 1992). NFIL-6α, a member of the C/EBP family (Akira & Kishimoto, 1992), was originally identified as a DNA-binding protein responsible for IL-1-stimulated human IL-6 gene induction (Akira et al., 1990). This gene was found to be rapidly and dramatically induced by LPS or inflammatory cytokines, such as IL-1, TNF, and IL-6 itself (Isshiki et al., 1990). Homologs of NFIL-6α in man have been identified in the rat [IL-6DBP (Poli et al., 1990) or LAP (Descombes et al., 1990)] as well as in the mouse [AGP/EBP (Chang et al., 1990) or C/EBPβ (Cao et al., 1991)]. This nuclear factor also has been observed to be responsible for IL-6 induction of several acute phase proteins, such as α1-acid glycoprotein (Prowse & Baumann, 1988), haptoglobin (Oliviero & Cortese, 1989), and hemopexin (Poli & Cortese, 1989). Many other examples of liver-enriched transcription factors have been described, i.e., HNF-1α (Mendel & Crabtree, 1991), also referred to as LF-B1 (Frain et al., 1989), or APF (Chouard et al., 1990) which has been observed to activate a number of liver genes, such as fibrinogen (Courtois et al., 1987), α1-antitrypsin (Shen et al., 1987), albumin (Cereghini et al., 1988; Lichtsteiner & Schibler, 1989), and α-fetoprotein (Feuerman et al., 1989). Ultimately, the control of tissue-specific gene expression is manifested through the interactions of these specific DNA-binding proteins with each other and with the basal transcriptional complex (Zawel & Reinberg, 1993).

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[1] Abbreviations: CRP, C-reactive protein; IL-6, interleukin-6; C/EBP, CAAT/enhancer binding protein; NFIL-6, nuclear factor IL-6; HNF, hepatocyte nuclear factor; Octamer, DNA-binding protein recognizing ATGCAAAT; CAT, chloramphenicol acetyl transferase; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetate; oligomer, double-stranded oligonucleotide.

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We present here our results demonstrating that IL-6 inducible expression of the human CRP gene involves both positive and negative regulation, whereby several constitutive and IL-6 inducible *trans*-acting factors from the C/EBP, HNF-1, HNF-3 and Octamer superfamilies interact with two synergistic IL-6 responsive elements.

EXPERIMENTAL PROCEDURES

Cell Culture. Human hepatoma cell line, PLC/PRF/5 (obtained from J. Gerin, Georgetown University, Washington, DC), was grown in minimum essential medium (GIBCO) containing 10% heat-inactivated fetal bovine serum, penicillin (50 units/mL), streptomycin (50 μg/mL), and gentamicin (50 μg/mL). The cultures were incubated at 37 °C in a humidified incubator containing 5% carbon dioxide.

Isolation of Nuclei and Preparation of Nuclear Extracts. Nuclei from the hepatoma cells, either untreated or stimulated with 200 units of interleukin-6 (IL-6, Genzyme Corp.) per 10^6 cells, were isolated as previously described (Clayton et al., 1985). Nuclear extracts were prepared as described previously (Dignam et al., 1983). After extraction of the nuclei and removal of the DNA, proteins were precipitated by adding 0.3 g of $(NH)_2SO_4$ per mL of the supernatant, collected by centrifugation, and dialyzed against 500 vol of buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). The protein solution was clarified by centrifugation, and aliquots were frozen and stored in liquid nitrogen. The protein concentration was determined by the BCA protein assay (Pierce Chemical Company).

Gel Mobility Shift Assay. The analytical binding reaction and gel electrophoresis were performed as previously described (Singh et al., 1986). Each reaction contained 0.2 ng of 5′-end ^{32}P-labeled oligomer (approximately 10 000 cpm) and PLC/PRF/5 nuclear extract consisting of 8 μg of protein. The resulting DNA−protein complexes were resolved by electrophoresis in a 7% polyacrylamide gel with 0.25× TBE buffer. For the binding competition experiments, various unlabeled oligomers (50× molar ratio to the probe) were added as competitors to the binding reaction. Mutant competitive oligomers were produced by changing A to C or G to T at a specific site(s) in the wild-type oligomers. Binding reactions in the presence of antiserum were performed by incubation of varying amounts of antiserum with the nuclear extract for 1 h prior to addition of the ^{32}P-labeled oligomer.

Methylation Interference Assay. A 5′-end ^{32}P-labeled oligonucleotide was annealed with its complementary unlabeled oligonucleotide and partially methylated with dimethyl sulfate (Maxam & Gilbert, 1977). Preparative binding reactions were carried out by scaling up the analytical binding reactions 10- to 20-fold. After resolution by polyacrylamide gel electrophoresis, DNA−protein complexes were excised and eluted (Gilman et al., 1986). The oligomeric DNA was treated with 1 M piperidine and resolved by electrophoresis in a 15% polyacrylamide−8 M urea sequencing gel.

Construction of Site-Directed Mutant Plasmids for Transient Transfection. A 592 bp *Xba*I−*Eco*RI fragment from plasmid 5′Δ10 CAT3M (Li et al., 1990), which contains the 5′-flanking sequence of the CRP gene from −332 to +9, was cloned into a phagemid (pBlueScript II, SK+, Stratagene). The single-stranded DNA was isolated. Mutant oligonucleotides which changed the different binding sites were used in site-directed mutagenesis of the 5′-flanking sequence (Oligonucleotide-Directed *in Vitro* Mutagenesis System, Version 2.1, Amersham Corp.). Mutants in the 592 bp *Xba*I−*Eco*RI fragment were purified from the phagemid and cloned back into plasmid 1-1 CAT3M (Li et al., 1990). All mutant plasmids for transient transfection were sequenced to confirm the mutated sites.

Transient Transfection and CAT Assay. Transfections were performed by the calcium phosphate precipitation technique (Graham & van der Eb, 1973) at concentrations of 10 μg of plasmid DNA per 5×10^5 PLC/PRF/5 hepatoma cells. The cultures were incubated with DNA for 16 h, and half of the cultures were stimulated with IL-6 (200 units/10^6 cells) for 16−24 h. The cells were harvested and lysed by sonication, and an aliquot (50 μL) of the extract was tested in the standard CAT assay (Gorman et al., 1982). Prior to analysis, the concentration of protein in all cell extracts was adjusted to 1 mg/mL by the BCA protein assay (Pierce Chemical Company). The analysis for each plasmid construct was repeated in triplicate in at least three separate transfection assays. pSV-β-galactosidase plasmid (Promega Corp.) was co-transfected with plasmid constructs as an internal control for transfection efficiency of the CAT assays.

RESULTS

Gel Mobility Shift Pattern of trans-Acting Factors That Bind to the Two IL-6 Responsive Elements. Previously, utilizing multiple CAT reporter constructs containing 5′, 3′, and internal promoter deletions, we functionally identified the two IL-6 responsive elements located in the first 300 bp 5′-flanking sequence of human CRP gene (Li et al., 1990). To identify the *trans*-acting factors that bound to these two elements and were involved in IL-6 inducible human CRP gene expression, we synthesized two 50 bp oligomers sp35-36 (−245 to −196) and sp39-40 (−78 to −29), which represented the upstream and the downstream IL-6 responsive elements respectively (Figure 1A). The gel mobility shift analysis was performed utilizing the labeled oligomer sp35-36 or sp39-40 and nuclear extract from untreated or IL-6-stimulated hepatoma cells. The left panel of Figure 1B showed that the upstream probe sp35-36 formed a constitutive complex 3, with nuclear extract from IL-6 stimulated (+) and untreated (−) cells, while only nuclear extract from IL-6 stimulated (+) cells gave rise to complex 2. The right panel of Figure 1B showed that the downstream probe sp39-40 also appeared to form two complexes with mobilities identical to those of complexes 2 and 3. Likewise, complex 3 was seen with nuclear extract from IL-6 stimulated (+) and untreated (−) cells, whereas complex 2 was only seen with nuclear extract from IL-6 stimulated cells. Thus, using either the upstream probe sp35-36 or the downstream probe sp39-40, the common features were that the two IL-6 responsive elements bound the same constitutive (complex 3) and IL-6-inducible (complex 2) factor(s). Furthermore, using the downstream probe sp39-40, we observed that three additional novel complexes, 1, 4, and 5, were formed. Although complexes 1, 4, and 5 were formed with nuclear extract from IL-6-stimulated and untreated cells, some novel features were observed. Using nuclear extract from IL-6-stimulated (+) cells, complex 1 appeared to run with a slightly slower mobility as compared to complex 1 formed with nuclear extract from untreated (−) cells. In addition, the amount of complex 5 formed with nuclear extract from IL-6 stimulated (+) cells decreased 3−4-fold as compared

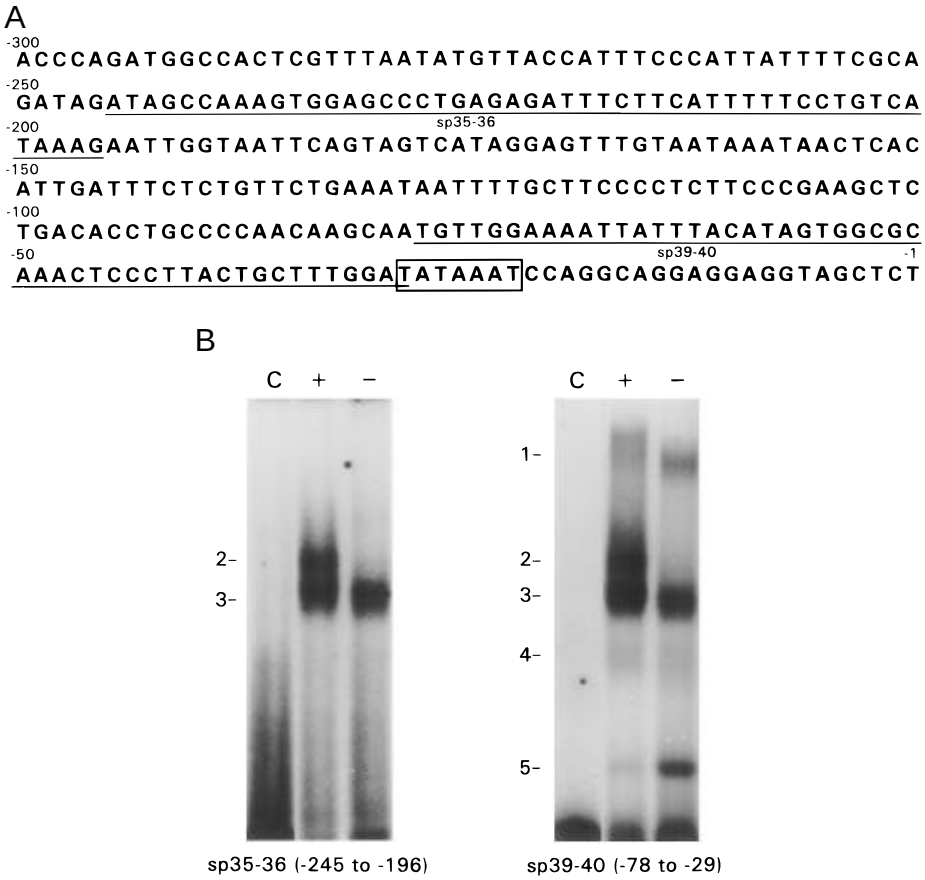

FIGURE 1: Interaction of the two IL-6 responsive elements in the human CRP gene promoter with nuclear protein from human hepatoma (PLC/PRF/5) cells. (A) The 300 bp 5′-flanking sequence of the human CRP gene. The locations of the two oligomers, sp35-36 (−245 to −196) and sp39-40 (−78 to −29), are underlined. The TATA box is framed. (B) Gel mobility shift analysis with ^{32}P-labeled oligomer sp35-36 (left panel) or sp39-40 (right panel). Labeled oligomer was incubated with nuclear extract from IL-6-stimulated (+ lane) or untreated (− lane) cells. Lane C represents the control reaction containing no nuclear extract. Electrophoretic analysis was carried out on a 7% native polyacrylamide gel. DNA−protein complexes 1−5 are indicated.

to nuclear extract from untreated (−) cells. Complex 4 was weakly seen as a doublet using nuclear extract from either IL-6-stimulated (+) or untreated (−) cells.

Identification of Mutual Binding Factors for Both IL-6 Responsive Elements. To characterize the factor(s) forming complexes 2 and 3 which appeared to be common to the two IL-6 responsive elements, methylation interference assays and competitive gel mobility shift analyses, as well as immunoinhibitory binding assays, were carried out. The results in Figure 2A of the methylation interference assay for complexes 2 and 3 formed using nuclear extract from IL-6-stimulated cells indicated that complexes 2 and 3 produced the identical protection pattern for both the upstream probe sp35-36 (left panel) and the downstream probe sp39-40 (right panel). Using the labeled (+) strand of sp35-36, it was observed with complexes 2 and 3 that a methylated G residue at −222 appeared to interfere slightly with protein binding, and methylated A residues at −213 and −221 also interfered with protein binding. Using the labeled (−) strand of sp35-36, it was observed with complexes 2 and 3 that a methylated G residue at −217 interfered strongly with protein binding, and, in addition, methylated A residues at −216 and −218 (and possibly at −211 and −212) also appeared to interfere with protein binding. Using the labeled (+) strand of sp39-40, it was observed with complexes 2 and 3 that methylated G residues at −52, −54, and −57 interfered strongly with protein binding, and a methylated A residue at −58 also interfered with protein binding. Lastly, using the labeled (−) strand of sp39-40, it was observed with complexes 2 and 3 that methylated G residues at −51 and −53 (and sometimes at −47) appeared to interfere slightly with protein binding, and methylated A residues at −46 and −56 also appeared to interfere with protein binding. When nuclear extracts from untreated cells were used, the methylation interference pattern for complex 3 was the same as that with nuclear extracts from IL-6-stimulated cells (data not shown). Thus, in the upstream IL-6 responsive element of the CRP promoter, it appeared that complexes 2 and 3 contained the same binding site between −211 and −222. Likewise, in the downstream IL-6 responsive element of the promoter, it appeared that complexes 2 and 3 contained the same binding site between −46 and −58. Having more precisely delineated the binding sequence we were able to perform competitive gel mobility shift analysis. The results in Figure 2B showed that the protein(s) which bind to either the upstream probe sp35-36 or the downstream probe sp39-40 to form both complex 2 and 3 recognized the consensus binding sequence of the C/EBP family. The labeled oligomer was incubated with the nuclear extract from IL-6-stimulated cells in the presence of different competitive oligomers. Reciprocal competition was observed for complexes 2 and 3 using both the upstream sp35-36 and the downstream sp39-40 wild-type oligomers, while no competition was seen with sp35-36 (Ma and Mb) and sp39-40 (Mα and Mβ) mutant oligomers containing nucleotide substitutions based on critical residues identified by the methylation interference results observed in Figure 1A. More importantly, complexes 2 and 3 also could be

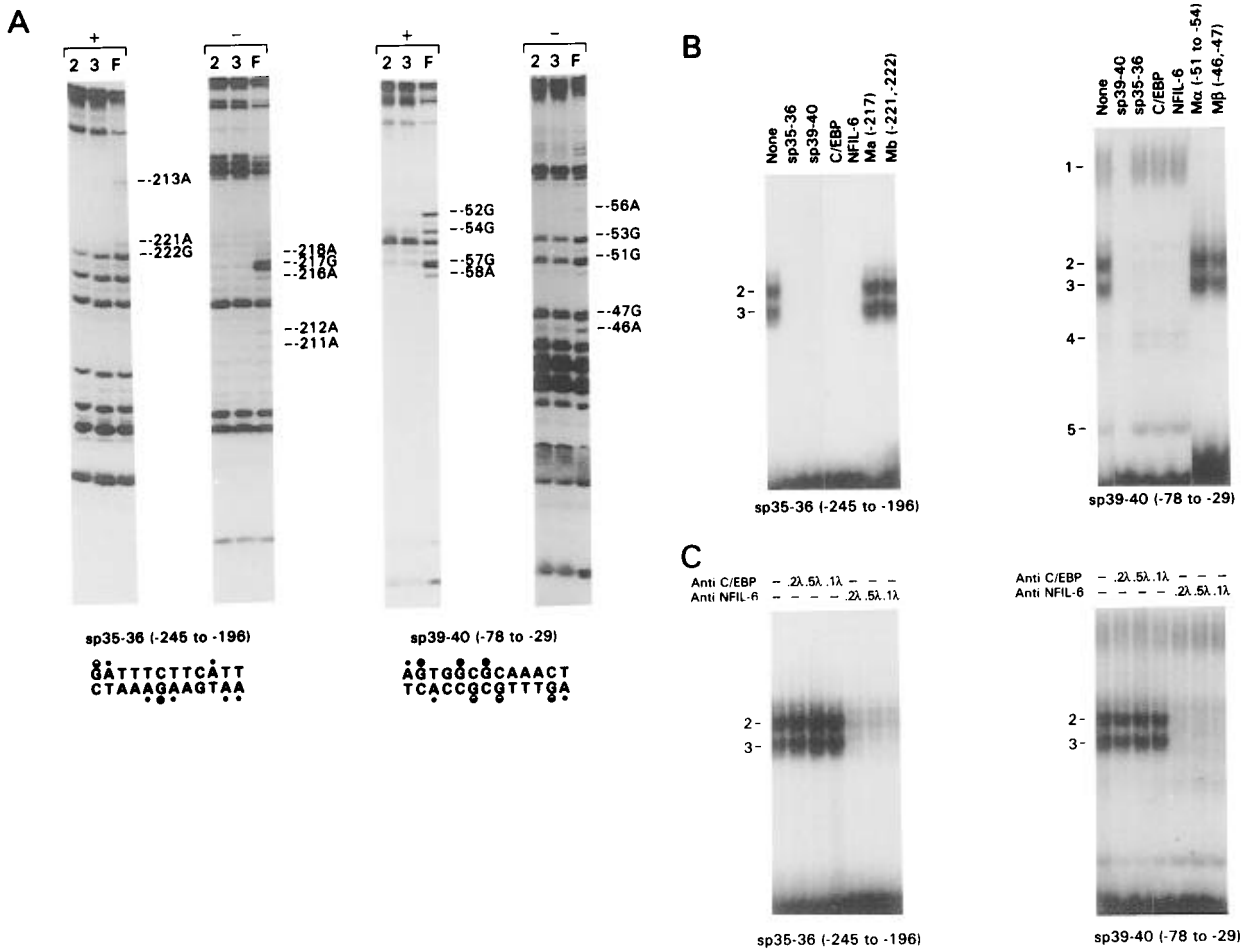

FIGURE 2: Characterization of the binding specificity of the two IL-6 responsive elements for NFIL-6α. (A) Methylation interference analysis for complexex 2 and 3. Oligomers sp35-36 (left panel) and sp39-40 (right panel) were labeled with ^{32}P on the 5′-end of the positive (+) and the negative (−) strands, annealed to their complementary strand, and partially methylated. These probes were incubated with nuclear extract from IL-6-stimulated cells and subjected to gel mobility shift analysis. The labeled complexes, 2 and 3, and free probe (lane F) were excised and treated with piperidine. These samples were analyzed on a 15% sequencing gel. Protected G and A residues are indicated. Below the figure is a summary of the methylation interference: (●) complete interference of a G residue; (◒) partial interference of a G residue; (•) interference of a A residue. (B) Competitive gel mobility shift analysis for complexes 2 and 3. ^{32}P-labeled oligomer sp35-36 (left panel) or sp39-40 (right panel) was incubated with nuclear extract from IL-6-stimulated cells in the presence of various unlabeled competitive oligomers (50× molar ratio to the probe) representing the wild-type sp35-36, wild-type sp39-40, mutant sp35-36 (Ma, Mb), mutant sp39-40 (Mα, Mβ), C/EBP binding site, and NFIL-6 binding site. The lane marked "None" represents no competitor added to the binding reaction. (C) Immunoinhibitory binding analysis of complexes 2 and 3. Various amounts of antiserum to C/EBP or NFIL-6α were added to the binding reaction containing nuclear extract from IL-6-stimulated cells and ^{32}P-labeled oligomer sp35-36 (left panel) or sp39-40 (right panel).

competed by an oligomer containing the binding site for C/EBP (Vinson et al., 1989) and NFIL-6α (Akira et al., 1990).

Finally, to distinguish whether complexes 2 and 3 were bound by either C/EBP or NFIL-6α (the two major members of the C/EBP family), an antiserum to C/EBP (Landschulz et al., 1988) and an antiserum to NFIL-6α (Akira et al., 1990) were used to test for inhibition of complex formation. Labeled oligomer was incubated with nuclear extract from IL-6-stimulated cells in the presence of various amounts of antiserum to C/EBP or NFIL-6α. We found in Figure 2C using either the upstream probe sp35-36 or the downstream probe sp39-40 that the antiserum to NFIL-6α inhibited the formation of complexes 2 and 3, while the antiserum to C/EBP showed no inhibition. When the nuclear extract from untreated cells was used, the antiserum to NFIL-6α inhibited the formation of complex 3 (data not shown). Thus, not only was the formation of the IL-6-inducible complex 2 and the constitutive complex 3 common to the two IL-6 responsive elements but both elements also bound the same factor, NFIL-6α.

Identification of Binding Factors Unique to the Downstream IL-6 Responsive Element. Since we could not ignore the other complexes which were found only with the oligomer representing the downstream IL-6 responsive element, we characterized the factors that formed complexes 1, 4, and 5 using the same methods as above for complexes 2 and 3. The results in Figure 3A of the methylation interference assay for complexes 1, 4, and 5 formed using nuclear extract from IL-6 stimulated cells indicated that complexes 4 and 5 produced the identical protection pattern, while complex 1 differed from them. Using the labeled (+) strand of sp39-40, it was observed with complexes 4 and 5 that methylated A residues at −58 (and possibly at −60 and −62) interfered with protein binding. Using the labeled (−) strand of sp39-40, it was observed with complexes 1, 4, and 5 that a methylated G residue at −61 interfered partially with protein binding, and methylated A residues at −63, −64, and −65 (and possibly at −67 and −68) interfered with protein binding. When nuclear extracts from untreated cells were used, the methylation interference pattern for the three complexes was the same as that with nuclear extracts from

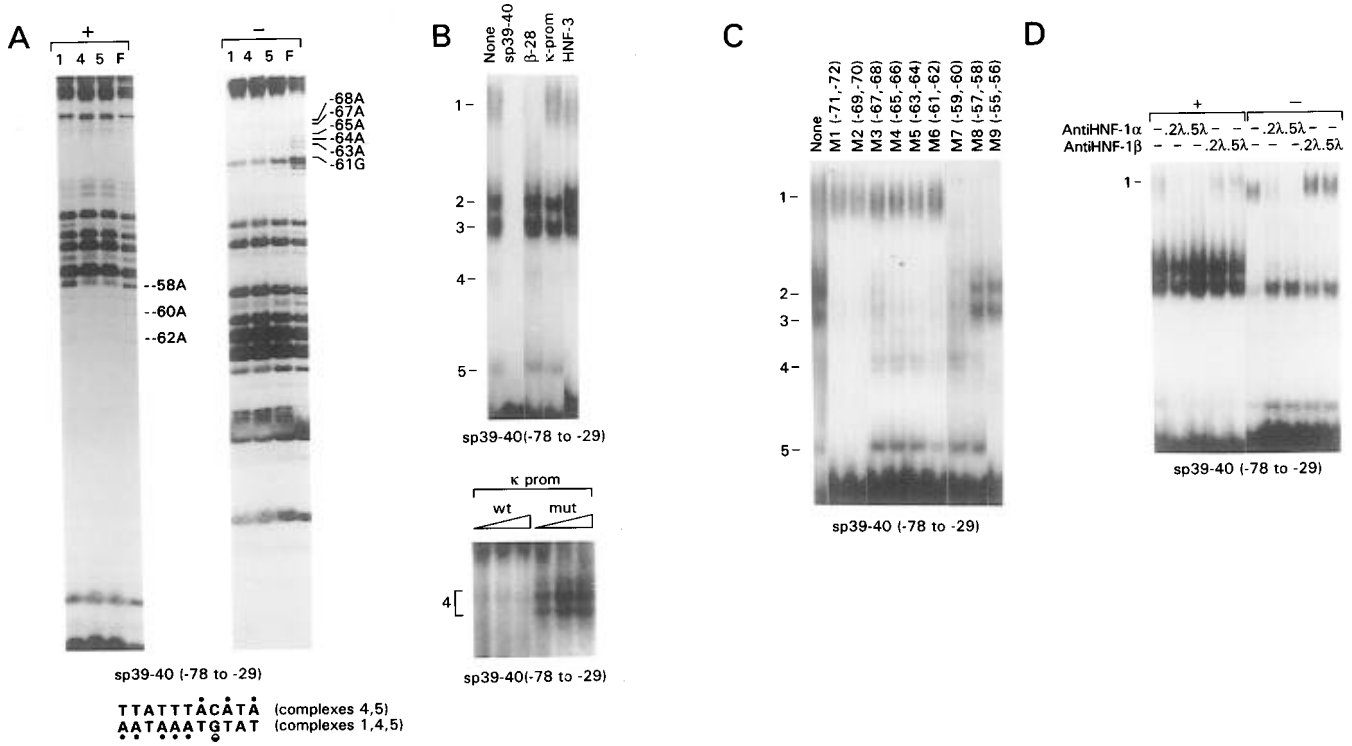

FIGURE 3: Characterization of the binding specificity of the downstream IL-6 responsive element for HNF-1α, Octamer-like, and HNF-3-like factors. (A) Methylation interference analysis for complexes 1, 4, and 5. Oligomer sp39-40 was labeled with ^{32}P on the 5′-end of the positive (+) and the negative (−) strands, and the remainder of the procedure was performed as described in the legend to Figure 2A. Protected G and A residues are indicated. Below the figure is a summary of the methylation interference: (◒) partial interference of a G residue; (•) interference of an A residue. (B) Competitive gel mobility shift analysis for complexes 1, 4, and 5 with known factor binding sites. ^{32}P-labeled oligomer sp39-40 was incubated with nuclear extract from IL-6-stimulated cells in the presence of various unlabeled oligomers (50× molar ratio to the probe) representing the HNF-1 binding site (*β*-28), Octamer binding site (*κ*-prom), HNF-3 binding site, and wild-type sp39-40. Since complex 4 showed up weakly, a darker exposure of this complex was attached to the bottom of the figure. Different amounts of wild-type (wt) or mutant (mut) *κ*-prom competitive oligomer (10×, 50× and 250× molar ratio to the probe) were added to the binding reaction. Lane marked "None" represents no competitor added to the binding reaction. (C) Competitive gel mobility shift analysis for complexes 1−5 with mutant sp39-40 oligomers. Various unlabeled mutant sp39-40 oligomers (M1 to M9, 50× molar ratio to the probe) were added as competitors to the binding reaction with nuclear extract from IL-6-stimulated cells. Lane marked "None" represents no competitor added to the binding reaction. (D) Gel mobility shift analysis using antibody for complex 1. Various amounts of antiserum to HNF-1α or HNF-1*β* were added to the binding reaction containing nuclear extract from IL-6-stimulated (+ lanes) or untreated (− lanes) cells and ^{32}P-labeled oligomer sp39-40.

IL-6-stimulated cells (data not shown). Thus, in the downstream IL-6 responsive element of the CRP promoter, it appeared that complexes 4 and 5 contained the same binding site between −58 and −68, while complex 1 contained a binding site between −61 and −68. By more precisely identifying the binding sites within the oligomer representing the downstream IL-6 responsive element which led to the formation of complexes 1, 4, and 5, we now were able to do competitive gel mobility shift analysis. The results in Figure 3B showed that the formation of complex 1 could be completely competed for by an oligomer (*β*-28) containing the binding site for HNF-1 (Courtois et al., 1987), while it could be competed for only slightly by an oligomer containing the binding site for HNF-3 (Lai et al., 1990). In contrast complex 5 could be completely competed for by an oligomer containing the binding site for HNF-3. Since complex 4 showed up weakly, a darker exposure of a competitive gel mobility shift analysis of complex 4 was shown in the insert panel below Figure 3B. Different amounts of wild-type or mutant oligomer (*κ*-prom) were added to the binding reaction. The results indicated that complex 4 consisted of a doublet which could be competed for by an oligomer (*κ*-prom) containing the binding site for Octamer factor (Rosner et al., 1990). In addition, competitive gel shift analysis using mutant oligomers of sp39-40 were performed (Figure 3C). No competition for complex 1 was seen with the mutant sp39-40 oligomers, M1 to M6, demarcating the HNF-1 site from −61 to −72. Little or no competition for complex 4 and 5 was seen with M3 to M8, demarcating the HNF-3 and Octamer sites from −57 to −68. Lastly, no competition for complexes 2 and 3 was seen with M8 and M9, demarcating the NFIL-6α site from −46 to −58 (also see Mα and M*β* in Figure 2B, right panel). Thus, it appeared that the 5′-end of the HNF-3 and Octamer sites overlapped with the HNF-1 site while the 3′-end of the HNF-3 and Octamer sites overlapped with the NFIL-6α site. There are two members in the HNF-1 family, HNF-1α and HNF-1*β*. Therefore, to determine the identity of the DNA-binding factor which formed complex 1, an antiserum to HNF-1α and an antiserum to HNF-1*β* (Mendel et al., 1991a) was used to test for inhibition of complex 1 formation. Labeled oligomer sp39-40 was incubated with nuclear extracts from either IL-6-stimulated (+) or untreated (−) cells in the presence of various amounts of antiserum to HNF-1α or HNF-1*β*. We found in Figure 3D that the antiserum to HNF-1α inhibited both the faster and the slower migrating forms of complex 1, while the antiserum to HNF-1*β* had no effect on either form of complex 1. Thus, complex 1 was formed by an HNF-1α factor which may be further modified following IL-6 treatment of cells (see Discussion).

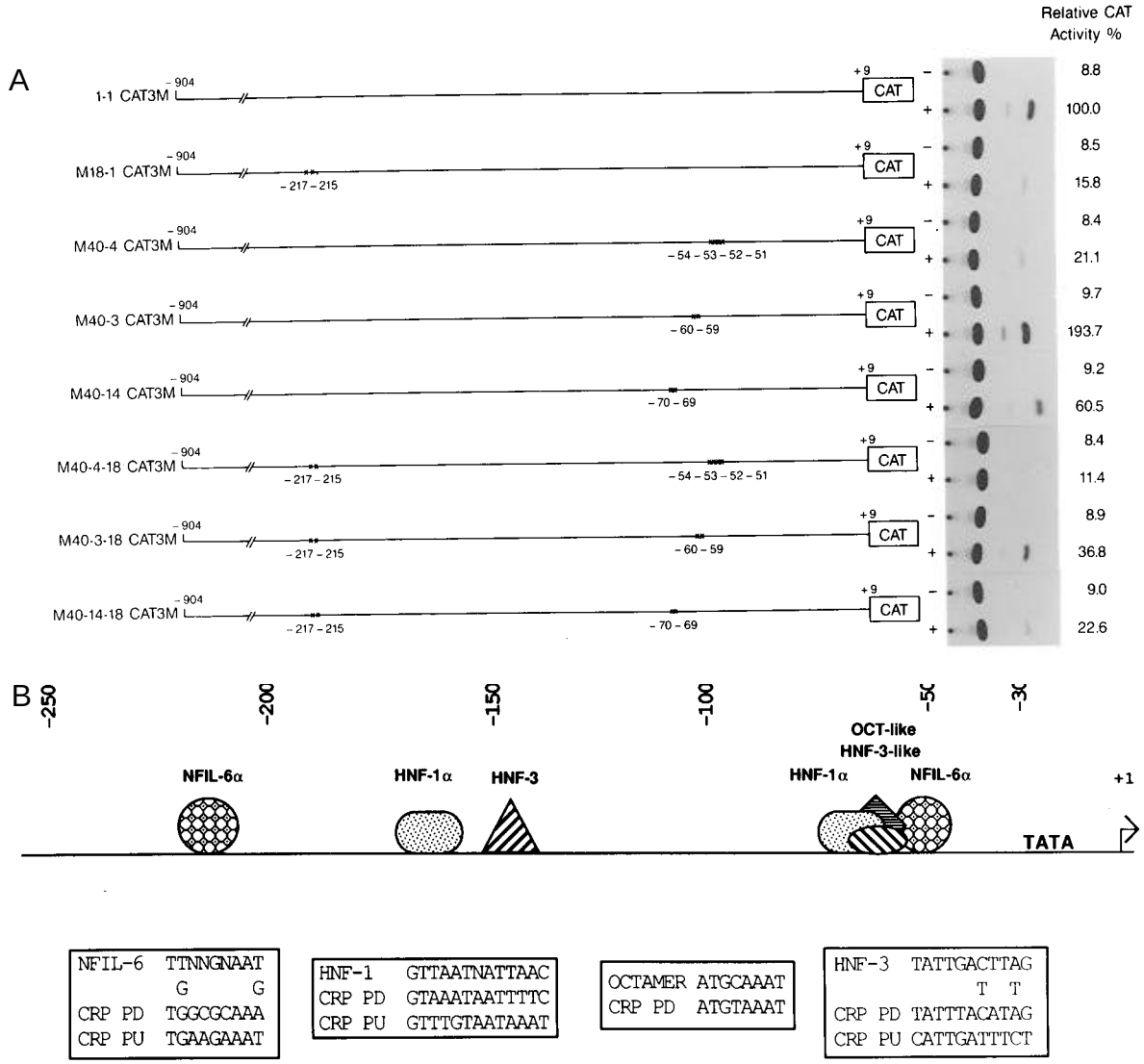

FIGURE 4: (A) *In vivo* analysis of IL-6-inducible transcription employing specific base pair mutations in the NFIL-6α, HNF-1α, and HNF-3/Octamer binding sites within the ~1 kbp 5′-flanking sequence of the CRP gene. Human hepatoma cells, PLC/PRF/5, were transfected with either the parental plasmid construct (1-1 CAT3M) or constructs containing mutations in one or two factor binding sites. Cultures were stimulated (+) with IL-6 or left untreated (−) and analyzed by the standard CAT assay. The percent relative CAT activity for each mutation was normalized to the result of the stimulated parent construct (1-1 CAT3M). (B) Summary of the *trans*-acting factors which bind to the 300 bp 5′-flanking region of the gene for human CRP. Specific binding sites for NFIL-6α, HNF-1α, HNF-3, and HNF-3/Octamer-like factors are located between −250 and −29 (TATA box). A comparison of the consensus sequences for the NFIL-6, HNF-1, HNF-3, and Octamer binding motifs with the binding site sequences in the human CRP promoter proximal upstream (PU) and proximal downstream (PD) regions is presented.

In Vivo Functional Analysis of the Factor Binding Sites in the Two IL-6 Responsive Elements. Having identified *trans*-acting factor binding sites, it was now possible to make point mutations in these sequences and functionally determine their effect on the level of transcription. We made both single and double binding site mutations in the parental plasmid construct 1-1 CAT3M, which contains the entire 904 bp 5′-flanking sequence of the human CRP gene (Li et al., 1990). Point mutations in the binding site sequence were based on the aforementioned mutant oligomers which we found would not bind specific factors *in vitro*. Plasmid constructs were transfected into hepatoma cells and 16 h later the cultures were left untreated (− lane) or stimulated (+ lane) with IL-6. Following incubation for 16−24 h, the cultures were harvested and the CAT activity determined (Figure 4A). A double NFIL-6α binding site mutant construct, M40-4-18 CAT3M, containing mutations in both the upstream (at −215, −217) and the downstream (at −51 to −54) NFIL-6α site, produced very little IL-6 inducible CAT activity, only about 10% of that of the parent (1-1 CAT3M). Single NFIL-6α binding site mutant constructs containing point mutations in either the upstream NFIL-6α site (M18-1 CAT3M, at −215, −217) or the downstream NFIL-6α site (M40-4 CAT3M, at −51 to −54) only produced 15%−20% of the parent activity after IL-6 induction. These results support the notion that both NFIL-6α binding sites are required for full IL-6 inducible activity and that the two binding sites do not just contribute additively but, instead, contribute synergistically. To determine the contribution of the other factor binding sites we identified, a construct containing point mutations within the HNF-3/Octamer-like site (M40-3 CAT3M, at −59, −60) resulted in a 2-fold increase in IL-6 inducible CAT activity compared to the parent (1-1 CAT3M). Likewise, with a construct (M40-3-18 CAT3M) containing point mutations within two factor binding sites, the upstream NFIL-6α site (at −215, −217) and the downstream HNF-3/Octamer-like site (at −59, −60), there was also a 2-fold increase as compared to the

upstream NFIL-6α site mutant construct alone (M18-1 CAT3M). Thus, it appeared that the factor(s) that bind to the HNF-3/Octamer-like binding site might function in a negative manner to silence expression. In addition, a construct (M40-14 CAT3M) containing point mutations in the downstream HNF-1α site (at −69, −70) produced less IL-6 inducible CAT activity (about 40% reduction) compared to the parental construct (1-1 CAT3M). In constract, a construct (M40-14-18 CAT3M) containing point mutations in both the upstream NFIL-6α site (at −215, −217) and the downstream HNF-1α site (at −69, −70) produced almost the same inducible CAT activity (i.e., no significant additional reduction) as compared to the upstream NFIL-6α site mutant construct alone (M18-1 CAT3M). Thus, the HNF-1α binding site appeared to behave as a positive regulatory factor whose activity was dependent on the synergistic activity of the two NFIL-6α binding sites. In summary, from an *in vivo* functional perspective it appeared that efficient IL-6 induction of the human CRP gene absolutely required the presence of two NFIL-6α binding sites. The presence of either one alone resulted in at least a 5-fold reduction in IL-6 inducible activity. Likewise, this IL-6 inducible activity was modulated by the presence of (1) a strong negative regulatory site which appeared to bind HNF-3/Octamer-like factor(s) and (2) a positive regulatory site which bound HNF-1α.

DISCUSSION

We presented here a topographical analysis of the two IL-6 responsive elements in the 5′-flanking region of the human CRP gene and an identification of the *trans*-acting protein factors which are bound to these two elements and are responsible for IL-6 inducible expression. From our gel mobility shift assays, methylation interference analyses, and *in vivo* site-specific mutation construct analyses, we found that both IL-6 responsive elements contained an IL-6-inducible NFIL-6α binding site and that the downstream IL-6 responsive element also contained a positive HNF-1α binding site and a negative HNF-3/Octamer-like factor binding site. In addition, by gel mobility shift analysis of the region between the two IL-6 responsive elements, we have found another HNF-1α and HNF-3 binding site (data not shown). Thus, in the 300 bp promoter proximal sequence of the CRP gene at least seven specific binding sites were identified which bound to five protein factors (see a summary in Figure 4B).

Due to the pleomorphism of the DNA-binding sequence for the various C/EBP family members, each binding site must be independently analyzed to determine the specific member in the nuclear extract of a particular cell type which will preferentially bind to it. Using specific antibody to C/EBP, NFIL-6α, and NFIL-6β (all three factors were observed by Western blot to be present in our nuclear extracts, data not shown), we found that the two IL-6 responsive elements only bound NFIL-6α. Nuclear extract from untreated hepatoma cells produced a single DNA−protein complex (complex 3) with either IL-6 responsive element, while nuclear extract from IL-6 stimulated cells produced two DNA−protein complexes (complexes 2 and 3). Both of these complexes contained NFIL-6α as determined by inhibition with specific antibody. In addition, we have found that the complex formation using nuclear extract from untreated or IL-6-stimulated cells did not change when these cells were previously incubated in the presence of cycloheximide (data not shown). These data supported the finding of others (Poli & Cortese, 1989; Poli et al., 1990) that NFIL-6α already existed in the cell and that it was post-translationally modified following IL-6 stimulation. It seemed that the NFIL-6α species forming the IL-6-inducible complex 2 could be the modified form of NFIL-6α, while the NFIL-6α in the constitutive complex 3 could be the truncated form of NFIL-6α which was thought to act as repressor (Descombes & Schibler, 1991; Natsuka et al., 1991). Thus, in PLC/PRF/5 human hepatoma cells the C/EBP family member, NFIL-6α, played a critical role in IL-6 induction of human CRP gene expression. In HepG2, human hepatoma cells, NFIL-6α was also found to form an IL-6-inducible complex with the haptoglobin IL-6 responsive element (Isshiki et al., 1991). In contrast, it has been reported that another member of the C/EBP family, the cycloheximide-sensitive NFIL-6β (Kinoshita et al., 1993), was observed in Hep3B human hepatoma cells to be the major IL-6-induced factor binding to an acute phase response element (Ramji et al., 1993). The reason for the differences between these results may be due to the different human hepatoma cell culture systems used.

The NFIL-6α binding motif in the two IL-6 responsive elements was flanked by additional sequences which appeared to confer specificity. Both the upstream NFIL-6α binding site (−211 to −222) and the downstream NFIL-6α binding site (−46 to −58) consisted of the NFIL-6 consensus sequence TT/GNNGNAAT/A (Akira et al., 1990). By methylation interference analysis, we identified a broad consensus sequence ANTGNNGNAANCT for the upstream site (AATGAAGAAATCT) and the downstream site (AGTGGCGCAAACT). By both competitive binding experiments and functional studies with site-specific mutations, it appeared that the upstream site was able to bind NFIL-6α slightly more tightly compared to the downstream site. In addition, we found other regions (−70 to −78, −129 to −137, and −162 to −170) which contained sequences representative of the above NFIL-6 core consensus sequence. These sequences could only weakly form complexes 2 and 3, and therefore could only weakly compete the upstream and the downstream NFIL-6α site bindings. None of these weak binding sites matched our broad consensus sequence. Thus, the two strong binding motifs which were functionally IL-6 responsive appeared to require additional specific flanking sequence for increased affinity.

Complete IL-6-inducible CRP gene transcription was dependent on the presence of an NFIL-6α binding site in both the upstream and the downstream elements. *In vivo* results using point mutations demonstrated functionally that the inactivation of both NFIL-6α binding sites completely abolished IL-6 induction. More importantly, inactivation of either site alone resulted in only 15%−20% of the total IL-6-inducible activity. Therefore, occupation of the two IL-6 responsive elements by NFIL-6α resulted in not just an additive (30%−40%) increase in IL-6 inducible activity, but a synergistic (100%) increase upon IL-6 induction. These results strongly suggested that the two binding sites acted synergistically and that together they were necessary for full IL-6-inducible activity.

The HNF-1 family of transcription factors has been found to be important for expression of a variety of hepatocyte-specific genes including the CRP gene (Toniatti et al., 1990). We have identified the HNF-1 site in the downstream IL-6 responsive element from −61 to −72. Using specific antibody to HNF-1α and HNF-1β, we have demonstrated that the protein factor which bound to this site was HNF-

1α. A mutant, M40-14 CAT3M, in the HNF-1α binding site no longer had the capacity to bind HNF-1α *in vitro*. IL-6-induced expression of this transient transfected construct resulted in a 40% reduction in CAT activity. This result confirmed that this HNF-1α site acted as a positive regulatory element whose activity was dependent on the NFIL-6α sites.

We also found that the HNF-1α complex formed with nuclear extract from IL-6-stimulated cells migrated a little slower in the native polyacrylamide gel. Likewise, nuclear extract from the hepatoma cells pretreated with cycloheximide and stimulated with IL-6 also demonstrated a slower migrating HNF-1α complex (data not shown). These results suggested that upon IL-6 induction, pre-existing HNF-1α may have undergone post-translational modification. HNF-1α was originally observed to exist as a homodimer (Frain et al., 1989; Nicosia et al., 1990). Shortly thereafter, a dimerization cofactor of HNF-1α (DcoH) was reported, which could form a tetramer with HNF-1α to stabilize the HNF-1α dimer and enhance its transcriptional activity (Mendel et al., 1991b). It appeared with our nuclear extract from untreated cells that the constitutive form may be reminiscent of the HNF-1α homodimer, while nuclear extract from IL-6-stimulated cells may give rise to a heterotetramer. If this is the case, the formation of a stable tetrameric DcoH−HNF-1α complex may be a direct result of IL-6 induction. Recently, it has been reported that different isoforms of HNF-1α may be generated by alternative RNA processing (Bach & Yaniv, 1993). Hence, further work needs to be performed to characterize the modification of HNF-1α subsequent to IL-6 induction.

The work in rat albumin gene expression had suggested that a negative regulatory element could exist which overlapped with the HNF-1 site in the albumin gene promoter region (Herbomel et al., 1989). It was postulated that a factor present in albumin-negative cells may have been extinguishing albumin expression. In the human CRP gene promoter, we observed an HNF-3/Octamer binding sequence, TTATTTACATAG, from −57 to −68 which overlapped at its 5′ sequence with the HNF-1α binding site (−61 to −72) and at its 3′-end with the NFIL-6α binding site (−46 to −58). Our competitive gel mobility shift analysis revealed that there was both an Octamer-like protein factor and a HNF-3-like protein factor in hepatoma cell nuclear extract which recognized this sequence. It appeared that the HNF-3-like factor may be a negative regulatory protein, since there was a significant decrease (2−3-fold) in its complex formation with nuclear extract from IL-6-stimulated cells as compared to nuclear extract from untreated cells. Previously, we reported a CRP promoter deletion construct, Δ15Δ6 CAT3M, which contained a deletion from −60 to −55, driving a CAT reporter gene (Li et al., 1990). The increased CAT activity from this transient transfected deletion construct suggested a potential negative element. Here we presented a site-specific mutant construct, M40-3 CAT3M (with point mutations in the HNF-3/Octamer site of the CRP promoter, driving a CAT reporter gene), that resulted in a 2-fold increase in IL-6-inducible CAT activity over that of its parental construct, 1-1 CAT3M. Likewise, a double binding site mutant construct, M40-3-18 CAT3M, containing point mutations at both the upstream NFIL-6α site and the HNF-3/Octamer site, also produced a 2-fold increase in IL-6-inducible CAT activity over that of its parental construct M18-1 CAT3M (with mutations only at the upstream NFIL-6α site). Therefore, the positive HNF-1α factor and the IL-6-inducible NFIL-6α factor may be regulated by protein−protein interaction with the negative HNF-3/Octamer-like factor(s) which overlapped with them. However, even in the presence of a mutated negative regulatory binding site, the positive HNF-1α site and the IL-6-inducible NFIL-6α sites cannot activate on their own but must await IL-6 induction to occur before enhancing transcription.

More work needs to be done to identify and characterize the factor(s) which bind to the negative regulatory element which overlaps the HNF-1α site and NFIL-6α site. Preliminary results indicate that the negative regulatory factor is neither HNF-3 (α, β, or γ) nor the Octamer proteins which bind to this same sequence, Oct 1 or Oct 2. The Octamer-like factors forming complex 4 have a greater mobility on gel shift analysis than complexes formed with Oct 1 or Oct 2 (data not shown). Hence, these Octamer-like factors may be more similar to Oct 4 or Oct 6, which are reported to function also as repressors (Scholer, 1991).

Proteins bound to widely separated promoter sites can still interact with each other resulting in DNA looping or bending which was hypothesized to play a role in the regulation of gene expression (Ptashne, 1986). DNA bending already has been observed to occur subsequent to binding of transcription factors [i.e. SRF (Gustafson et al., 1989), NF-κB (Schreck et al., 1990), AP-1 (Kerppola & Curran, 1991), LEF-1 (Giese et al., 1992), and NF-Y (Ronchi et al., 1995)] and the TATA-binding protein (TBP, Kim et al., 1993). Althrough transcription factors contact both the major and the minor grooves of DNA, it is believed that the minor groove interaction may strongly distort DNA and hence facilitate the protein−protein interactions necessary to build up a competent transcription initiation complex (Struhl, 1994). Recently a common theme has been confirmed by determining the three-dimensional structure of several protein−DNA complexes. The transcription factor's side chain interaction into the minor groove of DNA plays a pivotal role in the mechanism of kinking or bending the DNA (Werner et al., 1996). It has not been reported that the factors, NFIL-6α and HNF-1α, can induce the bending of the CRP promoter DNA. However, in both IL-6 responsive elements, methylated A residues interfered with the binding of NFIL-6α, HNF-1α, and HNF-3/Octamer-like factor(s). Interference of protein binding by methylated A residue(s) predicts protein interaction with the minor groove of DNA since dimethyl sulfate reacts with the N-3 of adenine (Maxam & Gilbert, 1977). Therefore, it is possible that NFIL-6α, HNF-1α, and HNF-3/Octamer-like factor(s), bound to the minor groove of DNA at separate sites of the CRP promoter, may further result in DNA bending to facilitate the formation of a loop structure. We, therefore, propose a potential model to explain the IL-6 induction mechanism resulting from interactions between various activators and repressors. In unstimulated liver cells, the human CRP promoter proximal region may be occupied by repressors: the truncated form of NFIL-6α and the HNF-3/Octamer-like factor(s). Potentially they may prevent the formation of a loop structure involving the two NFIL-6α sites which may be required to facilitate interaction of upstream transcription factors with the pre-initiation complex. Concurrently, the HNF-3/Octamer-like factor(s) may negatively inhibit HNF-1α from efficiently binding with its recognition site or interacting with other factors. Following IL-6 stimulation of liver cells, the truncated form of NFIL-6α may be replaced by a post-translationally modified full-length NFIL-6α molecule at both sites. The activation domain of each full-length NFIL-6α may interact separately with the pre-initiation complex to

form the active transcription loop. This transcription complex may further be enhanced by an interaction with the fully active DcoH−HNF-1α as the binding affinity of the HNF-3/Octamer-like repressor decreases. Thus, these *trans*-acting factors and *cis*-acting elements may work together to form a DNA−protein and protein−protein multiple component complex at the active transcription site which acts on the general transcription machinery to successfully induce expression of the gene.

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