Activation of the rat serine proteinase inhibitor 3 gene by interferon γ via the interleukin 6-responsive element

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Transcription of rat serine proteinase inhibitor 3 (SPI-3) gene is rapidly induced in the liver in response to inflammation. Treatment of rat hepatoma H-35 cells with interferon γ (INFγ) results in the immediate induction of this gene, with its 147 bp-long promoter being sufficient for activation. Within this promoter we have identified an IFNγ-responsive element which maps to the signal transducer and activator of transcription (Stat)3-binding site. Mutation of this element causes a loss of responsiveness to IFNγ, whereas fusion to a heterologous promoter confers a positive response on IFNγ. The latter apparently induces the binding of a protein, identified as Stat1, to the described element, which gradually decreases within 24 h. Thus the induction of the SPI-3 gene by IFNγ correlates with the binding of Stat1 to a specific element which, in turn, binds Stat3 in response to interleukin 6.

INTRODUCTION

The binding of cytokines and growth factors to their specific surface receptors triggers the activation of a common transduction pathway and results in the activation of gene expression [1]. Interleukin 6 (IL-6) and interferon γ (IFNγ) are cytokines with pleiotropic activities, each playing an important role in the host defence system. Infection of a tissue leads to the activation of the cells of the immune system, including T-lymphocytes which secrete IFNγ. This cytokine, in turn, induces the expression of a number of genes, including those encoding the intracellular adhesion molecule 1, interferon regulatory factor 1, high-affinity $F_{c\gamma}$ receptor, guanylate-binding protein and MHC class I and II genes [2]. On the other hand, infection also results in the acute-phase response, which leads to a return to normal function through the activation of so-called acute-phase genes in the hepatocytes by IL-6, a major mediator of this process [3,4]. However, other cytokines, including IFNγ, also regulate expression of acute-phase genes [3]. Binding of IL-6 and IFNγ to their individual receptors is associated with the activation of both protein tyrosine kinases of the Janus family and latent cytoplasmic proteins called Stats (signal transducers and activators of transcription) [5–7]. In response to IFNγ, Stat1 binds to the IFNγ-activation site (reviewed in ref. [8]), whereas Stat3 binds to the acute-phase response element after activation by IL-6 [9]. Recently, Stat1 and Stat3 were shown to bind to the same regulatory elements in the promoters of interferon regulatory factor 1, intracellular adhesion molecule 1 and α_2-macroglobulin (α_2M), and this binding correlated with the responsiveness to both IFNγ and IL-6 [10–12].

In this report we show that rat serine proteinase inhibitor 3 (SPI-3) gene is activated by IFNγ in rat hepatoma H-35 cells. This activation correlates with the rapid binding of Stat1 to the IL-6-responsive element within the SPI-3 promoter. Furthermore, we have found that the Stat1/Stat3-binding element confers the responsiveness to IFNγ on to a heterologous promoter and is indispensable for the responsiveness of the SPI-3 promoter to IFNγ. These results suggest that both IFNγ and IL-6 might be involved in the induction of the SPI-3 gene as well as for long-term maintenance of its expression *in vivo*.

MATERIALS AND METHODS

Cell culture and stimulation

Human hepatoma HepG2 cells and rat hepatoma H-35 cells, a gift from Dr. H. Baumann (Buffalo, NY, U.S.A.), were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Cells were grown to 70–90% confluency and stimulated with 500 units/ml human recombinant IL-6 (a gift from Immunex, Seattle, WA, U.S.A.), 200 units/ml human recombinant IFNγ (Boehringer) or 200 units/ml rat recombinant IFNγ (Gibco).

RNA preparation and Northern-blot analysis

Total RNA was prepared using the phenol extraction method [13,14]. Samples of RNA (5 μg) were subjected to formaldehyde-gel electrophoresis using standard procedures [15] and transferred to Hybond-N membranes (Amersham), according to the manufacturer's instructions. The filters were prehybridized at 68 °C for 3 h in a solution consisting of 10% dextran sulphate, 1 M NaCl and 1% SDS, and hybridized in the same solution with cDNA fragments labelled by random priming [16]. After hybridization, non-specifically bound radioactivity was removed by washing in 2 × SSC (where 1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by two washes in 2 × SSC/1% SDS at 68 °C for 20 min.

Synthetic oligonucleotides and plasmids

The oligonucleotides used in gel-retardation assays were designed to contain single-stranded 5′ overhangs of four bases at both ends after annealing (top strands are shown): rat SPI-3 Stat1/Stat3 site, 5′-GATCTGTTCCCAGAAA-3′; mutated rat SPI-3 Stat1/Stat3 site, 5′-GATCTGTTCGCTCAAATC-3′;

Abbreviations used: CAT, chloramphenicol acetyltransferase; IFNγ, interferon γ; IL-6, interleukin 6; SPI-3, serine proteinase inhibitor 3; Stat, signal transducer and activator of transcription; α_2M, α_2-macroglobulin; 1 × SSC, 0.15 M NaCl plus 0.015 M sodium citrate; ACT, α_1-antichymotrypsin.

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human haemopexin A site [17], 5′-AGCTTATTTGCAGTGA-TGTAATCAGCA-3′. Details of the construction of plasmids are obtainable from J.T. on request. Plasmid pS3CATΔ7 contained 147 bp of the 5′ flanking region of SPI-3 gene linked to the chloramphenicol acetyltransferase (CAT) gene. Plasmid pS3(mutStat3)CAT is analogous to the pS3CATΔ7 but carries point mutations introduced in the Stat1/Stat3 element. Plasmids pStatCAT, pRStatCAT, p2 × StatCAT, p3 × StatCAT and p4 × StatCAT contain double-stranded oligonucleotide(s) from the SPI-3 promoter (134 to −123) inserted in front of the herpes virus thymidine kinase (*tk*) promoter driving the transcription of CAT. The plasmid containing mouse contrapsin cDNA was kindly provided by Dr. Hyogo Sinohara (Osaka, Japan) [19].

Transient transfections

HepG2 cells were grown to 30% confluency in 6 cm dishes and transfected in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum using calcium phosphate precipitates [20], with 5 μg of plasmid DNA and 2 μg of internal control plasmid pCH110 (Pharmacia). Cells were incubated with precipitate for 6 h, washed twice with PBS and media were changed. One day after transfection, cells were stimulated, then cultured for another 24 h and harvested. Protein extracts were prepared by freeze–thawing [21], and protein concentration was determined by the bicinchoninic acid method (Sigma). CAT and β-galactosidase assays were performed as described [22,23]. All experiments were performed at least three times and gave reproducible results. CAT activities were normalized to the internal control β-galactosidase activities.

Extract preparation and gel-retardation assays

Nuclear extracts were prepared as described by Wegenka et al. [9]. Double-stranded oligonucleotides were labelled by filling in 5′ protruding ends using Klenow enzyme and [α-^{32}P]dCTP (3000 Ci/mmol). Gel-retardation assays were carried out by published procedures [24,25] using 5 μg of nuclear extracts and approx. 10 fmol (10000 c.p.m.) of probe. For supershift experiments, nuclear extracts were preincubated with antisera for 30 min at room temperature.

Antibodies

Anti-Stat3c (directed against the C terminal) and anti-Stat1 rabbit anti-mouse sera (described in ref. [26]) were kindly provided by Dr. J. E. Darnell, Jr. (The Rockefeller University, New York, NY, U.S.A.).

RESULTS

Induction of SPI-3 gene expression by IFNγ

Rat hepatoma H-35 cells express most of the acute-phase genes [27]. The regulation of SPI-3 gene expression by IL-6 and dexamethasone in these cells correlates with that observed in hepatocytes in primary culture [18,28]. We stimulated the H-35 cells with IFNγ and performed Northern-blot analysis. As shown in Figure 1, activation of SPI-3 gene expression by IFNγ was time- and dose-dependent. Accumulation of SPI-3 mRNA could be seen as soon as 1 h after IFNγ treatment of the cells, and this accumulation was sustained for 64 h. Induction of SPI-3 expression was observed at 1 unit/ml IFNγ and was maximal at 500 units/ml. Thus it can be clearly seen that IFNγ regulates SPI-3 gene expression.

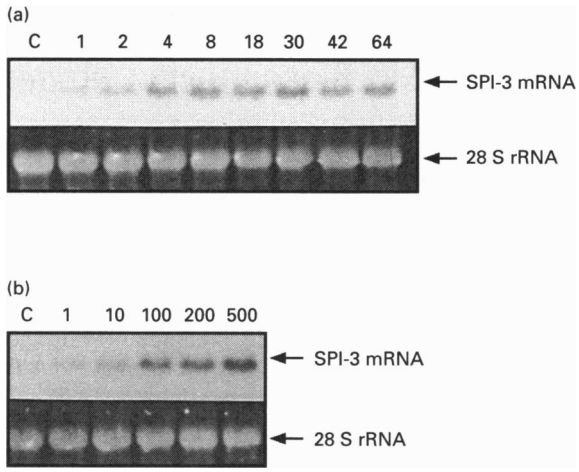

Figure 1 Time- and dose-dependent activation of SPI-3 expression by IFNγ

H-35 cells were stimulated with 200 units/ml IFNγ for the indicated time periods (h) (**a**) or with the indicated amounts of IFNγ (units/ml) for 18 h (**b**) C, control. RNA was isolated and analysed by Northern blotting using mouse contrapsin cDNA as a probe. The positions of SPI-3 mRNA are indicated. The lower panels show the 28 S rRNA stained with ethidium bromide on the membrane.

Identification of the IFNγ-responsive element

In order to identify the element(s) involved in the regulation of the SPI-3 gene by IFNγ, we transfected human hepatoma HepG2 cells with the construct containing the bacterial CAT gene under the control of the 147 bp-long promoter of the SPI-3 gene. The transiently transfected cells were stimulated with IFNγ, and after 24 h CAT activity was assayed. As shown in Figure 2, the 147 bp promoter of SPI-3 gene conferred responsiveness to IFNγ. This 5–6-fold induction by IFNγ was also observed when cells were transfected with longer constructs harbouring the 1021 bp of the 5′ flanking region of the SPI-3 gene (results not shown). To

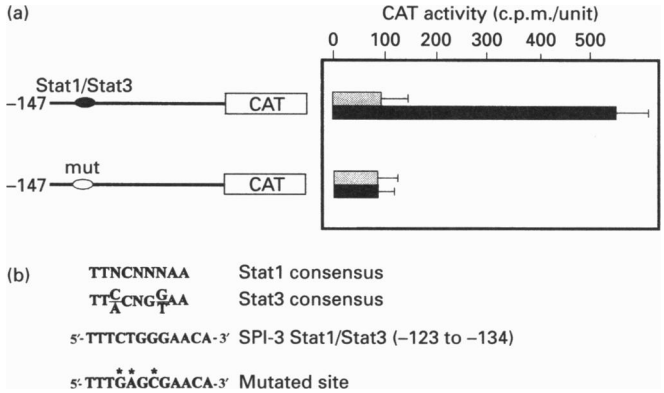

Figure 2 Stat1/Stat3-binding element triggers the response of SPI-3 gene to IFNγ

(**a**) HepG2 cells were transiently transfected with 5 μg of plasmids pS3CATΔ7 or pS3(mutStat3)CAT and 2 μg of plasmid pCH110 as an internal control of transfection efficiency. One day after transfection, cells were stimulated with 200 units/ml IFNγ, cultured for another 24 h, and harvested. CAT activities were normalized to β-galactosidase activities (c.p.m./unit) and are means ± S.E.M. (3 determinations). ▨, Control; ■, IFNγ. (**b**) Point mutations introduced within the Stat1/Stat3-binding element.

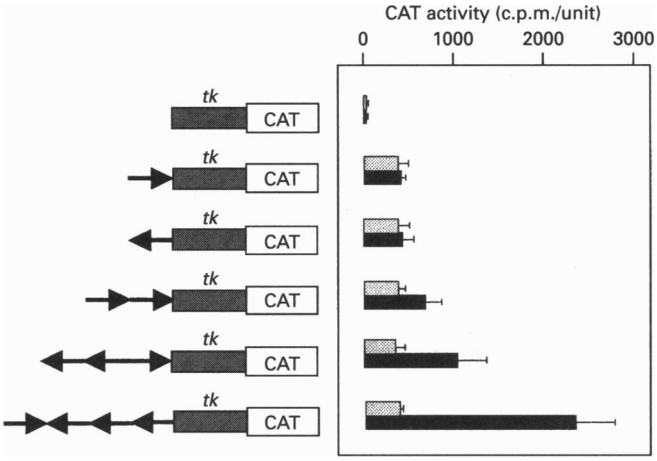

Figure 3 Stat1/Stat3-binding element confers responsiveness to IFNγ on the *tk* promoter

HEPG2 cells were transfected with 5 μg of plasmid carrying Stat1/Stat3-binding element(s) linked to the *tk* promoter and 2 μg of plasmid pCH110, as described in the Materials and methods section. Cells were stimulated with IFNγ, and lysates were prepared 24 h later. CAT activities were normalized to β-galactosidase activities (c.p.m./unit) and are means ± S.E.M. (3 determinations). →, Stat1/Stat3-binding site; ▨, control; ■, IFNγ.

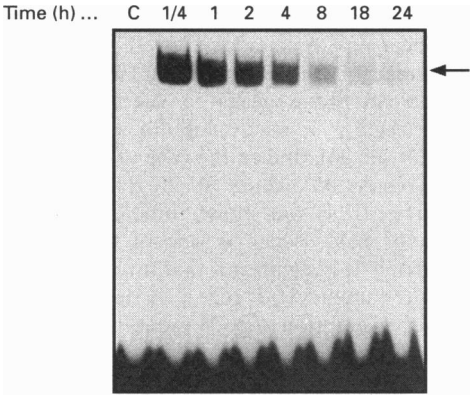

Figure 4 Time-dependent binding of IFNγ-induced protein

H-35 cells were stimulated with 200 units/ml IFNγ. After the times indicated, nuclear extracts were prepared and analysed in the gel-retardation assays using the double-stranded oligonucleotide probe covering the sequence at −124 to −132. The arrow indicates the position of the retarded band. C, Control.

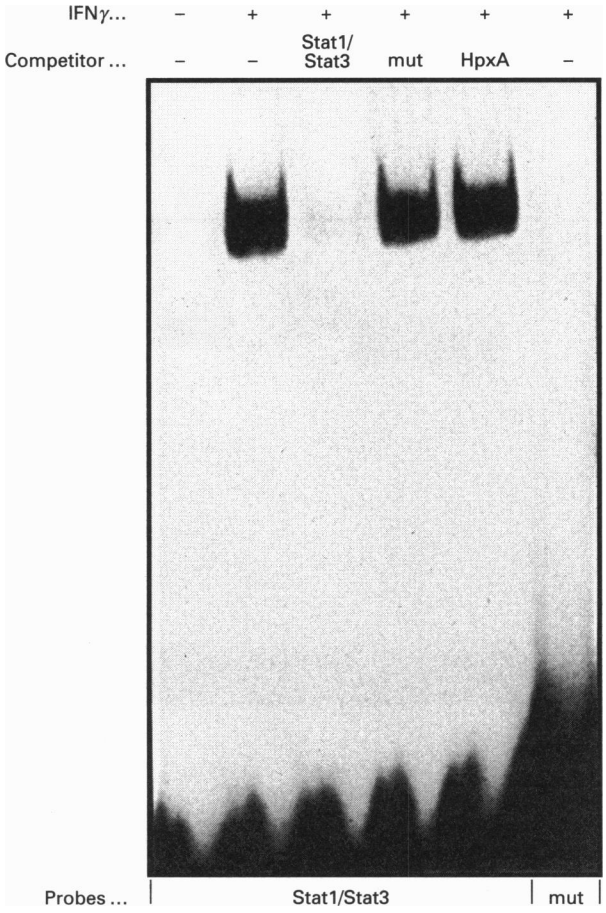

Figure 5 Competition gel-retardation assays

H-35 cells were stimulated with 200 units/ml INFγ. After 15 min, nuclear extracts were prepared and analysed in the gel-retardation assays using the ^{32}P-labelled SPI-3 Stat1/Stat3 or mutated SPI-3 Stat1/Stat3 double-stranded oligonucleotide probes. As competitors, a 100-fold excess of each of the following unlabelled double-stranded oligonucleotides was added to the binding reaction: SPI-3 Stat1/Stat3), mutated SPI-3 Stat1/Stat3 (mut) and human haemopexin A site (HpxA).

discover whether the IFNγ-induced transcription of SP-3 gene results from the binding of an IFNγ-activated transcription factor(s) to a previously identified IL-6-responsive element (−124 to −132), we transfected HepG2 cells with the construct harbouring the mutated IL-6-responsive element. As shown in Figure 2, the mutated promoter was no longer responsive to IFNγ, which proves that the −124 to −132 element is indispensable for responsiveness to IFNγ.

Next, we performed experiments to show that the identified element is an IFNγ-responsive enhancer. We transfected the HepG2 cells with the constructs containing this element (or its multiple copies) fused to the herpes virus thymidine kinase (*tk*) promoter, which itself is not responsive to IFNγ. Figure 3 shows that these constructs were responsive to IFNγ, and this was particularly evident when two or more copies of the identified element were linked to the *tk* promoter. Thus we believe that the −124 to −132 element confers the responsiveness to IFNγ.

Protein binding to the −124 to −132 element

We used a double-stranded synthetic oligonucleotide covering the sequence at −124 to −132 in gel-shift experiments to identify the protein binding to this region. Rat H-35 and human HepG2 cells were stimulated with rat and human IFNγ respectively for various time periods. Then nuclear extracts were prepared and used in gel-retardation assays. Using extracts from control cells we observed a weak band which previously was shown by us to be recognized by anti-Stat3c serum (T. Kordula and J. Travis, unpublished work). Treatment of the cells with IFNγ resulted in rapid binding of a different protein, and this binding gradually decreased up to 24 h after stimulation. Figure 4 shows typical time-dependent binding of IFNγ-induced protein in H-35 cells to the −124 to −132 element. Similar binding was observed with extracts from human HepG2 cells (results not shown). To demonstrate the specificity of the binding, we performed competition gel-retardation assays. As shown in Figure 5, the IFNγ-induced binding to the Stat1/Stat3

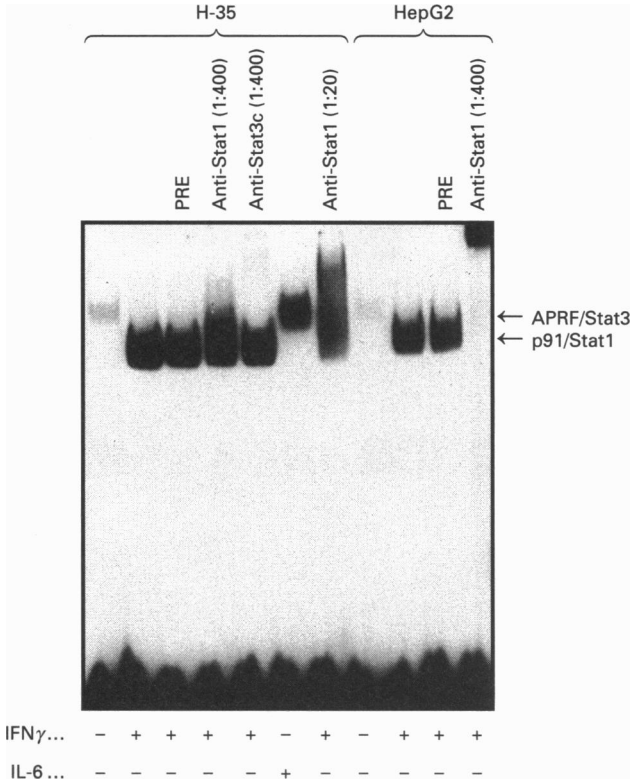

Figure 6 INFγ-induced protein is recognized by anti-Stat1 antibodies

H-35 and HepG2 cells were stimulated for 15 min with 200 units/ml INFγ (rat and human respectively) or 500 units/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) (1:20 dilution), anti-Stat1 or anti-Stat3c serum for 30 min (1:400 or 1:20 dilution as indicated). Binding to the double-stranded oligonucleotide probe covering the sequence at −124 to −132 was then analysed by gel-retardation assay. Positions of acute-phase response factor (APRF)/Stat3 and p91/Stat1 are indicated.

oligonucleotide can be competed for with excess of the unlabelled probe but not with an oligonucleotide harbouring mutated site or a non-specific binding site (HpxA). Moreover, mutated Stat1/Stat3 oligonucleotide did not produce any retarded bands.

Binding of IFNγ to its receptor results in phosphorylation of the Stat1 protein, its dimerization, translocation to the nucleus, and binding to the specific regulatory elements within promoters of target genes [8,29]. The element at −124 to −132 in the SPI-3 promoter possesses one mismatch to the Stat1 consensus sequence (TTNCNNNAA) (Figure 2b). In addition, previously described IFNγ-induced time-dependent binding of Stat1 in many cells correlates with binding to the IFNγ-responsive element from the SPI-3 gene [8,12]. To discover whether the protein that binds to this element is Stat1, we preincubated the nuclear extracts from H-35 and HepG2 cells treated with IFNγ with a preimmune serum, anti-Stat1 or anti-Stat3c serum (using comparable amounts of antisera) and performed gel-retardation assays. In addition, we stimulated cells with IL-6 to show the migration of a probe retarded by binding of a Stat3 protein. As shown in Figure 6, the anti-Stat1 serum produced supershifted bands when incubated with nuclear extracts from IFNγ-treated cells, whereas preimmune serum and anti-Stat3c antibodies had no effect. The higher concentrations of Stat1 antibodies required to produce supershifted bands from nuclear extracts of H-35 cells might result from lower reactivity of these antibodies to rat Stat1. All of the data presented show that the protein that binds to the −124 to −132 element is Stat 1 and that its binding correlated with IFNγ-induced transcription of the SPI-3 gene.

DISCUSSION

The rat SPI gene family consists of at least three genes, SPI-1, SPI-2 and SPI-3 [30,31]. The protein sequences of all three encoded proteins are very similar, but they differ in the reactive site which accounts for their different specificities towards target proteinases. The SPI-1 and SPI-2 genes are co-regulated. They are expressed in the liver of healthy animals and their expression is inhibited during inflammation. Both genes are induced by growth hormone and glucocorticoids, and totally repressed in hypophysectomized rats [30,31]. In contrast, the SPI-3 gene is barely expressed in healthy animals, seems to escape the regulation by growth hormone, and is strongly induced during the acute-phase reaction. IL-6 and glucocorticoids have been shown to regulate the expression of the SPI-3 gene in rat hepatocytes, and this regulation occurs at the level of transcription ([28]; T. Kordula and J. Travis, unpublished work). Recently, we identified the Stat3-binding element in the promoter of the SPI-3 gene which confers a responsiveness of IL-6 and enhancement by glucocorticoids (T. Kordula and J. Travis, unpublished work). Now, we show that IFNγ is capable of inducing SPI-3 gene expression (Figure 1). This induction is rapid and sustained for several days. Two other members of the SPI gene family (SPI-1 and SPI-2) appear not to be induced by IFNγ (Figure 1).

IFNγ has already been shown to regulate the expression of some human acute-phase genes in the liver, e.g., α_1-anti-chymotrypsin (ACT), α_2-macroglobulin (α_2M), haptoglobin and C1 inhibitor [32,33], and in the case of ACT, α_2M and C1 inhibitor this regulation occurs at the pretranslational level [33,34]. Moveover, IFNγ was shown to be the only regulator so far recognized of α_2M, which is one of the most abundant proteins in humans. It is significant that rat SPI genes show high homology to the human ACT gene [31]. However, in contrast with SPI-3, the transcription of ACT is diminished in response to IFNγ [32], even though two functional binding sites for Stat3 are present in the ACT promoter. The mechanism of inhibition by IFNγ remains unclear.

Recently, the rat α_2M promoter was shown to respond to IFNγ; however, induction of α_2M protein synthesis or accumulation of α_2M mRNA after IFNγ treatment of the cells was not demonstrated. The response of the rat α_2M promoter correlates with the binding of Stat1 protein to the Stat3-binding site [12]. We have shown that the same mechanism applies to SPI-3 gene activation. The Stat1/Stat3-binding element is indispensable for IFNγ induction of the SPI-3 gene (Figure 2), as mutations introduced within this element resulted in loss of responsiveness. The corresponding elements in the promoters of SPI-1 and SPI-2 genes, which on the mRNA level appeared not to respond to IFNγ, are identical and differ from the element found in the SPI-3 promoter by two point mutations [31]. The identified Stat1-binding site overlaps with a possible binding site for the CAAT-enhancer-binding β, and the binding of this protein to this element was demonstrated using footprinting analysis [35]. However, we did not observe binding of factors from the family of CAAT-enhancer-binding proteins in gel-retardation assays.

Although cytokines and growth factors bind to their specific receptors and activate specific sets of transcription factors, they activate overlapping sets of target genes. Proteins of the Stat family were recently found to be activated in response to many

cytokines and growth factors [1,8]. More recently, it was found that the binding of some cytokines and growth factors leads to activation of more than one type of Stat, and these different Stats are able to bind to the same discrete element in target genes [11,12]. Our data clearly show that the −124 to −132 element, which is known to bind Stat3, also binds Stat1 and this binding correlates with the transcriptional activation of the SPI-3 gene.

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