STAT3 Participates in Transcriptional Activation of the C-reactive Protein Gene by Interleukin-6*

(Dongxiao Zhangt, Ming Sunt, David Samolšt, and Irving Kushner§)

From the tDepartment of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106 and the §Department of Medicine, MetroHealth Medical Center, Case Western Reserve University, Cleveland, Ohio 44109

Interleukin-6 (IL-6) is the major cytokine inducing transcription of human C-reactive protein (CRP) during the acute phase response. STAT (signal transducers and activators of transcription) family members, recently shown to be important mediators of the effects of many cytokines including IL-6, generally induce their effects by binding to palindromic sequences with TT(N)₅AA motifs. We report an IL-6 responsive element in the proximal region of the human CRP 5' flanking region that bears a TT(N)₄AA motif, which we have termed CRP acute phase response element (CRP-APRE). In Hep3B cells, IL-6 but not interferon-γ was capable of activating CAT constructs driven by the CRP promoter containing CRP-APRE. Overexpressed STAT3 was able to transactivate CRP-chloramphenicol acetyltransferase constructs through the CRP-APRE and was able to enhance endogenous CRP mRNA accumulation in response to IL-6. STAT3 (or an antigenically related molecule) bound to the CRP-APRE in response to IL-6. Overexpression of STAT3 in the presence of IL-6 was capable of inducing expression of a construct consisting of the CRP-APRE and a minimal thymidine kinase promoter lacking a C/EBP site. Taken together, these findings indicate that STAT3 participates in the transcriptional activation of CRP in response to IL-6.

A large number of systemic and metabolic changes, collectively referred to as the acute phase response (APR), begin to occur within hours after an inflammatory stimulus (1–3). Among these changes is a reprogramming of the pattern of plasma protein gene expression in hepatocytes, with consequent changes in blood concentrations of these proteins. C-reactive protein (CRP) is a major acute phase protein in humans, its concentration increasing more than 1000-fold in severe inflammatory states.

Interleukin-6 (IL-6) appears to be the major cytokine inducing transcription of human C-reactive protein (CRP) during the acute phase response. STAT (signal transducers and activators of transcription) family members, recently shown to be important mediators of the effects of many cytokines including IL-6, generally induce their effects by binding to palindromic sequences with TT(N)₅AA motifs. We report an IL-6 responsive element in the proximal region of the human CRP 5' flanking region that bears a TT(N)₄AA motif, which we have termed CRP acute phase response element (CRP-APRE). In Hep3B cells, IL-6 but not interferon-γ was capable of activating CAT constructs driven by the CRP promoter containing CRP-APRE. Overexpressed STAT3 was able to transactivate CRP-chloramphenicol acetyltransferase constructs through the CRP-APRE and was able to enhance endogenous CRP mRNA accumulation in response to IL-6. STAT3 (or an antigenically related molecule) bound to the CRP-APRE in response to IL-6. Overexpression of STAT3 in the presence of IL-6 was capable of inducing expression of a construct consisting of the CRP-APRE and a minimal thymidine kinase promoter lacking a C/EBP site. Taken together, these findings indicate that STAT3 participates in the transcriptional activation of CRP in response to IL-6.

The binding of IL-6 to its receptor complex leads to phosphorylation of Janus kinase kinases, with subsequent rapid (15–60 min) phosphorylation, dimerization, and nuclear translocation of a transcription factor originally named acute phase response factor (8) and since designated STAT3 (6). STAT3 then binds to specific response elements in the promoter regions of cytokine responsive genes. The promoter regions of a number of human and rat acute phase genes contain TT(N)₄AA sequences (10–13) capable of binding STAT proteins. STAT1 (14) also appears to be activated by IL-6 as well as by IFN-γ with similar rapid kinetics and binds to a similar consensus motif called the γ interferon activation site (GAS) (15–17). Both STAT3 and STAT1 have been found to be activated by a broad spectrum of cytokines and growth factors, including IFN-γ, epidermal growth factor, and IL-6-related cytokines (17, 18).

We have previously shown in the human hepatoma cell line Hep3B that IL-6 activates transcription of CRP, that IL-1β, which has no effect alone, synergistically enhances CRP transcription in the presence of IL-6, and that the proximal 157 bp of the 5'-flanking region of CRP was sufficient to confer IL-6 induction and IL-1β synergistic activation on CRP-CAT (19). Several cis-elements and trans-activators that were required for CRP transcription in response to monocyte conditioned medium and IL-6 have been characterized. Two C/EBP binding sites in the proximal region of the CRP promoter have been shown to bind IL-6-inducible C/EBP family members (20), and two HNF-1 sites have been shown to be adjacent to the C/EBP sites and to be necessary for CRP transcription (21, 22). Other regions containing positive and negative cis-elements have also been found in the 5'- and 3'-flanking regions of the CRP gene (23, 24).

We report here the finding of a STAT3 response element in the human CRP promoter with the sequence TTCCCGAA, which is necessary for optimal IL-6-induced transcription of CRP. Oligonucleotides with the TT(N)₄AA motif have recently been reported to specifically bind STAT3 (25). This finding indicates that STAT3 or a closely related molecule participates in mediation of the transcriptional effect of IL-6 on human CRP.
EXPERIMENTAL PROCEDURES

Materials—Recombinant human IL-1β and human IL-6 were from BioSource International (Camarillo, CA). RCmCMV-STAT3, pMNC-91 expression vectors, and antibody against sheep STAT5 were generous gifts from Dr. J. E. Darnell (Rockefeller University) (6, 26). pCAT(An) and pTK-CAT (27) were gifts from Dr. R. Eckert of Case Western Reserve University. C-20 anti-STAT3 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). DNA probe PCR5 for human CRP was generously provided by Dr. H. R. Colten (Washington University). Restriction enzymes, T4 DNA polymerase, DNA polymerase I (Klenow Fragment), and DNA ligase were from Boehringer Mannheim. pRSV-β-galactosidase control vector (pRSV-β-Gal) was from Promega (Madison, WI).

Cell Culture—Human hepatoma Hep3B cells were kindly provided by Dr. G. J. Darlington (Baylor College of Medicine, Houston, TX). HepG2 cells were gifts from Dr. R. Hanson (Case Western Reserve University). Hep3B cells and HepG2 cells were maintained in RPMI 1640 medium and Dulbecco’s modified Eagle’s medium/F12, respectively, supplemented with 10% fetal bovine serum. Cells were subcultured weekly after trypsinization.

Transfection and Cytokine Induction—Electroporation was performed according to published procedures (28). Briefly, 10^6 cells subcultured 3 days earlier (80–90% confluent) were transfected and mixed with 20 μg of DNA (15 μg of CAT reporter DNA, 5 μg of RSV-β-Gal, and 2 μg of expression DNA or control vector) plus 200 μg of carrier DNA (Herring Testis DNA, Sigma) in total volume of 0.5 ml of Hepes-buffered saline. Electroporation was performed at V = 260 V, C = 960 microfarads using Gene Pulser Apparatus (Bio-Rad, Richmond, CA). Following electroporation, cells were plated onto eight 35-mm-diameter dishes for 6 h in RPMI 1640 medium supplied with 10% fetal bovine serum and for 12 h in RPMI 1640 medium without serum.

For cytokine induction, the transfected cells were incubated with serum-free medium and exposed to 10 ng (100 units)/ml IL-6, 10 ng (100 units)/ml IL-1β, or 10 ng (100 units)/ml IFN-γ for up to 24 h. STAT3 stable cell lines were generated by transfecting Hep3B cells with RCmCMV-STAT3 by electroporation as described above, followed by G418 (400 μg/ml) selection. Single colonies were picked and expanded.

Assay for CAT—CAT assay was performed using the phase extraction method (29). Cell extracts were prepared by three cycles of freezing and thawing. 15 μl from 100 μl of the cell extract (for 10^6 cells) was used to determine β-galactosidase activity as described (30). The remainder of the cell extract was assayed for CAT activity in a reaction containing 0.25 μg/ml of nitroblue tetrazolium and 0.02 μc of 3H chloramphenicol. The β-Gal activity was used to normalize CAT activity to control for transfection efficiency.

Synthetic Oligonucleotides and Plasmid Construction—157/-3CRP-CAT was constructed as described (19) and contains 157 bp 5' to the transcription start site and 3 bp of the 5' untranslated region of the CRP promoter. Three deletion constructs, −123/-3CRP-CAT, −85/-3CRP-CAT, and −50/-3CRP-CAT, were constructed using −157/-3CRP-CAT as template employing a partial digestion protocol for deletion mutagenesis (31). Four block mutations A, B, C, and D of the 3CRP-CAT, and CRP+157, 5′-AACATTCCGAGGAACAGCCGACGAGCAG-3′, and CAT-15, 5′-AATCTCGCCAAGCTC-3′.

Northern Analysis—Northern blotting was performed as described previously (19).

Preparation of Nuclear Extracts—Nuclear extracts were prepared according to Shapiro et al. (33) with the following modifications. Hep3B or HepG2 cells (3 × 10^7) were washed with cold phosphate-buffered saline, collected, and pelleted by centrifugation at 4,000 x g for 5 min, at 4°C. The cell pellet was resuspended in 3 × packed cell volume of buffer A (20 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM NaVO₃, 1 mM EDTA, 0.2% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The suspension was rocked gently for 30 min at 4°C followed by centrifugation at 12,000 x g for 5 min, after which the supernatant was collected and stored at −70°C.

EMSA—Complementary oligonucleotides were annealed and labeled by filling in its 5' protruding ends with Klenow enzyme, using [α-32P]dCTP (3000 Ci/mM). EMSA was carried out according published procedures (34). Nuclear extract (5 μg of protein) was incubated with 200 fmol (1,000 cpm) of probe in gel shift incubation buffer (40 mM KCl, 20 mM Hepes, pH 7.9, 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4% Ficoll, 0.1 μg of poly(dI-dC)-poly(dI-dC); Pharmacia Biotech Inc.) for 15 min at room temperature. In supershift experiments, antiserum was added at the same time as the probe. In competition experiments, an excess of cold oligonucleotides was added to the binding reactions. The DNA-protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel in 0.25× TBE (1× TBE = 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA) at 10 V/cm. The gels were dried and autoradiographed.

RESULTS

The −123 to −85 Region Is Required for Optimal IL-6 Response—A series of 5′ deletion constructs of CRP-CAT were transiently transfected into Hep3B cells, followed by the addition of IL-1β, IL-6, or their combination. IL-1β alone had no effect on CAT expression, whereas IL-6 caused a 4-fold increase in CAT activity (Fig. 1). Deletion of the region between −123 to −85 (see top Table II) markedly diminished. Basal CAT activity was also decreased to about one-third of that seen with −123/-1 CRP-CAT, and IL-1β synergy was not observed. Deletion of −85 to −50 abolished any measurable cytokine effect and decreased basal activity to that of the vector alone (not shown). These results were consistent with the existence of known IL-6 responsive cis-elements upstream of −123 and downstream of −85 (see top of Fig. 1) but also suggested that a cis-element(s) in the region of −123 to −85 was necessary for constitutive expression of CRP, for IL-6 induction, and for IL-1β synergy.

A GAS-like Sequence (CRP-APRE) Is Localized in the Region of −123 to −85—Four “block mutations” were constructed such that 5′-7 nucleotides were replaced by irrelevant nucleotides at four positions in the interval between −123 and −85 (Table II). These mutant constructs, designated A, B, C, and D, were

pBLCAT2 (32), a construct containing 105 bp of thymidine kinase (TK) promoter including a C/EBP binding site between −105 and −83, we subsequently constructed a truncated vector named pTK(−77)/CAT, based on the parental pTK-CAT but with the C/EBP site deleted. Briefly, a polymerase chain reaction fragment containing −77 to +55 of the TK gene was amplified using pTK-CAT as template, an upstream primer containing CTTC (++) which extends TK sequence (−77 to −60) flanked by a PstI site, and a downstream primer CAT1-15, which is complementary to the first 15 bp of CAT gene. This DNA fragment was cloned into PstI-XhoI sites of pCAT(An) to generate pTK(−77)/CAT. Two copies of CRP-APRE were released from pAPRE-TK-CAT by HindIII digestion and ligated into the HindIII site of pTK(−77)-CAT with CRP-APRE having the same direction as in the CRP promoter, and the resulting plasmid is pAPRE-TK(−77)-CAT. The primers used were the following: −77TK(+), 5′-AAACCTGACGCGACGAACACCGGTTAAGCAG-3′; and CAT1-15, 5′-AATCTCCGCCAAGCTC-3′.

pCAT1–15, 5′-AGCTCTTCCGAAGCTAAGAGG-3′; mutant B, 5′-AGCTCTTCCGAGGAACAGCCGACGAGCAG-3′; mutant C, 5′-AGCTCTTCCGAGGAACAGCCGACGAGCAG-3′; and mutant D, 5′-AGCTCTTCCGAGGAACAGCCGACGAGCAG-3′. The 3′ primer was 5′-ATTCTCCGAGTAAGCTCCTCCGTCCGC-3′. The underlined sequences indicate the heterologous sequences in each mutant. Antisense STAT3 was constructed by releasing the STAT3 DNA fragment with HindIII, followed by ligation of the DNA fragment in an inverted orientation into RCmCMV vector. Oligonucleotides were used in EMSA following: CRP-APRE(−), −123/-3CRP-CAT; CRP-APRE(−), −5'-GATACGTGGGAGGAAAGC-3'; rat α2m (+), −5'-AGCTTCTTGGAGGAAAGC-3'; rat α2m (+), −5'-AGCTTCTTGGAGGAAAGC-3'; rat α2m (+), −5'-AGCTTCTTGGAGGAAAGC-3'; and CRP-APRE(−), −5'-AGCTTCTTGGAGGAAAGC-3'. To construct pAPRE-TK-CAT constructs, HindIII sites were designed to flank the sequence, and the annealed oligonucleotides were inserted into HindIII site of pTK-CAT (27). The annealing oligonucleotides were 5′-AGCTCTTCCGAGGAACAGCCGACGAGCAG-3′.
transfected into Hep3B cells, which were then studied for their responses to IL-6 (Table I). Although some decreased IL-6 response was seen with every mutant, the B mutant showed substantially diminished IL-6 inducibility as well as decreased basal activity.

The sequence disrupted in the B mutant, TTCCCGAA, was found to be homologous to members of the family of acute phase response elements (APREs) first identified in the rat α2M gene as well as to the GAS (Table II). It is noteworthy that the CRP element has only four nucleotides between the conserved TT . . . AA, although the surrounding sequences closely resemble α2M APRE. Therefore, we named the element disrupted in mutant B CRP-APRE.

STAT3 but Not STAT1 Transactivates CRP-CAT in Response to IL-6—Because both STAT3 and STAT1α have been shown to bind to GAS-like sequences in response to both IL-6 and IFN-γ, we evaluated the possible roles of these transcription factors in activating CRP transcription (Fig. 2). Overexpression of STAT3 in the absence of cytokines was sufficient to increase CAT expression 2–3-fold in −157/+3 CRP-CAT. The effect of overexpressed STAT3 was moderately increased by the addition of IL-1β and was greatly enhanced by the addition of IL-6. In contrast, overexpression of STAT1α had no effect on CAT expression in the presence or the absence of cytokines. As a control experiment, overexpression of STAT1α was able to enhance the IFN-γ response of a CAT reporter construct containing the interferon regulatory factor 1 promoter about 3-fold (data not shown). Studies employing −123/+3 CRP-CAT showed similar results, whereas −85/+3 CRP-CAT was not influenced by co-transfection of either STAT3 or STAT1α (data not shown).

Similar observations were made for the endogenous CRP gene in Hep3B cells. In cells stably transfected with a STAT3 expression construct, CRP responses to IL-6 and to the combination of IL-6 + IL-1β were markedly increased (Fig. 3).

In contrast, overexpressed STAT1α was unable to increase CAT expression of −123/+3 CRP-CAT even in the presence of IFN-γ (Fig. 4). It was of interest that IFN-γ as well as IL-6 was able to enhance transactivation of −123/+3 CRP-CAT by STAT3 and that the combination of IL-6 and IFN-γ had some additive effect in the presence of overexpressed STAT3.

To verify the hypothesis that STAT3 transactivation was exerted through CRP-APRE, the four block mutants of −123/+3 CRP-CAT were employed in co-transfection experiments (Fig. 5). As expected, the response to cytokines in cells overexpressing STAT3 was greatly abolished in mutant B, while other mutants were only moderately affected. In another approach, we employed an antisense STAT3 expression construct in which the direction of the cDNA was reversed in the expression vector in the co-transfection experiments and found that antisense STAT3 was able to suppress IL-6 induction of CRP-CAT expression to one-third of vector-transfection control levels (data not shown).

STAT3 Binds to CRP-APRE in an IL-6-inducible Manner—EMSAs were employed to determine whether STAT3 binds to CRP-APRE. Because HepG2 cells have been shown to display abundant STAT3 activity in response to IL-6, we initially used nuclear extracts from this cell line to explore this possibility. As shown in Fig. 6, several complexes were observed when nuclear extracts from IL-6-treated (15 min) or untreated cells were incubated with a CRP-APRE probe. Formation of Complex I was induced by IL-6 and was competed by excess amounts (10–50×) of either self-oligonucleotides or rat α2M APRE but not by “CRP α” oligonucleotides, which have been shown to bind both C/EBP β and C/EBP δ (20). This finding indicated that the binding activity was specific for CRP-APRE and that it shared the binding properties of STAT3, which has been shown to bind rat α2M APRE. Antibody against STAT3 abolished the formation of Complex I, whereas a complex with a slower migration rate formed, presumably a “supershifted” antibody-containing complex. Neither rabbit IgG against β-galactosidase nor antibody against STAT5 had any effect. This result demonstrated that STAT3 (or an antigenically related protein) was present in Complex I. Two other complexes (II and in Hep3B cells, III, see below) were observed which were not IL-6-inducible. The significance of these complexes is not clear. A similar pattern of complexes in EMSA was observed for Hep3B cells, although STAT3 binding activity was lower than was seen with

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Activation of CRP-CAT 5′ deletion constructs by IL-6 and IL-1β in Hep3B cells. A diagrammatic representation of the CRP promoter (−157 to +3) is shown at the top. The indicated C/EBP binding sites and HNF-1 site have been described by others (20, 21). CRP-CAT constructs were transiently co-transfected with pRSVβ-Gal into Hep3B cells. Cells were treated with cytokines (IL-1β, 100 units/ml; IL-6, 100 units/ml) for 24 h. CAT activity (percentage of butyrylated chloramphenicol) was measured as described under “Experimental Procedures.” CAT activities were normalized by β-galactosidase activity to control for differences in transfection efficiencies. The values are the averages of four independent experiments performed in duplicate. The error bars indicate one standard error.

| Mutant | Sequence substitutions in the region of −123 to −85 region | Relative CAT activity | −IL-6 | +IL-6 |
|--------|----------------------------------------------------------|----------------------|-------|-------|
| A      | −123 TGTTCCCCCTCTCCCCGAAGCTCTGACACCTGCCCCAAC −85        | −123/ +3 CRP-CAT     | 3.8   | 24.0  |
| B      | −123/ +3 CRP-CAT                                       | −123/ +3 CRP-CAT     | 3.5   | 16.3  |
| C      | −123/ +3 CRP-CAT                                       | −123/ +3 CRP-CAT     | 2.0   | 5.6   |
| D      | −123/ +3 CRP-CAT                                       | −123/ +3 CRP-CAT     | 2.2   | 10.5  |
| E      | −123/ +3 CRP-CAT                                       | −123/ +3 CRP-CAT     | 3.3   | 21.7  |
STAT3 Activates CRP Transcription

**Table II**

| Name                | Sequence                  | Reference |
|---------------------|---------------------------|-----------|
| Rat α2 macroglobulin APRE | GAATTCCCCAGAAGGA          | 13        |
| Human α2 macroglobulin APRE | CCATCCCCGTAAGAG          | 8         |
| Rat α2-acid glycoprotein C | TTTTCCCCAGAACCC          | 10        |
| Human α1-antichymotrypsin | CTTTCACCTAAACAA          | 11        |
| Ly-6E GAS            | CTTACCAGAAATT              | 12        |
| GAS consensus        | ATATCCCTGTAAAGT           | 17        |
| CRP-APRE             | CTCTTCCCCGAAGCT           |           |

**Fig. 2.** Transactivation of -157/+3 CRP-CAT by STAT3 in response to IL-6 and IL-1β. -157/+3 CRP-CAT (15 μg) was transiently co-transfected with STAT3 expression plasmid pRc/CMV-STAT3 (2 μg) or STAT1α expression plasmid pMNC-91 (2 μg) plus pRSV-βGal (5 μg). 2 μg of pRc/CMV vector DNA were employed where neither expression plasmid was used. Cells were treated with cytokines (IL-1, 100 units/ml; IL-6, 100 units/ml) for 24 h. CAT activity was determined and normalized to β-galactosidase activity. Two independent experiments with duplicate samples were performed, and similar patterns of expression were obtained. CAT activity of the means of duplicate samples in each of these experiments is plotted.

**Fig. 3.** Northern analysis of endogenous CRP mRNA accumulation in cells stably transfected with pRc/CMV-STAT3. Cells were treated with IL-1 (100 units/ml), IL-6 (100 units/ml), and IL-1β (100 units/ml) for 24 h. mRNA was loaded at 15 μg/lane. pCRP5 (for CRP) was used as a probe. CRP signals are shown in the lower panel, and actin signals are shown in the lower panel. The results from a single lane of pRc/CMV-STAT3 stably transfected Hep3B cells are shown. The signal in Hep3B treated with IL-6 only was visible with longer exposure (not shown).

**DISCUSSION**

Our major findings were that overexpressed STAT3 but not STAT1α was able to transactivate CRP-CAT constructs in response to IL-6 stimulation through a GAS-like sequence that we have termed CRP-APRE, that overexpressed STAT3 activated the endogenous CRP gene in response to IL-6, that CRP-APRE was able to bind STAT3 in an IL-6 inducible fashion, and that CRP-APRE, in the presence of overexpressed STAT3, conferred IL-6 inducibility on a heterologous promoter lacking C/EBP binding sites in Hep3B cells. Taken together, these findings indicate that STAT3 participates in the transcriptional activation of C-reactive protein in response to IL-6.

Although both STAT3 and STAT1α have been shown to bind the same GAS-like elements and activate transcription of a number of genes (9, 35), this was not the case for CRP-APRE. Only STAT3 and not STAT1α transactivated CRP-CAT containing wild-type CRP-APRE: IL-6 markedly enhanced this transactivation. IFN-γ in the absence of STAT3 overexpression had no effect on either CRP-CAT constructs or on the endogenous CRP gene (data not shown). Accordingly, we conclude that the ability of IFN-γ to enhance CAT expression that we observed in STAT3-transfected cells was due to activation of overexpressed STAT3 in Hep3B cells. Similarly, EMSA demonstrated that CRP-APRE bound to STAT3 in a 1L-6-dependent manner. This selective response to IL-6 but not IFN-γ has not been reported for promoters containing other APREs, including α2M APRE (36). STAT3 is known to be the major STAT member mediating IL-6 signaling, whereas STAT1α is the major STAT activated by IFN-γ (17). It is likely that the selective binding of STAT3 determines the specific response of the CRP gene to IL-6 and not to IFN-γ.

This specificity of CRP-APRE for STAT3 may be explained by its structure. TT(N)5AA, which differs from the TT(N)5AA motif found in other APREs. In a recent study (25) in which the effects of the spacing between the TT and AA core half-sites on the binding of the STAT complexes were examined using synthetic oligonucleotides, it was found that TT(N)5AA elements displayed general STAT binding, whereas in contrast the TT(N)5AA motif bound only to complexes containing STAT3. Interestingly, one of their synthetic TT(N)5AA oligonucleotides...
STAT3 Activates CRP Transcription

The binding of STAT3 to CRP-APRE in Hep3B cells was found to last at least 18 h, which contrasts to the duration of 1 h reported in HepG2 cells with the α2M APRE (8). This difference may arise from differences in the cell lines used or the assay systems employed or may be due to distinctive binding properties of each DNA oligonucleotide. Long-term activation of STAT proteins has been reported using GAS-like elements such as pRE of the interferon regulatory factor 1 gene in the human breast carcinoma cell line T47D (38). It is conceivable that in our system, continuous activation of STAT3 contributes to the "prolonged" binding detected in EMSA. Alternatively, inactivation of STAT3 signaling (e.g. dephosphorylation of STAT3) may be slow in our cells.

Our findings suggest that the CRP-APRE may also participate in the maintenance of basal expression of CRP. Mutation of CRP-APRE not only decreased IL-6 induction of CRP-CAT but also lowered the basal level of CAT expression. We observed additional IL-6-independent complexes not abolished by antibody against STAT3 in EMSA using CRP-APRE as a probe (Fig. 6 and 7). This finding raises the possibility that binding of an unidentified transcription factor(s) other than STAT3 may account for constitutive basal level expression of CRP-CAT.

The observations presented here should not be taken to imply that STAT3 is the major transcription factor participating in mediation of the CRP response to IL-6. The role of C/EBP binding sites in the CRP response to IL-6 is well documented (22). Our finding that two copies of CRP-APRE in the absence of overexpressed STAT3 were not sufficient to confer IL-6 responsiveness on a truncated minimal TK promoter, although subject to other interpretations, raises the possibility that there is need for one or more other response elements. The fact that a GAS-like IL-6 responsive element exists in close proximity to the two C/EBP binding sites in the CRP promoter (and other IL-6 responsive promoters) points to possible cooperative effects between STAT and C/EBP family members. Relevant to this issue are our preliminary findings, in studies of reporter
constructs containing mutated C/EBP or CRP-APRE sites, which suggest that these sites are cooperative rather than functionally independent. The latter observation, if confirmed, would be consistent with the abundant evidence supporting the fundamental role of interactions between transcription factors in gene-specific transcriptional regulation (39). Among acute phase proteins, for example, an element in the human hemopexin promoter has been shown to bind a complex which contains STAT3 in response to IL-6 (40). Physical interaction between C/EBP and NF-κB has been demonstrated in numerous cases (41–43). C/EBPβ has also been shown to physically interact and functionally synergize with the glucocorticoid receptor in the induction of the rat α1 acid glycoprotein promoter (44). Whether C/EBP members have similar interaction with STAT3 is under investigation. These examples indicate how difficult it may prove to be to assign relative importance to interacting transcription factors participating in the full CRP response.

Similarly, our findings should not be taken to indicate that other as yet unidentified transcription factors do not participate in CRP induction by IL-6. Several studies indicate that unidentified activity other than STAT3 may activate IL-6-induced transcription (45–47). Although STAT3 has been shown to bind to three CTGGGA elements in the γ-fibrinogen gene (48), a similar CTGGGA element in Aα-fibrinogen was reported to associate with an unidentified protein that was not STAT3 (47). In another recent report, STAT3 was found to contribute to but not to be sufficient to up-regulate specific IL-6 response element-containing reporter constructs (46). It is likely that various combinations of STAT3, C/EBP, and other as yet undefined IL-6 responsive factors and elements in their unique promoter contexts determine the activation mechanisms for each IL-6 responsive acute phase gene. Our finding that CRP-APRE(2X) is not sufficient to confer IL-6 responsiveness on the 105-bp TK promoter (which contains a C/EBP site) raises the possibility that an optimal response may require elements other than STAT and C/EBP, although other explanations for these findings clearly exist.

Finally, it is premature to conclude, as some have, that IL-6-induced expression of all acute phase response genes requires STAT3 (49). Thus far, as indicated above, it is known that both STAT and C/EBP family members can mediate IL-6-induced gene transcription and that other unidentified proteins may have such capabilities as well. Theoretically there may be genes whose response to IL-6 is dependent on transcription factors other than STAT3. This possibility is supported by our continuing studies of Hep3B cells stably transfected with STAT3 in which CRP demonstrated an enhanced response to IL-6 + IL-1β (Fig. 3). Preliminary studies in these cells suggest that serum amyloid A does not display such a response, implying that STAT3 does not play a substantial role in the serum amyloid A response to these cytokines.

It should also be noted that the minimal elements required for inducible expression of the CRP gene in hepatoma cells are not sufficient to control expression of the human CRP gene in the presence of other factors.
transgenic mice following LPS treatment (24). The reasons for this discrepancy are unclear. One possibility is that the cytokine milieu induced in vivo by LPS may be more complex than the defined medium and defined cytokines used in our cell culture experiments. In addition, many genes are dependent for expression on the presence of distant regulatory elements (both positive and negative), which may be thousands of base pairs away. It should therefore not be surprising that findings of gene regulation employing relatively short DNA sequences cannot be replicated in vivo, where many more regulatory elements, both positive and negative, come into play.

Acknowledgments—We thank Dr. J. Darnell and Z. Zhong (Rockefeller University) for providing us the valuable reagents used in this study and for discussions of the work.

REFERENCES

1. Kushner, I. (1982) Ann. N. Y. Acad. Sci. 389, 39–48
2. Kushner, I., and Mackiewicz, A. (1993) in Acute Phase Proteins: Molecular Biology, Biochemistry, and Clinical Applications (Mackiewicz, A., Kushner, I., and Baumann, H., eds) pp. 3–19, CRC Press, Inc., Boca Raton, FL
3. Baumann, H., and Gauldie, J. (1994) J. Biol. Chem. 269, 12003–12008
4. Taga, T., and Kishimoto, T. (1992) J. Immunol. Today 15, 74–80
5. Rezzonica, R., Ponzio, G., Loubat, A., Lallemand, D., Proudfoot, A., and Rossi, B. (1995) J. Biol. Chem. 270, 1261–1268
6. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) Science 264, 95–98
7. Akira, S., Nishio, Y., Inoue, M., Wang, X., Wei, S., Matusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994) Cell 77, 63–71
8. Wegenka, U. M., Buschmann, J., Lutticken, C., Heinrich, P. C., and Horn, F. (1993) Mol. Cell. Biol. 13, 276–288
9. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
10. Won, K. A., and Baumann, H. (1993) Mol. Cell. Biol. 10, 3965–3978
11. Oliveira, S., and Cortese, R. (1989) EMBO J. 8, 1145–1151
12. Bao, J. J., Sifers, R. N., Kidd, V. J., Ledley, F. D., and Woo, S. L. C. (1987) Biochemistry 26, 7755–7759
13. Kunz, D., Zinnemonn, M., Heisig, M., and Heinrich, P. C. (1989) Nucleic Acids Res. 17, 1121–1138 (abstr.)
14. Shuai, K., Zievecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z., and Darnell, J. E., Jr. (1993) Nature 366, 580–583
15. Hill, C. S., and Treisman, R. (1995) Cell 80, 299–311
16. Poli, V., and Ciliberto, G. (1994) in Liver Gene Expression (Tronche, F., and Yaniv, M., eds) pp. 131–151, R. G. Landes Company, Austin, TX
17. Sadowski, H. B., Shuai, K., Darnell, J. E., Jr., and Gilman, M. Z. (1993) Science 261, 1739–1744
18. Raz, R., Durbirn, J. E., and Levy, D. E. (1994) J. Biol. Chem. 269, 24391–24395
19. Zhang, D., Jiang, S., Rzewnicki, D., Samols, D., and Kushner, I. (1995) Biochem. J. 310, 145–148
20. Ramji, D. P., Vitelli, A., Tronche, F., Cortese, R., and Ciliberto, G. (1993) Nucleic Acids Res. 21, 289–294
21. Toniatti, C., Demartis, A., Monaci, P., Nicosia, A., and Ciliberto, G. (1990) EMBO J. 9, 4467–4475
22. Majello, B., Arcone, R., Toniatti, C., and Ciliberto, G. (1990) EMBO J. 9, 457–465
23. Li, S. P., Liu, T. Y., and Goldman, N. D. (1990) J. Biol. Chem. 265, 4136–4142
24. Murphy, C., Beckers, J., and Ruther, U. (1995) J. Biol. Chem. 270, 704–708
25. Seidel, H. M., Milocco, L. H., Lamb, P., Darnell, J. E., Jr., Stain, R. B., and Rosen, J. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3041–3045
26. Shuai, K., Stark, G. R., Kerr, I. M., and Darnell, J. E., Jr. (1993) Science 261, 1744–1746
27. Jacoby, D. B., Zilz, N. D., and Towle, H. C. (1989) J. Biol. Chem. 264, 17623–17626
28. Neumann, E., Schaefer-Riddler, M., Wang, Y., and Hofschneider, P. H. (1982) EMBO J. 1, 842–844
29. Seed, B., and Sheen, J. Y. (1988) Gene (Amst.) 72, 271–277
30. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. (1983) J. Mol. Biol. 172, 341–399
31. Zhang, D., Xia, D., and Samols, D. (1995) BioTechniques 18, 750–752
32. Luckow, B., and Schutz, G. (1987) Nucleic Acids Res. 15, 5490
33. Shapiro, D. J., Sharp, P. A., Wahl, W. W., and Keller, M. J. (1988) DNA (N. Y.) 7, 47–55
34. Levy, D. E., Kessler, D. S., Pine, R., and Darnell, J. E., Jr. (1989) Genes & Dev. 3, 1362–1371
35. Caldenhoff, E., Coffield, P., Yuan, J., Van de Stolpe, A., Horn, F., Krujiver, W., and Van der Saag, P. T. (1994) J. Biol. Chem. 269, 21146–21154
36. Yuan, J., Wegenka, U. M., Lutticken, C., Buschmann, J., Decker, T., Schindler, C., Heinrich, P. C., and Horn, F. (1994) Mol. Cell. Biol. 14, 1657–1668
37. Coffield, P., Lutticken, C., van Puijenbroek, A., Kloof-de Jonge, M., Horn, F., and Krujiver, W. (1995) Nucleic Acids Res. 23, 4097–4103
38. Harroch, S., Revel, M., and Chebath, J. (1994) EMBO J. 13, 1942–1949
39. Kel, O. V., Romashchenko, A. G., Kel, A. E., Wingender, E., and Kudchodkar, N. A. (1995) Nucleic Acids Res. 23, 4097–4103
40. Immenschuh, S., Nagao, Y., Satoh, H., Baumann, H., and Muller-Eberhard, U. (1994) J. Biol. Chem. 269, 12654–12661
41. Stein, B., Copogwell, P. C., and Baldwin, A. S. J. (1993) Mol. Cell. Biol. 13, 3964–3974
42. LeClair, K. P., Bianar, M. A., and Sharp, P. A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8145–8149
43. Matsumura, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matushima, K., Kishimoto, T., and Akira, S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10193–10197
44. Nishio, Y., Ishihuki, H., Kishimoto, T., and Akira, S. (1993) Mol. Cell. Biol. 13, 1854–1862
45. Morella, K. K., Lai, C. F., Kumaki, S., Kumaki, N., Wang, Y., Bluman, E. M., Witthuhn, B. A., Ihle, J. N., Giri, J., Gearing, D. P., Cosman, D., Ziegler, S. F., Tweardy, D. J., Campos, S. P., and Baumann, H. (1995) J. Biol. Chem. 270, 8298–8310
46. Lai, C. F., Ripperger, J., Morella, K. K., Wang, Y., Gearing, D. P., Fey, G. H., and Van der Saag, P. T. (1994) J. Biol. Chem. 269, 21146–21154
47. Liu, Z., and Fuller, G. M. (1995) J. Biol. Chem. 270, 7580–7586
48. Zhang, Z., Fuentes, N. L., and Fuller, G. M. (1995) J. Biol. Chem. 270, 24287–24291
49. Ihle, J. N. (1995) Nature 377, 591–594
STAT3 Participates in Transcriptional Activation of the C-reactive Protein Gene by Interleukin-6
Dongxiao Zhang, Ming Sun, David Samols and Irving Kushner

J. Biol. Chem. 1996, 271:9503-9509.
doi: 10.1074/jbc.271.16.9503

Access the most updated version of this article at http://www.jbc.org/content/271/16/9503

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 24 of which can be accessed free at http://www.jbc.org/content/271/16/9503.full.html#ref-list-1