The role of Stat and C/EBP transcription factors in the synergistic activation of rat serine protease inhibitor-3 gene by interleukin-6 and dexamethasone

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The rat serine proteinase inhibitor 3 gene is activated by interleukin 6 (IL-6) and glucocorticoids in hepatic cells. We report here that a 147 bp promoter is sufficient for both IL-6 stimulation and glucocorticoid enhancement of IL-6 induced transcription. Within this region we identified two functional elements binding transcription factors from the C/EBP (CCAAT/enhancer binding proteins) and Stat (signal transducers and activators of transcription) families. Mutations introduced into the Stat binding site resulted in a loss of responsiveness, showing that this element is indispensable for activation. In contrast, the promoter containing the mutated C/EBP binding site was still responsive to IL-6 and glucocorticoids; however, the magnitude of the induction was decreased by 50 %. The Stat binding element is an enhancer capable of conferring both responsiveness to IL-6 and partial enhancement of glucocorticoids on to a heterologous promoter. In response to IL-6 this element rapidly binds acute-phase response factor (APRF/Stat3) and, later, the protein(s) that require ongoing protein synthesis and is recognized by anti-Stat3 antibodies. In addition, long-term treatment with IL-6 results in sustained phosphorylation of APRF/Stat3.

INTRODUCTION

Infection, injury or trauma of a tissue causes a series of complex host reactions that lead to a return to normal function. During this process, known as the acute-phase reaction, cytokines released by different cell types transmit their signals to the hepatocytes, triggering the induction of synthesis of so-called acute-phase proteins (APP) [1,2], with interleukin-6 (IL-6), interleukin-1 (IL-1) and glucocorticoids playing the central role (reviewed in [1]).

The 5′ flanking regions of many APP genes have been studied in detail to identify regulatory elements mediating cytokine induction. Two types of IL-6 response element (IL-6RE) can be found in APP genes, the type I with the consensus sequence T(T/G)NNGNAA(T/G) [3] and the type II with the consensus sequence TT(C/A)CNG(G/T)AA [4,5]. The nuclear factors that bind to the type I IL-6RE belong to the C/EBP (CCAAT/enhancer binding proteins) family. The type II IL-6REs were first identified in some of the APP genes (i.e. α_2-macroglobulin, fibrinogen) [4,6] and later found in several immediate-early genes (*junB*, *IRF*-1, *ICAM*-1) [7,8]. The binding of two proteins was demonstrated, with the first being described as the factor termed IL-6 response element binding protein (IL-6REBP), which was shown to bind to the rat α_2-macroglobulin IL-6RE after IL-6 stimulation of various human cell lines [6]. This factor was maximally induced 4 h after IL-6 treatment and required ongoing protein synthesis. The acute-phase response factor (APRF) was shown to bind transiently to the same element within minutes after IL-6 induction, and this activation occurred in the absence of ongoing protein synthesis [5]. The latent APRF is rapidly activated by phosphorylation at the tyrosine residue by the Janus family of kinases (JAK) after IL-6, LIF, OSM, IL-11 and CNTF binding to their complex receptors [9,10]. Recently, serine phosphorylation was shown to be a requirement for formation of APRF–promoter complexes [11,12]. The mouse and human APRF/Stat3 have been cloned and found to be related to the family of proteins referred as Stat(s) (signal transducers and activators of transcription), which are activated by cytokines and growth factors [13–16].

Three highly similar serine proteinase inhibitors (SPI-1, SPI-2 and SPI-3) (contrapsins) are synthesized in rat liver [17] but only the expression of SPI-3 is strongly up-regulated during acute-phase response [18]. In healthy rats the expression of the SPI-3 gene is barely detectable; however, within 2 h after intraperitoneal injection of lipopolysaccharide the induction of SPI-3 mRNA can be observed. Moreover, the induction of SPI-3 expression could be observed as soon as 1 h after IL-6 stimulation of cultured primary rat hepatocytes, and maximal levels of SPI-3 mRNA were achieved 24 h later [19]. Therefore the SPI-3 gene represents a potentially very interesting model system to study induction by IL-6

The aim of this study was to show the contribution of Stat and C/EBP binding elements in the induction of target promoter(s) and to correlate the binding of induced factors with the activation of gene expression in response to IL-6. We identify two elements present in the promoter of SPI-3 gene binding transcription

Abbreviations used: APP, acute-phase proteins; APRF/Stat3, acute-phase response factor; CAT, chloramphenicol acetyltransferase, C/EBP; CCAAT/enhancer binding proteins; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; IL-6RE, IL-6 response element; IL-6REBP, IL-6 response element binding protein; SPI, serine proteinase inhibitor; Stat, signal transducers and activators of transcription.

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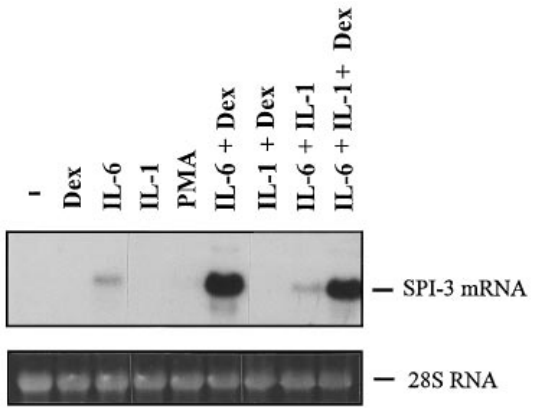

Figure 1 Northern blot analysis of SPI-3 mRNA in H-35 cells

H-35 cells were stimulated with IL-6 (50 ng/ml), IL-1 (10 ng/ml), phorbol 12-myristate 13-acetate (100 ng/ml) and Dex (100 nM) for 18 h. RNA was isolated and analysed by Northern blotting, with mouse contrapsin complementary DNA as a probe. The lower panel shows the 28 S rRNA stained with ethidium bromide on the membrane.

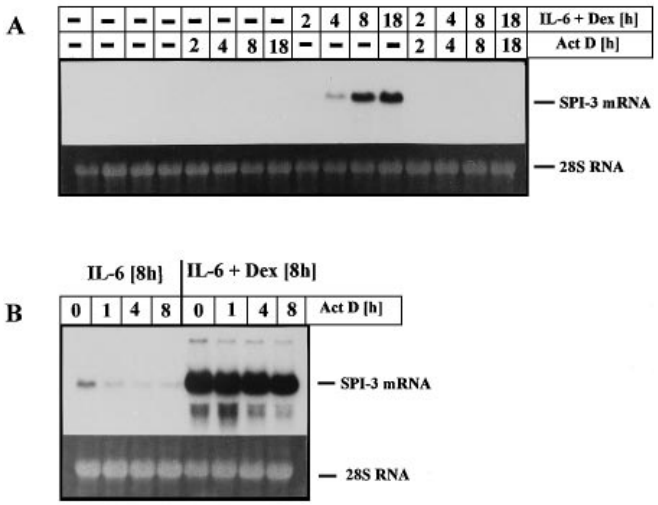

Figure 2 Effect of actinomycin D on the IL-6- and Dex-induced SPI-3 gene expression

(**A**) H-35 cells were treated with IL-6 (50 ng/ml), Dex (100 nM) and (or) actinomycin D (5 μg/ml). After the times indicated, RNA was isolated and subjected to Northern blot analysis. (**B**) H-35 cells were prestimulated with 50 ng/ml IL-6, alone or with 100 nM Dex for 8 h, and actinomycin D (5 μg/ml) was added. RNA was isolated at the indicated times and Northern blot analysis was performed. The lower panels show the 28 S rRNA stained with ethidium bromide on the membrane.

factors from the Stat and C/EBP families and showed that their role is not equal. The Stat binding site is indispensable for SPI-3 induction by IL-6. In contrast, the C/EBP binding site is not necessary for this induction, but mutation of the site decreased the induction by 50%. We also show that IL-6REBP is at least antigenically related to APRF/Stat3 and that long-term treatment of the cells with IL-6 results in sustained phosphorylation of APRF/Stat3-related protein.

EXPERIMENTAL

Cell culture and stimulation

Primary rat hepatocytes were prepared by liver perfusion [20]. Human hepatoma HepG2 cells, rat hepatoma H-35 cells (a gift from Dr. H. Baumann, Buffalo, NY, U.S.A.) and primary rat hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/v) fetal calf serum and antibiotics. Cells were stimulated with 50 ng/ml (10^7 units/mg) hrIL-6 (a gift from Immunex, Seattle, WA, U.S.A.), 10 ng/ml (10^7 units/mg) hrIL-1 (a gift of Dr. D. Schenk from Athena Neurosciences Inc., San Francisco, CA, U.S.A.), 100 ng/ml phorbol 12-myristate 13-acetate (Sigma) or 100 nM dexamethasone (Dex) (Sigma). Cycloheximide (10 μg/ml) was added to the medium 1 h before IL-6 treatment.

RNA preparation and Northern blot analysis

Total RNA was prepared by using the phenol extraction method [21,22]; 5 μg samples of RNA were subjected to formaldehyde gel electrophoresis by standard procedures [23] and transferred to Hybond-N membranes (Amersham), according to the manufacturer's instructions. The filters were prehybridized at 68 °C for 3 h in 10% (w/v) dextran sulphate, 1 M sodium chloride and 1% SDS, and hybridized in the same solution with complementary DNA fragments labelled by random priming [24]. After the hybridization, non-specifically bound radioactivity was removed by washing in 2 × SSC at room temperature, followed by two washes in 2 × SSC/1% (w/v) SDS at 68 °C for 20 min.

Synthetic oligonucleotides

The following oligonucleotides were used to obtain the 1021 bp 5′ region of the SPI-3 gene: 5′-ATGATCTAGAAGTTCTTAGCATGGG-3′ (−1027 to −1001) and 5′-GTTGGGATCCCCAGTGCACAGGGAT-3′ (+3 to −22). To obtain a deletion mutant containing 147 bp of 5′ region we used the following PCR primer: 5′-AGAATCTAGAGACTATGAGTCCA-3′. Mutants containing point mutations in the Stat3/APRF and C/EBPβ recognition sequences were generated by PCR, using as primers: 5′-CGACTCTAGAGACTATGAGTCCATGTTCGCTCAAATCA-3′, 5′-CAGCGAATTCTAAGAGGAGGGA-3′ and 5′-CTTAGAATTCGCTGGCATGGACAAG-3′ respectively. All oligonucleotides used for gel retardation assays were designed to contain single-stranded 5′ overhangs of four bases at both ends after annealing. The following double-stranded oligonucleotides were used as labelled probes or competitors in gel retardation assays (top strands are shown): rat SPI-3 APRF/Stat3 site, 5′-GATCTGTTCCCAGAAA-3′; rat α_2-macroglobulin APRE core site [5], 5′-GATCCTTCTGGGAATTCCTA-3′; human haemopexin A site [25], 5′-AGCTTATTTGCAGTGATGTAATCAGCA-3′ and human C-reactive protein gene promoter α site (CRPα) [26], 5′-AGCTTCATAGTGGGGAAACTCCCTTACTGA-3′.

Plasmid construction

A DNA fragment from the 5′ flanking region of the SPI-3 gene (−1021 to +3) was synthesized by PCR with oligonucleotides described above as primers, Sprague–Dawley rat liver genomic DNA as a template, and $Vent_R$ DNA Polymerase (NEB). Plasmid ptkCATΔEH (a gift of Dr. F. Horn, Aachen, Germany) is a derivative of pBLCAT2 [27] from which the *Eco*O109I–*Hin*dIII fragment (harbouring the cryptic enhancer) was deleted. The plasmid pS3CAT(−1021) was obtained by insertion of the

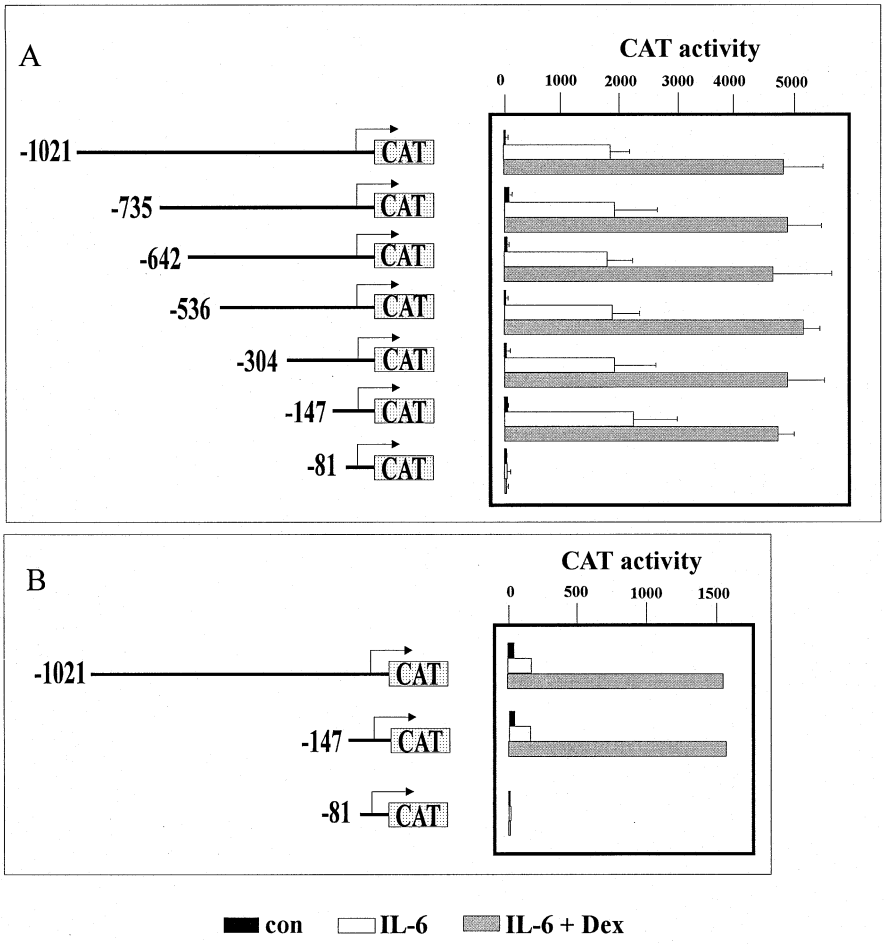

Figure 3 Localization of the IL-6 response region in the SPI-3 promoter

HepG2 cells (**A**) or primary rat hepatocytes (**B**) were transiently transfected with 5 μg of plasmid pS3CAT (-1021), pS3CATΔ1, pS3CATΔ2, pS3CATΔ3, pS3CATΔ4, pS3CATΔ7 or pS3CATΔ8 and 2 μg of β-galactosidase expression vector pCH110 as an internal control for transfection efficiency. One day after transfection, cells were stimulated with 50 ng/ml IL-6, alone or with 100 nM Dex, cultured for another 24 h and harvested. CAT activities were normalized to β-galactosidase activities (c.p.m. per unit) and are means $\pm$ S.E.M. for HepG2 cells (three to seven determinations); a representative example is shown for primary rat hepatocytes.

*Xba*I/*Bam*HI-digested PCR product into *Xba*I and *Bgl*II sites of ptkCATΔEH. Plasmids pS3CATΔ1, pS3CATΔ2, pS3CATΔ3 and pS3CATΔ4 containing 735 bp, 642 bp, 536 bp and 304 bp of the 5′ region of the SPI-3 gene were generated by exonuclease III progressive digestion [28]. Plasmids pS3CATΔ7, containing 147 bp of 5′ flanking region, pS3(mutStat3)CAT and pS3(mutC/EBPβ)CAT analogous to the pS3CATΔ7 but with introduced point mutations in the Stat3/APRF or C/EBPβ binding elements, were generated by insertion of *Xba*I/*Bam*HI-digested PCR products into *Xba*I and *Bgl*II sites of ptkCATΔEH. Plasmid pS3CATΔ8 (containing 81 bp of the 5′ region of the SPI-3 gene) was obtained by insertion of an *Alu*I–*Xho*I fragment from SPI-3 promoter into *Bam*HI/blunt and *Xho*I sites of ptkCATΔEH. Plasmids pStatCAT, pRStatCAT, p2xStatCAT, p3xStatCAT and p4xStatCAT were generated by insertion of double-stranded oligonucleotide(s) from SPI-3 promoter (-132 to -124) with *Bgl*II ends into the *Bam*HI site of ptkCATΔEH. All constructs were sequenced on both strands. The plasmid containing mouse contrapsin complementary DNA was kindly provided by Dr. Hyogo Sinohara (Osaka, Japan) [29].

Transient transfections

HepG2 cells were grown to 30% confluency, whereas primary rat hepatocytes were cultured for 2 days and used for transfections on the third day. Cells were transfected in DMEM supplemented with 10% fetal calf serum by using calcium phosphate precipitates [30], with 5 μg of plasmid DNA and 2 μg of internal control plasmid pCH110 (Pharmacia). Cells were incubated with precipitate for 6 h and washed twice with PBS, and the media was changed. One day after transfection, cells were stimulated, then cultured for another 24 h and harvested. Protein extracts were prepared by freeze–thawing [31], and protein concentration was determined by the BCA method (Sigma). Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were per-

formed as described [32,33]. CAT activities were normalized to the internal control β-galactosidase activity and are means $\pm$ S.E.M. for HepG2 cells (three to seven determinations). A representative example of one out of at least three independent transfections that gave reproducible hormonal effects is shown for primary rat hepatocytes (see Figure 5A). A 2–3-fold variation in measured CAT activities was observed for any given construct, presumably reflecting differences between cell preparations.

Extract preparation and gel retardation assays

Nuclear and cytosolic extracts were prepared as described by Shapiro et al. [34] as modified by Wegenka et al. [5]. Double-stranded DNA fragments or oligonucleotides were labelled by filling in 5′ protruding ends with Klenow enzyme using [α-^{32}P]dCTP (3000 Ci/mmol). Gel retardation assays were performed according to published procedures [35,36]; 2–5 μg of nuclear extracts and approx. 10 fmol (10000 c.p.m.) of probe were used.

Immunoprecipitation and immunoblotting

The polyclonal rabbit anti-mouse anti-Stat3c antiserum (directed against C-terminus) and anti-Stat1 antiserum were kindly provided by Dr. J. E. Darnell, Jr. (The Rockefeller University, New York, NY, U.S.A.), and the monoclonal anti-Stat3 antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.). Lysis of the cells (native and denaturing conditions), immunoprecipitations and Western blotting were performed according to the protocols of Transduction Laboratories.

RESULTS

The 147 bp of the SPI-3 promoter is responsive and sufficient for IL-6 and Dex stimulation

SPI-3 gene expression is strongly activated by IL-6 and Dex in primary cultures of rat hepatocytes [19] and, as shown in Figure 1, in H-35 cells. We stimulated rat H-35 cells with IL-6, Dex, IL-1 and phorbol 12-myristate 13-acetate and performed Northern blot analysis. IL-6 was able to induce the production of SPI-3 mRNA. Treatment of the cells with a combination of IL-6 and Dex led to the dramatic activation of SPI-3 expression. As shown in Figure 2A, actinomycin D, a known inhibitor of transcription, blocks the induction of the SPI-3 gene by IL-6 and Dex (or IL-6 alone; results not shown). The half-life of IL-6-induced SPI-3 mRNA is at least 8 h, and treatment with Dex seems to have some limited positive effect on the stabilization of SPI-3 mRNA (Figure 2B). To identify the regulatory elements responsible for this stimulation we amplified the 1021 bp of the 5′ flanking region of the SPI-3 gene by PCR, based on the published gene sequence [37], cloned it into the reporter plasmid encoding the bacterial CAT gene and transiently transfected HepG2 cells. Figure 3 shows that transfected cells expressed barely detectable levels of CAT, and treatment of these cells with IL-6 or a combination of IL-6 and Dex resulted in a profound increase of CAT activity, whereas Dex alone had no effect (results not shown). Next we generated a series of 5′ deletion mutants and measured their activities in the transfection experiments. We found that the construct containing 147 bp of the SPI-3 promoter was responsive to IL-6 and Dex in the same manner as all of the longer constructs (Figure 3). However, further truncation abolished responsiveness to both IL-6 and Dex enhancement. Because the enhancement by Dex was lower than observed on the mRNA level, we transfected primary rat hepatocytes. Again, it appeared that the 147 bp promoter is sufficient for IL-6 responsiveness whereas the 81 bp promoter is not. Furthermore the Dex enhancement was similar to that observed on the mRNA level.

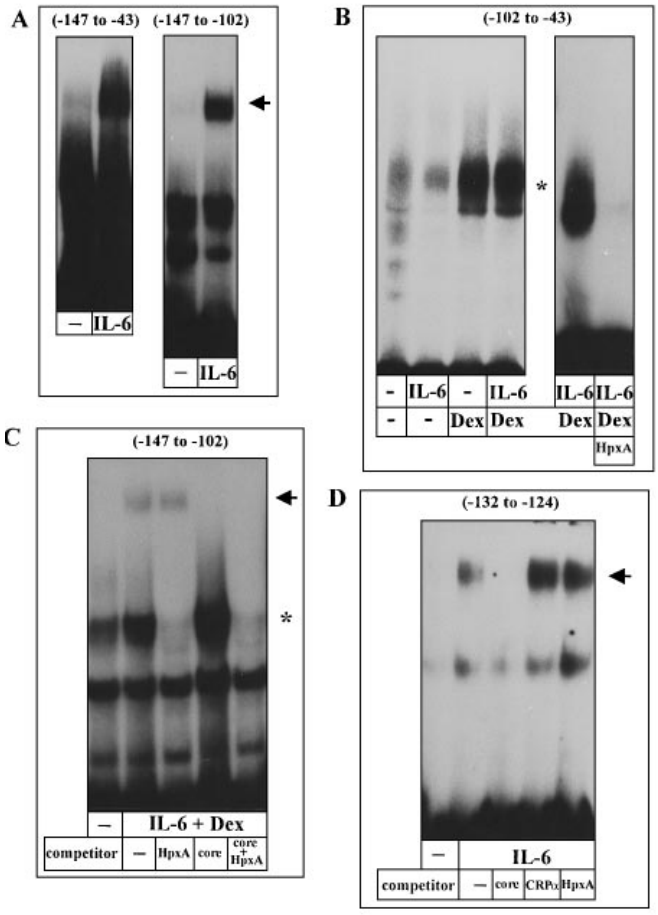

Figure 4 Gel retardation assay of H-35 cell nuclear extracts using fragments derived from the SPI-3 promoter

H-35 cells were incubated with 50 ng/ml IL-6 for 15 minutes (**A**, **D**) or 50 ng/ml IL-6 and (or) 100 nM Dex for 24 h (**B**, **C**). Cells were harvested and nuclear extracts prepared. Nuclear protein (1–5 μg) was incubated with ^{32}P-labelled double-stranded fragments derived from the SPI-3 promoter. As competitors, a 100-fold excess of each of the following unlabelled double-stranded oligonucleotides was added to the binding reaction: rat α_2-macroglobulin core site (core), human haemopexin A site (HpxA) and C-reactive protein gene α site (CRPα). The DNA–protein complexes formed were separated on a native 4% (w/v) polyacrylamide gel. After drying, the gel was exposed to X-ray film. The arrow indicates protein binding to APRF/Stat3 element; an asterisk marks the protein binding to the C/EBP elements.

Identification of IL-6REs in the promoter of the SPI-3 gene

Next we addressed the question of which elements are responsible for this induction. Nuclear extracts were prepared from H-35 cells treated with IL-6 or Dex, or both factors together for short (15 min) and long (12–24 h) time periods (optimal for activation of Stat proteins IL-6REBP and C/EBP respectively) and analysed by gel retardation assays using various fragments of SPI-3 promoter. Using (-147 to -3) and (-147 to -43) fragments as probes we observed strong constitutive binding and a weaker IL-6-induced band (Figure 4A, and results not shown) whereas the (-43 to -3) fragment did not produced any retarded bands (results not shown). Shorter fragments were used in gel retardation assays, and this analysis showed that the fragment

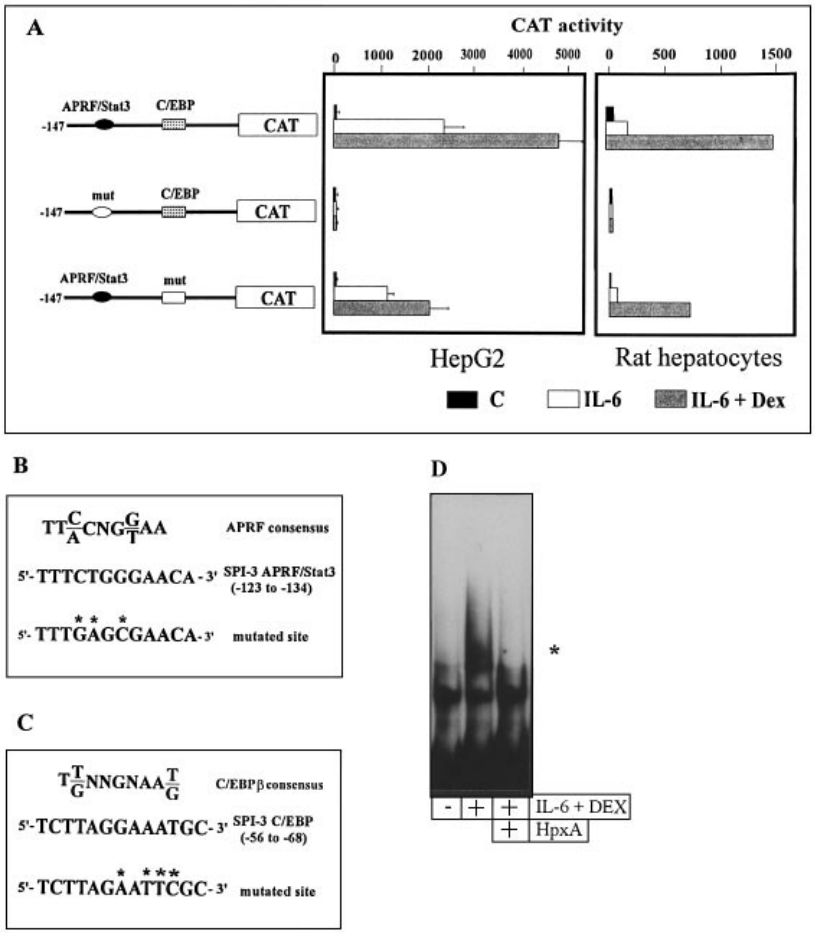

Figure 5 Effect of point mutations in the APRF/Stat3 and C/EBP elements

Point mutations (indicated by stars) were introduced into the SPI-3 APRF/Stat3 (**B**) or C/EBPβ elements (**C**). (**A**) HepG2 cells or primary rat hepatocytes were transiently transfected with 5 μg of plasmid pS3CATΔ7, pS3(mutStat3)CAT or pS3(mutC/EBPβ)CAT and 2 μg of plasmid pCH110 as described in the legend to Figure 3. Cells were stimulated with 50 ng/ml IL-6 or (and) 100 nM Dex, and lysates were prepared 24 h later. CAT activities were normalized to β-galactosidase activities (c.p.m. per unit) and are means $\pm$ S.E.M. for HepG2 cells (three determinations); a representative example is shown for primary rat hepatocytes. (**D**) H-35 cells were incubated with 50 ng/ml IL-6 and 100 nM Dex for 24 h, harvested and nuclear extracts were prepared. Nuclear protein (2 μg) was incubated with the ^{32}P-labelled (-147 to -102) fragment derived from the SPI-3 promoter containing the mutated APRF/Stat3 element. As competitor, a 100-fold excess of unlabelled double-stranded oligonucleotide containing the human haemopexin A site (HpxA) was added to the binding reaction. The DNA–protein complexes formed were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed to X-ray film. An asterisk indicates binding of a C/EBP protein.

(-147 to -102) was bound by the IL-6 induced protein, with the latter also binding to a double-stranded oligonucleotide covering the sequence -124 to -132 (Figures 4A and 4D). In contrast, the (-102 to -43) fragment was bound by a protein induced by Dex treatment of the cells and not by IL-6. This binding was competed for by a human haemopexin A site oligonucleotide containing the binding site for C/EBP proteins (Figure 4B). The ($-$ 124 to -132) element possesses one mismatch to the consensus sequence of APRF/Stat3 and two mismatches to the consensus sequence of C/EBPβ. To identify the IL-6 induced protein we performed competition gel retardation assays. The binding of IL-6-induced protein to the oligonucleotide probe (-124 to -132) or DNA fragment (-147 to -102) was competed for by a 'core' oligonucleotide containing the APRF/Stat3 binding site but not by oligonucleotides containing the C/EBP binding site (HpxA and CRPα) (Figures 4C and 4D). A strong IL-6-induced binding was observed 15 min after IL-6 treatment of cells, in contrast with much weaker binding after 12–24 h (Figures 4A and 4C, and results not shown). Additionally the (-147 to -102) fragment was bound by a Dex- (or Dex and IL-6)-induced protein and this binding was competed for by an HpxA oligonucleotide.

Functional analysis of APRF/Stat3 and C/EBP binding elements

To evaluate the contribution of both elements in the IL-6- and Dex-induced response of the SPI-3 gene we introduced point mutations. The mutation introduced into the (-132 to -124) element did not change the consensus for the possible C/EBPβ binding site but destroyed the binding site for APRF/Stat3 (Figure 5B). As shown in Figure 5D, the IL-6 induced protein was no longer able to bind to the mutated site, whereas binding of a C/EBP protein was not influenced (compare with Figure 4C, where the same nuclear extract was used). Figure 5A shows that this mutation resulted in a total loss of responsiveness to IL-6 and Dex. In contrast, mutation of the (-58 to -66) element (Figure 5C) binding C/EBP did not abolish the responsiveness to

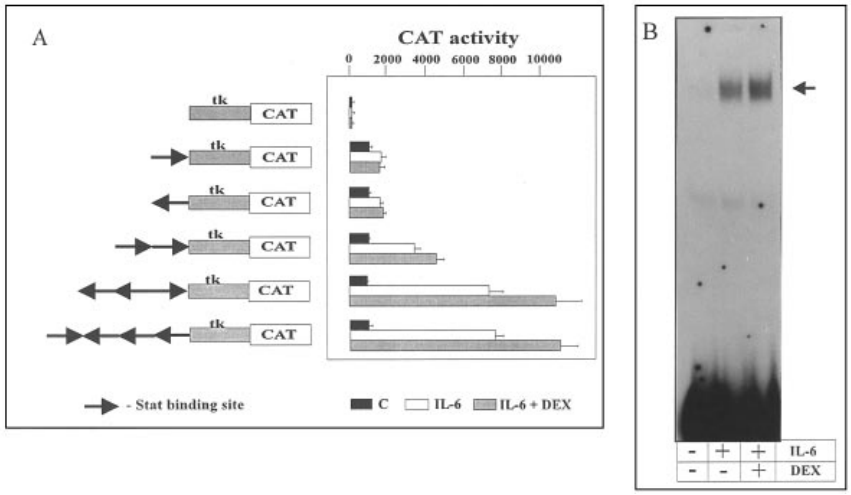

Figure 6 The APRF/Stat3 element confers responsiveness to IL-6 and enhancement to Dex on to the *tk* promoter

(**A**) APRF/Stat3 binding element(s) (−132 to −124) was (were) cloned on the front of the *tk* promoter. HepG2 cells were transfected with 5 μg of obtained plasmids and 2 μg of plasmid pCH110 as described in the legend to Figure 3. Cells were stimulated with 50 ng/ml IL-6 or (and) 100 nM Dex, and lysates were prepared 24 h later. CAT activities were normalized to β-galactosidase activities (c.p.m. per unit) and are means ± S.E.M. (three determinations). (**B**) H-35 cells were incubated with 50 ng/ml IL-6 or 50 ng/ml IL-6 and 100 nM Dex for 24 h and harvested, and nuclear extracts were prepared. Nuclear protein (5 μg) was incubated with the ^{32}P-labelled rat SPI-3 APRF/Stat3 double-stranded oligonucleotide. The DNA–protein complexes formed were separated on a native 4% polyacrylamide gel. After drying, the gel was exposed to X-ray film. The arrow indicates protein binding to APRF/Stat3 element.

IL-6 alone or together with Dex but resulted in a decrease by approx. 50% of the transcription of the CAT gene. To show that the APRF/Stat3 element is a real IL-6-responsive enhancer we inserted several copies of this element in the front of the thymidine kinase promoter linked to the CAT gene. Figure 6A shows that insertion of the SPI-3 APRF/Stat3 element resulted in a 10-fold higher basal transcription of the CAT gene. Moreover, this element was able to confer responsiveness to IL-6 and partial enhancement to Dex, which was more evident when using its multiple copies. The amount of the IL-6-induced protein binding to this element was slightly higher when cells were additionally treated with Dex (Figure 6B).

APRF/Stat3 and IL-6REBP

APRF/Stat3 and IL-6REBP have been described as binding to rat 'core' element from the α_2-macroglobulin promoter. To determine whether the APRF/Stat3 element from the SPI-3 gene binds both proteins we performed gel retardation assays on extracts from HepG2 cells. We observed very strong binding 15 min after IL-6 treatment, which gradually decreased up to 2 h after IL-6 stimulation. The first increase in binding was followed by a weaker peak at about 4–8 h (results not shown). Next we addressed the question of whether the observed binding requires ongoing protein synthesis. We pretreated HepG2 and H-35 cells with 10 μg/ml of cycloheximide for 1 h to inhibit protein synthesis and then stimulated the cells with IL-6. As shown in Figure 7A, cycloheximide did not prevent the rapid appearance of the IL-6-induced band, but the long-term IL-6-induced binding was inhibited. Moreover, cycloheximide was unable to inhibit induction of the SPI-3 gene by IL-6 when measured after 2.5 h (although the magnitude of this induction was decreased by approx. 50%) but totally inhibited this activation after 10 h (Figure 7B), and this correlates with the results from the binding experiments. We then preincubated nuclear extracts with either anti-Stat3c or anti-Stat1 antiserum (both 1:400) and subjected them to the gel retardation assay. The results obtained are shown in Figure 8. As expected, the protein binding 15 min after IL-6 treatment was recognized by anti-Stat3c antibodies but not by a preimmune serum. The anti-Stat1 antibodies produced a weak, retarded band but the major part of the IL-6-induced band was not retarded. The protein (IL-6REBP), which binds later to the same element, was also specifically recognized by the anti-Stat3c antibody. In all our experiments we observed a weak, constitutive binding in the extracts from control cells. The protein present in these extracts was recognized by anti-Stat3c antibody as well. These results suggest that IL-6REBP is at least antigenically related to APRF/Stat3. Because APRF/Stat3 is phosphorylated at a tyrosine residue we prepared cell lysates from HepG2 cells treated with IL-6 for 15 min and 12 h, immunoprecipitated the proteins with the anti-Stat3c antibody and analysed them in immunoblot experiments. As expected, with anti-phosphotyrosine antibody we observed a strong band, representing the tyrosine-phosphorylated APRF/Stat3, 15 min after IL-6 treatment of the cells. Hardly detectable bands were observed when using extracts from control cells and cells treated with IL-6 for 12 h (Figure 9). When the blot was probed for APRF/Stat3 we noticed one additional weaker band present in the lysates from control cells but not in the lysates from IL-6-treated cells. This band, which migrated slower than the APRF/Stat3 band (molecular mass approx. 91 kDa), was identified as p91/Stat1 while probing for this protein (Figure 9). The simplest explanation would be that the anti-Stat3c antibodies recognize p91/Stat1; however, after IL-6 treatment p91/Stat1 is phosphorylated and forms homodimers [9,10] that can no longer be recognized by these antibodies. To detect very low levels of phosphorylated APRF/Stat3 we lysed the IL-6 treated cells under denaturing conditions, immunoprecipitated proteins with antiphosphotyrosine antibodies and analysed them in immunoblot experiments with antiphosphotyrosine and monoclonal anti-Stat3 antibodies. This analysis clearly shows (Figure 9B) that control cells already contain very low levels of phosphorylated APRF/Stat3. Stimulation of the cells with IL-6 for 15 min resulted in a profound increase of phosphorylated APRF/Stat3.

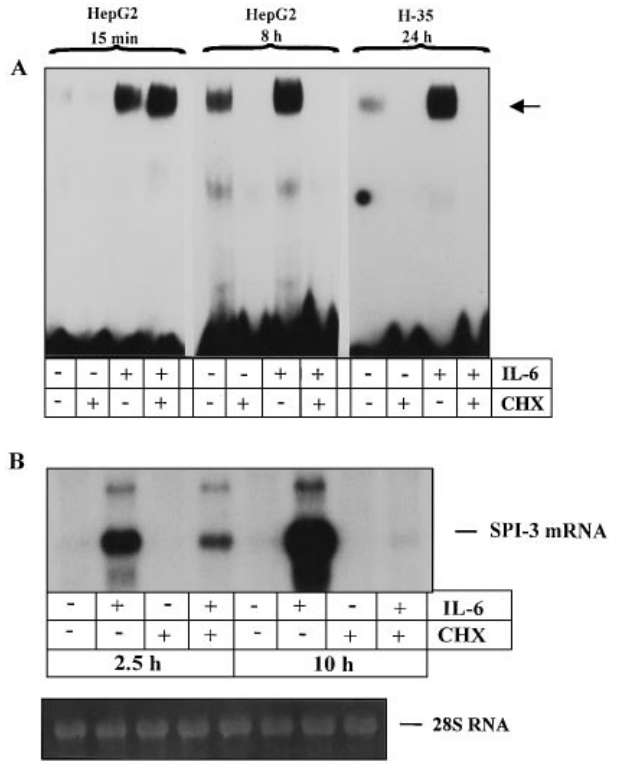

Figure 7 Effect of cycloheximide on IL-6-induced binding to APRF/Stat3 element from the SPI-3 gene and IL-6-induced transcription of the SPI-3 gene

HepG2 or H-35 cells were preincubated with 10 μg/ml of cycloheximide for 1 h where indicated (CHX). Then 50 ng/ml IL-6 was added to the medium. After the times indicated, nuclear extracts were prepared and RNA was isolated. (**A**) Gel retardation assays performed as described in the legend to Figure 4, using 5 μg of nuclear protein and ^{32}P-labelled double-stranded rat SPI-3 APRF/Stat3 oligonucleotide probe. The arrow indicates the IL-6-induced protein(s). Gels were exposed for 12, 48 and 12 h respectively. (**B**) Northern blot analysis of SPI-3 mRNA. The lower panel shows the 28 S rRNA stained with ethidium bromide on the membrane.

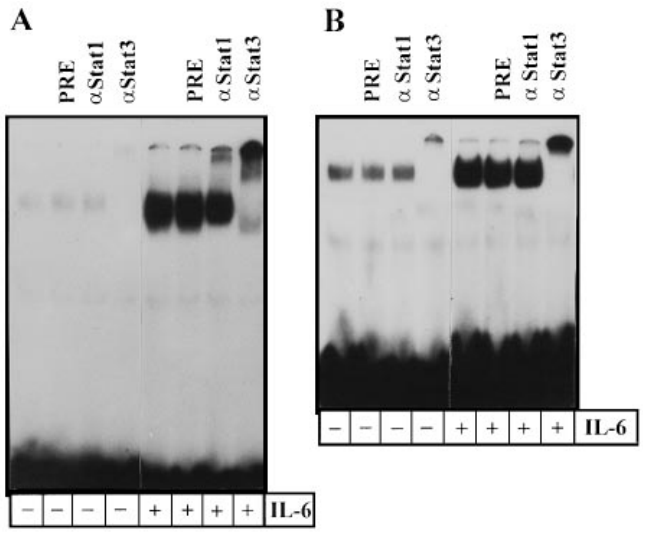

Figure 8 APRF/Stat3 and IL-6REBP are recognized by anti-Stat3c antibody

HepG2 cells were stimulated with 50 ng/ml IL-6 and nuclear extracts were prepared. Nuclear proteins (5 μg) were incubated in the gel retardation assay cocktail with the rat preimmune serum (PRE), anti-Stat1 antiserum or anti-Stat3c antiserum for 30 min (1 : 400 dilution). Binding to the SPI-3 APRF/Stat3 site was then analysed by gel retardation assay. Nuclear extracts were prepared 15 min (**A**) and 12 h (**B**) after IL-6 treatment of the cells. Autoradiograms were exposed for 12 h (**A**) and 48 h (**B**).

Finally, treatment of cells with IL-6 for 12 h evidently resulted in an increase of the phosphorylated APRF/Stat3 in comparison with control cells.

DISCUSSION

Cis-acting elements regulating the expression of SPI-3 gene

The SPI-3 gene is not expressed in control rats but is strongly activated during inflammation [17], with IL-6 and glucocorticoids as major activators [19]. Several regulatory elements located 5′ from the 147 bp promoter were suggested to be involved in the regulation of the SPI-3 gene [38]. We clearly show that such sequences are not involved in the IL-6-induced stimulation of the SPI-3 gene because the 147 bp SPI-3 promoter in which these were deleted was fully responsive to IL-6 (Figure 3). Within 147 bp of the SPI-3 promoter, we identified two new elements (Stat and C/EBP) that bind both constitutive and IL-6-induced proteins. Finally, we have shown the function of Stat and C/EBP elements in the SPI-3 promoter. The C/EBP element seems to enhance the response to IL-6 and Dex but is not necessary for this activation. In contrast, the Stat element plays a crucial role in the IL-6 induction, whereas the promoter with the mutated site does not function (Figure 5). The same binding site is indispensable for the activation of SPI-3 gene by interferon-γ [39].

Proteins binding to the SPI-3 promoter

Using competitors we showed binding of factors of the C/EBP family to the (-58 to -66) element. On the other hand, the (-124 to -132) element formed complexes with the factors of the Stat and C/EBP families. These data are in part contrary to the observations of others describing binding only of a factor from the C/EBP family to the sequence overlapping the Stat binding site, but Rossi et al. were unable to show any difference in the binding of nuclear proteins from the extracts prepared from control and inflamed animals [38]. However, *in vivo*, this binding (if it occurs) seems not to be involved in IL-6-induced transcription because the mutation in the APRF/Stat3 element that did not influence the binding of C/EBP protein resulted in unresponsiveness to IL-6. Thus after IL-6 stimulation this site is normally occupied by the factors from the Stat family.

Effect of glucocorticoids on IL-6-induced expression of SPI-3 gene

Glucocorticoids were shown to stimulate directly the expression of several APP genes and enhance the effect of IL-6 and IL-1 type cytokines. The observed effect of glucocorticoids could be explained by several possible sites of their action, i.e. the up-regulation of the 80 kDa IL-6 receptor, the induction of C/EBPβ, the binding of glucocorticoid receptors to the 5′ flanking sequences or the stabilization of the mRNA of APP genes. The enhancement of IL-6-induced activation of the SPI-3 gene probably involves several mechanisms. First, treatment of the H-35 cells with Dex resulted in an increase of binding (approx. 5-

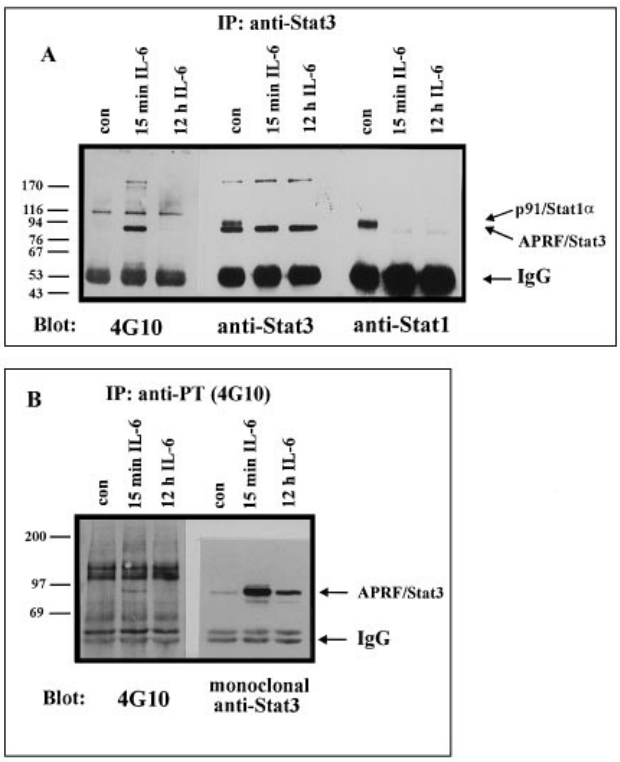

Figure 9 Immunoblot analysis of IL-6-treated HepG2 cells

HepG2 cells were treated with 50 ng/ml IL-6 for the times indicated. (**A**) Cells were lysed under native conditions and proteins were immunoprecipitated from lysates with anti-Stat3c antibodies. The immune complexes formed were separated by SDS/PAGE (7.5% gel) and electroblotted to nitrocellulose membrane. Blots were probed with anti-phosphotyrosine monoclonal antibodies 4G10 (1:1000). The blot was stripped of the 4G10 antibodies and probed with anti-Stat3c (1:2000). It was stripped again and probed with anti-Stat1α (p91) antibodies (1:1000). (**B**) Cells were lysed under denaturing conditions and proteins were immunoprecipitated from lysates with anti-phosphotyrosine antibodies 4G10. The immune complexes formed were separated by SDS/PAGE (7.5% gel) and electroblotted to nitrocellulose membrane. Blots were probed with 4G10 antibodies (1:1000). The blot was stripped of the 4G10 antibody and probed with monoclonal anti-Stat3 (1:250). The positions of molecular mass markers (kDa) are indicated on the left.

fold) to the localized C/EBP element (Figure 4B). The induction of C/EBPβ mRNA in H-35 cells by Dex was reported [3], and the observed 3–5-fold increase supports our finding. However, this mechanism seems to play only a very limited role because point mutations introduced into C/EBPβ binding element did not result in a loss of enhancement (Figure 5A). Secondly, some stabilization of SPI-3 mRNA can be seen after Dex stimulation, but this effect cannot explain the difference between the amount of SPI-3 mRNA present after stimulation with IL-6 and Dex in comparison with IL-6 alone when measured after 8 h (Figure 2B, lanes 1 and 5). Thirdly, two or more copies of the Stat binding element confer partial enhancement of Dex onto the *tk* promoter and we observed somewhat higher levels of IL-6-induced protein in the H-35 cells treated with Dex for 24 h (Figure 6). Thus an additional site of glucocorticoid action could be the IL-6-induced protein binding to the APRF/Stat3 element. Recently it was suggested [40] that the binding site for the glucocorticoid receptor was present in the SPI-2 gene at (-88 to -74), but this region in the SPI-3 gene differs by two nucleotides. A different magnitude of enhancement of Dex in transient transfection experiments when using HepG2 cells in contrast with rat hepatocytes seems to be the feature of this human cell line. Such a different magnitude of induction was reported for HepG2 and H-35 cells by others [41].

APRF/Stat3 and IL-6REBP

APRF/Stat3 and IL-6REBP were described as proteins binding to the 'core' element from the rat α_2M promoter. In the supershift experiments we showed that both proteins were recognized by the anti-Stat3c antibodies. Thus it seems that IL-6REBP contains the protein, which is at least antigenically related to Stat3 (Figure 8). Additionally, we show that long-term treatment of cells with IL-6 leads to a sustained increase in the amount of phosphorylated APRF/Stat3 (Figure 9B), and APRF/Stat3 is the only band recognized by anti-Stat3c antibodies when immunoblots are performed (Figure 9A). These observations and the data from the experiments with cycloheximide imply that it is very likely that IL-6REBP contains a newly synthesized APRF/Stat3, which is probably indispensable for sustained transcription of IL-6-induced genes. The 5-fold increase in APRF/Stat3 mRNA in the mouse liver 3 h after injection of IL-6 has already been reported [13]. However, the final answer on whether APRF/Stat3 and IL-6REBP are the same or different proteins requires the sequencing of the latter.

Recently, there has been growing evidence suggesting that many cytokines and growth factors rapidly activate members of a family of Stat proteins (reviewed in [14]). Although Stat1 is immediately activated in response to interferon-γ, binding of Stat1 to target sequences decreases within hours. Similarly, APRF/Stat3 is activated within minutes, but after 1 h the binding was reported to be only slightly higher then before activation [5]. On the other hand, the interferon-γ- and IL-6-induced transcription of many genes continues for much longer. The present experiments were performed in an attempt to characterize the contribution of Stat- and C/EBP-binding elements to the activation of the SPI-3 promoter and to correlate the binding of induced factors to these elements with the observed transcriptional activity. The Stat element is indispensable for IL-6 activation, whereas the C/EBP element merely enhances this activation. The immediate activation of latent APRF/Stat3 in response to IL-6 seems to correlate with the rapid induction of the SPI-3 gene, and the sustained transcriptional activity might be the result of binding of IL-6-induced protein, which most probably contains a newly synthesized APRF/Stat3.

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