MOLECULAR AND CELLULAR BIOLOGY, Mar. 1993, p. 1854–1862
0270-7306/93/031854-09$02.00/0

Vol. 13, No. 3

A Nuclear Factor for Interleukin-6 Expression (NF-IL6) and the Glucocorticoid Receptor Synergistically Activate Transcription of the Rat α1-Acid Glycoprotein Gene via Direct Protein-Protein Interaction

YUKIHIRO NISHIO,[1] HIROSHI ISSHIKI,[1] TADAMITSU KISHIMOTO,[1,2] AND SHIZUO AKIRA[1]*

Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565,[1] and Department of Medicine III, Osaka University Medical School, Fukushima-ku, Osaka 553,[2] Japan

Received 17 August 1992/Returned for modification 2 October 1992/Accepted 14 December 1992

The acute-phase reaction is accompanied by an increase in a variety of serum proteins, named acute-phase proteins. The synthesis of these proteins is synergistically controlled by glucocorticoids and inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha. Recently, we have cloned nuclear factor–IL-6 (NF-IL6), a transcription factor that activates the IL-6 gene, and have demonstrated its involvement in the expression of acute-phase-protein genes. We report here an analysis of the molecular mechanisms by which inflammatory cytokines and glucocorticoid act synergistically to activate expression of the rat α1-acid glycoprotein (AGP) gene. We found that NF-IL6 and ligand-activated rat glucocorticoid receptor acted synergistically to transactivate the AGP gene and that maximal transcriptional activation of the AGP gene required expression of both intact NF-IL6 and rat glucocorticoid receptor. Surprisingly, however, transcriptional synergism was still observed even when one of the two factors lacked either its DNA-binding or transcriptional-activation function. We present evidence for a direct protein-protein interaction between these two distinct transcription factors and propose that this may be responsible for the synergistic activation of the rat AGP gene.

The acute-phase reaction is one of the essential events for host defense. It is accompanied by an increase in the level of several kinds of serum proteins, such as haptoglobin, hemopexin, C-reactive protein, and fibrinogen (29). These serum proteins increase very rapidly and dramatically in response to many kinds of stimuli that are damaging to the host, including tissue injuries, burns, and infections. Therefore, these are called positive acute-phase proteins (28). Some serum proteins, such as albumin and transthyretin, are downregulated during acute-phase reaction and are thus called negative acute-phase proteins. These regulated changes in protein synthesis are controlled by several cytokines (3, 17). The cytokines primarily involved in the regulation of the acute-phase reaction are interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha, collectively referred to as the inflammatory cytokines (1a). Tissue damage in one area of the host body triggers the local production of inflammatory cytokines, which are then transported to the liver. These cytokines ultimately augment or suppress the synthesis of acute-phase proteins at the transcriptional level. In fact, most acute-phase protein genes harbor a cytokine-responsive element(s) in their 5′-flanking regions (16, 36). So far, two types of elements responsible for mediating the IL-6 signal have been identified. Type I elements are found mainly in the haptoglobin, hemopexin, and α1-acid glycoprotein (AGP) genes (6, 36). The sequences found in these elements resemble the binding consensus sequence of nuclear factor–IL-6 (NF-IL6), a transcription factor for IL-6 expression. NF-IL6 was originally identified as an IL-1-induced transactivator of the IL-6 gene (25). The molecular cloning of NF-IL6 revealed that this activator belongs to the C/EBP family (2). Transcription of NF-IL6 is induced and the protein is posttranslationally modified in response to the IL-6 signal (24, 37). Our previous investigation (24) and the subsequent cloning and analysis of several NF-IL6 homologs (LAP [14], AGP/EBP [12], IL-6DBP [37], C/EBPβ [11], and CRP2 [44]) have revealed that type I elements are a target sequence of NF-IL6. Type II elements, on the other hand, are found mainly in the rat α2-macroglobulin and fibrinogen genes and are bound by a *trans*-acting factor other than NF-IL6 (IL-6 response element-binding protein [RE-BP]) (10). Two of the acute-phase proteins mentioned above, AGP and α2-macroglobulin, are synthesized in response to stimulation by glucocorticoids (4, 21). Interestingly, the synthesis of these proteins is synergistically augmented by inflammatory cytokines and glucocorticoids (4–7, 21, 22, 45), although the molecular mechanism underlying this synergism remains unknown. We investigated this mechanism of synergistic induction with the rat AGP gene as a model system. A glucocorticoid-responsive element (GRE) exists between positions −120 and −107 in the 5′-flanking region of this gene. However, maximal induction by glucocorticoid requires another sequence located immediately downstream of this GRE (27, 38). Using this latter sequence as a probe (positions −121 to −93 of the AGP promoter), Chang et al. isolated AGP/EBP, a mouse homolog of NF-IL6, by direct screening (12). Recently, Williams et al. reported that AGP/EBP is required for maximal AGP gene activation (43). In the present paper, we describe the protein-protein interaction between two distinct transcription factors, glucocorticoid receptor (GR) and NF-IL6. This interaction may explain the synergistic induction of some acute-phase-protein genes by inflammatory cytokines and glucocorticoids.

* Corresponding author.

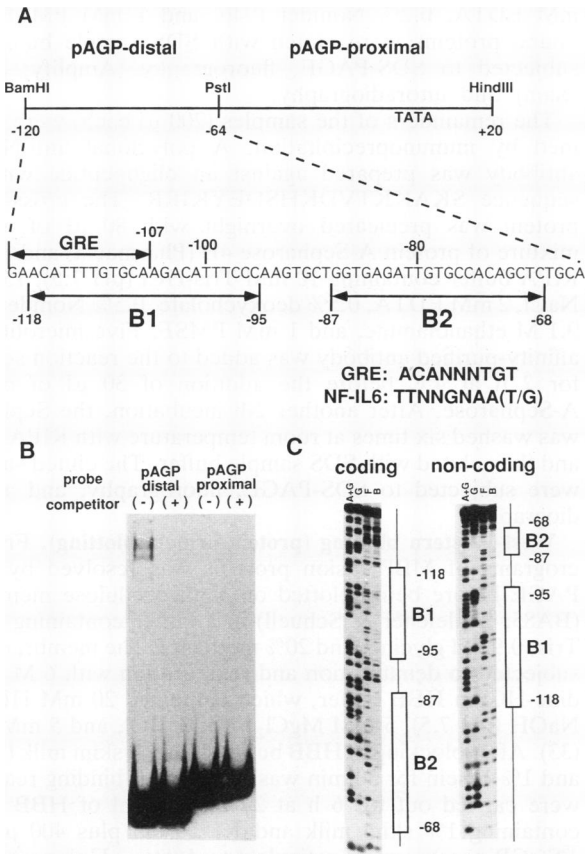

FIG. 1. NF-IL6 binds to the promoter region of the rat AGP gene. (A) Schematic diagram of the rat AGP gene promoter. The double-stranded promoter sequences (pAGP-distal and pAGP-proximal) were prepared by a DNA synthesizer (Millipore). The numbers under the line indicate the distances from the transcription initiation site. The binding sites occupied by either NF-IL6 or rGR are shown by arrows. The putative binding consensus sequences of NF-IL6 and rGR are presented at the bottom. (B) Gel shift competition assay. Bacterially expressed NF-IL6 fusion protein was incubated with the end-labeled pAGP-d or pAGP-p oligonucleotide in the absence or in the presence of a 100-fold excess of the unlabeled fragment. An extract from wild-type *E. coli* did not generate specific complexes. (C) DNase I footprinting of the AGP gene promoter. An end-labeled probe encompassing pAGP-d was partially digested by DNase I in the absence of (lanes F) or in the presence of (lanes B) the bacterial NF-IL6 fusion protein used in the gel shift assay. Nucleotide sequence markers were also electrophoresed (lanes A+G and G). Specific protected windows are indicated to the right (B1 and B2).

MATERIALS AND METHODS

Construction of plasmids. Two chemically synthesized double-stranded oligonucleotides, pAGP-distal (pAGP-d) and pAGP-proximal (pAGP-p), which contain sequences between positions −120 and ~+20 of the rat AGP gene promoter region, were linked in tandem at the *Pst*I site located at position −64 (Fig. 1A). This promoter fragment was subcloned into pUC18 for use in the gel shift assay and into an enhancer-promoterless chloramphenicol acetyltransferase (CAT) vector for CAT assays. In the mutant reporter gene constructs, pAGP-d was replaced with oligonucleotides containing the mutations listed in Fig. 3A. The nucleotide sequence of each fragment was confirmed by dideoxy sequencing (39). The vectors expressing NF-IL6 and its related genes were constructed as previously described (2, 35). 6RGR, GR(Δ525-795), and GR(F463S), expression vectors of the rGR and its mutant, are generous gifts from K. R. Yamamoto (18, 40). GR(Δ77-262) and GR(Δ428-490), expression vectors of human GR mutants, were kindly provided by R. M. Evans (23). The interferon regulatory factor 1 (IRF-1) expression vector was kindly provided by T. Taniguchi.

Gel shift assay and footprinting. A pBluescript vector carrying the NF-IL6 cDNA was expressed in bacteria after induction with 0.3 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. *Escherichia coli* cells were disrupted by freezing and thawing, and ~3 μg of crude extract was incubated with ^{32}P-labeled probe at room temperature for 30 min. Binding reactions were carried out in a buffer consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.9), 50 mM NaCl, 5 mM Tris-HCl (pH 7.0), 1 mM dithiothreitol (DTT), 15 mM EDTA, 10% glycerol, and various amounts of the carrier polymer poly(dI-dC) (Pharmacia). The DNA-protein complex was resolved by electrophoresis on a 5% polyacrylamide gel containing 7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 3.8 mM β-mercaptoethanol, and 1 mM EDTA prior to autoradiography. The same bacterial extract was subjected to a DNase I footprint analysis. A ^{32}P-labeled fragment containing pAGP-d was added to a 50-μl reaction mixture containing 25 mM HEPES-HCl (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg of double-stranded poly(dI-dC) (Pharmacia). Bacterial crude extract (~5 μg) was added last, and the binding reaction was allowed to proceed for 30 min at room temperature. One microliter of DNase I (5 μg/ml), freshly diluted in 8 mM $MgCl_2$, was added to the reaction mixtures. Digestion of DNA-protein complexes was allowed to proceed for 1 min at 25°C. The digestions were stopped by the addition of 100 μl of a solution containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), 10 mM EDTA (pH 8.0), and 25 μg of salmon sperm DNA per ml. The samples were extracted twice with phenol-chloroform and precipitated once with ethanol. The DNA pellets were dried and resuspended in formamide-dye (80% formamide, 1% bromophenol blue, 1% xylene cyanol), and the suspensions were heated at 90°C for 2 min and loaded onto a 10% polyacrylamide-6 M urea sequencing gel. The gel was dried and autoradiographed. Nucleotide sequence markers were prepared by the standard Maxam-Gilbert reaction (39).

DNA transfection and CAT assay. A murine teratocarcinoma cell line, F9, was cultured in 10% fetal calf serum containing alpha-minimal essential medium (GIBCO). A total of 5×10^5 cells per 10-cm diameter collagen-coated dish were plated 24 h before transfection. Ten micrograms of CAT reporter plasmid, 1 μg of GR (or mutant) expression vector, and 5 μg of NF-IL6 (or mutant) were cotransfected by the standard calcium phosphate precipitation method (39). The total amount of plasmid DNA was adjusted to 20 μg with pUC18. Twenty-four hours later, the cells were washed once with Hanks solution and refed with fresh medium containing 1 μM dexamethasone, if needed. The cells were grown for an additional 24 h before being harvested for CAT assays. CAT assays were performed as described by Gorman et al. (19). Conversion ratios of [^{14}C]chloramphenicol were measured by an image analyzer system (BAS2000; Fujix, Tokyo, Japan).

Detection of transiently expressed nuclear proteins. F9 cells (10^7), which had been transfected with the expression vec-

tors 36 h previously, were metabolically labeled for 2 h with [^{35}S]methionine (Dupont, NEN). The cells were lysed in 500 μl of hypotonic buffer consisting of 10 mM HEPES (pH 7.5), 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Nonidet P-40, and 1 mM PMSF. After centrifugation, nuclear proteins were eluted for 30 min at 4°C with 500 μl of buffer containing 15 mM HEPES (pH 7.9), 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT. The supernatants were diluted fivefold with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 1 mM PMSF). Immunoprecipitations were performed either with a polyclonal anti-NF-IL6 antibody directed against an oligopeptide with the sequence RRERNNIAVRKSRDKAK or with a monoclonal anti-rGR antibody provided by J.-Å. Gustafsson. The immune complexes bound to protein A-Sepharose 4B (Pharmacia) were washed six times with the RIPA buffer described above and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Expression and affinity purification of MBP fusion proteins. The maltose-binding protein (MBP) expression and purification system was purchased from New England Biolabs Inc. Truncated cDNA fragments, ΔSplNFIL6 and ΔSplΔSacNFIL6, were inserted at the *Stu*I-*Sal*I polylinker sites of an MBP expression vector, pIH902. Transformed *E. coli* cells were grown in 1 liter of culture and harvested after induction with 0.3 mM IPTG for 2 h at 37°C. *E. coli* cells were lysed in lysis buffer containing 10 mM sodium acetate, 30 mM NaCl, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM PMSF, and 1 mM sodium azide by five successive sonication pulses, each for 1 min at 4°C. After separation from the insoluble fraction by centrifugation, the crude extract was loaded on an amylose resin affinity chromatography column, which was equilibrated in a column buffer containing 10 mM sodium acetate, 500 mM NaCl, 1 mM EGTA, 1 mM PMSF, and 1 mM sodium azide. After the column was washed with column buffer containing 0.25% Tween 20, the MBP fusion protein was eluted with column buffer containing 10 mM maltose. The eluted protein solution was dialyzed overnight in phosphate-buffered saline (PBS) to remove the maltose before use in the succeeding experiments.

Affinity precipitation and immunoprecipitation. A cDNA fragment derived from 6RGR was subcloned into the vector pBluescriptII to synthesize rGR mRNA in vitro. rGR mRNA was synthesized by using the Megascript system (Ambion) according to the supplier's instructions. ^{35}S-labeled rGR was synthesized in vitro by using a rabbit reticulocyte lysate (Promega) under conditions suggested by the manufacturer. Usually, 0.1 to ~0.5 μg of in vitro-transcribed rGR mRNA per synthesis reaction was used. Twenty-five-microliter samples of the lysates containing ^{35}S-rGR were incubated with 2.5 μg of various affinity-purified MBP fusion proteins at 37°C for 30 min in a buffer containing 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 0.6 mM EDTA, and 12% glycerol, prior to addition of the cross-linking agent dithiobis(succinimidyl propionate) (DSP) (Pierce) to a final concentration of 2 mM, in a total volume of 250 μl. The cross-linking reaction was carried out at room temperature for 1 h and stopped by the addition of ethanolamine to a final concentration of 0.1 M.

Fifty microliters of each cross-linked protein sample was incubated overnight at 4°C in a rotary shaker with 30 μl of a (1:1) mixture of amylose resin and MBP column buffer. The resin was then washed five times at room temperature with buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 1 mM PMSF. The bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE, fluorography (Amplify; Amersham), and autoradiography.

The remainders of the samples (200 μl each) were examined by immunoprecipitation. A polyclonal anti-NF-IL6 antibody was prepared against an oligopeptide with the sequence SKAKKTVDKHSDEYKIRR. The cross-linked protein was precleared overnight with 80 μl of a (1:1) mixture of protein A-Sepharose 4B (Pharmacia) and PBS in RIPA buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% deoxycholate, 0.5% Nonidet P-40, 0.1 M ethanolamine, and 1 mM PMSF. Five microliters of affinity-purified antibody was added to the reaction solution for 2 h at 4°C before the addition of 30 μl of protein A-Sepharose. After another 2-h incubation, the Sepharose was washed six times at room temperature with RIPA buffer and then eluted with SDS sample buffer. The eluted samples were subjected to SDS-PAGE, fluorography, and autoradiography.

West-Western blotting (protein-protein blotting). Five micrograms of MBP fusion proteins was resolved by SDS-PAGE before being blotted on a nitrocellulose membrane (BA85; Schleicher & Schuell) in a buffer containing 0.1 M Tris, 0.92 M glycine, and 20% methanol. The membrane was subjected to denaturation and renaturation with 6 M guanidine-HCl in HBB buffer, which contained 20 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM KCl, and 5 mM DTT (33). After blocking in HBB buffer with 5% skim milk (Difco) and 1% casein for 30 min was performed, binding reactions were carried out for 6 h at 20°C in 25 ml of HBB buffer containing 1% skim milk and 1% casein plus 400 μl of a ^{35}S-rGR-programmed reticulocyte lysate. Unincorporated [^{35}S]methionine had been removed prior to use with a Sephadex-G25 column. The membrane was washed five times for 10 min each in the buffer that was used for the affinity precipitation of MBP and then dried and autoradiographed.

RESULTS

NF-IL6 binds to the sequence downstream of the GRE in the 5′-flanking region of the rat AGP gene. A GRE is present in the rat AGP gene at a position between −120 and −107 bp upstream of the transcription initiation site (7). We noticed that just downstream of that sequence there are two NF-IL6-binding consensus sequences. To analyze whether NF-IL6 really binds to these elements, the region of the rat AGP promoter from positions −120 to +20 was prepared by chemical synthesis of two double-stranded oligonucleotides, named pAGP-distal (pAGP-d) and pAGP-proximal (pAGP-p), respectively (Fig. 1A). Each oligonucleotide was end labeled with [^{32}P]dCTP and subjected to a gel shift assay with a recombinant β-galactosidase–NF-IL6 fusion protein. As shown in Fig. 1B, slowly migrating specific complexes were detected only when pAGP-d was used as a probe. We next performed a DNase I footprinting assay to exactly identify the regions bound. This experiment revealed two protected regions, one from −118 to −95 and the other from −87 to −68 (Fig. 1C).

NF-IL6 is a positive transactivator of the rat AGP gene and activates synergistically with the rGR. Next, we examined whether these NF-IL6-binding sequences are transcriptionally functional. For this purpose, a reporter gene plasmid, pAGP(WT)CAT, which contained pAGP-d and pAGP-p just upstream of the CAT gene, was constructed.

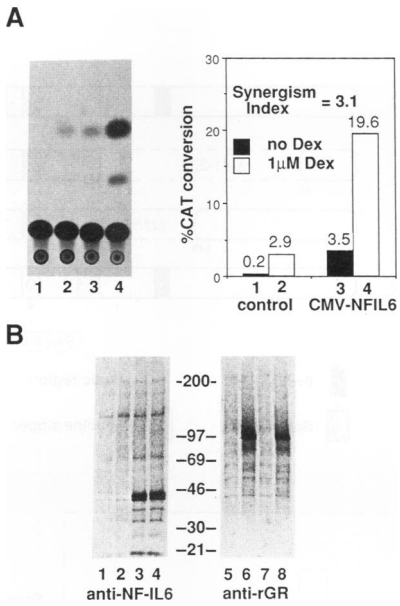

FIG. 2. Synergistic activation of the rat AGP gene by rGR and NF-IL6. (A) Ten micrograms of the reporter plasmid, pAGP(WT)-CAT, was transfected into F9 cells together with 1 μg of 6RGR. Lanes: 1, control; 2, dexamethasone (Dex) stimulation (1 μM) without CMV-NFIL6 cotransfection; 3, CMV-NFIL6 cotransfection (5 μg) without dexamethasone stimulation; 4, dexamethasone stimulation (1 μM) with CMV-NFIL6 cotransfection (5 μg). The synergism index is defined as the ratio of the percent CAT conversion rates obtained after cotransfection with CMV-NFIL6 and 6RGR (lane 4) and the sum of the rates obtained after transfection with each vector separately (lanes 2 and 3). This experiment was repeated four times, and similar results were obtained. The results of one representative experiment are shown. (B) Both NF-IL6 and rGR are equally expressed despite cotransfection. Lanes 1 and 5, control DNA (20 μg); 2 and 6, 6RGR (10 μg); 3 and 7, CMV-NFIL6 (10 μg); 4 and 8, CMV-NFIL6 and 6RGR (10 μg each). The amount of transfected DNA was adjusted to 20 μg with control DNA. The cells were metabolically labeled for 2 h with [^{35}S]methionine 36 h after transfection. The nuclear extracts were subjected to immunoprecipitation with polyclonal peptide antibody against NF-IL6 or anti-rGR monoclonal antibody.

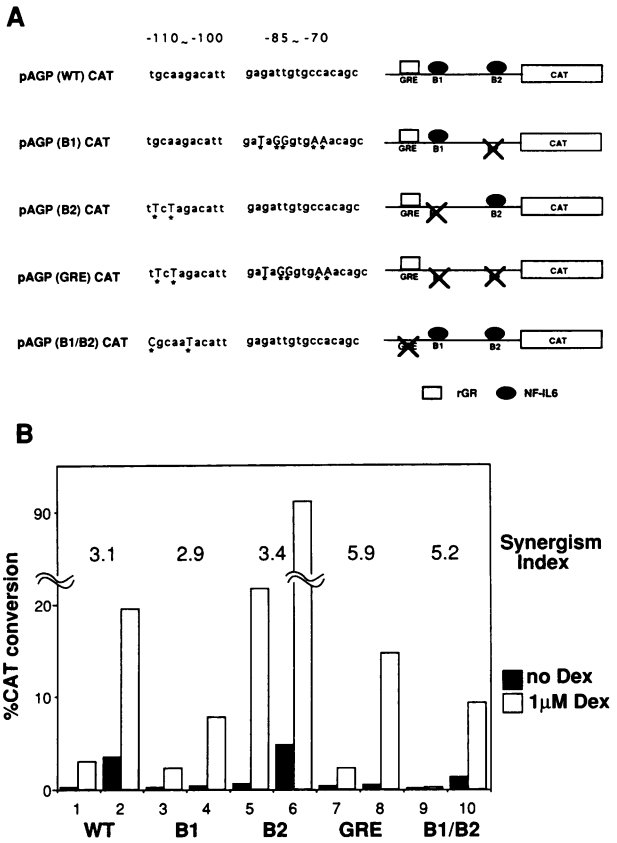

FIG. 3. Functional analyses of the NF-IL6- and rGR-binding sites. (A) Construction of mutant reporter genes. Substituted nucleotides are indicated by capital letters and asterisks. Schematic diagrams of each mutant are shown to the right. (B) Functional differences between each binding site in their transcriptional activities and synergism. F9 cells are transfected with reporter plasmid (10 μg) and GRGR (1 μg). Lanes 1, 3, 5, 7, and 9 show cotransfection of control plasmid (5 μg). Lanes 2, 4, 6, 8, and 10 show cotransfection of CMV-NFIL6 (5 μg). Other conditions are the same as those described in the legend to Fig. 2. This experiment was repeated three times, and similar results were obtained. The results of one representative experiment are shown. Dex, dexamethasone; WT, wild type.

Murine teratocarcinoma F9 cells, which do not express NF-IL6, were cotransfected with pAGP(WT)CAT, a reporter gene, and 6RGR, an rGR expression vector. pAGP-(WT)CAT was activated when cells were both cotransfected with 6RGR and stimulated with 1 μM dexamethasone for 24 h (Fig. 2A, lane 2) but was not activated by either 6RGR cotransfection (lane 1) or dexamethasone stimulation alone (data not shown). Cotransfection of a cytomegalovirus promoter-driven NF-IL6 expression vector (CMV-NFIL6) transactivated the rat AGP gene to an extent that was almost similar to that in the case of the ligand-activated rGR (Fig. 2A, lane 3). Therefore, both NF-IL6 and ligand-activated rGR were shown to be positive transactivators for the rat AGP gene. Interestingly, when these two factors were expressed simultaneously, pAGP(WT)CAT was transactivated more than three times as much as the sum of the activation by each factor (Fig. 2A, lane 4 and synergism index). In addition, it was demonstrated by immunoprecipitation that the amount of NF-IL6 or rGR did not change as a result of cotransfection (Fig. 2B). Hence, we conclude that NF-IL6 and rGR transcriptionally synergize with each other.

Mutagenesis in the AGP promoter reveals functional differences between two NF-IL6-binding sites. To obtain more detailed information on the function of each NF-IL6-binding site, four mutant reporter plasmids, in which one or more rGR-binding and NF-IL6-binding sites were disrupted by multiple nucleotide sequence substitutions, were constructed (Fig. 3A). Assays of the activities of these promoters are indicated in Fig. 3B. Disruption of the distal NF-IL6-binding site (B1) enhanced the CAT activity of the wild-type promoter more than threefold (Fig. 3B, lanes 5 and 6). Therefore, this site was antagonistic for AGP gene transactivation. On the contrary, disruption of the proximal NF-IL6-binding site (B2) diminished the CAT activity of the same reporter gene to less than 50% (Fig. 3B, lanes 3 and 4), indicating that B2 was agonistic for AGP gene transcription. Thus, we conclude that B2 is necessary for maximal rat AGP gene transactivation by glucocorticoid. When B1 and B2 were disrupted at the same time, the total CAT activity was also diminished to approximately the same extent as in the

case of the B2 disruption. Interestingly, however, the synergism between NF-IL6 and rGR was still observed (Fig. 3B, lanes 7 and 8). This phenomenon was also observed when the GR-binding site was disrupted (Fig. 3B, lanes 9 and 10), but neither activation nor synergism was observed when all binding sites for NF-IL6 and rGR were disrupted (data not shown). These results suggest that the transcriptional synergism between NF-IL6 and rGR can still occur even when only one of the two factors is bound to the target sequence.

DNA-binding-deficient or transcriptionally inactive NF-IL6 mutants are still capable of augmenting activation by rGR. Next, we examined whether the DNA-binding or transcriptional activity of NF-IL6 is dispensable for synergism by using truncated and site-directed mutants of NF-IL6 (Fig. 4A). ΔSplNFIL6 and ΔStyNFIL6 are truncated mutants of NF-IL6 which can bind to DNA but are transcriptionally inactive by themselves. NFIL6(S288A) is a site-directed mutant of NF-IL6 in which the serine residue at position 288 in the center of the basic region is replaced by alanine. This mutant is unable to bind to the NF-IL6 recognition sequences of pAGP-d (data not shown). Although each of these mutants was less transcriptionally active than the wild-type NF-IL6, all were capable of activating transcription synergistically with rGR (Fig. 4B). This result implies that the transcriptional and DNA-binding activities of NF-IL6 are dispensable for the synergism between NF-IL6 and rGR. As a control, IRF-1 and Fos, which do not bind to the AGP promoter, were examined for their abilities to synergize with rGR; these results were negative (data not shown). This demonstrates that the ability of NF-IL6 mutants that lack DNA-binding function to synergize with rGR is specific. To further ascertain that direct binding of both factors to DNA is not essential for synergistic activation, a reporter plasmid, pAGP(GRE)CAT, in which two NF-IL6-binding sites were disrupted, was used instead of pAGP(WT)CAT (Fig. 4C). In this experiment, NFIL6(WT) and NFIL6 (S288A) synergized equally well with rGR, although they were transcriptionally inactive by themselves. From the results described above, we conclude that the synergism between NF-IL6 and rGR does not require transcriptional activity or direct DNA binding.

The DNA-binding domain of GR is required for synergistic activation with NF-IL6. To characterize the domains of GR involved in synergism, we carried out a cotransfection experiment with three kinds of GR truncated mutants: GR(Δ77-262) (transacting-region-deleted mutant), GR(Δ428-490) (DNA-binding-region-deleted mutant), and GR(Δ525-795) (ligand-binding-region-deleted mutant) (Fig. 5A). These mutants all showed a marked attenuation in their abilities to transactivate pAGP(WT)CAT when expressed alone as well as when expressed together with NF-IL6 (Fig. 5B). Interestingly, however, GR(Δ77-262) still retained its capacity to activate synergistically with NF-IL6. In contrast, GR(Δ428-490) and GR(Δ525-795) displayed no such synergism. These data suggest that the DNA-binding domain of GR is required for synergism with NF-IL6. In fact, GR(F463S), which contains a site-directed mutation in the first zinc finger region of GR, also failed to activate synergistically with NF-IL6, as had been observed with GR(Δ428-490) (Fig. 5B).

NF-IL6 physically interacts with rGR in vitro through its basic leucine zipper (bZip) structure. The results described above prompted us to examine the possibility that NF-IL6 and rGR physically interact with one another. As depicted in Fig. 4B, synergism between rGR and NF-IL6 does not require intact NF-IL6. Therefore, two truncated mutants of NF-IL6 were used for the following experiments.

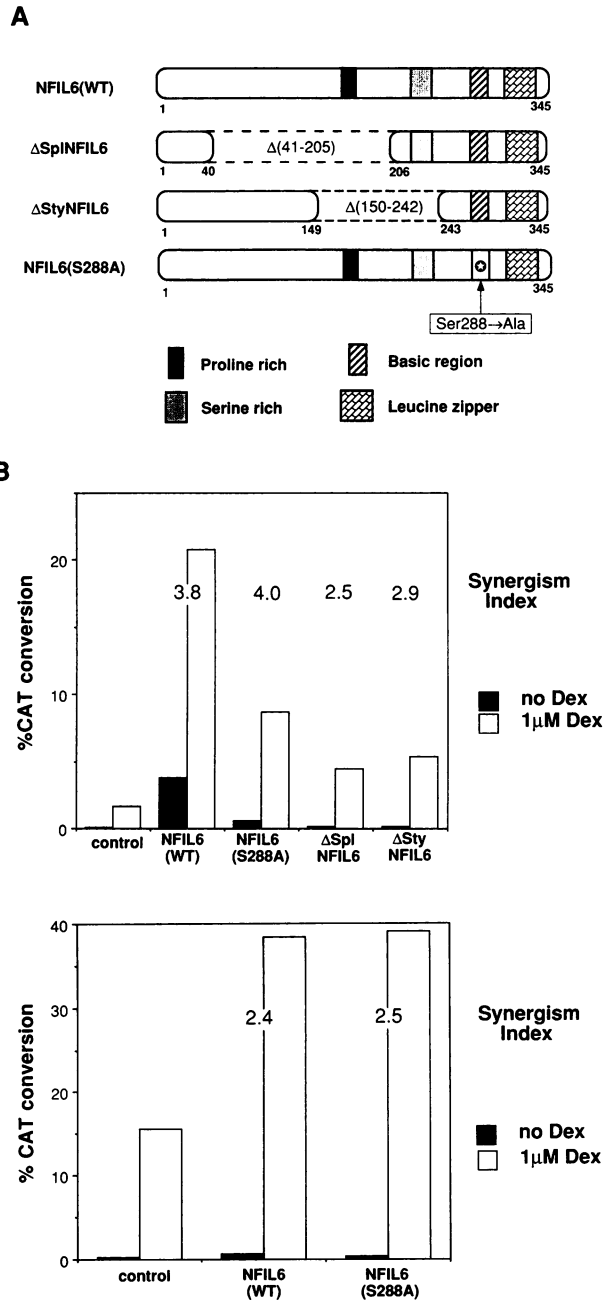

FIG. 4. DNA-binding-deficient or transcriptionally inactive NF-IL6 still confers synergism. (A) Structures of wild-type (WT) NF-IL6 and its mutants. ΔSplNFIL6 and ΔStyNFIL6 are truncated mutants which delete the *SplI* and *StyI* fragments within the coding sequence, respectively. NFIL6(S288A) is constructed by site-directed mutagenesis which replaces serine 288 of NF-IL6 with alanine, leading to a total loss of DNA binding. The numbers shown under the molecules indicate the positions of amino acids from the NH_2 terminus. (B) Effects of NF-IL6 mutants with wild-type GR. The assay conditions are the same as those described in the legend to Fig. 2. (C) Direct DNA binding of NF-IL6 is dispensable for the synergism. pAGP(GRE)CAT, in which two NF-IL6-binding sites are mutated, was used as a reporter plasmid instead of pAGP-(WT)CAT. The experiment was repeated three times, and similar results were obtained. The results of one representative experiment are shown.

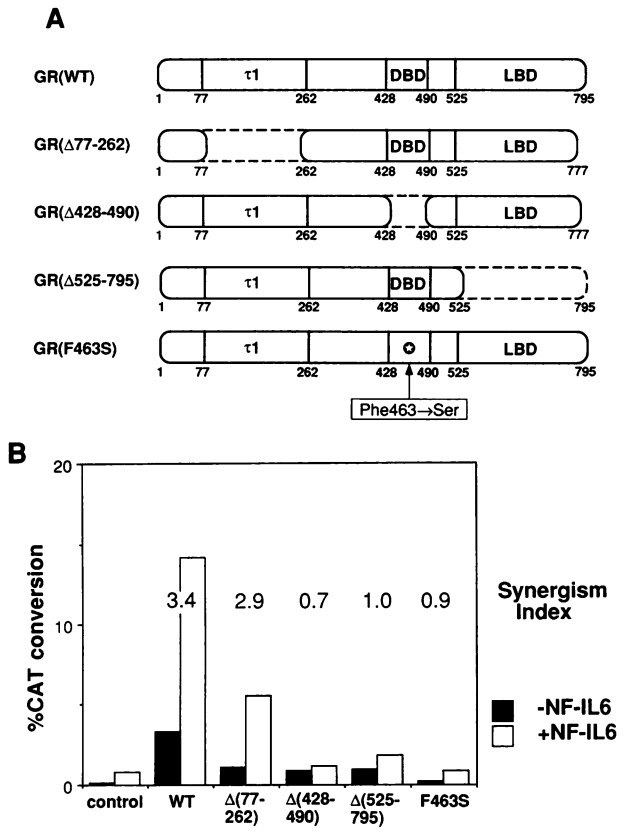

FIG. 5. (A) Structures of wild-type (WT) GR and its mutants. GR(WT), GR(Δ525-795) (ligand-binding-region-deleted mutant), and GR(F463S) (site-directed mutant in the zinc finger region) are rGR derivatives, while GR(Δ77-262) (transacting-region-deleted mutant) and GR(Δ428-490) (DNA-binding-region-deleted mutant) are derivatives of human GR. τ1, transactivating domain; DBD, DNA-binding domain; LBD, ligand-binding domain. (B) The DNA-binding domain of GR is required for synergism. pAGP(WT)CAT was used as a reporter plasmid. Each assay was done in the presence of 1 μM dexamethasone. Other conditions are the same as those described in the legend to Fig. 2. The experiment was repeated three times, and similar results were obtained. The results of one representative experiment are shown.

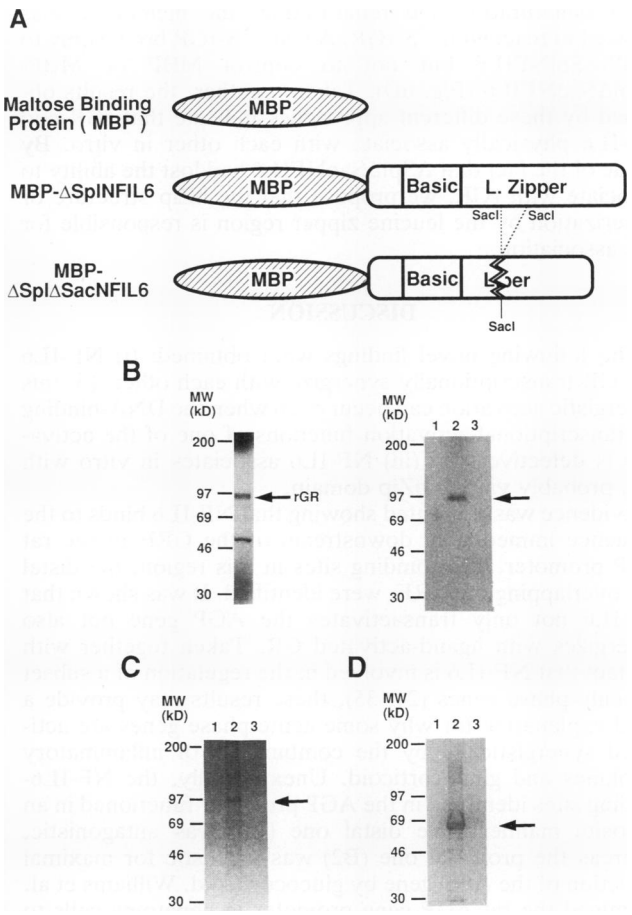

FIG. 6. Direct protein-protein interaction between rGR and NF-IL6. (A) Structure of bacterially expressed fusion proteins. MBP, which is a ligand of amylose resin, is fused to the NF-IL6 mutants ΔSplNFIL6 and ΔSplΔSacNFIL6. ΔSplΔSacNFIL6 is a derivative of ΔSplNFIL6 that is truncated from threonine 307 to leucine (L) 327 and that consequently has lost its dimerizing function. (B [left]) ^{35}S-labeled rGR programmed in rabbit reticulocyte lysate. The rGR product is indicated by an arrow. The band at 47 kDa represents an artifact protein which is labeled by [^{35}S]methionine as a result of the addition of a methionine residue to its N terminus by an enzyme that uses methionyl tRNA. (B [right]) Affinity coprecipitation of ^{35}S-labeled rGR with the bZip region of NF-IL6. Programmed reticulocyte lysate was cross-linked with various MBP fusion proteins by DSP, precipitated with amylose resin, washed, eluted, and analyzed by SDS-PAGE. Lanes: 1, MBP; 2, MBP-ΔSplNFIL6; 3, MBP-ΔSplΔSacNFIL6. (C) Coimmunoprecipitation of ^{35}S-labeled rGR with the bZip region of NF-IL6. The cross-linked proteins that were used in the experiment depicted in panel B were mixed with polyclonal peptide antibody against NF-IL6, precipitated with protein A-Sepharose, washed, eluted, and analyzed by SDS-PAGE. (D) West-Western blotting. The MBP fusion proteins that were used in the experiment depicted in panel B were blotted to a nitrocellulose membrane after SDS-PAGE. The membrane was treated under denaturation and renaturation conditions by reducing the concentration of guanidine-HCl and was hybridized with ^{35}S-rGR, washed, and autoradiographed. MW, molecular weight.

One mutant, ΔSplNFIL6, is defective in its transcriptional-activation function and was described earlier (Fig. 4A). A second mutant, ΔSplΔSacNFIL6, is a derivative of ΔSplNFIL6 in which the NH_2-terminal half of the leucine zipper region has been deleted and is defective in its dimerization function. These two mutant constructs were fused to the MBP for use in affinity purification (Fig. 6A) (20, 34). ^{35}S-labeled rGR was synthesized in a rabbit reticulocyte lysate. After the chemical cross-linking of ^{35}S-rGR and these MBP fusion proteins with the reversible cross-linker DSP, we performed two types of coprecipitation experiments.

First, we performed an affinity precipitation experiment by immobilizing cross-linked proteins to amylose resin. The bound complexes were eluted after being washed and were analyzed by SDS-PAGE (Fig. 6B [right]). We found that MBP-ΔSplNFIL6 coprecipitated ^{35}S]rGR but that control MBP and MBP-ΔSplΔSacNFIL6 did not. Next, we performed immunoprecipitation with an anti-NF-IL6 antibody and the same protein samples. The only protein able to coprecipitate ^{35}S-rGR was MBP-ΔSplNFIL6 (Fig. 6C).

In addition, this interaction was monitored by the West-Western (protein-protein) blotting method. Three forms of MBP fusion proteins (MBP, MBP-ΔSplNFIL6, and MBP-ΔSplΔSacNFIL6) were separated on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane.

After denaturation and renaturation, the membrane was allowed to react with ^{35}S-rGR. Again, ^{35}S-rGR bound only to MBP-ΔSplNFIL6 but not to control MBP or MBP-ΔSplΔSacNFIL6 (Fig. 6D). Taken together, the results obtained by these different approaches indicate that rGR and NF-IL6 physically associate with each other in vitro. By virtue of the fact that ΔSplΔSacNFIL6 had lost the ability to associate with rGR, we propose that the bZip structure or dimerization by the leucine zipper region is responsible for this association.

DISCUSSION

The following novel findings were obtained: (i) NF-IL6 and GR transcriptionally synergize with each other; (ii) this synergistic activation can occur even when the DNA-binding or transcriptional-activation functions of one of the activators is defective; and (iii) NF-IL6 associates in vitro with GR, probably via the bZip domain.

Evidence was presented showing that NF-IL6 binds to the sequence immediately downstream of the GRE in the rat AGP promoter. Two binding sites in this region, the distal one overlapping the GRE, were identified. It was shown that NF-IL6 not only transactivates the AGP gene but also synergizes with ligand-activated GR. Taken together with the fact that NF-IL6 is involved in the regulation of a subset of acute-phase genes (24, 35), these results may provide a good explanation for why some acute-phase genes are activated synergistically by the combination of inflammatory cytokines and glucocorticoid. Unexpectedly, the NF-IL6-binding sites identified in the AGP promoter functioned in an opposite manner. The distal one (B1) was antagonistic, whereas the proximal one (B2) was agonistic for maximal induction of the AGP gene by glucocorticoid. Williams et al. examined the rat AGP gene promoter in hepatoma cells to identify the sequences responsible for maximal induction by glucocorticoid (43). Their results also indicated the antagonism of B1 as well as agonism of B2. The reason why B1 shows antagonism was not determined. However, considering the fact that B1 completely overlaps the GRE, competition for the same target sequence by NF-IL6 and rGR may be involved in this antagonism (32), although the biological significance of this competition remains unclear.

Through a series of experiments with NF-IL6 or GR mutants to activate the AGP wild-type or mutated reporter genes, we concluded that the synergism between NF-IL6 and GR persists even when one of the two factors loses its DNA-binding ability. This result can be interpreted in two ways: NF-IL6 and rGR are mutually transactivated either by direct protein-protein interaction or by an indirect effect mediated by some unidentified cofactor(s). Recently, Hocke and coworkers examined the synergistic activation of the rat α2-macroglobulin gene by IL-6 and glucocorticoids (10, 21, 22). They identified a nuclear protein (IL-6RE-BPs) which is involved in this synergism but which is distinct from NF-IL6. Interestingly, an 18-bp element which contains the IL-6 response element on the α2-macroglobulin promoter is capable of conferring synergism between IL-6 and glucocorticoid, in spite of the absence of an obvious consensus GRE. This result resembles our finding that NF-IL6 can activate synergistically without direct DNA binding to the rat AGP gene. Hocke et al. also speculated on the possible direct interaction between these two transcription factors, although no direct evidence was presented.

Prior to coprecipitation experiments, we attempted to detect an interaction between NF-IL6 and rGR by a gel shift assay, without success (data not shown). Hocke et al. also reported that attempts to detect a complex between IL-6RE-BPs and the DNA-binding domain of rGR by this same experimental method failed (22). However, these results do not rule out a possible mutual interaction, since this complex could be too unstable to be detected under standard assay conditions.

In spite of these initial negative results, we were convinced that NF-IL6 and GR could interact directly, since several truncated, transcriptionally inactive mutants of either factor were still able to synergize with a normal version of the counterpart factor. In fact, three different methods subsequently demonstrated a specific protein-protein interaction via the bZip portion of NF-IL6 in vitro. On the basis of the fact that a DNA-binding-domain-deleted mutant of GR could not synergize with NF-IL6, we infer that the interacting portion of GR resides near its zinc finger region. In the case of the bZip family transcription factors, this leucine zipper region has been implicated in mediating factor dimerization (30); however, our results suggest that this region may also mediate interactions with other factors. Recently, Wegner et al. reported that C/EBPβ, a mouse homolog of NF-IL6, is phosphorylated in its leucine zipper region in a calcium-dependent manner (42). Although the functional significance of this modification has not been determined, it is conceivable that phosphorylation regulates the interactions of NF-IL6 with other transcription factors.

Recently, several groups have reported that interactions between dissimilar transcription factors at the protein level are involved in the modulation of some gene expression. For example, Diamond et al. have demonstrated that the GR acts agonistically when it associates with the Jun-Jun homodimer but antagonistically with the Jun-Fos heterodimer (15). Other groups have reported that the direct interaction between GR and various AP-1 complexes can mutually inhibit their abilities to bind to DNA, thus interfering with transcription activation (26, 41, 46). We might expect the domain which mediates the interaction between AP-1 and GR to be located within the bZip domain of Jun and within the DNA-binding domain of GR, consistent with what we observe in the case of NF-IL6 and GR and with results reported in the case of interactions between Jun and the retinoic acid receptor or MyoD (8, 47). As far as we know, our observation of an interaction between NF-IL6 and GR is the first such case reported for a member of the C/EBP family.

Courey et al. demonstrated that even a fingerless (DNA-binding-deficient) Sp1 transcription factor could synergistically augment the basal activity of wild-type Sp1 (13). These authors propose that the fingerless Sp1 may mediate synergistic activation by contributing to the stability of a transcriptionally active complex formed between wild-type Sp1 and an adaptor. Similarly, we might imagine that NF-IL6 and GR also combine with an adaptor to form a complex that stimulates transcription of the AGP gene. This complex would presumably be more stable when both NF-IL6 and GR are present than when either is present alone and thus would lead to the synergistic activation of AGP.

LeClair et al. recently reported that the NF-κB p50 subunit physically interacts with the bZip domain of NF-IL6 (31). An NF-κB-binding site is frequently present in the promoter sequences of several acute-phase genes, such as IL-6, IL-8, and angiotensinogen (9, 25). In fact, analyses of the gene expression of IL-6 and IL-8 have demonstrated that NF-IL6 and NF-κB transcriptionally synergize with each other (1). Taken together, these observations may help to

explain the coordinated expression of genes involved in inflammatory reaction in terms of direct protein-protein interactions between NF-IL6 and other transcription factors of distinct families. Such interactions could serve to integrate stimuli transmitted by several independent signalling pathways into a coordinated, effective response.

ACKNOWLEDGMENTS

We thank M. Lamphier for reviewing the manuscript and K. Kubota and K. Ono for secretarial assistance. We also thank K. R. Yamamoto and R. M. Evans for GR expression vectors, T. Taniguchi for the IRF-1 expression vector, N. Miura for the c-Fos expression vector, and J.-Å. Gustafsson for anti-rGR monoclonal antibody. We thank H. Hirai for his technical advice on nuclear-protein extraction and immunoprecipitation.

This work was supported by grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. **Akira, S., K. Fujikawa, N. Mukaida, K. Matsushima, and T. Kishimoto.** Unpublished data.
1a. **Akira, S., T. Hirano, T. Taga, and T. Kishimoto.** 1990. Biology of multifunctional cytokines: IL6 and related molecules (IL1 and TNF). FASEB J. **4**:2860–2867.
2. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
3. **Andus, T., T. Geiger, T. Hirano, T. Kishimoto, and P. C. Heinrich.** 1988. Action of recombinant human interleukin 6, interleukin 1β and tumor necrosis factor α on the mRNA induction of acute-phase proteins. Eur. J. Immunol. **18**:739–746.
4. **Baumann, H., G. L. Firestone, T. L. Burgess, K. W. Gross, K. R. Yamamoto, and W. A. Held.** 1983. Dexamethasone regulation of α1-acid glycoprotein and other acute phase reactants in rat liver and hepatoma cells. J. Biol. Chem. **258**:563–570.
5. **Baumann, H., and J. Gauldie.** 1990. Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. Mol. Biol. Med. **7**:147–159.
6. **Baumann, H., G. P. Jahreis, and K. K. Morella.** 1990. Interaction of cytokine- and glucocorticoid-response elements of acute-phase plasma protein genes. J. Biol. Chem. **265**:22275–22281.
7. **Baumann, H., and L. E. Maquat.** 1986. Localization of DNA sequences involved in dexamethasone-dependent expression of the rat α1-acid glycoprotein gene. Mol. Cell. Biol. **6**:2551–2561.
8. **Bengal, E., L. Ransone, R. Scharfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma.** 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. Cell **68**:507–519.
9. **Brasier, A. R., D. Ron, J. E. Tate, and J. F. Habener.** 1990. A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1α induced, NF-κB mediated *trans*-activation of the angiotensinogen gene acute-phase response element. EMBO J. **9**:3933–3944.
10. **Brechner, T., G. Hocke, A. Goel, and G. H. Fey.** 1991. Interleukin 6 response factor of the rat α2-macroglobulin gene. Mol. Biol. Med. **8**:267–285.
11. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5**:1538–1552.
12. **Chang, C.-J., T.-T. Chen, H.-Y. Lei, D.-S. Chen, and S.-C. Lee.** 1990. Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. Mol. Cell. Biol. **10**:6642–6653.
13. **Courey, A. J., D. A. Holzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59**:827–836.
14. **Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler.** 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcription activator protein. Genes Dev. **4**:1541–1551.
15. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive and negative regulation from a single DNA element. Science **249**:1266–1272.
16. **Ganter, U., R. Arcone, C. Toniatti, G. Morrone, and G. Cili berto.** 1989. Dual control of C-reactive protein gene expression by interleukin-1 and interleukin-6. EMBO J. **8**:3773–3779.
17. **Geiger, T., T. Andus, J. Klapproth, T. Hirano, T. Kishimoto, and P. C. Heinrich.** 1988. Induction of rat acute-phase proteins by interleukin 6 *in vivo*. Eur. J. Immunol. **18**:717–721.
18. **Godowski, P. J., S. Rusconi, R. Miesfeld, and K. R. Yamamoto.** 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature (London) **325**:365–368.
19. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
20. **Guan, C. D., P. Li, P. D. Riggs, and H. Inouye.** 1988. Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene **67**:21–30.
21. **Hattori, M., L. J. Abraham, W. Northemann, and G. H. Fey.** 1990. Acute-phase reaction induces a specific complex between hepatic nuclear proteins and the interleukin 6 response element of the rat α2-macroglobulin gene. Proc. Natl. Acad. Sci. USA **87**:2364–2368.
22. **Hocke, G. M., D. Barry, and G. H. Fey.** 1992. Synergistic action of interleukin-6 and glucocorticoids is mediated by the interleukin-6 response element of the rat α2 macroglobulin gene. Mol. Cell. Biol. **12**:2282–2294.
23. **Hollenberg, S. M., V. Giguere, P. Segui, and R. M. Evans.** 1987. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell **49**:39–46.
24. **Isshiki, H., S. Akira, T. Sugita, Y. Nishio, S. Hashimoto, T. Pawlowski, S. Suematsu, and T. Kishimoto.** 1991. Reciprocal expression of NF-IL6 and C/EBP in hepatocytes: possible involvement of NF-IL6 in acute phase protein gene expression. New Biol. **3**:63–70.
25. **Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto.** 1990. Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene. Mol. Cell. Biol. **10**:2757–2764.
26. **Jonat, C., H. J. Rahmsdorf, K.-K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62**:1189–1204.
27. **Klein, E. S., D. DiLorenzo, G. Posseckert, M. Beato, and G. M. Ringold.** 1988. Sequences downstream of the glucocorticoid regulatory element mediate cycloheximide inhibition of steroid induced expression from the rat α1-acid glycoprotein promoter: evidence for a labile transcription factor. Mol. Endocrinol. **2**:1343–1351.
28. **Koj, A.** 1985. Liver response to inflammation and synthesis of acute-phase plasma proteins, p. 139–232. *In* A. H. Gordon et al. (ed.), Acute-phase response to injury and infection: the roles of interleukin one and other mediators, vol. 10. Elsevier, New York.
29. **Kushner, I.** 1982. The phenomenon of the acute phase response. Ann. N.Y. Acad. Sci. **389**:39–48.
30. **Landschultz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**:1681–1688.
31. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89**:8145–8149.
32. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59**:405–408.
33. **Macgregor, P. F., C. Abate, and T. Curran.** 1990. Direct cloning of leucine zipper proteins: Jun binds cooperatively to the CRE with CRE-BP1. Oncogene **5**:451–458.
34. **Maina, C. V., P. D. Riggs, A. G. Grandea III, B. E. Slatko, L. S.**

Moran, J. A. Tagliamonte, L. A. McReynolds, and C. D. Guan. 1988. An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. Gene **74:**365–373.

35. **Natsuka, S., H. Isshiki, S. Akira, and T. Kishimoto.** 1991. Augmentation of haptoglobin production in Hep3B cell line by a nuclear factor NF-IL6. FEBS Lett. **291:**58–62.
36. **Poli, V., and R. Cortese.** 1989. Interleukin 6 induces a liver-specific nuclear protein that binds to the promoter of acute-phase genes. Proc. Natl. Acad. Sci. USA **86:**8202–8206.
37. **Poli, V., F. P. Mancini, and R. Cortese.** 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell **63:**643–653.
38. **Ratajczak, T., P. M. Williams, D. DiLorenzo, and G. M. Ringold.** 1992. Multiple elements within the glucocorticoid regulatory unit of the rat α1-acid glycoprotein gene are recognition sites for C/EBP. J. Biol. Chem. **267:**11111–11119.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Schena, M., L. P. Freedman, and K. R. Yamamoto.** 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev. **3:**1590–1601.
41. **Schüle, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans.** 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62:**1217–1226.
42. **Wegner, M., Z. Cao, and M. G. Rosenfeld.** 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. Science **256:**370–373.
43. **Williams, P. M., T. Ratajczak, S. C. Lee, and G. M. Ringold.** 1991. AGP/EBP(LAP) expressed in rat hepatoma cells interacts with multiple promoter sites and is necessary for maximal glucocorticoid induction of the rat alpha-1 acid glycoprotein gene. Mol. Cell. Biol. **11:**4959–4965.
44. **Williams, S. C., C. A. Cantwell, and P. F. Johnson.** 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev. **5:**1553–1567.
45. **Won, K.-A., and H. Baumann.** 1990. The cytokine response element of the rat α1-acid glycoprotein gene is a complex of several interacting regulatory sequences. Mol. Cell. Biol. **10:** 3965–3978.
46. **Yang-Yen, H.-F., J.-C. Chambard, Y.-L. Sun, T. Smeal, T. J. Schmidt, J. Drouin, and M. Karin.** 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell **62:**1205–1215.
47. **Yang-Yen, H.-F., X.-K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl.** 1991. Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation. New Biol. **3:**1206–1219.