The Functional Role of an Interleukin 6-inducible CDK9-STAT3 Complex in Human γ-Fibrinogen Gene Expression

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The signal transducer and activator of transcription 3 (STAT3) is an IL-6-inducible transcription factor that mediates the hepatic acute phase response (APR). Using γ-fibrinogen (FBG) as a model of the APR, we investigated the requirement of an IL-6-inducible complex of STAT3 with cyclin-dependent kinase 9 (CDK9) on γ-FBG expression in HepG2 hepatocarcinoma cells. IL-6 induces rapid nuclear translocation of Tyr-phosphorylated STAT3 that forms a nuclear complex with CDK9 in nondenaturing co-immunoprecipitation and confocal colocalization assays. To further understand this interaction, we found that CDK9-STAT3 binding is mediated via both STAT NH2-terminal modulatory and COOH-terminal transactivation domains. Both IL-6-inducible γ-FBG reporter gene and endogenous mRNA expression are significantly decreased after CDK9 inhibition using the potent CDK inhibitor, flavopiridol (FP), or specific CDK9 siRNA. Moreover, chromatin immunoprecipitation (ChIP) experiments revealed an IL-6-inducible STAT3 and CDK9 binding to the proximal γ-FBG promoter as well as increased loading of RNA Pol II and phospho-Ser2 CTD Pol II on the TATA box and coding regions. Finally, FP specifically and efficiently inhibits association of phospho-Ser2 CTD RNA Pol II on the γ-FBG promoter, indicating that CDK9 kinase activity mediates IL-6-inducible CTD phosphorylation. Our data indicate that IL-6 induces a STAT3-CDK9 complex mediated by bivalent STAT3 domains and CDK9 kinase activity is necessary for licensing Pol II to enter a transcriptional elongation mode. Therefore, disruption of IL-6 signaling by CDK9 inhibitors could be a potential therapeutic strategy for inflammatory disease.

The hepatic acute phase response (APR) is a coordinated response to tissue injury, infection or malignancy that initiates a global switch in the transcription of secreted proteins expressed by the vertebrate liver (1). Here, cytokines produced at the site of injury activate the de novo expression of genes encoding acute phase proteins (APPs), proteins important in homeostasis, opsonization, and wound repair. Of the APPs, fibrinogen (FBG) is known to play a key role in the APR by mediating hemostasis, participating in clot formation, platelet aggregation and clot retraction, processes important in promoting tissue repair at the site of injury. The FP heterocomplex is encoded by three chains; of these, the FBG γ chain (γ-FBG) is of interest because it contains binding sites for platelet integrin α1β3 and leukocyte integrin αMβ2, leading to platelet aggregation and leukocyte recruitment in inflammation (2, 3). In addition, its high binding affinities for vascular endothelial growth factor (5), fibroblast growth factor-2 (6), and interleukin-1β (7) contribute to wound healing. Finally, γ-FBG contains a fibrin polymerization site, which is involved in fibrin clot formation and platelet aggregation. Because of the crucial role of γ-FBG in multiple processes, the transcriptional mechanisms controlling inducible γ-FBG expression have been extensively investigated.

The cytokine interleukin-6 (IL-6) has emerged as a major mediator of de novo acute phase reactants synthesis, and for γ-FBG in particular (8–11). Here, IL-6 produced and secreted at the site of injury, circulates and binds to the hepatic high-affinity IL-6 receptor (IL-6R)-α. The liganded IL-6Rα then forms an oligomeric complex with gp130 (transducin) and subsequently activates the Janus- (JAK) and Tyk tyrosine kinases. Tyrosine-phosphorylated gp130, in turn, binds the cytoplasmic signal transducer and activator of transcription (STAT)-1 and -3 isoforms via their Src homology (SH)-2 domains. As additional substrates of the JAK/Tyk kinases, STATs are tyrosine-phosphorylated, allowing them to dimerize via intramolecular SH3 interactions, whereupon they translocate into the nucleus (12). Gene deletion experiments have shown that the actions of STAT3 are necessary for inducible expression of a network of APP genes, including C-reactive protein, serum amyloid A, angiotensinogen, and γ-FBG (13–15).

The mechanism of how tyrosine-phosphorylated STAT3 (pTyr-STAT3) induces gene expression is partly understood. Upon entry into the nucleus STAT3 associates with the p300/ogen; FP, flavopiridol; IL-6, interleukin-6; IL6 RE, IL-6 response elements; NE, nuclear extract; Pol II, RNA polymerase II; Q-RT-gPCR, quantitative real time genomic PCR; STAT, signal transducers and activators of transcription; PBS, phosphate-buffered saline; nt, nucleotides; Ab, antibody; FITC, fluorescein isothiocyanate.
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CBP coactivator, a protein that acetylates STAT3 on its NH₂-terminal modulatory domain to stabilize the STAT3-p300/CBP complex (16, 17). This complex, in turn modifies chromatin via its histone acetyl transferase activity, serving as an intermolecular bridge to recruit other chromatin modifying proteins like BRG1 (16, 18, 19). The recruitment of an enhansome results in enzymatic modifications of core nucleosomes, affecting chromatin structure and inducing preinitiation complex formation. By this mechanism, STAT3 controls genes important in diverse cellular functions such as cell cycle control (20, 21), antioxidant cellular defenses (22), and the APR (13–15).

In this study, we observed that IL-6 strongly induces a nuclear complex of STAT3 with cyclin-dependent kinase 9 (CDK9) and sought to further understand its role in APP regulation using γ-FBG as a model gene. CDK9 is a major component of a complex known as the positive transcriptional elongation factor (P-TEFb) involved in derepression and activation of RNA Pol II (23). We found IL-6 induces STAT3 binding to CDK9 in a mechanism mediated by both the NH₂- and COOH-terminal domains of STAT3. Inhibition of CDK9 activity or its expression decrease IL-6-inducible γ-FBG transcription. Chromatin immunoprecipitation (ChIP) experiments provide direct evidence that IL-6 induces CDK9 recruitment to the γ-FBG promoter along with enhanced RNA Pol II and phosphorylated Ser² CTD Pol II loading on the coding region. Moreover, IL-6-inducible Pol II binding is abolished by the CDK9 inhibitor, flavopiridol (FP). Taken together, our data indicate that the IL-6-inducible STAT3–CDK9 complex is essential for γ-FBG induction during the APR. This phenomenon suggests STAT3 promotes transcription elongations as an additional mechanism for induction of APPs.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-CDK9 (c-20), STAT3 (c-20), anti-phospho-Tyr STAT3 (B7), cyclin T1 (H-245), and RNA polymerase II (N-20) Abs were purchased from Santa Cruz Biotechnology. Anti-V5 and FLAG Abs were obtained from Invitrogen and Sigma, respectively. Monoclonal anti-phospho-Ser²—the COOH-terminal domain (CTD) Pol II Ab (H5) was from Covance. Anti-Ac-Lys⁸⁷ STAT3 Ab was described (16). Recombinant human IL-6 was from Peprotech.

Cell Culture and Stimulation—The human hepatoblastoma cell line HepG2 (ATCC, Manassas, VA) was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and penicillin (100 units/ml)/streptomycin (100 µg/ml). Cells are maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were serum-starved for at least 16 h before treatment. FP was added 1 h before IL-6 stimulation.

Plasmid Construction—For the γ-FBG-LUC reporter, 643 bp of the 5’-flanking human γ-FBG promoter was amplified from HepG2 cell genomic DNA by PCR using the forward primer 5’-CGCGGGATCCCTCTGGAGAGCCGCTTA-3’ and reverse primer 5’-GCCCAAGC TTGAGCTCGCCAGCTTGG-3’. The PCR product was digested with BamH1 and HindIII, producing pcDNA-FLAG-Straw. Second, the full-length human CDK-9 cDNA was amplified using oligo-dT-primed cDNA from HeLa cell RNA. The sequence of the primers was: 5’-CCCTGCTCTGGATCCAGCTTTCTTGT-AGACGTTCCATGCC-3’ (upstream), 5’-GTCACAAAGCTTGTGGATCCAGCTTTCTTGT-ACAGTCTCGTCCATGCC-3’ (downstream). The mStraw PCR product was digested with BglII and HindIII endonucleases, producing pcDNA-mStraw-CDK9. The DN-CDK9 expression plasmid was constructed in two steps. First, the mStraw plasmid (a generous gift of R. Tsien (25)) was amplified using primers to introduce a BglII restriction site upstream of the initiator methionine, remove the stop codon and insert multiple cloning sites. The sequence of these primers was: 5’-CAGTCAGATCTATGTTGAGAAGCTTGTGGATCCAGCCCAATGGAATCAGCT-3’ (upstream), and 5’-GGAAATGGCCCAATGGAATCAGCT-3’ (downstream). The mStraw cDNA was then restricted with HindIII and XbaI and cloned into the pcDNA-FLAG-Straw (15) digested with BamH1 and HindIII, producing pcDNA-FLAG-Straw. Second, the full-length human CDK-9 cDNA was produced by amplification of oligo-dT-primed cDNA from HeLa cell RNA. The sequence of the primers was: 5’-CATGCAAGCTTGTGAGAAGCTTGTGGATCCAGCCCAATGGAATCAGCT-3’ (upstream), and 5’-GTCATCTGAGACCCGCTGAACAGCCTCGTCCATGCC-3’ (downstream). The CDK9 cDNA was then restricted with HindIII and XbaI and cloned into the pcDNA-FLAG-Straw plasmid restricted with the same endonucleases, producing pcDNA-FS-CDK9. The DN-CDK9 (Asp⁶⁷ to Asn, D167N) was produced from wild-type CDK9 as a template using site-directed mutagenesis (QuikChange, Stratagene (26)). The sequence of the primers used was: mutations underlined: 5’-CCTGAGCAGCTTGTGGATCCAGCCCAATGGAATCAGCT-3’ (sense) and 5’-CCCGGGCCAGCCCAATGGAATCAGCT-3’ (antisense). All plasmids were purified by ion exchange chromatography and sequenced prior to transfection.

Transfection and Luciferase Activity Assay—Transient transfection was performed using Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer’s instruction. For reporter assay, HepG2 cells were plated into 6-well plates and cotransfected with γ-FBG-LUC reporter gene and the transfection efficiency control plasmid pSV2PAP. Twenty-four hours later, cells were stimulated with IL-6 with or without FP pretreatment. Both luciferase and alkaline phosphatase activities were measured 48 h after transfection. All transfections are carried out in three independent experiments. For co-immunoprecipitation, indicated expression plasmids were co-transfected into 10-cm² dishes using the same protocol. Cells were treated with IL-6 24 h after transfection prior to protein extraction.
Preparation of Subcellular Extracts—Sucrose cushion-purified nuclear extract (NE) was prepared as described (16). In brief, cells were harvested in PBS and centrifuged to collect pellets. Pellets were resuspended in double cell volume of solution A and centrifuged to obtain the supernatants as cytoplasmic fraction. The nuclear pellets were purified on sucrose cushions (16). After high salt extraction in buffer C (16), the nuclei were centrifuged at 12,000 × g at 4 °C for 20 min. The supernatants were saved as NE. The protein concentrations were measured using Coomassie dye binding (Protein Reagent, Bio-Rad).

Western Blotting—Proteins were fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 5% milk for 0.5–1 h and incubated with indicated primary Ab at 4 °C overnight. Membranes were washed in TBS-0.1% Tween 20 and incubated with secondary Ab at 20 °C for 1 h. Signals were detected by the enhanced chemiluminescence assay (ECL; Amersham Biosciences) or visualized by the Odyssey Infrared Imaging system. β-Actin is used as a loading control.

Co-immunoprecipitation—1–2 mg of HepG2 NE was precleared with 40 μl of protein A-Sepharose beads (Sigma) for 1 h at 4 °C. Immunoprecipitation was performed in the presence of 5 μg of the indicated primary Ab at 4 °C overnight. Immune complexes were captured by adding 50 μl of protein A-Sepharose beads and rotated at 4 °C for 2 h. After the supernatant was discarded, protein A-Sepharose beads were washed with cold PBS for 4–5 times, and immunoprecipitates were fractionated by SDS-PAGE.

siRNA Transfection—HepG2 cells were plated into 6-well plates at a density of 2.5 × 10° cells/well. On the following day, the cells were transfected with either siRNA targeting human CDK9 or the negative control siRNA (Ambion, Austin, TX) using TransIT-siQUEST transfection reagent (Mirus, Madison, WI) according to the manufacturer’s instructions. 48 h later, the transfected cells were exposed to IL-6 stimulation prior to total cellular RNA extraction for Real-Time (RT)-PCR.

Two Step ChIP Assay—Two step ChIP was performed as described (27). In brief, 4–6 × 10° HepG2 cells per 100-mm dish were washed twice with PBS after stimulation. Protein-protein cross-linking was first performed with disuccinimidyl glutarate (DSG, Pierce) followed by protein-DNA cross-linking with formaldehyde. After cells were washed and collected in 1 ml of PBS, pellets were lysed by SDS lysis buffer and sonicated 4 times, 15 s each at setting 4 with 10 s break on ice until DNA fragments lengths were between 200 and 1000 bp. Equal amounts of DNA were immunoprecipitated overnight at 4 °C with 4 μg of the indicated Ab in ChIP dilution buffer. Immunoprecipitates were collected with 40 μl of protein A magnetic beads (Dynal Inc., Brown Deer, WI), and washed sequentially with ChIP dilution buffer, high-salt buffer, LiCl wash buffer, and finally in 1× TE buffer. DNA was eluted in 250 μl of elution buffer for 15 min at room temperature. Samples were de-cross-linked in de-cross-linking mixture at 65 °C for 2 h. DNA was phenol/chloroform extracted, precipitated by 100% ethanol and used for RT-PCR.

Quantitative Real-time PCR (Q-RT-PCR)—Total cellular RNA was extracted by Tri Reagent (Sigma). 2 μg of RNA was used for reverse transcription using SuperScript III First-Strand Synthesis System from Invitrogen. 2 μl of cDNA products were amplified in a 20-μl reaction system containing 10 μl of iQ SYBR Green Supermix (Bio-Rad) and 400 nm primer mix. All the primers were designed by PrimerExpress v2.0. For γ-FBG mRNA expression, the primers 5′-GGCAACTGTGCTGAACAGGATTG-3′ and 5′-GATGGCCACGGTGACACTT-3′ were used. The sequences of primer sets used in genomic assays are shown in Table 1. All the reactions were processed in MyiQ Single Color Real-Time PCR thermocycler using two step plus melting curve program, and the results were analyzed by IQ5 program (Bio-Rad). For quantitative real-time genomic PCR (Q-gPCR), a standard curve was generated using a dilution series of genomic DNA (from 40 ng to 25 μg) for each primer pair. The fold change of DNA in each immunoprecipitate was determined by normalizing the absolute amount to input DNA reference and calculating the fold change relative to that amount in unstimulated cells.

Immunofluorescent Staining—HepG2 cells were grown in 6-well tissue culture plates containing sterile coverslips (Fisher). After treatment, cells on coverslips were rinsed by PBS twice and fixed with 4% paraformaldehyde for 20 min at room temperature. After fixation, cells were washed with PBS three times and treated with 0.2% Triton X-100 for 15 min at room temperature prior to immunostaining. All slips were blocked with blocking buffer containing 1% bovine serum albumin and 0.1% Triton X-100 for 1 h and then washed once with PBS. The primary antibody was diluted in blocking buffer (1:50 for anti-pTyr705 STAT3, 1:100 for anti-STAT3 (c-20), and 1:200 for anti-CDK9 (c-20)) and applied to the cells on coverslips. Cells were incubated in the primary antibody at 4 °C overnight. Next day, all slips were washed three times for 10 min each with PBS and then incubated with the diluted fluorescence-labeled secondary antibody (1:100, secondary antibodies for STAT3 and pTyr705STAT3 were from Jackson ImmunoResearch, secondary antibody for CDK9 staining was from Invitrogen) in blocking buffer for 1 h at room temperature in the dark. Cells were washed three times with PBS in low lighting and dried at room temperature. Coverslips were mounted on slides using Dako-Cytomation Fluorescent Mounting Medium and observed under confocal microscope (Zeiss LSM510 META system).

RESULTS

IL-6-inducible γ-FBG Expression Is Mediated by STAT3—Previous studies showed that IL-6 potently up-regulates γ-FBG expression in the human hepatoma cell line, HepG2 (8–11). To confirm this finding, γ-FBG expression was measured over a time course of IL-6 stimulation by Q-RT-PCR. A 2.5-fold increase in γ-FBG mRNA abundance was detected as early as 2 h after IL-6 treatment, and the mRNA level continued to increase until a plateau of 12-fold relative to control was observed at 24 h (Fig. 1A). To determine the transcriptional contribution, a luciferase reporter driven by 643 bp of the γ-FBG promoter containing three functional type II IL-6 response elements (IL-6REs) was constructed. This −607/+36 γ-FBG-LUC plasmid was transiently transfected into HepG2 cells and stimulated in the absence or presence of various doses of IL-6 (10 and 50 ng/ml) for two different times (12 and 24 h).
For the cells stimulated with 10 ng/ml IL-6, we observed an
8-fold induction of normalized luciferase reporter activity
relative to control after 12 h, and 13-fold over control after 24 h of
stimulation (Fig. 1B). At 50 ng/ml IL-6, the inducible activity of
the γ-FBG promoter was increased by 15-fold at 12 h and
24-fold at 24 h (Fig. 1B). To determine the contribution of IL-6-
inducible transcription mediated by STAT3, increasing con-
centrations of dominant-negative (DN-) STAT3 (Tyr705 to Phe,
Ref. 15) were co-transfected with the −607/+36 γ-FBG-LUC
reporter gene. As little as 0.1 μg of DN-STAT3 decreased IL-6-
inducible reporter activity by more than 70% (Fig. 1C). Co-
transfected DN-STAT3 had no significant effects on the basal
activity of the γ-FBG-LUC reporter gene. Together these data
indicated that γ-FBG is an IL-6-inducible gene, mediated at
least in part by STAT3-dependent transcriptional induction.

**IL-6 Induces a Nuclear STAT3-P-TEFb Complex**—A series
of studies have shown that P-TEFb can interact with various tran-
scription factors or nuclear receptors, such as nuclear factor-
kappa B (NF-κB) (28), c-Myc (29), androgen receptor (30), and
peroxisome proliferator-activated receptor-γ (PPAR-γ) (31). To
determine whether STAT3 associates with P-TEFb, HepG2
cells were stimulated in the presence or absence of IL-6 and NE
was subjected to nondenaturing co-immunoprecipitation (Co-
IP) assay using anti-CDK9 as the primary Ab. The immune
complexes were fractionated on an SDS-PAGE, and STAT3
was detected by Western blot (Fig. 2A, upper panel). We
observed STAT3 binding only in the IL-6-stimulated NE. By
contrast, cyclin T1 was also observed in the complex, but there
was no difference in cyclin T1 abundance between IL-6-stimu-
lated and unstimulated NEs (Fig. 2A, middle panel), indicating
that the CDK9-cyclin T1 complex formation is independent of
IL-6 stimulation.

Because IL-6 was required for the association of STAT3 and
CDK9, we next asked whether activated STAT3 isoforms were
interacting with CDK9. After non-denaturing CDK9 IP, the
immune complexes were probed with an antibody that specifi-
cally recognized phospho-Tyr705 STAT3. A strong signal was
specifically detected in the IL-6-stimulated CDK9 complexes
(Fig. 2B, upper panel). By contrast, inactive cytosolic STAT3
failed to interact with CDK9 although CDK9 is expressed con-
stitutively in both the cytosol and nucleus (data not shown).
Together, these data suggest that Tyr phosphorylation and
nuclear translocation are essential for the formation of the
STAT3-CDK9 complex.

After STAT3 is translocated into the nucleus, it recruits the
p300/CBP coactivator, an enzyme with histone acetyltrans-
ferase activity. p300/CBP acetylates two lysines (Lys49, Lys87)
localized at the NH2 terminus of STAT3, thereby stabilizing
STAT3-p300/CBP interaction and facilitating downstream
gene expression (16). To see whether acetylated STAT3 (Ac-
STAT3) associates with CDK9, proteins present in the immune
complexes precipitated by CDK9 antibody were revealed by
immunoblotting with anti-Ac-Lys87STAT3 Ab (16). We
observed that Ac-STAT3 binds CDK9 only in IL-6-stimulated
NE (Fig. 2A, lower panel). We confirmed this
factual because of biochemical fractionation, we confirmed this
interaction using confocal colocalization assays. For this pur-
pose, HepG2 cells were transfected with a plasmid encoding
CDK9 fused to a monomeric strawberry fluorescence protein
pcDNA-FStraw-CDK9, and stimulated with IL-6 prior to fixa-
CDK9-STAT3 Complex in γ-FBG Transcription

A

IL-6: 

- +

IP: CDK9

IgG

IB: STAT3

Cyclin T1

CDK9

B

IL-6: 

- +

IP: CDK9

IgG

IB: p-STAT3

(Y705)

Cyclin T1

CDK9

d

D

DAPI END-O-CDK9 overlay

DAPI straw-CDK9 overlay

IL-6 (+)

E

DAPI STAT3 straw-CDK9 overlay

IL-6 (+)

F

DAPI p-STAT3 Tyr705 straw-CDK9 overlay

IL-6 (+)

FIGURE 2. Activated STAT3 complexes with CDK9 in HepG2 nuclear in the presence of IL-6. A and B, STAT3 and CDK9 form a complex in nuclear in an IL-6-dependent manner. Serum-starved HepG2 cells were treated with IL-6 (10 ng/ml) for 30 min, and NEs were isolated using sucrose gradient fractionation. 2 mg of NE were immunoprecipitated by anti-CDK9 Ab or normal rabbit IgG as control. The immune complexes were fractioned by 10% SDS-PAGE and immunoblotted with anti-STAT3 (A), anti-phospho-STAT3 (Y705) (B), or anti-Ac-K87 STAT3 Abs (C). The blots were then reprobed with cyclin T1 and CDK9 Abs. Because the polyvinylidene difluoride membrane was reprogred with anti-cyclin T1 antibody without stripping, the STAT3 band (indicated by * in A, middle panel, second lane) remained in IL-6-stimulated cells. D, the distribution of straw-CDK9 is similar to that of endogenous CDK9. Endogenous CDK9 (upper panel) was stained by a polyclonal rabbit IgG directed against CDK9 and Alexa 568 goat anti-rabbit secondary Ab. For straw-CDK9 (lower panel), HepG2 cells were transfected with pcDNA3-straw-CDK9 and 24-h later split into 6-well plates containing sterile coverslips. Nuclei were stained by DAPI. E and F, activated STAT3 colocalizes with CDK9 under IL-6 stimulation. Cells were treated with IL-6 (10 ng/ml) for 30 min and then stained for STAT3 or pTyr(705)-STAT3. STAT3 was detected by a rabbit Ab directed against STAT3 (c-20) and a FITC-conjugated goat anti-rabbit secondary Ab. pTyr(705)-STAT3 was recognized by a mouse anti-phospho-Tyr(705) STAT3 Ab (B7) and a FITC-conjugated goat anti-mouse secondary Ab. Straw-CDK9 was transfected and expressed as described in D. Shown is confocal immunofluorescent imaging of representative cells. Empty arrows indicated the co-localization of STAT3 or pTyr(705)-STAT3 with CDK9.

activation. Cells were then stained with anti-STAT3 Ab and secondary FITC-labeled Ab. In the transfected cells, straw-CDK9 is diffusely and constitutively localized in nucleus but excluded from the nucleoli (Fig. 2D, bottom middle). This distribution pattern of Straw-CDK9 is identical to that of the endogenous CDK9 by immunofluorescence labeling (Fig. 2D, top middle). In the unstimulated cells, the majority of STAT3 was detected in cytoplasm (Fig. 2E, top). By contrast, after IL-6 treatment, there is an obvious accumulation of nuclear STAT3 (Fig. 2E, bottom). STAT3-CDK9 co-localization is indicated by the merged overlay (Fig. 2E, bottom right). A similar assay was performed staining for phospho-Tyr(705) STAT3. By contrast with anti-STAT3 labeling, no detectable phospho-Tyr(705) STAT3 was observed in unstimulated cells (Fig. 2F, top). Upon IL-6 stimulation, phospho-Tyr(705) STAT3 was strongly localized to the nucleus, where it colocalized with nuclear CDK9 (Fig. 2F, bottom right). These data confirmed the Co-IP studies and indicated that activated STAT3 co-localizes with CDK9 in the nucleus.

The STAT3 NH2 Terminus Is Sufficient for CDK9 Complex Formation—To map the domain(s) of STAT3 responsible for CDK9 interaction, we first sought to confirm that ectopically expressed V5 epitope-tagged STAT3 (V5-STAT3) associated with endogenous CDK9 in an IL-6-dependent manner. For this purpose, an expression vector encoding full-length V5-STAT3 was transiently transfected into HepG2 cells. NEs were prepared in the absence or presence of IL-6 stimulation, and subjected to nondenaturing Co-IP assays. As seen in Fig. 3A, V5-STAT3 is captured by nondenaturing IP of CDK9 in an IL-6-dependent manner. Note that no complex is seen using IgG as the immunoprecipitating Ab, demonstrating assay specificity. Fig. 3B shows that cyclin T1 is also captured by nondenaturing IP of V5-tagged STAT3. These data indicated that the ectopic V5-STAT3 also inducibly associated with endogenous P-TEFb, containing CDK9 and cyclin T1.

To identify the regions of STAT3 interacting with CDK9, a series of expression vectors encoding COOH domain-deleted V5-STAT3 proteins were constructed (the relevant domains are schematically shown in Fig. 3C). The V5-STAT3 deletion mutants were then expressed in HepG2 cells and the CDK9-bound mutated STAT3 proteins were detected using nondenaturing Co-IP. We observed that all of the STAT3 COOH-terminal deletion mutants (containing amino acids 1–320, 1–465, 1–585, and 1–688) complexed with endogenous CDK9 (Fig. 3D). To further dissect the domain in the NH2 terminus, an expression vector encoding STAT3-(1–130) was constructed and tested for binding by Co-IP (Fig. 3F). STAT3-(1–130) bound endogenous CDK9 in a manner similar to STAT3-(1–320). This finding suggests that NH2-terminal domain of STAT3 is sufficient for the association of STAT3 and CDK9. To determine if the NH2 terminus was necessary for CDK9 interaction, we tested whether the NH2-terminal-deleted STAT3 containing amino acids 131–770 (termed Δ130) could still
interact with CDK9. As seen in Fig. 3F, STAT3 (Δ130) still bound CDK9. Previous work has indicated that the STAT3 COOH transactivation domain, spanning residues 716–770 could bind to in vitro-translated CDK9 (20). We therefore tested whether the STAT3-CDK9 interaction depended on the NH2- and COOH-terminal domains. For this purpose, we constructed an expression vector encoding STAT3-(130–688), missing both NH2 and COOH-terminal activation domains. As we expected, STAT3-(130–688) did not bind CDK9 (Fig. 3G). Together these data indicated that although the STAT3 NH2 terminus was sufficient for CDK9 complex formation, both NH2 and COOH termini participate in complex formation.

To further explore the importance of the NH2-terminal domain for the STAT3 transcriptional activation function, STAT3-(Δ130) was co-transfected with γ-FBG-LUC reporter gene and luciferase reporter activity measured. As shown in Fig. 3H, 0.5 μg of STAT3-(Δ130) decreased IL-6-inducible reporter activity by nearly 30% and 1.0 μg dramatically reduced reporter activity by 97% compared with empty vector controls. In addition, the basal level of luciferase activity was also inhibited by STAT3-(Δ130) in a dose-dependent manner. This result indicates that the NH2 terminus is essential for STAT3 to activate the transcription of downstream genes. Our interpretation of this finding is that STAT3-(Δ130) competes with endogenous STAT3 for promoter binding, and is unable to effectively activate transcription because of its reduced binding affinity for transcriptional elongation factors.

CDK9 Activity Is Required for IL-6-induced Expression of γ-FBG—To investigate the functional role of CDK9 in IL-6-induced expression of γ-FBG, we inhibited CDK9 kinase activity by a chemical inhibitor, flavopiridol (FP). FP is a highly selective P-TEFb inhibitor with a Ki of 3 nM (32). We first tested the effect of FP on IL-6-inducible γ-FBG transcription. HepG2 cells transfected with the γ-H253 FBG-LUC reporter plasmid were pretreated with either vehicle (Me2SO) or FP (500 nM) for 1 h prior to IL-6 stimulation. Both the basal and IL-6-induced activities of γ-FBG-LUC reporter were dramatically decreased when FP was added (Fig. 4A). In addition, FP also inhibited IL-6-induced endogenous γ-FBG mRNA (Fig. 4B). Consistent with these results, expression of a kinase-deficient DN-CDK9 also inhibited the induction of
γ-FBG-LUC reporter in a dose-dependent manner, with greater than 50% inhibition seen with 0.25 μg of expression vector (Fig. 4C). These results indicated that IL-6-inducible expression of γ-FBG was highly dependent on CDK9 kinase activity.

To more specifically confirm the essential role of CDK9 in the γ-FBG induction, short interfering RNA (siRNA) transfection was used to specifically silence endogenous CDK9 expression. As seen in Fig. 4D, transfection of CDK9 siRNA (siCDK9) significantly reduced CDK9 protein levels to less than 20% compared with control siRNA. To examine the effect of CDK9 knockdown on IL-6-inducible γ-FBG expression, abundance of γ-FBG mRNA was measured in a time course of stimulation by Q-RT-PCR. In contrast to control transfectants, the induction of γ-FBG mRNA in siCDK9-transfected cells was significantly decreased at every time point (Fig. 4E). From these results, we concluded that IL-6-induced γ-FBG expression requires CDK9 expression and activity. Although CDK9 is considered to be a general regulator of Pol II-dependent gene transcription, siRNA against CDK9 did not affect GAPDH mRNA expression (data not shown). This indicates that γ-FBG and the housekeeping genes may have different requirements for CDK9.

**IL-6 Induces P-TEFb Recruitment to the γ-FBG Gene**—Three functional type II IL-6 response elements (REs) have been identified on the about 600 bp of the human γ-FBG promoter upstream of the transcription initiation site, and all contribute to full IL-6 inducibility (8). To identify the interaction of STAT3 with these sites, three sets of primers spanning distinct STAT3-responsive region in γ-FBG promoter (RE1, RE2, and RE3) were designed (sequences in Table 1) and optimized by quantitative real time

**TABLE 1**

| Amplified | Primer sequences | Reverse | Primer sequences |
|-----------|------------------|---------|------------------|
| region    | Forward          |         | TGGGCAAGAGGAGACTATGCTTC AG  |
| IL-6RE1   | CTAACGTAGATTGAGACTATGCTTC AG  | AGCTGCCGCTGAGGAGACTATGCTTC AG  |
| IL-6RE2   | ATGGAGAGGAGACTATGCTTC AG  | GAGCTGCCGCTGAGGAGACTATGCTTC AG  |
| TATA      | GTGGAGAGGAGACTATGCTTC AG  | CAGGGGCTGAGGAGACTATGCTTC AG  |
| Exon 5    | ACGAGAGGAGACTATGCTTC AG  | ACTGCCGCTGAGGAGACTATGCTTC AG  |
| Exon 7    | ACGAGAGGAGACTATGCTTC AG  | ACCGCCGCTGAGGAGACTATGCTTC AG  |

**FIGURE 4.** CDK9 activity is required for IL-6-stimulated expression of γ-FBG. A, A CDK9 inhibitor, FP, inhibits γ-FBG-LUC reporter gene activity. HepG2 cells were transfected with γ-FBG-LUC reporter gene and pSV2PAP as an internal control. 24 h after transfection, cells were stimulated with IL-6 alone (Neg) or pretreated with FP (500 nM) for 1 h followed by IL-6 stimulation. The control cells were pretreated with the vehicle dimethyl sulfoxide. 24 h after IL-6 stimulation, cells were collected to measure reporter gene activity. Shown is normalized reporter activity. B, FP blocks IL-6-induced γ-FBG mRNA expression. HepG2 cells were pretreated with FP or dimethyl sulfoxide as described above before 24 h of IL-6 stimulation. γ-FBG and GAPDH mRNA expressions were assayed by Q-RT-PCR. The fold change of γ-FBG in IL-6-treated cells over IL-6-unstimulated control was obtained after correction for the amount of GAPDH. C, DN-CDK9 inhibits the IL-6 induction of the γ-FBG reporter gene. Different amounts of DN-CDK9 were cotransfected with γ-FBG-LUC reporter gene. Cells were then treated with IL-6 (10 ng/ml) for 24 h or left unstimulated prior to reporter gene assay. Data shown are means ± S.D. from three independent transfections. D, CDK9 siRNA transfection efficiently inhibits CDK9 expression. HepG2 cells grown in 6-well plates were transiently transfected with 100 nM CDK9 siRNA (siCDK9), control siRNA (Con) or transfection reagent alone (Neg). 72 h after transfection, equivalent amounts of protein from the whole cell lysates were used for immunoblot. Top panel, CDK9 staining; bottom, β-actin staining as a loading control. E, siRNA transfection was performed as described in D. 48 h after siRNA transfection, cells were treated with IL-6 (10 ng/ml) for indicated times. The γ-FBG mRNA expression and the fold of induction were assayed by Q-RT-PCR. The results from Q-RT-PCR are expressed as means ± S.D. from duplicates. The data were analyzed by Student’s t test. *, p value <0.05; **, p value <0.01.
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A

B

C

D

E

F

G

H

I

Figure A: Schematic representation of the γ-FBG promoter region.

Figure B: IP DNA (% of input) for IL-6 RE1, IL-6 RE2, and IL-6 RE3 under 0 and 30m conditions.

Figure C: IP DNA (% of input) for IL-6 RE1, TATA, exon5, and exon7 under 0 and 30m conditions.

Figure D: IP DNA (% of input) for TATA, exon5, and exon7 under 0 and 30m conditions.

Figure E: IP DNA (% of input) for TATA, exon5, and exon7 under 0 and 30m conditions.

Figure F: IP DNA (% of input) for TATA, exon5, and exon7 under FP pretreatment and RNA pol II conditions.

Figure G: IP DNA (% of input) for TATA, exon5, and exon7 under FP pretreatment and phospho-RNA pol II conditions.

Figure H: IP DNA (% of input) for IL-6 RE1, IL-6 RE2, and IL-6 RE3 under 0, 30m, FP, FP-0, and FP-30m conditions.

Figure I: IP DNA (% of input) for IL-6 RE1, TATA, exon3, and exon5 under 0, 30m, FP, FP-0, and FP-30m conditions.
We next examined the effect of IL-6 on inducible CDK9 binding to the γ-FBG promoter. In a pattern similar to that observed for STAT3, IL-6 induces 2-fold increase of CDK9 binding to the upstream γ-FBG IL6 RE1 (Fig. 5C), but no significant recruitment was observed on the RE2 and RE3 (data not shown). The same DNA was examined for changes in CDK9 binding to the TATA box region (spanning nt −66 to +6), exon 5 (nt +2524 to +2606), and exon 7 (nt +4148 to +4239). Here, IL-6 induced 2.3-fold binding to the TATA box, a 4-fold increase on exon 5 and a 2.5-fold increase on exon 7 within 30 min after stimulation, suggesting that CDK9 may accompany the elongating polymerase during transcription (Fig. 5C).

IL-6 Induces Pol II Recruitment to the γ-FBG Gene—To further understand the role of CDK9 recruitment in IL-6 stimulation, the binding of RNA Pol II and phospho-Ser2 CTD Pol II was examined by two step ChIP assay. IL-6 induced a 3-fold increase in total Pol II binding on the γ-FBG TATA box, and strongly induced Pol II loading on the coding sequences (Fig. 5D). Because CDK9 is a kinase for serine 2 of the Pol II CTD, two step ChIP was performed using an anti-phospho-Ser2 CTD Pol II Ab. We observed a 3-fold increase of phospho-Ser2 CTD Pol II binding to the TATA box, and greater than 8-fold increase on exon 5 (Fig. 5E). We noted that the distribution pattern of CDK9 was similar to that of phospho-Ser2 CTD Pol II, supporting the notion that CDK9 is the IL-6-inducible Ser2-CTD kinase.

To further establish this relationship, we investigated the effects of FP on IL-6-inducible total and phospho-Ser2 CTD Pol II recruitment. In this experiment, HepG2 cells were pretreated with FP (500 nM) before IL-6 stimulation. The chromatin was processed for two step ChIP assay using anti-Pol II (Fig. 5F) and phospho-Ser2 CTD Pol II (Fig. 5G) Abs. We found that IL-6-induced occupancy of the TATA box, exon 5, and exon 7 by RNA Pol II as well as phospho-Ser2 CTD Pol II was significantly inhibited by FP. These results suggest that CDK9 is required for RNA pol II recruitment and licensing it to enter transcription elongation mode, thereby promoting IL-6-inducible γ-FBG gene expression. To exclude the possibility that FP interferes with STAT3 and CDK9 recruitment, their binding to the γ-FBG gene in the presence of FP was assayed by two step ChIP. IL-6-induced STAT3 and CDK9 occupancy of the γ-FBG promoter and coding region were not significantly affected by FP pretreatment (Fig. 5, H and I).

FP Inhibits Basal and IL-6-inducible Phospho-Ser2 CTD Pol II Formation—Because RNA Pol II is not the only substrate of CDK9 (33–37), we tested whether CDK9 could also phosphorylate STAT3. HepG2 cells treated in the absence or presence of FP were then IL-6-stimulated. Western blots were performed using an anti-phospho-Tyr705 and anti-phospho-Ser727 STAT3 Abs (the latter modification is known to be essential for maximal transcriptional activity of STAT3 (38)). We observed the strong IL-6-inducible STAT3 Tyr and Ser phosphorylation were unaffected by FP (Fig. 6, A and B). As an additional determination, we observed that the IL-6-inducible STAT3:CDK9 association was unaffected by FP treatment (Fig. 6C), suggesting that CDK9 kinase activity is not essential for complex formation. Because CDK9 is thought to be a major Pol II Ser2-CTD kinase we examined its effect on total Pol II- and phospho-Ser2 CTD Pol II abundance. Although FP did not affect total Pol II abundance (Fig. 6D), the general levels of phospho-Ser2 CTD

**FIGURE 5. Recruitment of CDK9 and Pol II to the γ-FBG gene after IL-6 stimulation.** A, schematic diagram of Q-gPCR primers on the γ-FBG promoter. Primer pairs spanning the IL-6REs, TATA box, and exons 5 and 7 were designed and optimized (See Table 1 for sequence information). B to I, serum-starved HepG2 cells were treated with IL-6 (10 ng/ml) alone for 30 min (from B to E) or FP (500 nM) pretreated for 1 h (from F–I), and two step ChIP assay was performed as described under “Experimental Procedures.” The sequences in the promoter or coding region of the γ-FBG gene in the immunoprecipitates were amplified by Q-gPCR using specific primer sets as shown in Table 1. B, STAT3 recruits to IL-6 REs on γ-FBG promoter after IL-6 stimulation. C, IL-6 induces CDK9 recruitment to the IL-6 RE1, TATA box as well as exons 5 and 7. D and E, IL-6 increases the Pol II and phospho-Ser2 CTD Pol II loading to the endogenous γ-FBG gene. F and G, FP inhibits the recruitment of RNA pol II and phospho-Ser2 CTD Pol II to the TATA box and coding region. H and I, FP pretreatment does not affect the inducible STAT3 and CDK9 binding to the γ-FBG gene. The results are expressed as means ± S.D. from duplicates. The data were analyzed by Student's t test. *, p value < 0.05; ***, p value <0.01.
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Pol II was significantly decreased (Fig. 6E). We therefore conclude that FP specifically inhibits phospho-Ser2 CTD Pol II formation without affecting STAT3 activation or complex formation with CDK9.

DISCUSSION

FBG is an APP that plays key roles in fibrin clot formation, platelet aggregation and wound repair by binding to cell surface receptors or growth factors through its γ-chain. Previous studies have shown the acute phase induction of γ-FBG in liver cells is mainly regulated by the cytokine-inducible STAT3 transcription factor. In this study, we further investigated the molecular mechanism by which IL-6-inducible γ-FBG transcription is regulated by the STAT3-P-TEFb complex. We found that IL-6 induces a formation of STAT3-CDK9 complex mediated by both the STAT3 NH2 and COOH termini. Moreover, activated pTyr705 STAT3 and Ac-Lys87 STAT3 were preferentially complexed with CDK9. Quantitative two step ChIP assays indicate that IL-6 induces STAT3, CDK9, Pol II, and phospho-Ser2 CTD Pol II recruitment to the γ-FBG gene. Finally, our studies indicate that CDK9 is required for γ-FBG expression because siRNA transfection and inhibition of CDK9 kinase activity both inhibit IL-6-inducible transcription. These studies indicate that P-TEFb is a critical regulator of STAT3-dependent gene activation in the APR.

STAT3 is a central transcription factor in IL-6-induced hepatic APRs. The transcription of APPs controlled by STAT3 is regulated at multiple levels. First, IL-6 induces tyrosine phosphorylation of STAT3 in its COOH terminus, leading to its dimerization and nuclear translocation. The activated STAT3 then recognizes specific motifs in the promoters of target genes and initiates assembly of the basal transcriptional apparatus (39). At this point, STAT3 recruits p300/ CBP coactivators containing histone acetyltransferase activity (HAT) and the BRG1 chromatin-remodeling complex. HAT regulates transcription by acetylating the amino-terminal histone tail, increasing accessibility of chromatin-condensed templates to the transcriptional machinery (19), whereas chromatin-remodeling complexes function by altering nucleosomal structure and increasing the accessibility of Pol II to the proximal promoter (40). Both these activities promote target gene activation by relieving repression and facilitating the loading of the pre-initiation complex. The findings of our study add a new dimension to how STAT3 mediates gene expression. STAT3 not only induces transcription initiation, but also regulates transcription elongation through its association and recruiting P-TEFb to its target genes.

The P-TEFb complex has been shown to play an important role in Pol II-dependent transcription by its ability to release RNA pol II from transcriptional arrest, allowing production of full-length mRNA transcripts (41). Experiments using CDK9 inhibitors strongly indicate that CDK9 activity is required for both HIV transcription and the expression of many cellular genes. Tat, a viral transactivator encoded by HIV and other retroviral genomes (42, 43), is able to recruit the CDK9-cyclinT1 complex to the TAR element of the HIV promoter (44), and position CDK9 to phosphorylate the negative elongation factors as well as the RNA Pol II CTD, thereby enabling transcriptional elongation (43, 45–47). In addition to Tat, recent studies have identified other cellular transcription factors that associate with P-TEFb, including CIITA, NF-κB, c-Myc, and p53 (28, 29, 37, 48, 49). The involvement of CDK9 in regulating STAT3-dependent cell cycle regulatory genes was first reported for the p21waf1 gene (20). The authors found that DRB, another P-TEFb inhibitor, inhibits p21waf1 expression as well as RNA Pol II recruitment to p21waf1. Although these results indicated that CDK9 was required for STAT3 ability to control expression of cell cycle regulatory genes, there may be significant heterogeneity in the mechanisms of transcriptional induction between different classes of STAT3-responsive genes and to what extent the CDK9 was required for activation of the APPs was unknown. Using γ-FBG as a model gene of the APR, our study here extends the requirement of CDK9 in STAT3-dependent APP activation. Transient transfection assays revealed that CDK9 activity is required for the activation of γ-FBG promoter transcription (Fig. 4, A and C), and Q-RT-PCR showed that CDK9 knockdown significantly suppresses the endogenous γ-FBG expression during the 24-h time course (Fig. 4E).

Although the STAT3-CDK9 interaction was reported before (20), our study extends this previous work by: 1) demonstrating the interaction using an independent technique of confocal colocalization; 2) demonstrating that tyrosine-phosphorylated and Lys-acetylated STAT3 is found in the complex with CDK9; and, 3) discovering that the STAT3 NH2 terminus participates in CDK9 complex formation and transcriptional activation.

Co-IP and confocal colocalization showed that the inducible STAT3-CDK9 complex is rapidly formed in the nucleus within 30 min after IL-6 stimulation (Fig. 2). Moreover, only the activated, nuclear translocated STAT3 complexes with CDK9, even though CDK9 is also found in the cytoplasm. We interpret this finding to mean that the tyrosine phosphorylation produces a conformational change in STAT3, exposing the CDK9-interacting domains at NH2 and COOH termini. Currently our data do not prove that the NH2 terminus of STAT3 binds to CDK9 through a direct protein-protein interaction. Therefore, another possibility could be that the STAT3-CDK9 interaction is indirectly mediated through other protein-protein interactions that are mapped to the STAT3 NH2 terminus. In this regard, a recent finding from our laboratory shows that the STAT3 NH2 terminus is sufficient for the interaction with p300/CBP, an enzyme that acetylates two lysine residues (Lys48, Lys87) in this domain (16). These acetylations increase the stability of the STAT3-p300/CBP complex, and are indispensable for STAT3-dependent target gene expression (16). This finding indicates the possibility that STAT3 NH2 terminus-CDK9 interaction is indirectly mediated by p300/CBP. The NH2-terminal domain is highly conserved in STAT members. According to previous studies, NH2 terminus is required for cooperative binding of STAT4 dimers to adjacent recognition sites on DNA (50). It also regulates multiple protein-protein interactions important for the functions of STAT1 and STAT2 (51–55). However, little is known about the function of NH2 terminus in STAT3. Our findings reveal that the NH2 terminus is involved in the interactions between STAT3, the p300/CBP coactivator and the P-TEFb transcriptional elongation com-
plex. The important role of the NH₂ terminus for STAT3 function can also be seen in the finding that NH₂-terminal-deleted mutant (Δ130) repressed both the basal and IL-6-inducible activities of γ-FBG-LUC reporter gene (Fig. 3H). Although STAT3-Δ130 has promoter binding activity, it could not effectively induce transcription because it is unable to successfully recruit coactivators or transcriptional elongation factors.

Although it is known that P-TEFb is generally required for transcription elongation, an unanswered question is whether P-TEFb is recruited to all promoters and regulates downstream gene transcription by similar mechanisms. The existence of eight potential P-TEFb complexes resulting from different combinations of two CDK9 isoforms (56) and four types of cyclins (57) suggest the possibility that unique P-TEFb complexes might be differentially recruited by inducible transcription factors for different genes. Consistent with this notion, the requirement for CDK9 varies widely among genes. For example, HIV replication can be inhibited by FP at concentrations that have no detectable effect on cellular genes transcription (32, 58). Also, a recent study found that some p53 target genes, including p21 and PUMA, are activated when CDK9 activity is inhibited, suggesting a specific subset of p53 target genes can bypass the requirement of CDK9 activity for expression (59).

P-TEFb is recruited to all promoters and regulates downstream transcription elongation, an unanswered question is whether CDK9 an appealing target for therapeutic intervention. Although it is known that P-TEFb is generally required for transcription elongation of the γ-FBG gene, CDK9 regulates IL-6-induced γ-FBG transcription via a mechanism involving increased binding of total and phosphorylated RNA Pol II to γ-FBG. Considering the important roles of FBG in inflammation and cancer, this finding has functional significance, making CDK9 an appealing target for therapeutic intervention.

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REFERENCES

1. Jiang, S. L., Samols, D., Sipe, J., and Kushner, I. (1992) Folia Histochem. Cytobiol. 30, 133–135
2. Rooney, M. M., Parise, L. V., and Lord, S. T. (1996) J. Biol. Chem. 271, 8553–8555
3. Hawiger, J. (1995) Semin Hematol. 32, 99–109
4. Ugarova, T. P., Lishko, V. K., Podolnikova, N. P., Okumura, N., Merkulov, S. M., Yakubenko, V. P., Yee, V. C., Lord, S. T., and Haas, T. A. (2003) Biochemistry 42, 9365–9373
5. Sahni, A., and Francis, C. W. (2000) Blood 96, 3772–3778
6. Sahni, A., Altland, O. D., and Francis, C. W. (2003) J. Thromb. Haemost. 1, 1304–1310
7. Sahni, A., Guo, M., Sahni, S. K., and Francis, C. W. (2004) Blood 104, 409–414
8. Duan, H. O., and Simpson-Haidaris, P. J. (2003) J. Biol. Chem. 278, 41270–41281
9. Mizuguchi, J., Hu, C. H., Cao, Z., Loeb, K. R., Chung, D. W., and Davie, E. W. (1995) J. Biol. Chem. 270, 28350–28356
10. Ray, A. (2000) J. Immunol. 165, 3411–3417
11. Duan, H. O., and Simpson-Haidaris, P. J. (2006) J. Biol. Chem. 281, 12451–12457
12. Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Dave, L. (1998) Biochem. J. 334, 297–314
13. Ramji, D. P., Vitelli, A., Tronche, F., Cortese, R., and Ciliberto, G. (1993) Nucleic Acids Res. 21, 289–294
14. Hagihara, K., Nishikawa, T., Sugamata, Y., Song, J., Isole, T., Tabi, T., and Yoshizaki, K. (2005) Genes Cells 10, 1051–1063
15. Sherman, C. T., and Brasier, A. R. (2001) Mol. Endocrinol. 15, 441–457
16. Ray, S., Boldogh, I., and Brasier, A. R. (2005) Gastroenterology 129, 1616–1632
17. Ray, S., Sherman, C. T., Lu, M., and Brasier, A. R. (2002) Mol. Endocrinol. 16, 824–836
18. Ni, Z., and Brenner, R. (2007) J. Immunol. 178, 345–351
CDK9-STAT3 Complex in γ-FBG Transcription

19. Kouzarides, T. (1999) Curr. Opin. Genet. Dev. 9, 40–48
20. Giraud, S., Hurlstone, A., Avril, S., and Coqueret, O. (2004) Oncogene 23, 7391–7398
21. Hirano, T., Ishihara, K., and Hibi, M. (2000) Oncogene 19, 2548–2556
22. Haga, S., Terui, K., Zhang, H. Q., Enosawa, S., Ogawa, W., Inoue, H., Okuyama, T., Takeda, K., Akira, S., Ogino, T., Irani, K., and Ozaki, M. (2003) J. Clin. Investig. 112, 989–998
23. Garriga, J., and Grana, X. (2004) Gene 337, 15–23
24. Brasier, A. R., Tate, J. E., and Habener, J. F. (1989) BioTechniques 7, 1116–1122
25. Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004)Nat. Biotechnol. 22, 133–142
26. Madore, S. J., and Cullen, B. R. (1993) J. Virol. 67, 3703–3711
27. Nowak, D. E., Tian, B., and Brasier, A. R. (2005) BioTechniques 39, 715–725
28. Barboric, M., Nissen, R. M., Kanazawa, S., Jabrane-Ferrat, N., and Peterlin, B. M. (2006)J. Biol. Chem. 281, 175–185
29. Eberhardy, S. R., and Farnham, P. J. (2002) J. Biol. Chem. 277, 40156–40162
30. Lee, D. K., Duan, H. O., and Chang, C. (2001) J. Biol. Chem. 276, 9978–9984
31. Iankova, I., Petersen, R. K., Annicotte, J. S., Chavey, C., Hansen, J. B., Strehlow, I., and Schindler, C. (1998) EMBO J. 17, 7056–7065
32. Shuster, S. H., Fujinaga, K., Marion, J. E., Taube, R., Sausville, E. A., Senderowicz, A. M., Peterlin, B. M., and Price, D. H. (2000) J. Biol. Chem. 275, 28345–28348
33. Grana, X., De Luca, A., Sang, N., Fu, Y., Claudio, P. P., Rosenblatt, J., Morgan, D. O., and Giordano, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3834–3838
34. Song, A., Wang, Q., Goebi, M. G., and Harrington, M. A. (1998) Mol. Cell. Biol. 18, 4994–4999
35. Kim, J. B., and Sharp, P. A. (2001)J. Biol. Chem. 276, 12317–12323
36. Radhakrishnan, S. K., and Gartel, A. L. (2006)Cell Cycle 5, 519–521
37. Claudio, P. P., Cui, J., Ghafouri, M., Mariano, C., White, M. K., Safak, M., Sheffield, J. B., Giordano, A., Khalili, K., Amini, S., and Sawaya, B. E. (2006)J. Cell. Physiol. 208, 602–612
38. Wen, Z., Zhong, Z., and Darnell, J. E., Jr. (1995) Cell 82, 241–250
39. Levy, D. E., and Darnell, J. E., Jr. (2002)Nat. Rev. Mol. Cell. Biol. 3, 651–662
40. Roberts, C. W., and Orkin, S. H. (2004) Nat. Rev. Cancer 4, 133–142
41. Price, D. H. (2000) Mol. Cell. Biol. 20, 2629–2634
42. Rana, T. M., and Jeang, K. T. (1999)Arch. Biochem. Biophys. 365, 175–185
43. Wimmer, J., Fujinaga, K., Taube, R., Cujec, T. P., Zhu, Y., Peng, J., Price, D. H., and Peterlin, B. M. (1999)Virology 255, 182–189
44. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998)Cell 92, 451–462
45. Zhu, Y., Pe’ery, T., Peng, J., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997)Genes Dev. 11, 2622–2632
46. Ping, Y. H., and Rana, T. M. (2001)J. Biol. Chem. 276, 12951–12958
47. Bieniasz, P. D., Girdina, T. A., Bogerd, H. P., and Cullen, B. R. (1998)EMBO J. 17, 7056–7065
48. Kanazawa, S., Okamoto, T., and Peterlin, B. M. (2000)Immunity 12, 61–70
49. Eberhardy, S. R., and Farnham, P. J. (2001)J. Biol. Chem. 276, 48562–48571
50. Xu, X., Sun, Y. L., and Hoye, T. (1996)Science 273, 794–797
51. Zhang, J. J., Vinkemeier, U., Gu, W., Chakravarti, D., Horvath, C. M., and Darnell, J. E., Jr. (1996)Proc. Natl. Acad. Sci. U. S. A. 93, 15092–15096
52. Vinkemeier, U., Cohen, S. L., Moarefi, I., Chait, B. T., Kuriyan, J., and Darnell, J. E., Jr. (1996)EMBO J. 15, 5616–5626
53. Leung, S., Qureshi, S. A., Kerr, I. M., Darnell, J. E., Jr., and Stark, G. R. (1995)Mol. Cell. Biol. 15, 1312–1317
54. Murphy, T. L., Geissal, E. D., Farrar, J. D., and Murphy, K. M. (2000)Mol. Cell. Biol. 20, 7121–7131
55. Streith, I., and Schindler, C. (1998)J. Biol. Chem. 273, 28049–28056
56. Shore, S. M., Byers, S. A., Maury, W., and Price, D. H. (2003)Gene 307, 175–182
57. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998)Genes Dev. 12, 755–762
58. Chao, S. H., and Price, D. H. (2001)J. Biol. Chem. 276, 31793–31799
59. Gomes, N. P., Bjerke, G., Llorente, B., Szostek, S. A., Emerson, B. M., and Espinoza, J. M. (2006)Genes Dev 20, 601–612
60. Yang, X., Gold, M. O., Tang, D. N., Lewis, D. E., Aguilar-Cordova, E., Rice, A. P., and Herrmann, C. H. (1997)Proc. Natl. Acad. Sci. U. S. A. 94, 12331–12336
61. Garriga, J., Peng, J., Parreno, M., Price, D. H., Henderson, E. E., and Grana, X. (1998)Oncogene 17, 3093–3102
62. Herrmann, C. H., Carroll, R. C., Wei, P., Jones, K. A., and Rice, A. P. (1998)J. Virol. 72, 9881–9888
63. Ghose, R., Liou, L. Y., Herrmann, C. H., and Rice, A. P. (2001)J. Virol. 75, 11336–11343
64. Marshall, R. M., Salerno, D., Garriga, J., and Grana, X. (2005)J. Immunol. 175, 6402–6411
65. Fu, J., Yoon, H. G., Qin, J., and Wong, J. (2007)Mol. Cell. Biol. 27, 4641–4651