Functional Analysis of Interleukin 6 Response Elements (IL-6REs) on the Human γ-Fibrinogen Promoter

BINDING OF HEPATIC Stat3 CORRELATES NEGATIVELY WITH TRANSACTIVATION POTENTIAL OF TYPE II IL-6REs*  

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Several families of transcription factors play important roles in modulating liver-specific gene expression during an acute phase response (APR). Stat3/APR factor is the main transactivator of gene expression by the interleukin (IL)-6 family of cytokines signaling through gp130. During an APR, fibrinogen (FBG) genes are coordinately up-regulated by IL-6 and glucocorticoids. Except for rat γFBG, attempts to demonstrate direct binding of IL-6-activated Stat3 to FBG CTGGGAA promoter elements have not been successful. Herein we show the presence of three functional type II IL-6 response elements (IL-6REs) on the human γFBG promoter and that the magnitude of Stat3 binding to these elements correlates negatively with their functional activity in reporter gene assays. Stat3-specific binding to γFBG IL-6REs was confirmed by cross-competition with α2-macroglobulin IL-6RE and specific interactions with anti-Stat3 in electrophoretic mobility shift assays. All type II IL-6REs contributed to full promoter activity; however, transactivation from Site II at −306 to −301 was strongest. In contrast to a previous report, IL-6 failed to induce activation of serum amyloid A-activating factor-1/c-Myc-associated zinc finger protein (SAF-1/MAZ), and mutation of the SAF-1RE had little effect on IL-6 induction of γFBG promoter activity. In the absence of a functional glucocorticoid receptor response element, dexamethasone potentiated IL-6-induced γFBG promoter activity 2-fold, requiring promoter-proximal Site I and Site II; the promoter-distal Site III had no effect on dexamethasone potentiation of IL-6-induced promoter activity. Notably the propensity for Stat3 binding to human γFBG IL-6REs was low compared with Stat3 binding to the α2-macroglobulin IL-6RE. Together these data suggest that Stat3 transactivation via IL-6REs on FBG promoters likely involves participation of additional transcription factors and/or coactivators to achieve optimal coordinated up-regulation during an APR.

Fibrinogen (FBG) and fibrin are the targets of two complex and opposing biochemical pathways, the coagulation and fibrinolytic cascades, respectively, that together preserve vascular integrity and maintain the hemostatic balance (1, 2). In addition, the fibrin clot provides a critical provisional matrix at sites of injury, inflammation, or infection in which cells can proliferate, organize, and carry out specialized functions (3–5). FBG is a dimeric molecule with each half composed of three polypeptide chains, Αα, Ββ, and γ. Three separate single copy genes encode the FBG polypeptide chains, and each gene is separately transcribed and translated. The FBG chains are rapidly assembled in a sequential series of steps by the independent attachment of the preformed Αα and γ chains, drawn from intracellular pools, to the nascent Ββ chain (6). These chains, and each half-molecule, are linked by a network of disulfide bonds to form the circulating 340-kDa protein. Expression of the FBG genes is transcriptionally regulated, and in response to inflammation, hepatic transcription of the three genes is coordinately up-regulated (7).

The acute phase response is the mechanism by which the host responds to disruption of homeostasis by infection, tissue injury, or neoplasia. The major regulatory cytokines of the acute phase response are tumor necrosis factor-α, interleukin (IL)-1, and IL-6, which act on the liver to change the expression of a number of plasma proteins collectively known as the acute phase proteins (APPs). Change in APP gene expression is either positive or negative depending on the relative -fold up- or down-regulation of each gene, respectively. The positive APPs are further divided into three groups depending on the magnitude of their induction. Group 1 APPs such as C3 and C4 are increased by 50%, Group 2 APPs such as FBG and haptoglobin are increased by 2–10-fold, and Group 3 APPs including C-reactive protein and serum amyloid A (SAA) are increased by 100–1000-fold in plasma. In addition, APP genes are classified into two categories based on their cytokine responsiveness. Type I APP genes are up-regulated by IL-1, IL-6, tumor necrosis factor-α, and glucocorticoids (GCs) or various combinations thereof; whereas type II genes respond to IL-6 and GCs (8). In contrast, neither tumor necrosis factor-α nor IL-1β induce expression of type II APP genes but instead frequently down-regulate their expression even in the presence of high levels of IL-6 (9, 10). The FBG genes are classified as type II in that GCs C/EBP, CCAAT/enhancer-binding protein; NF-κB, nuclear factor-κB; Stat, signal transducer and activator of transcription; RE, response element; SAF-1, SAA-activating factor-1; MAZ, c-Myc-associated zinc finger protein; EMSA, electrophoretic mobility shift assay; αM, α2, macroglobulin; CAT, chloramphenicol acetyltransferase; GR, glucocorticoid receptor; GRE, glucocorticoid receptor response element; CBP, CAMP-response element-binding protein (CREB)-binding protein; DEX, dexamethasone; wt, wild type.

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act synergistically with IL-6 in up-regulation of FBG gene expression (11, 12).

Several families of transcription factors play important roles in modulating liver-specific APP gene expression including the CCAAT/enhancer-binding protein (C/EBP), nuclear factor (NF)-κB, and the signal transducer and activator of transcription (Stat). NF-κB and C/EBP transcription factors are important in regulation of type I APP genes. IL-1β and IL-6 induce expression of C/EBP-β and C/EBP-δ, while IL-1β and tumor necrosis factor-α activate NF-κB. These transcription factors, in turn up-regulate IL-6 production, which activates the Stat family of transcription factors; activated Stat3/acute phase response factor binds to IL-6 response elements (REs) on promoters of type II APP genes. Basal promoter elements and enhancers sequences for constitutive and IL-6-regulated expression of the human and rat FBG genes have been described (13).

Unlike the rat γFBG promoter, which contains three functional type II IL-6REs (14), only one such IL-6RE was found on the human γFBG promoter (15). Furthermore, unlike the IL-6REs on the human Aα and Bβ chain promoters, IL-6 induction of human γFBG promoter activity is not responsive to the presence of elevated levels of either β or δ isoforms of C/EBP (e.g. type I IL-6REs). Recently another transcription factor, SAA-activating factor-1 (SAF-1/MAZ), was shown to contribute to IL-6 regulation of γFBG gene expression in human hepatoma cells (15).

Although rat and human FBG genes are clearly responsive to IL-6, the identity of the cognate transcription factors that bind to IL-6 enhancer elements on promoter regions of the FBG genes has remained elusive. IL-6-activated Stat3 binds to the type II cis-acting elements of the rat γFBG promoter (16); however, no such complexes have been identified on the human γFBG promoter (15, 16) or the rat and human Aα and Bβ chain promoters (7, 16, 17). Because IL-6-mediated Stat3 signaling plays critical roles in essential biological functions including the immune response, inflammation, hematopoiesis, oncogenesis, and embryogenesis by regulating cell growth, survival, and differentiation (for reviews, see Refs. 16 and 19), we investigated the role of Stat3 binding to IL-6REs and transactivation of the human γFBG promoter as described in this report.

EXPERIMENTAL PROCEDURES

Cell Culture, Reagents, and Antibodies—HepG2 cells (HB-8065) from the ATCC were grown in Eagle’s minimal essential medium containing 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, penicillin, streptomycin, and 10% fetal bovine serum; cell culture reagents were obtained from Promega (Madison, WI), [32P]ATP and [14C]chloramphenicol were obtained from PerkinElmer (Boston, MA). The following antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA): goat polyclonal anti-actin (sc-1615), which recognizes multiple actin isoforms; goat anti-Stat1 (sc-7986-X); and mouse monoclonal anti-Stat3 (sc-8019-X). Dexamethasone (DEX), ethyl acetate, and o-nitrophenyl β-D-galactopyranoside were purchased from Sigma, and recombinant human IL-6 was from Research Diagnostics (Flanders, NJ).

Western Blot Immunodetection—HepG2 cells were grown to near confluence and then incubated in medium containing various concentrations of IL-6 for 24 h; the cells were washed with phosphate-buffered saline and lysed as described previously (16). After determination of the protein concentration by Bradford assay, equivalent amounts of protein from the cell lysates were resolved after reduction by SDS-10% PAGE. After transfer to nitrocellulose membranes, the γ chain was detected by Western blotting with monoclonal antibody 88B, which specifically recognizes the γ chain of human FBG (20). The blots were stripped and reprobed with antibody to actin to control for variability in protein loaded on the gel. Immune complexes were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (PerkinElmer Life Sciences).

**Superfect Transfections and Luciferase Assays—**Transfection of HepG2 cells was performed using Superfect (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Cells were grown to 40–50% confluence in 35-mm culture dishes and transfected with reporter construct, 5 ng of pRL-SV40 as the internal control for transfection efficiency, and pSG5 plasmid DNA to a total of 2 μg in each transfection reaction. The amount of reporter and expression vectors used in each experiment is indicated in the figure legends. After incubation with Superfect-DNA complexes for 16–18 h, fresh medium with various concentrations of IL-6 (0 ± 1 μM DEX) was added to the cells and incubated for 24 h. Luciferase activity in cell lysates was determined by the Dual-Luciferase reporter assay system according to the manufacturer’s protocol (Promega). The wtStat3 construct and Stat3Δ355C mutant were used as dominant inhibitory controls in transfections of Dr. H. Baumann (Roswell Park Cancer Institute, Buffalo, NY).

Luciferase Reporter Plasmid Construction—Two constructs in pGL2-basic vector containing either 1359 or 954 bp of the 5’-flanking region of the human γFBG promoter were kindly provided by Dr. M. L. Tenchini from the University of Milan, Italy (21). The 954-bp promoter fragment was subcloned into pGL3-basic vector (Promega). Progressively truncated DNA segments were prepared by PCR using different appropriate 5’-end sense primers, a constant 3’-end antisense primer, and pGL2–1359 DNA as template. The sense primer contained MluI and the antisense primers contained BglII restriction recognition sequences to facilitate cloning. The antisense primer was 5′-AGATCTAGCTTCGTATGAACTAG-3′ and the sense primers were as follows: 5′-AGCCGCTAGAAGTGGAGCCTATGTA-3′; pGL3-450, 5′-GACGGGT- TGGTATTCAGGTTAT-3′; pGL3-300, 5′-GACGGCGTCACTACACA-GCTCCAG-3′; pGL3-200, 5′-GACGGCGTACTGGGCGGACAAAAGG-3′; pGL3-100, 5′-GACGGCTCGTCCACCTTCTGTC-3′. Site-directed deletions of the putative type II IL-6REs on the human γFBG promoter were generated by PCR as described previously (22). Primer sets for A1 (promoter-proximal) were sense 5′-GTTGTCGAACTCCTGACATGTTAGGTTGTTGGGC-3′/antisense 5′-CTATACTGCTGAGTTTGGTGACTGCTACAGGTTGAAGCTCTCCCTCC-3′, for A2 (middle) were sense 5′-CAGTCTGAGATGTTGCTACACAGGCTCCTGAG-3′/antisense 5′-GATGCAGACCTCTCAGCTAGTGTTGACACAAACAGATATGGCC-3′, and for A3 (promoter-distal) were sense 5′-GTCGATCAGGTTAGGTTGTTGGGC-3′/antisense 5′-AGCTTCTACAGGTAAGTGGTTACTTCG-3′. PCR site-directed mutations in the SAF1RE were made sense 5′-GACGTGCTGATGCTACACAGCACTGGATAGTAGTGGTTGTTGGGC-3′/antisense 5′-GCTGTGATGCTACACAGCACTGGATAGTAGTGGTTGTTGGGC-3′.

**Preparation of Nuclear Extracts—**HepG2 nuclear extracts were prepared as described previously (23). After the desired treatment, the cells were washed twice with ice-cold phosphate-buffered saline, resuspended into 15-mL conical tubes, and then centrifuged for 5 min at 1000 × g at 4 °C. Cell pellets were resuspended in 10 μL HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), allowed to swell on ice for 10 min, and then vortexed for 10 s. After centrifugation for 3 min at 1300 × g at 4 °C, the supernatant was discarded, and the pellet was resuspended in cold hypotonic buffer (20 mM HEPES-KOH, pH 7.9, 250 mM glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. Cellular debris were removed by centrifugation at 14000 × g for 15 min. The supernatant fractions containing nuclear proteins were stored at −70 °C until use.

**Electrophoretic Mobility Shift Assays (EMSAs) —**The sense strand of the double-stranded probe corresponding to the human γFBG IL-6REs for EMSAs is given in Fig. 3A. The rat αm-macro globulin (αm) type II IL-6RE that binds with high affinity to Stat3 served as a positive control (9). Double-stranded probes were end labeled with [γ-32P]ATP by T4 polynucleotide kinase. DNA-protein binding reactions were performed in 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM MgCl2, 5 μM β-mercaptoethanol, 0.5 μM phenylmethylsulfonyl fluoride, 100 ng of poly(dI-dC)/μL, and 1 μg of bovine serum albumin/μL as described previously (24). Two to 10 μg of HepG2 nuclear extract and 20 fmol of probe were incubated at room temperature for 10 min and then the reaction was stopped by adding loading buffer. For supershift, the reaction was incubated at room temperature for 10 min following addition of probe at 1 μM concentration and incubating an additional 10 min. Nondenaturing 6% polyacrylamide gels were precleared by electrophoresis at 20 mA for 30 min at 4 °C in 1 × running buffer (7 mM Tris-HCl, pH 7.5, 3 mM sodium acetate, 1 mM EDTA); free DNA and DNA-protein complexes were resolved at 30 mA.
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RESULTS

Dose-dependent IL-6 Up-regulation of γFBG Production—Previously we reported that IL-6 up-regulates FBG expression in both HepG2 and A549 cell lines (27–29). To confirm this expression and determine whether increasing concentrations of IL-6 would result in elevated production of γFBG protein, Western blotting of IL-6-treated HepG2 cell lysates was performed using monoclonal antibody J88B to specifically detect the γ chain. After 24 h of treatment, IL-6 induced γFBG production in HepG2 cells in a dose-dependent manner. The magnitude of γFBG induction with 50 ng/ml IL-6 was >10-fold over control in HepG2 cells (Fig. 1A). Functional assays using the Dual-Luciferase reporter assay system were performed to test the response of the human γFBG promoter to IL-6 treatment. Increasing concentrations of IL-6 induced luciferase activity driven by the human γFBG promoter construct pGL3-954 in transiently transfected HepG2 cells (Fig. 1B). At 50 ng/ml IL-6, the γFBG promoter was transactivated −30-fold over control. These data confirm that cis-element(s) within the 954-bp promoter region efficiently mediate IL-6-induced transactivation of the human γFBG promoter in HepG2 cells.

Additional Type II IL-6RE Identified on the Human γFBG Promoter—While Mizuguchi et al. (13) demonstrated the lack of type I (C/EBP) IL-6RE on the human γFBG promoter region, a functional type II IL-6RE was localized to −306 to −301. The type II consensus motif serves as a docking site for IL-6-activated Stat3 to transactivate expression of Group 2 APP genes (i.e., positive APP up-regulated by 2–10-fold in response to IL-6 and also possessing the type II IL-6RE) such as the rat αM (24) and rat γFBG (14) genes. However, past attempts to show Stat3 binding to the functional IL-6REs of the human γFBG promoter were unsuccessful (13, 15). IL-6 is a definitive inducer of expression of all three FBG genes across species (7, 13, 15–17); however, the role of Stat3 binding to the IL-6REs on FBG promoter elements and functional transactivation remains unresolved. Therefore, we examined whether there might be additional IL-6-responsive elements on the human γFBG promoter by analysis of the promoter region up to −1000 bp from the +1 site of transcription initiation. By computer-assisted analysis, we looked for the type II IL-6 consensus motif CTGG(G)AA found in most Group 2 APP genes (30) and the SAF-1 consensus sequence GGGRAGGR where R represents any purine (31). In addition to the functional type II IL-6REs previously identified (13), two more putative type II IL-6REs were found. We designated these sites from promoter-proximal to promoter-distal as Sites I, II, and III, corresponding to the nucleotide positions on the human γFBG promoter: Site I, −157 to −151; Site II, −306 to −301; and Site III, −531 to −525 (Fig. 2A). No additional SAF-1 binding sites were found on the human γFBG promoter other than the one at position −271 to −262 (15).

Functional Analysis by Luciferase Reporter Assays of the 5′-Flanking Region of the Human γFBG Promoter in Response to IL-6—To test whether each putative IL-6RE plays a role in mediating IL-6 induction of the human γFBG gene, progressive deletions of the 5′-flanking promoter region were cloned in front of the luciferase reporter gene in pGL3-basic (Fig. 2B). These constructs were transiently expressed in HepG2 cells, and the luciferase activity with IL-6 was compared with that without IL-6 to get the relative -fold induction, which represents the capacity of each promoter construct to respond to IL-6 treatment. For purposes of comparison, the promoter activity of pGL3-600, which contains three putative type II IL-6REs and one SAF-1 site, is defined as 100% activity (Fig. 2B). The loss of the distal IL-6RE in pGL3-400 resulted in a 25% loss of promoter responsiveness to IL-6 treatment (p = 0.0004). Deletion of an additional 100 bp, which, in addition to Site III, eliminated the middle IL-6RE corresponding to Site II, led to greater than 85% reduction in promoter activity in response to IL-6 (p < 0.0001). Although the deletion of the next 100 bp resulted in loss of the SAF-1 site as well as Sites II and III, the promoter activity of pGL3-200 in response to IL-6 treatment was similar to that of pGL3-300 (p = 0.8501) (Fig. 2B). Only 4% promoter activity remained in response to IL-6 in pGL3-100 in which all putative IL-6-responsive elements were deleted. These results indicate that the three putative type II IL-6REs on the human γFBG 600-bp promoter show differing degrees of responsiveness to IL-6 induction; Site II plays the most significant role in IL-6-induced gene expression, which is consistent with previous data (13). About 25% of the transactivation potential of the γFBG promoter construct resides in the −600 to −400 region, which contains the distal IL-6RE (Fig. 2A). These results sug-
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Fig. 2. Transient transfections indicate the relative functional importance of the putative IL-6REs for IL-6 induction of γFBG expression. A, schematic representation of 600 bp of the 5’-flanking region of the human γFBG promoter showing the relative positions of three putative IL-6REs and one SAF-1RE based on nucleotide numbering of the human γFBG gene derived from GenBankTM accession number M10014 (drawn approximately to scale). B, the progressively deleted segments prepared by PCR were directly cloned into the pGL3-basic vector. Replicate reporter gene assay experiments were performed (n = 4). HepG2 cells were treated with 50 ng/ml IL-6 for 24 h following transfection. For purposes of comparison, the promoter activity of wild type γFBG (pGL3-600) is defined as 100%.

Functional Analysis by Luciferase Reporter Assays of Site-directed Internal Deletions of the Human γFBG Promoter in Response to IL-6—Although the results from functional analysis of the deletion constructs suggest that Sites II and III account for the majority of the wild type promoter activity defined by pGL3-600. While Site I has minimal transactivation potential in response to IL-6, its loss (pGL3-200 to pGL3-100) resulted in a statistically significant reduction in luciferase activity (p = 0.0110). Deletion of −300 to −200, which includes the SAF-1 IL-6RE, did not affect luciferase expression in response to IL-6 treatment (Fig. 2B).

Overexpressed Wild Type Stat3 Potentiates IL-6-induced γFBG Promoter Activity.—The functional data indicate that all three type II IL-6REs contribute to IL-6-induced expression of the γFBG 600-bp promoter in HepG2 cells (Figs. 2 and 3). In addition, the EMSA analyses suggest that IL-6-activated Stat3 likely plays a role in transactivation of luciferase gene expression driven by the γFBG promoter (Fig. 4). Because the relative affinity of Stat3 for the γFBG IL-6REs is low, we determined whether overexpression of wtStat3 in HepG2 cells would lead to IL-6-enhanced transactivation. In response to 50 ng/ml IL-6, the relative luciferase activity using the pGL3-600 reporter construct was increased 25-fold, whereas, using pGL3-600 ΔII,III, a residual induction by IL-6 was observed. In the presence of increasing concentrations of wtStat3 transiently expressed in IL-6-treated HepG2 cells, the relative -fold luciferase activity was further enhanced 37-fold above the not IL-6-treated pGL3-600, resulting in 1.5-fold potentiation of
yFBG promoter activity over IL-6-treated pGL3-600 due to Stat3 overexpression (Fig. 5). However, overexpression of wtStat3 did not potentiate IL-6-induced luciferase expression of pGL3-600-ΔII,III suggesting that no additional or cryptic Stat3 binding sites remain in the this 600-bp promoter region. Although, due to the high levels of endogenous Stat3 in HepG2 cells, the use of the transdominant Stat3Δ55C as a competitive inhibitor of IL-6-induced Stat3 transactivation was limited (32), in the presence of increasing concentrations of Stat3Δ55C, the 1.5-fold potentiation in the yFBG promoter activity of pGL3-600 induced by wtStat3 was inhibited (not shown). Taken together, these data indicate that type II IL-6REs on the human yFBG promoter are required for IL-6-mediated wtStat3 potentiation of gene expression in HepG2 cells.

Induction of Human yFBG Promoter Activity Is IL-6 Concentration-dependent—EMSA analysis indicated that the degree of Stat3 complex formation with each of the yFBG IL-6REs was dependent on the concentration of IL-6 used to stimulate HepG2 cells (Fig. 4). Therefore, we investigated whether the transactivation potential of each of the type II yFBG IL-6REs was affected by increasing concentrations of IL-6. Using the wild type human yFBG 600-bp promoter as control, we tested the promoter responsiveness of single-site (Fig. 6A), two-site (Fig. 6B), or three-site (Fig. 6C) internal deletions to different concentrations of IL-6. The results showed that the magnitude of luciferase activity promoted by each single or multiple site-deleted construct was responsive to increasing concentrations of IL-6; however, each IL-6RE on the yFBG promoter showed the same rank order of transactivation potential (Site II ≫ Site I > Site III) as determined by the data shown in Fig. 3B regardless of the IL-6 concentration.

Dexamethasone Potentiates IL-6-induced Activation of the Human yFBG Promoter in the Absence of a Functional GRE—GCs synergistically enhance IL-6 induction of Group 2 APP genes even in the absence of a functional GRE (7). The human yFBG 600-bp promoter region lacks the functional GRE at −1116 to −1102 as described by Asselta et al. (21). Therefore, we compared the functional activity of the wild type pGL3-600 promoter construct to pGL3-600-ΔII,III, in which all Stat3 binding sites were deleted, in response to 50 ng/ml IL-6, 0.1 μM DEX, or both. Whereas IL-6 induced the wild type promoter activity 30-fold as expected, DEX alone induced pGL3-600 luciferase activity by 25-fold over pGL3-600 activity in the absence of IL-6 treatment (Fig. 7A, white bars) (p < 0.001). The 2.25-fold induction in wild type promoter activity by 0.1 μM DEX in the presence of 50 ng/ml IL-6 compared with IL-6 treatment of pGL3-600 alone was statistically significant (p = 0.0063) (Fig. 7A, white bars), consistent with a previous report (33). In contrast, when all three type II IL-6REs were deleted, 0.1 μM DEX failed to potentiate the residual promoter activity in the presence of IL-6 (Fig. 7A, black bars), suggesting that the SAF-1RE does not contribute to DEX enhancement of yFBG
FIG. 4. The γFBG type II IL-6REs form protein-DNA complexes with HepG2 nuclear extracts treated with IL-6. A, HepG2 cells were treated with IL-6 (2.5 ng/ml) for 0, 20, 40, 80, and 160 min, and cells were collected for nuclear extract preparation. The sequences of oligos used for EMSA probes are as depicted in Fig. 3A. The rat α2M IL-6RE that binds strongly to activated Stat3 was used as a positive control. Nine μg of IL-6-treated HepG2 nuclear extracts were used in each binding reaction. B, the specificity of Stat3 binding to α2M IL-6RE was tested by competition with 100× nonlabeled oligo and by supershift analysis with antibody against Stat3 or Stat1. HepG2 cells were treated with IL-6 (2.5 ng/ml) for 40 min, and cells were collected for nuclear extract preparation. Two μg of nuclear extracts were used for each binding reaction. C, each nonlabeled γFBG IL-6RE-containing oligo was used at 100× concentration for cross-competition with labeled α2M probe in binding to Stat3 from 2 μg of nuclear extract from IL-6-treated HepG2 cells. D, HepG2 cells were treated with increasing concentrations of IL-6 (2.5, 10, and 50 ng/ml) for 0, 15, 30, and 90 min, and cells were collected for nuclear extract preparation. The α2M probe was used as a positive control (first four lanes). The capacity of each putative γFBG IL-6RE-containing oligo to form a protein-DNA complex with nuclear extracts from HepG2 cells treated with increasing concentrations of IL-6 was tested. Nine μg of nuclear extracts were used in each lane; the concentrations of IL-6 used and the time points are depicted at the top of the panel. Specificity controls for complex formation were performed using anti-Stat3 and anti-Stat 1 antibodies and are shown in the last four lanes of each subpanel.
Expressed in response to IL-6 transactivation, the SAF-1RE of SAA as a positive control. A representative thin layer chromatogram of [14C]chloramphenicol acetyltransferase assay, and performing EMSAs with the γFBG SAF-1 sequence compared with the SAF-1RE of SAA as a positive control. A representative thin layer chromatogram of [14C]chloramphenicol acetylated by CAT expressed in response to IL-6 transactivation of the various human γFBG pCAT3 constructs is shown in Fig. 8A; the relative CAT activity reported as the mean and S.E. of three independent experiments is shown in Fig. 8B. A comparison in expression efficiency of the human γFBG 600-bp element promoter element in pCAT3-basic, designated pCAT3-600, shows that IL-6 induced CAT activity by 6.4-fold using calcium phosphate, whereas IL-6 induced CAT activity by 18.5-fold using Superfect for transient transfection (Fig. 8B, compare data set 2 to data set 10) (p < 0.0001). This 3-fold difference in transfection efficiency would account for the overall higher levels in γFBG promoter activity induced by IL-6 using the pGL3-luciferase expression system. Furthermore the luciferase reporter system is more sensitive than the CAT reporter system as shown by comparing the 18.5-fold IL-6-induced expression of pCAT3-600 (Fig. 5B, data set 10) to the 25–30-fold IL-6-induced expression of pGL3-600 (Figs. 5–7) by Superfect-mediated transfection. Therefore, the increased sensitivity of the luciferase reporter system and the more efficient expression of pCAT3-600 (Fig. 5B, black bars). Deletion of the promoter-distal IL-6RE (Site III) resulted in an equivalent potentiating effect by DEX as that observed for the wild type promoter. Whereas all three type II γFBG IL-6REs contributed to the full activity of the 600-bp promoter in response to IL-6, the data suggest that together Sites I and II but not Site III are required for DEX-enhanced expression of IL-6-induced γFBG promoter activity.

Elucidation of Human γFBG IL-6RE Promoter Elements Using CAT Reporter Constructs Compared with Luciferase Reporter Constructs—Because the IL-6-responsive sites identified on the human γFBG promoter using the luciferase reporter constructs described in this report differ considerably from the site identified by A. Ray (15) using CAT gene reporter constructs, we repeated the reporter gene assays following the methods used by Ray (15). This involved subcloning our human γFBG promoter constructs into the pCAT3-basic vector, performing transient transfections by the calcium phosphate method, measuring relative changes in promoter activity induced by IL-6 using the [14C]chloramphenicol acetyltransferase assay, and performing EMSAs with the γFBG SAF-1 sequence compared with the SAF-1RE of SAA as a positive control. A representative thin layer chromatogram of [14C]chloramphenicol acetylated by CAT expressed in response to IL-6 transactivation of the various human γFBG pCAT3 constructs is shown in Fig. 8A; the relative CAT activity reported as the mean and S.E. of three independent experiments is shown in Fig. 8B. A comparison in expression efficiency of the human γFBG 600-bp element promoter element in pCAT3-basic, designated pCAT3-600, shows that IL-6 induced CAT activity by 6.4-fold using calcium phosphate, whereas IL-6 induced CAT activity by 18.5-fold using Superfect for transient transfection (Fig. 8B, compare data set 2 to data set 10) (p < 0.0001). This 3-fold difference in transfection efficiency would account for the overall higher levels in γFBG promoter activity induced by IL-6 using the pGL3-luciferase expression system. Furthermore the luciferase reporter system is more sensitive than the CAT reporter system as shown by comparing the 18.5-fold IL-6-induced expression of pCAT3-600 (Fig. 5B, data set 10) to the 25–30-fold IL-6-induced expression of pGL3-600 (Figs. 5–7) by Superfect-mediated transfection. Therefore, the increased sensitivity of the luciferase reporter system and the more efficient expression of reporter constructs by the Superfect method of transient transfection in HepG2 cells allowed us to measure smaller changes in reporter gene activity.

Aside from the lower sensitivity of the CAT assay and efficiency of expression by the calcium phosphate precipitation method of transfection, the results we obtained with the human γFBG 600-bp promoter-CAT reporter constructs were essentially the same as the results we observed with the luciferase reporter system. 
reporter constructs (Fig. 8B and Table I). We showed by EMSA that protein from HepG2 cell nuclear extracts binds to the positive control probe derived from the SAA promoter as well as to the yFBG probe containing the SAF-1RE at nucleotides −271 to −262 as described by Ray (15). The specificity of SAF-1 binding was confirmed by competition with excess nonlabeled oligo (Fig. 8C). However, IL-6 treatment did not alter the amount of SAF-1 that bound to either the SAA or yFBG probes (Fig. 8, C and D). Furthermore, in contrast to the results of Ray (15), the HepG2 cells used in this study express high constitutive levels of SAF-1 (Fig. 8C). Using increasing concentrations of nonlabeled probes and 2 µg of nuclear extracts, cross-competition of protein binding to labeled SAA probe with cold yFBG SAF-1 oligo and vice versa was performed. Although the yFBG SAF-1 sequence competitively inhibited binding of nuclear protein to the labeled SAA probe, it failed to completely inhibit this binding. In contrast, nonlabeled SAA probe completely abolished the binding of protein from HepG2 nuclear extracts to the yFBG SAF-1 probe, suggesting that SAF-1 binds with lower affinity to the yFBG SAF-1 promoter element (Fig. 8D). Deletion or mutation of the yFBG SAF-1 site had minimal effect on IL-6 induction of the human yFBG promoter containing the type II IL-6-responsive sites, in particular Site II (Figs. 2B, 3B, and 8B). Taken together, the data in this report suggest that the y SAF-1 site contributes little to the human yFBG promoter activity in response to IL-6.

**DISCUSSION**

In addition to the IL-6 family of cytokines, Stat3 is activated by leptin, granulocyte colony-stimulating factor, and epidermal growth factor. Targeted disruption of the **stat3** gene results in lethality between embryonic days 6.5 and 7.5, demonstrating that Stat3 is essential early in embryonic development (34). Analyses of conditional cell type- or tissue-specific Stat3-deficient mice indicate that Stat3 plays a crucial role in a variety of biological functions including cell growth, cell motility, and suppression and induction of apoptosis (18, 35, 36). In addition, targeted disruption of genes involved in Stat3 activation such as IL-6 and gp130, as well as Stat3 target genes including members of the C/EBP transcription factor family, leads to dysregulation of innate immunity, metabolism, hematopoiesis, or embryogenesis (37–41). Stat3 is the main mediator of APP gene induction downstream of IL-6 family cytokines and gp130 signaling (42). IL-6 activation of Stat3 is essential for inducing APP gene expression including the FBG Aα, Bβ, and γ chain genes in response to lipopolysaccharide, IL-6, or localized tissue damage in conditionally Stat3-deficient mice (38, 39). Furthermore, the functional importance of the Stat3 cognate binding sites in promoting expression of rat and human Aα, Bβ, and γ chain genes is well established. However, the inability to demonstrate direct binding of Stat3 to wild type promoter elements on each of these genes (13, 15–17, 43), except the rat γ chain IL-6REs (14), remains unexplained. Similarly attempts to demonstrate binding of the mouse haptoglobin IL-6RE to Stat3 by EMSA failed for unknown reasons, although functional assays demonstrated it as the **cis**-acting element required for regulation by Stat3 (32).

We demonstrate for the first time that, like the rat promoter, the human yFBG promoter contains three functional type II IL-6REs within 600 bp upstream of the transcription initiation site. The relative position of promoter elements on the human 600-bp promoter is compared with corresponding elements on the rat yFBG promoter region in Fig. 9. We confirmed that the functional IL-6RE identified at −306 to −301 (13) (designated Site II in Fig. 9) is the major IL-6-responsive site on the human yFBG promoter and provide evidence that Sites I (−157 to −151) and III (−531 to −525) contribute to full promoter activity in response to IL-6. In contrast to the report by Ray (15), our results do not show that the SAF-1 site is the major IL-6-responsive element on the human yFBG promoter; therefore, we repeated a number of experiments using the CAT reporter gene expression system to verify the positions of the IL-6REs. The results we obtained with the human yFBG promoter-CAT reporter constructs were essentially the same as the results we observed with the luciferase reporter constructs and did not replicate the work of Ray (15). Although we cannot fully explain the reasons for this discrepancy, one major difference between our report and that of Ray is that we see high levels of constitutive expression of SAF-1 in our HepG2 cells, whereas Dr. Ray did not (Fig. 5 of Ref. 15). Furthermore EMSA results showed that IL-6 did not induce binding of a HepG2 nuclear protein to labeled probes corresponding to the SAF-1 consensus motif of either SAA or yFBG. These data suggest that high constitutive levels of SAF-1 may abrogate or mask IL-6 induction of low levels of SAF-1. SAF-1 is a zinc finger transcription factor originally cloned from a rabbit brain cDNA library (25) and is >95% identical to the deduced primary structure of mouse Pur-1 (44) and human MAZ (e-Myc-associated zinc finger protein) (45) orthologs, which are ubiquitously expressed housekeeping genes. Interestingly SAF-1 mRNA is abundantly expressed in mouse heart, liver, lung, brain, skeletal muscle, testis, and kidney (25), suggesting that SAF-1 protein is ubiquitously expressed as well. In light of these data, we cannot...
FIG. 8. Functional analysis of IL-6REs on the human γFBG 800-bp promoter by site-directed deletions or mutations using CAT reporter assays. A, a representative CAT assay thin layer chromatogram is shown. Data are grouped in pairs (data sets 1–10) based on the promoter construct tested (as identified in B) without (–) or with (+) 50 ng/ml IL-6. B, relative -fold change in CAT activity without (white bars) or with (black bars) 50 ng/ml IL-6 for 24 h (n = 3). pCAT3-Δ.Δ represents pCAT3-ΔI,II,III,ΔSAF-1. Data set 10 represents transient transfection of HepG2 cells using Superfect to compare the transfection efficiency to calcium phosphate-mediated transfection (data set 2) with pCAT3-600. C, EMSA using the SAF-1 cis-element from SAA and γFBG as probes. HepG2 cells were incubated with medium with or without IL-6 (50 ng/ml) for 24 h. Labeled SAF-1 site probes were incubated with 10 μg of nuclear extracts for EMSA. The specificity of binding was determined by incubation of 10 μg of HepG2 nuclear extracts with 100× nonlabeled “self” probe. D, the specificity of the DNA-protein binding interactions was further tested by cross-competition using 2 μg of nuclear extracts, labeled SAA or γFBG probe, and increasing concentrations of the heterologous nonlabeled probe as competitor.
IL-6-activated Stat3 Binds to the Human ϴFBG Promoter

Comparison of the functional analysis of the human ϴFBG 600-bp promoter element with previously published results and by reporter gene and transfection method

| ϴFBG promoter construct | + SAF-1RE | Percent CAT activity, calcium phosphate method, previous report | Percent CAT activity, calcium phosphate method, this report | Percent luciferase activity, Superfect method, this report |
|-------------------------|----------|---------------------------------------------------------------|-----------------------------------------------------------|----------------------------------------------------------|
| p600                    | +        | 100                                                           | 100                                                       | 100                                                      |
| p400                    | +        | 109.1                                                         | 75                                                        |
| p300                    | -        | 110.9                                                         | 15                                                        |
| p200                    | -        | 22.7                                                         | 15                                                        |
| p100                    | -        | 21.8                                                         | 4                                                         |
| ΔI,II,III               | +        | ND                                                           | 14                                                       |
| ΔSAF-1                  | -        | 28.8                                                        | 70                                                       |
| ΔI,II                   | -        | ND                                                           | 10                                                       |

+ Ref. 15.
* Data derived from results presented in Fig. 2B of Ref. 15 where -fold CAT activity of p600 is set to 100%.
 negligible.
+ Data derived from results presented in Fig. 4C of Ref. 15 where -fold CAT activity of p600 is set to 100%.

pCAT-3ΔI,II,IIIΔSAF-1

Comparison of human and rat ϴFBG 600-bp promoter elements.

The basal promoter and enhancer sequences identified to date are shown on the ~600 bp portion of human (H, top strand) and rat (R, bottom strand) ϴFBG promoter regions. Both human and rat ϴFBG promoters contain a TATA-like sequence, one CAAT box, and one upstream stimulatory factor (USF) binding site to constitute the minimal promoter. An Sp1 site is found in the rat promoter that is lost in the human ϴFBG basal promoter region. The previously identified type II IL-6-responsive element is located at ~306 to ~301 on the human ϴFBG promoter and is designated as Site II; the positions of the two additional type II IL-6REs identified as Sites I and III in this report are shown; the three type II IL-6REs previously identified on the rat ϴFBG promoter are indicated (references cited in text). No SAF-1 site has been identified in the rat ϴFBG promoter. The NF-κB site that overlaps the rat Stat3-II site is not conserved in the human ϴFBG promoter. Negative response regions previously identified are indicated. hStat3, human Stat3; rStat3, rat Stat3.

Taken together, the data in this report show the following rank order of Stat3-DNA complex formation from strongest to binding to Sites I and III appears stronger than that with Site II. In addition, the degree of complex formation was IL-6 dose-dependent. However, compared with the magnitude of Stat3 binding to the α2M type II core element, Stat3 complex formation with the human ϴFBG type II IL-6RE probes of the same specific activity occurred with much lower intensity. The specificity of Stat3 binding to α2M and ϴFBG IL-6REs was confirmed by cold competition with excess probe and either supershift (α2M) or ablation of complex formation (ϴFBG Sites I and III) with anti-Stat3 but not anti-Stat1 antibodies. In cross-competition studies, 100-fold excess nonlabeled probes for ϴFBG Sites I and III successfully competed off most of the Stat3 binding to the labeled α2M probe, whereas excess ϴFBG Site II probe only partially inhibited Stat3-α2M complex formation. In addition, overexpression of wild type Stat3 potentiated the IL-6-induced activity of the wild type 600-bp promoter 1.5-fold, which could be inhibited by expression of the transdominant Stat3Δ55C construct, whereas site-directed deletion of all three type II ϴFBG IL-6REs abrogated this potentiation. Furthermore, we show that Sites I and II on the ϴFBG 600-bp promoter, which lacks a GRE, are critical for DEX potentiation of IL-6-induced promoter activity. The promoter-distal Site III, which lies upstream of a negative response region identified by Mizuguchi et al. (Fig. 9), had no effect on DEX potentiation of IL-6 activity.

Figure 9. Comparison of human and rat ϴFBG 600-bp promoter elements.

The basal promoter and enhancer sequences identified to date are shown on the ~600 bp portion of human (H, top strand) and rat (R, bottom strand) ϴFBG promoter regions. Both human and rat ϴFBG promoters contain a TATA-like sequence, one CAAT box, and one upstream stimulatory factor (USF) binding site to constitute the minimal promoter. An Sp1 site is found in the rat promoter that is lost in the human ϴFBG basal promoter region. The previously identified type II IL-6-responsive element is located at ~306 to ~301 on the human ϴFBG promoter and is designated as Site II; the positions of the two additional type II IL-6REs identified as Sites I and III in this report are shown; the three type II IL-6REs previously identified on the rat ϴFBG promoter are indicated (references cited in text). No SAF-1 site has been identified in the rat ϴFBG promoter. The NF-κB site that overlaps the rat Stat3-II site is not conserved in the human ϴFBG promoter. Negative response regions previously identified are indicated. hStat3, human Stat3; rStat3, rat Stat3.
IL-6-activated Stat3 Binds to the Human γFBG Promoter

The binding of Stat3 complexes with ligand-activated GR to the IL-6RE at −531 to −525 may place this complex too far from the promoter-proximal Sites II and I, both of which are required for DEX potentiation of IL-6-induced promoter activity. Such an interaction would effectively remove the distal site from interactions with the putative enhancer complex formed with Stat3 and GR on Sites II and I as well as with the basal transcription apparatus including coactivators such as p300/CBP. Fuller and Zhang (7) have proposed further that chromatin remodeling by nuclear cofactor acetylation of histones upstream of the γFBG gene, which is positioned at the 5′ proximal end on both the human and rat FBG loci, may facilitate the coordinated transcriptional activation of the Ao and Bβ chain genes during an acute phase response. Because elevated levels of circulating FBG correlate with increased risk for cardiovascular disease and stroke, elucidation of the molecular mechanisms that regulate FBG expression becomes important for control of diseases associated with high levels of FBG (50, 51). Thus, in addition to furthering our understanding of how FBG is up-regulated in response to inflammatory stimuli, this work’s considerable insight in elucidating the mechanisms that down-regulate FBG gene expression will allow the hopes of identifying agents that will normalize elevated plasma FBG in patients at risk for cardiovascular disease.

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REFERENCES

1. Bini, A., Simpson-Haidaris, P. J., and Kudryk, B. J. (2000) in Encyclopedic Reference of Molecular Biology & Biotechnology (Bikfalvi, A., ed.) p 372, Springer-Verlag, Berlin

2. Esmon, C. T., Vekich, Y. I., Gerkin, O. V., Medved, L. V., Nieuwenhuizen, W., and Weitzel, J. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1–26

3. Francis, C. W., Bunce, L. A., and Sporn, L. A. (1993) Blood Cells 19, 291–306

4. Naito, M., Funaki, C., Hayashi, T., Yamada, K., Asai, K., Yoshimine, N., and Sano, M. (1992) Atherosclerosis 96, 227–234

5. Brown, L. F., Laniz, N., McDonagh, J., Tegnazzi, N., Dvorak, A. M., and Dvorak, H. F. (1995) Ann. J. Pathol. 142, 273–282

6. Rodman, C. M., and Xia, H. (2001) Ann. N. Y. Acad. Sci. 936, 480–495

7. Fuller, G. M., and Zhang, Z. (2001) Ann. N. Y. Acad. Sci. 969, 469–479

8. Castell, J. V., Gomez-Lechon, M. J., David, M., Andus, T., Geiger, T., Trulenque, R., Rubra, B., and Heinrich, P. C. (1989) FEBS Lett. 242, 257–259

9. Bode, J. G., Fischer, R., Hausinger, D., Graewe, L., Heinrich, P. C., and Schaper, F. (2001) J. Immunol. 167, 1469–1481

10. Zhang, Z., and Fuller, G. M. (1997) Biochem. Biophys. Res. Commun. 237, 90–94

11. Otto, J. M., Grenett, H. E., and Fuller, G. M. (1987) J. Cell Biol. 105, 1067–1072

12. Simpson-Haidaris, P. J. (1997) Blood 89, 873–882

13. Mizuguchi, J., Hu, C. H., Cho, L. K., Chung, D. W., and Davie, E. W. (1995) J. Biol. Chem. 270, 28350–28356

14. Zhang, Z., Fuentes, N. L., and Fuller, G. M. (1995) J. Biol. Chem. 270, 24287–24291

15. Ray, A. (2000) J. Immunol. 165, 3411–3417

16. Dalmon, J., Laurent, M., and Courtioux, G. (1993) Mol. Cell. Biol. 13, 1183–1193

17. Hu, C. H., Harris, J. E., Davie, E. W., and Chung, D. W. (1995) J. Biol. Chem. 270, 28342–28349

18. Akira, S. (2000) Oncogene 19, 2607–2611

19. Hirano, T., Ishihara, K., and Hibi, M. (2000) Oncogene 19, 2548–2556

20. Conlon, T. M., Hyarrazzay, C. W., Lawrence, S. E., Hamaguchi, M., and Simpson-Haidaris, P. J. (1996) Biochem. Biophys. Acta 1298, 69–77

21. Asseleta, R., Duga, S., Modugno, M., Malcovati, M., and Tenchini, M. L. (1998) J. Biol. Chem. 273, 11440–11450

22. Hemes, A., Arheim, N., Toney, M. D., Cortecassey, G., and Galas, D. J. (1989) Nucleic Acids Res. 17, 6455–6458

23. Andrews, N. C., and Fuller, G. M. (1991) Nucleic Acids Res. 19, 2499

24. Wegenka, U. M., Buschman, J., Lutticken, C., Heinrich, P. C., and Horn, F. (1995) Mol. Cell. Biol. 13, 276–288

25. Ray, A., and Ray, B. K. (1994) Mol. Cell. Biol. 14, 4324–4332

26. Guadiz, G., Sporn, L. A., and Simpson-Haidaris, P. J. (1997) Blood 90, 2644–2653

27. Guadiz, G., Sporn, L. A., Goss, R. A., Lawrence, S. O., Marder, V. J., and Simpson-Haidaris, P. J. (1997) Am. J. Respir. Cell Mol. Biol. 17, 60–69

28. Simpson-Haidaris, P. J., and Courtney, M. A. (1992) Blood 79, 1218–1224

29. Hattori, M., Abraham, L. J., Northemann, W., and Fey, G. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2964–2968

30. Ray, A., and Ray, B. K. (1996) Mol. Cell. Biol. 16, 1584–1594

31. Kim, H., and Baumann, H. (1997) J. Biol. Chem. 272, 14571–14579

32. Zhang, Z., Jones, S., Hagen, J. S., Fuentes, N. L., and Fuller, G. M. (1997) J. Biol. Chem. 272, 30607–30610

33. Takada, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3801–3804

34. Alonzi, T., Maritano, D., Gorgoni, B., Rizzuto, G., Libert, C., and Poli, V. (2001) Mol. Cell. Biol. 21, 1621–1632

35. Chapman, R. S., Lourenco, P., Toner, E., Flint, D., Selbert, S., Takeda, K., Akira, S., Clarke, A. R., and Watson, C. J. (2000) Adv. Exp. Med. Biol. 480, 129–138

36. Burgess-Beusse, B. L., and Darlington, G. J. (1998) Mol. Cell. Biol. 18, 7269–7277

37. Alonzi, T., Gorgoni, B., Screpani, L., Gulino, A., and Poli, V. (1997) Immunol. 198, 144–156

38. Alonzi, T., Fattori, E., Cappelletti, M., Ciliberto, G., and Poli, V. (1998) Cytochrome C 13, 13–18

39. Cantwell, C. A., Sterneck, E., and Johnson, P. F. (1998) Mol. Cell. Biol. 18, 2108–2117

40. Akira, S., Yoshida, K., Tanaka, T., Taga, T., and Kishimoto, T. (1985) Immu-
IL-6-activated Stat3 Binds to the Human γFBG Promoter

42. Lai, C. F., Ripperger, J., Morella, K. K., Wang, Y., Gearing, D. P., Fey, G. H., and Baumann, H. (1995) *J. Biol. Chem.* **270**, 14847–14850
43. Liu, Z., and Fuller, G. M. (1995) *J. Biol. Chem.* **270**, 7580–7586
44. Song, J., Murakami, H., Tsutsui, H., Uegai, H., Geltinger, C., Murata, T., Matsumura, M., Itakura, K., Kanaarawa, I., Sun, K., and Yokoyama, K. K. (1999) *Eur. J. Biochem.* **259**, 676–683
45. Song, J., Murakami, H., Tsutsui, H., Tang, X., Matsumura, M., Itakura, K., Kanaarawa, I., Sun, K., and Yokoyama, K. K. (1998) *J. Biol. Chem.* **273**, 20603–20614
46. Paulson, M., Pisharody, S., Pan, L., Guadagni, S., Mui, A. L., and Levy, D. E. (1999) *J. Biol. Chem.* **274**, 25343–25349
47. Giraud, S., Bienvenu, F., Avril, S., Gascon, H., Heery, D. M., and Coqueret, O. (2002) *J. Biol. Chem.* **277**, 8004–8011
48. Chan, H. M., and La Thangue, N. B. (2001) *J. Cell Sci.* **114**, 2363–2373
49. He, Y., Szapary, D., and Simons, S. S., Jr. (2002) *J. Biol. Chem.* **277**, 49256–49266
50. de Maat, M. P. (2001) *Ann. N. Y. Acad. Sci.* **936**, 509–521
51. de Maat, M. P., Knipscheer, H. C., Kastelein, J. J., and Klutz, C. (1997) *Thromb. Haemostasis* **77**, 75–79