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Convergence of TNFα and IFNγ signalling pathways through synergistic induction of IRF-1/ISGF-2 is mediated by a composite GAS/κB promoter element

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ABSTRACT

The molecular basis for the well known synergistic biological effects of tumor necrosis factor α (TNFα) and interferon γ (IFNγ) is still poorly understood. This report demonstrates that expression of interferon-regulatory factor 1 (IRF-1), also known as interferon-stimulated-gene factor 2 (ISGF-2), is synergistically induced by these cytokines. The induction is a primary transcriptional response that occurs rapidly without a requirement for new protein synthesis. Synergism is mediated by a novel composite element in the IRF-1 promoter that includes an IFNγ-activation site (GAS) overlapped by a non-consensus site for nuclear factor kappa B (NFκB). These sequences are bound strongly by signal transducer and activator of transcription 1 (STAT-1) and weakly by the p50/p65 heterodimer form of NFκB, respectively. However, the binding of STAT-1 and NFκB to the GAS/κB element *in vitro* seems to be mutually exclusive and independent. Synergistic induction of IRF-1 is likely to be an important early step in regulatory networks critical to the synergism of TNFα and IFNγ. The GAS/κB element may mediate synergistic transcriptional induction of IRF-1 by other pairs of ligands that together activate NFκB and STAT family members. Other genes are likely to contain this motif and be regulated similarly.

INTRODUCTION

The effects of tumor necrosis factor α (TNFα) and interferon γ (IFNγ) on cell growth control are well known, and are related to inflammatory and immunomodulatory properties of these cytokines (reviewed in refs 1–6). In addition to their individual effects, there are notable synergistic responses to the combination of TNFα plus IFNγ (7–13). Many of these consequences apparently arise from the ability of both TNFα and IFNγ to change the cellular program of gene expression.

Each of these cytokines has led to a paradigm for signal transduction and transcriptional regulation. In both cases, receptor–ligand interaction initiates a rapid signal transduction cascade which leads to activation and translocation to the nucleus of preexisting cytoplasmic nuclear factor kappa B (NFκB) or signal transducer and activator of transcription 1 (STAT-1), in response to TNFα or IFNγ, respectively. Each of these two proteins leads to increased transcription of target genes when bound to specific DNA regulatory elements (14–20).

Specificity of the pathway that leads from a receptor to the particular transcription factors that are affected sets the stage for distinct patterns of gene expression induced by different cytokines. However, the final determinants of specificity for transcriptional regulation of gene expression are the particular combinations of regulatory elements in the promoters of different genes and the combinatorial features of transcription factor function (for example, 21,22). Synergism of TNFα and IFNγ provides a particularly relevant example of this principle. Synergistic induction of the chemokine IP-10, the cell adhesion molecule ICAM-1, and certain MHC class I antigens results from the combined effects of transcription factors that are induced predominantly or exclusively by one or the other cytokine at the time the synergism occurs. Those transcription factors then bind to distinct, separate sites in the promoters of the genes that encode those proteins (9,10,13). Pairs of transcription factors so far implicated in the synergistic induction include NFκB plus STAT-1, or NFκB plus interferon regulatory factor 1 (IRF-1).

IRF-1 has been studied in the contexts of inflammation and immunity and of cell growth control. It was discovered in the course of work on virus induced interferon β (IFNβ) gene expression, and independently as interferon-stimulated gene factor 2 (ISGF-2), a transcription factor induced by interferon α (IFNα) (23–25). TNFα-induced accumulation of IRF-1 mRNA and DNA binding activity has also been reported (26,27). It was ultimately found that IFNγ is a more potent inducer of IRF-1 expression than is IFNα, that IFNα and TNFα result in similar induction of IRF-1, and that virus infection is perhaps the least effective inducer of IRF-1 expression. (25,28; R. Pine, this report and unpublished observations). While several reports supported the hypothesis that IRF-1 regulates virus-induced transcription of the IFNβ gene (for example, 29,30), experiments done with HeLa cells suggested that IRF-1 plays at most only a small role in the production of IFNβ (25,31,32). Studies with mice homozygously deleted for IRF-1 subsequently corroborated this conclusion (33,34). However, IRF-1 does play an important role in resistance to both viral and bacterial infections (31,35,36), consistent with its role in cellular responses to IFNs and TNFα. Furthermore, IRF-1 can exert an antiproliferative effect on cells and can participate in some apoptotic pathways (37,38).

As the transcription factor product of a primary response gene, IRF-1 prolongs the expression of other primary response genes or

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activates secondary response genes. Activation of IRF-1 gene transcription by both IFNγ and IFNα was found to be mediated by an IFNγ activation site (GAS) in the IRF-1 promoter (28). As part of the overall response to IFNγ, IRF-1 is required for protein-synthesis-dependent transcriptional induction of the murine *GBP* gene (39) and for synergistic induction of the murine inducible nitric oxide synthetase (*iNOS* or NOS2) gene by IFNγ plus lipopolysaccharide or TNFα (35,40). Maximal TNFα-induced expression of the VCAM-1 gene requires induced synthesis of IRF-1, which then acts as a secondary regulator (27).

Since IRF-1 induced by TNFα or IFNγ functions in the molecular responses to each cytokine alone, and in the synergistic induction of MHC class I alleles and iNOS by TNFα plus IFNγ, it was of interest to determine if TNFα plus IFNγ caused synergistic induction of IRF-1. This report shows that transcription of the IRF-1 gene and accumulation of IRF-1 DNA-binding activity are synergistically induced by the combination of TNFα and IFNγ. A novel composite GAS/κB element alone can mediate this response. The synergistic induction of IRF-1 by TNFα plus IFNγ may involve both NFκB and STAT-1 even though they do not seem to bind simultaneously to the GAS/κB element *in vitro*. The results presented here raise the possibility that the composite GAS/κB element in the IRF-1 promoter might mediate other interactions among pathways that utilize members of the NFκB family and those that act through STAT proteins. Furthermore, GAS/κB regulatory elements are likely to occur and function similarly in other genes.

MATERIALS AND METHODS

Reagents

Recombinant human IFNγ and TNFα were from Amgen and Chiron, respectively. TNFα and IFNγ were used at 5 ng/ml. All antisera were polyclonal, from rabbits. The anti-ISGF2 antiserum was raised against the human protein purified from HeLa cells, and immobilized on polyvinylidene difluouride membrane (25). An irrelevant immune serum was obtained after immunization with duck metallothionein (gift of P. C. Huang) by the same protocol. Anti-STAT-1 and anti-STAT-2 antisera (gift of Chris Schindler) have been previously described (41). Antibodies against NFκB family members and from normal rabbit serum (gift of Hsiou-Chi Liou) have been previously described (42). Nitrocellulose was from Schleicher and Schuell. Radioisotopes were from ICN. Poly (dIdC:dIdC) was from Pharmacia. Enzymes were from New England Biolabs or Boehringer Mannheim. All other chemicals were from Boehringer Mannheim, Sigma or Fisher Scientific, except as specifically indicated.

Plasmid constructs and oligonucleotide sequences

The IRF-1-luciferase exon fusion constructs, and the GAS/κB WT, GAS/κB 5M and GAS/κB 3M heterologous promoter constructs have been described before (28). The –199/–16, –199/–89 and –89/–16 plasmids contained the respective *Nar*I fragments from the IRF-1 promoter in the heterologous promoter luciferase reporter. The CMVβ-GAl plasmid was from California Biotechnology, Inc.

The interferon-stimulated response element (ISRE) oligonucleotide (CTCGGGAAA**GGGAAACCGAAACTG**AAGCC) and its complement, synthesized with *Bam*HI cohesive termini at the 5′ end of each strand, spans from –117 to –89 of the ISG15 promoter (24,43). The ISRE homology is shown in bold. A non-specific oligonucleotide (CTCTCTGCAAGGGTCATCAGTAC) and its complement, synthesized with *Hin*dIII cohesive termini at the 5′ end of each strand, includes the distal HNF4 site from the transthyretin promoter (44). The GAS/κB WT oligonucleotide (TACAACAGC-CTGAT**TTCCCCGAA**ATGACGGC) and its complement, synthesized with *Hin*dIII cohesive termini at the 5′ end of each strand, spans from –137 to –107 of the IRF-1 promoter. The GAS homology is shown in bold and the non-consensus NFκB site reverse complement is underlined. The GAS/κB 5M and GAS/κB 3M oligonucleotides had TT→GG or AA→CC mutations (top strand; –122 and –123, or –115 and –116, respectively). A shorter version of the IRF-1 GAS oligonucleotide (GAT**TTCCCCGAA**-AT) and its complement, synthesized with *Bam*HI cohesive termini at the 5′ end of each strand, spans from –126 to –113 of the IRF-1 promoter. The GAS homology is shown in bold.

Cell culture and transfection assays

HepG2 cells (ATCC HB 8065) were maintained as subconfluent monolayer cultures in Dulbecco-modified Eagle's medium (Bio-Whittaker) plus 10% defined supplemented calf serum (HyClone). Cells were transfected in suspension with a calcium phosphate protocol, essentially as described (45), except that only 8 μg of DNA (40 μg/ml) was used for ~10^6 cells. Luciferase reporter constructs that included sequences from the IRF-1 promoter were mixed with an expression vector that encoded β-galactosidase as an internal standard for transfection efficiency. Cells transfected in a single tube were diluted with culture medium then subdivided into 35 mm wells for subsequent untreated control or cytokine treated samples. The monolayers were washed and refed with fresh culture medium ~24 h later, and then received no further treatment or were treated with TNFα, IFNγ or both together ~64 h after transfection for the next 4 h. Extracts were made as recommended by Promega. Reagent from Promega was used to assay extracts for luciferase with an OptoComp I luminometer from MGM Instruments. β-Galactosidase was assayed according to standard procedures (45), except that reactions were performed in 96 well plates and optical density was measured with a plate reader at various times without stopping the reactions. Luciferase activity was normalized to β-galactosidase activity, then fold induction was calculated. Each transfection was performed in triplicate, and at least two experiments were done with independent preparations of each plasmid.

Extract preparation and electrophoretic mobility shift assays

HepG2 monolayers at 60–90% confluence were untreated or treated with cytokines as indicated in the figure legends. All extracts were prepared at 0–4°C. Whole cell extracts were prepared essentially as previously described (31), except that the proportion of extraction buffer to the number of cells was reduced, so that 80, 250 or 500 μl was used for cells in 35, 60 or 100 mm plates, respectively. The extraction buffer is 0.5% NP-40, 0.3 M NaCl, 10 mM $Na_4P_2O_7$, 5 mM NaF, 0.1 mM EDTA, 20 mM Na·HEPES, pH 7.9 at room temperature, 10% glycerol; plus 1 mM dithiothreitol, 100 μM Na_3VO_4, 0.4 mM phenylmethylsulfonyl fluoride, 3 μg aprotinin per ml, 1 μg leupeptin per ml and 2 μg pepstatin per ml, each freshly added before each use (buffer A + 0.3 M NaCl). For preparation of cytoplasmic and nuclear extracts, cell monolayers from 100 mm plates were scraped into phosphate buffered saline, recovered by centrifugation and lysed in 250 μl of buffer A + 0.1 M NaCl.

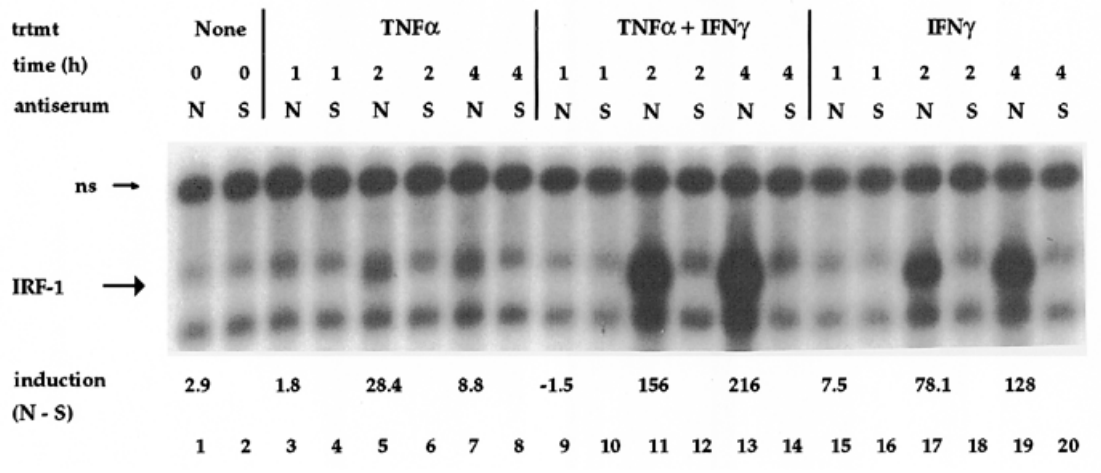

Figure 1. IRF-1 DNA-binding activity is synergistically induced by TNFα plus IFNγ. EMSAs were done with an ISRE oligonucleotide as the probe. Cells were untreated or treated with the indicated cytokine(s) for the indicated length of time, as shown. Odd-numbered or even-numbered lanes were loaded with reactions that included non-specific (N) or anti-IRF-1 (S) antiserum, respectively. The complex of IRF-1 bound to the ISRE oligonucleotide and of a non-specific complex (ns) detected by autoradiography are indicated. The results were quantitated with a gas-ionization detector (Packard Instant Imager) and are shown in arbitrary units. The signal from IRF-1 was normalized to the signal from the respective non-specific complex as an internal standard, then induction (N-S) was determined as the amount of complex removed by inclusion of the anti-IRF-1 antiserum, relative to the signal in the presence of the non-specific antiserum.

Nuclei were recovered by centrifugation at 1000 *g* for 5 min, then resuspended in 50 µl of buffer A + 0.3 M NaCl for extraction. After a 30 min incubation, debris was removed by centrifugation at 12 000 *g* for 10 min and the supernatant was recovered as the nuclear extract. The supernatant from the cell lysate was clarified by centrifugation at 12 000 *g* for 10 min, and the supernatant was recovered as the cytoplasmic extract.

Approximately 10 000 d.p.m. of oligonucleotide labeled by pulse-chase fill-in of 5′ cohesive ends (0.4–2 ng, depending on time since labeling) was used as probe for electrophoretic mobility shift assays (EMSAs) with ~10 µg of extract protein (typically 2–3 µl of extract). In addition to the contribution from the extract, binding reactions included 1× binding buffer (4% ficoll, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 20 mM Na·HEPES, pH 7.9 at room temperature). The final volume was 12.5 µl. For the ISRE probe, 0.5 µg of pGEM-1 and 1 µg of poly (dIdC:dIdC) were included as non-specific DNA. For the GAS/κB probe, 0.75 µg of poly (dIdC:dIdC) was used as non-specific DNA. Reactions were incubated for 20–30 min at room temperature. If indicated, 2.5 µl of 1× binding buffer containing 0.5 µl of antiserum or antibody was added after the binding reaction was complete, and then incubation was continued for 40–60 min at 4°C. Samples were electrophoresed on 6% polyacrylamide gels run at 4°C with 20 mM Tris-borate (pH 8.3 at room temperature), 0.4 mM EDTA. Radioactivity in protein–DNA complexes was quantitated by two dimensional gas-ionization beta particle detection (Packard Instant Imager) and visualized by autoradiographic exposure.

Determination of transcription rates

Cells were untreated or treated with cytokines as indicated in the legend to Figure 2. Run-on assays were performed with isolated nuclei to determine relative rates of transcription as previously described (46). Radiolabeled RNA was recovered and hybridized to excess plasmid DNA fixed to nitrocellulose. IRF-1 transcription was measured with a cDNA probe (25). A β-tubulin pseudogene (47) was used as a positive control and internal standard. pGem-1 (Promega) was used as a negative control probe. The results were quantitated directly with a Molecular Dynamics phosphorimager, and visualized by autoradiographic exposure.

RESULTS

IRF-1 expression is synergistically induced by TNFα plus IFNγ

Since it was already known that transcription of the IRF-1 gene is activated by IFNγ, that IRF-1 expression is induced by TNFα, and that TNFα and IFNγ often act synergistically, the combined effect of TNFα and IFNγ on induction of IRF-1 was examined (Fig. 1). Irrelevant immune antiserum or specific anti-IRF-1 antiserum was included in EMSAs that used the ISG15 ISRE as a probe for binding by IRF family members. TNFα plus IFNγ led to a synergistic increase in the amount of IRF-1 DNA binding activity after 2 or 4 h of treatment (compare lanes 1–8 and 15–20 with lanes 9–14). Synergism is defined here as a quotient greater than one for the response to the combined treatment divided by the sum of the responses to the respective individual treatments (i.e., the whole is greater than the sum of the parts). These quotients are 1.5 or 1.6 for 2 or 4 h of treatment with TNFα plus IFNγ.

Nuclear run-on assays were performed to directly determine whether transcriptional regulation of the IRF-1 gene could account for or contribute to the synergistic induction of the DNA-binding activity. The results from experiments that compared the constitutive rate of transcription to the rate after 0.5 or 2 h of cytokine treatment are shown in Figure 2A and B, respectively. The transcriptional synergism of TNFα plus IFNγ for activation of the IRF-1 gene was sufficient to account for the level of induced DNA-binding activity, taking into account that there is a lag of 1–2 h between transcriptional activation and accumulation of DNA binding activity. After 0.5 and 2 h, the quotients for transcriptional induction by the combined treatment divided by the sum of the responses to individual treatments were 2.6 and 1.7, respectively. Inhibition of protein synthesis for 30 min by cycloheximide does not activate IRF-1 transcription, or alter its activation by IFNγ during that time (25; R. Pine,

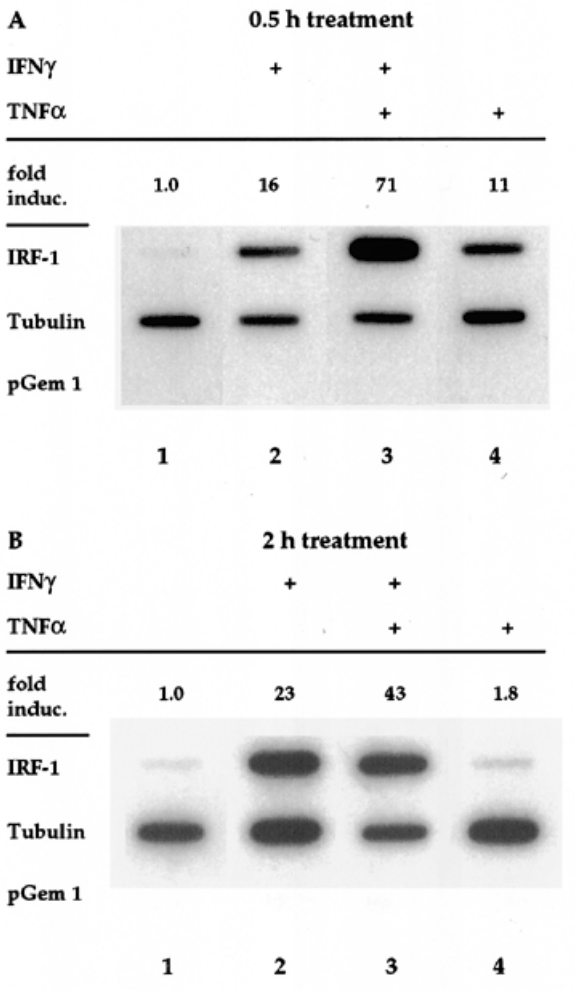

Figure 2. Transcription of the IRF-1 gene is synergistically induced by TNFα plus IFNγ. A nuclear run-on assay was done to measure transcription rates of the indicated genes. Tubulin serves as a positive control and internal standard for normalization, and pGEM-1 serves as a negative control for specificity of hybridization and background subtraction. Cells were untreated, treated for 0.5 h, or treated for 2 h, as shown. The image was detected by autoradiography and quantitated with a phosphor storage screen (Molecular Dynamics Phosphorimager).

unpublished observations). Cycloheximide in the presence of both TNFα and IFNγ had little effect on the synergistic induction of IRF-1 at 0.5 h (data not shown), thus, it did not require protein synthesis.

A GAS/κB promoter element mediates the synergistic transcriptional response to TNFα plus IFNγ

Transient transfection was used to identify the regulatory elements in the IRF-1 promoter that mediated synergism of TNFα with IFNγ (Fig. 3) Successive 5′ deletions made in the context of the IRF-1 transcription start site and first exon fused to a luciferase reporter were examined, and it was found that sequences between –3.4 kb and –160 bp did not contribute to synergism. Constructs with IRF-1 upstream sequence fused to a minimal thymidine kinase promoter-luciferase reporter were then tested to determine the effects of additional 5′ and 3′ deletions. A significant response to TNFα was conferred by the –199/–89 fragment, though TNFα induction was primarily effected by regions downstream from –89. As expected from previous studies with HeLa and K562 cells (28,48), the –199/–89 fragment gave substantial response to IFNγ, which ranged from 50 to 100% of the level obtained with the –199/–16 fragment in this and other experiments. The –89/–16 fragment did not produce a response to IFNγ. All induced responses were similarly elevated compared to those seen with the exon fusion constructs. Thus, there may be a negative regulatory element between –16 and +168 in the IRF-1 gene which affects both TNFα and IFNγ induction. Alternatively, the interaction of the upstream elements of the IRF-1 promoter with the minimal thymidine kinase promoter may be more effective than the interaction with the native TATA-less IRF-1 basal promoter sequences. Of greatest interest for the present studies, the –199/–89 fragment did mediate a synergistic response to TNFα plus IFNγ, while the –89/–16 fragment did not.

The GAS consensus that mediates response to IFNγ overlaps a sequence previously recognized as a possible NFκB site (48). An oligonucleotide that includes this region of the IRF-1 promoter, referred to here as a GAS/κB element, as well as oligonucleotides that had site-specific double point mutations in either half of the GAS dyad symmetry (Fig. 3 bottom, GAS/κB WT, 5M and 3M), were tested for response to TNFα, IFNγ or both. It was found that a heterologous promoter plus the GAS/κB WT oligonucleotide mediated essentially the same response to TNFα or IFNγ as the –199/–89 fragment linked to the same heterologous promoter, and also mediated similar synergistic induction in response to TNFα plus IFNγ. The mutations in the 5M and 3M oligonucleotides were previously found to eliminate response to IFNs (28). The 5M sequence did not mediate induction by TNFα, while the 3M sequence reduced but did not usually eliminate the response to TNFα. Neither mutant oligonucleotide conferred a synergistic response to stimulation by TNFα plus IFNγ (data not shown).

Effect of treatment with TNFα plus IFNγ on protein binding to the GAS/κB element

To determine which proteins might mediate the synergistic induction of IRF-1 in response to TNFα plus IFNγ, the GAS/κB oligonucleotide was used as a probe for EMSA with extracts from cells that were untreated or had been treated with TNFα, IFNγ or both (Fig. 4). Two complexes formed with extracts from TNFα-treated cells (Fig. 4A, lane 4), and each complex contained both the p50 and p65 subunits of NFκB, as judged by the effect of antibodies specific for those proteins (Fig. 4A, lanes 5 and 6). Additional protein(s) are likely to be present in the slower mobility complex, but remain to be identified. Each antibody also reacted essentially completely with those two complexes when extracts from cells treated with both TNFα and IFNγ were assayed (Fig. 4A, compare lane 7 to lanes 8 and 9). Antibodies against other human Rel family members, p52, c-Rel and RelB, did not react detectably with either complex regardless of whether cells had been treated with TNFα only or both TNFα and IFNγ (data not shown). This result is partially obscured by the intensity of the major IFNγ-induced complex and what may be a minor IFNγ-induced complex that migrates very close to the lower TNFα-induced NFκB complex. However, extracts from cells treated with both cytokines or only with IFNγ clearly produced the same pattern in the presence of anti-p50 or anti-p65 antibodies (compare lanes 8 and 9 to lanes 11 and 12). All of the complexes induced by TNFα plus IFNγ were found to contain either STAT-1,

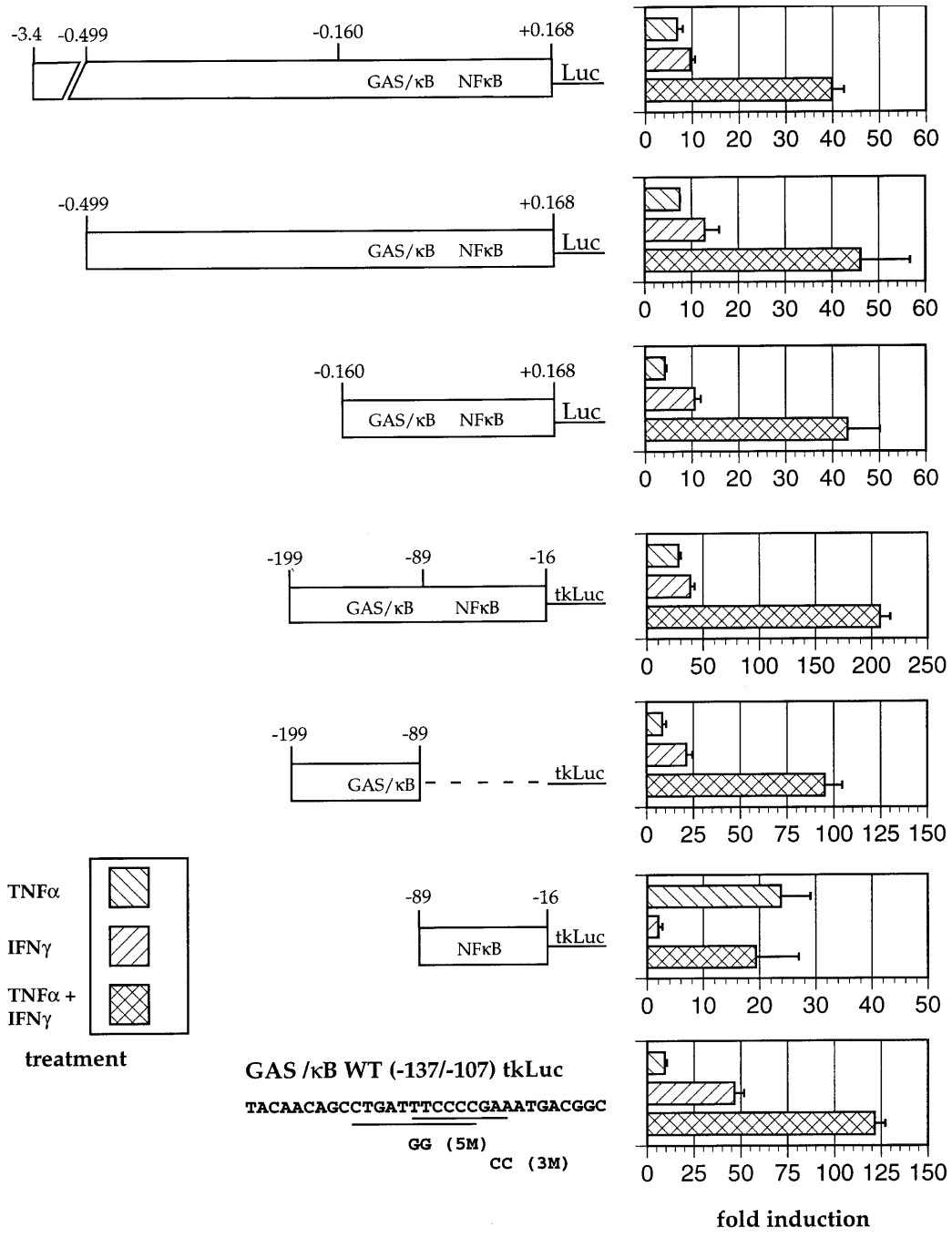

Figure 3. A GAS/κB element of the IRF-1 gene promoter mediates synergistic response to TNFα plus IFNγ. Transfected luciferase reporter constructs contained fragments of the IRF-1 gene with 5′ and 3′ endpoints as indicated. Constructs that included IRF-1 sequence to +168 bp were made with a promoterless luciferase reporter (indicated as Luc). The other constructs were made with a luciferase reporter that included a minimal promoter from the HSV thymidine kinase gene (indicated as tkLuc). Cells from a single transfection were divided and then left untreated or treated with cytokine(s) as indicated. Fold induction (triplicate mean and standard deviation) is shown for a typical experiment. The sequence of the GAS/κB wild type and mutant elements is shown. WT: the overlapping GAS consensus sequence and non-consensus NFκB sequence are underlined. The bases changed in the 5M and 3M mutants are written under the respective wild type bases, and the designations of the mutated sequences are written in parentheses alongside the base changes.

or p50 and p65, by use of the anti-STAT-1 antiserum together with either the anti-p50 or anti-p65 antibodies (Fig. 4A, lanes 13 and 14). Anti-STAT-1 antiserum alone had no effect on the NFκB complexes induced by treatment with TNFα alone (Fig. 4B, lanes 1–4), or by treatment with TNFα plus IFNγ (Fig. 4B, lanes 5 and 6). With extracts from cells treated with TNFα plus IFNγ (Fig. 4B, lanes 5 and 6) or with IFNγ alone (Fig. 4B, lanes 7 and 8), the major induced complex reacted specifically and completely with the anti-STAT-1 antiserum. Together, these results indicate that none of the complexes observed upon assay of extracts from cells treated with both cytokines contained heteromeric factors composed of Rel and STAT subunits.

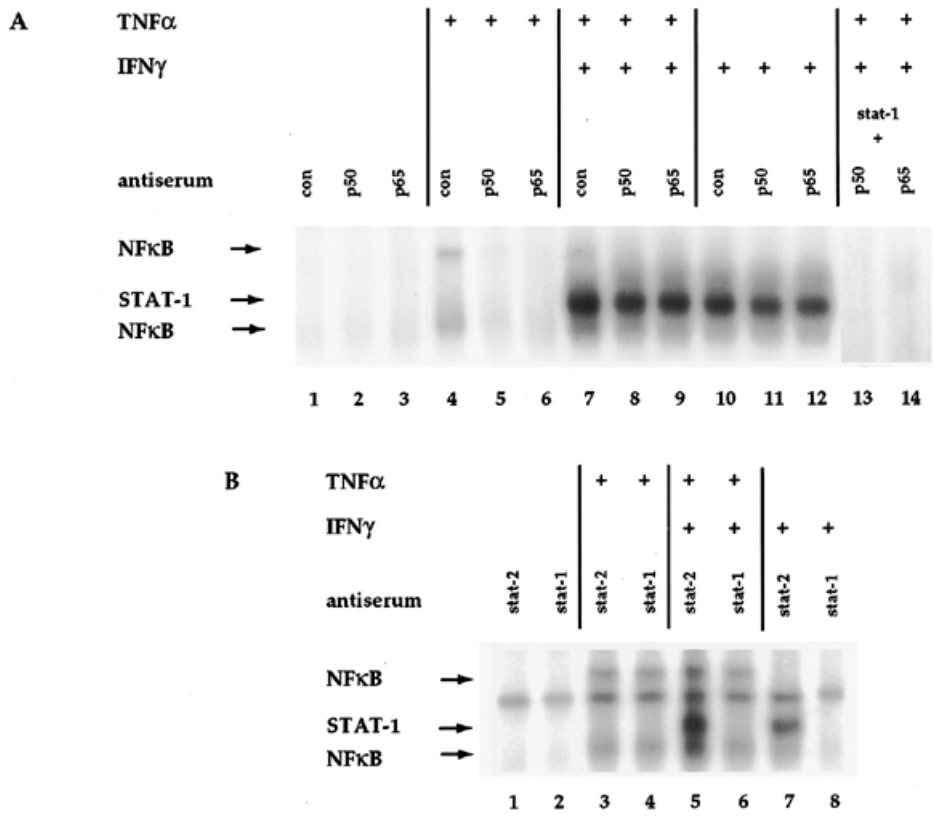

Figure 4. GAS/κB complexes induced by TNFα plus IFNγ include either the p50 and p65 subunits of NFκB or STAT-1, as do the corresponding complexes induced by TNFα or IFNγ. EMSAs were performed with the GAS/κB oligonucleotide as probe. The complexes that include NFκB (p50 and p65) or STAT-1 are indicated. (**A**) and (**B**) Cells were untreated or were treated with TNFα and/or IFNγ as indicated, for 30 min, prior to preparation of whole cell extracts. Non-specific (con), anti-p50 (p50), anti-p65 (p65), anti-STAT-1 (STAT-1) or anti-STAT- 2 (STAT-2) antisera were included in the assay reactions as indicated. The unlabeled constitutive complex seen in (B) is not detected in some sets of extracts.

When the kinetics of stimulation with either cytokine alone or both together was examined (Fig. 5A), it was found that all extracts from cells treated with both cytokines produced a pattern of complexes consistent with the superimposition of the results from each cytokine alone. This was confirmed by quantitation of the radioactivity in each complex (data not shown). In fact, when assays were done with the corresponding mixtures of extracts, the result was the same. Furthermore, extracts from cells treated with both cytokines did not produce any complexes that had mobility distinct from the complexes detected with extracts from cells treated with TNFα or IFNγ (data not shown). Consistent with the kinetics of IRF-1 transcription induced by TNFα or IFNγ alone, the NFκB complexes were most in evidence after 30 min of TNFα treatment (lanes 1–5), while the STAT-1 complex was nearly constant from 30 min through 4 h of treatment with IFNγ (lanes 6–9). Curiously, the NFκB complexes reappeared slightly after 2 or 4 h of treatment with TNFα, although they had almost vanished at 1 h. The biphasic induction of the NFκB complexes actually helped distinguish between the results obtained with extracts from cells treated with TNFα plus IFNγ and those obtained with extracts from cells treated with IFNγ alone. The differences again were somewhat obscured by the intensity of the IFNγ induced complex(es). However, the pattern of complexes detected in extracts from cells treated for 1 h with both cytokines was very similar to the pattern observed after any length of treatment with IFNγ alone (compare lane 7 with lanes 10–13), and that pattern is different from the one seen with the extracts from cells treated for 30 min, 2 h or 4 h with both cytokines together (lanes 6, 8 and 9, respectively). Thus, the combination of complexes detected by EMSA of extracts from cells treated with either cytokine alone and the complexes observed upon assay of extracts from cells treated with the combination of TNFα plus IFNγ exhibited no quantitative or qualitative differences at any time point examined. The same was true for nuclear extracts prepared after 30 min of treatment with TNFα, IFNγ or both, despite the fact that those extracts were 10 times more concentrated on a cell equivalent basis (data not shown).

Competition with unlabeled oligonucleotides was done as an alternative way to detect differences among the complexes of the GAS/κB element with NFκB or STAT-1 that were due to treatment with TNFα plus IFNγ compared to treatment with each cytokine alone (Fig. 5B). The presence of excess oligonucleotide should also reveal any interdependence in the formation of the complexes that might result from treatment with both cytokines. A non-specific sequence did not compete with any of the complexes (lanes 2, 7 and 12), while excess wild type GAS/κB oligonucleotide eliminated all the complexes (lanes 3, 8 and 13). The GAS/κB 5M mutant oligonucleotide also failed to compete with any of the complexes (lanes 4, 9 and 14). As expected, the GAS/κB 3M mutant oligonucleotide competed essentially completely for the NFκB complexes induced by TNFα alone or in cells treated with TNFα plus IFNγ. It did not compete with the

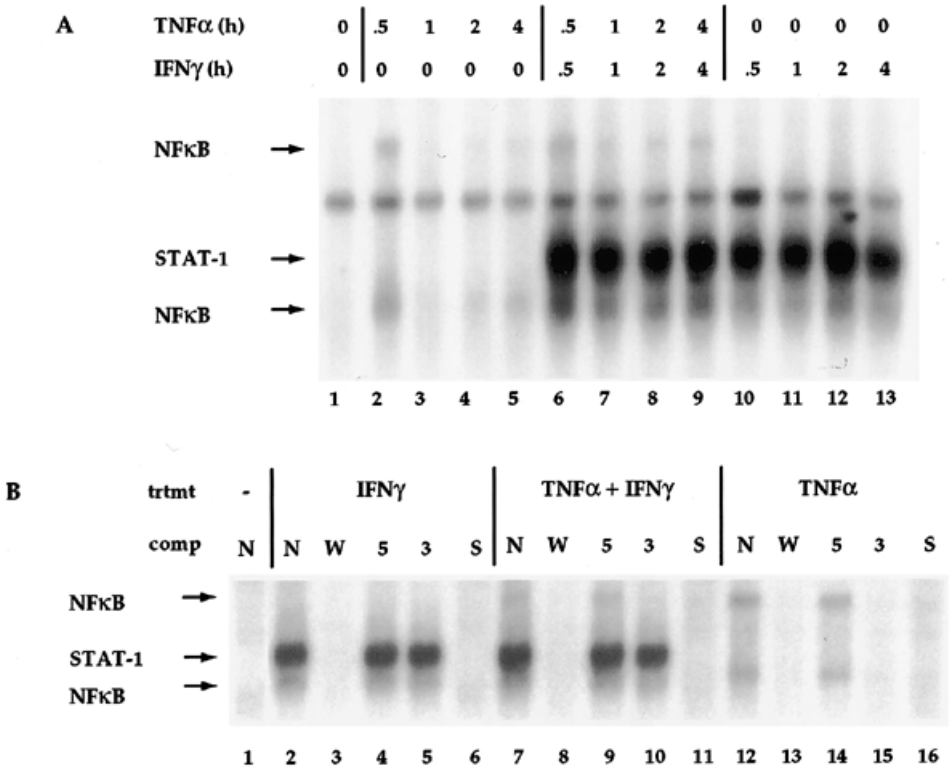

Figure 5. GAS/κB complexes induced by TNFα plus IFNγ have the same kinetics of induction and site specificity as the corresponding complexes induced by TNFα or IFNγ. EMSAs were done with the GAS/κB oligonucleotide as the probe. The complexes that include NFκB (p50 and p65) or STAT-1 are indicated. (**A**) Cells were untreated or were treated with TNFα and/or IFNγ as indicated, for the length of time indicated, prior to preparation of whole cell extracts. Electrophoresis was continued for more time than usual to increase separation of the specific complexes. The unlabeled constitutive complex is not detected in some sets of extracts. (**B**) Cells were untreated or were treated with TNFα and/or IFNγ as indicated, for 30 min, prior to preparation of whole cell extracts. A 200-fold molar excess of an unlabeled oligonucleotide was included in the assay reactions as indicated: non-specific (N), wild type GAS/κB (W), 5M mutated GAS/κB (5), 3M mutated GAS/κB (3), short wild type GAS (S).

STAT-1 complex induced in cells treated with IFNγ alone or TNFα plus IFNγ. An oligonucleotide referred to here as a short GAS element, that included the wild-type IRF-1 GAS consensus and reconstituted an alternative non-consensus NFκB site, was also tested. It competed completely for the STAT-1 complexes in extracts from cells treated with IFNγ alone or TNFα plus IFNγ, and competed to an extent similar to the GAS/κB 3M sequence for the NFκB complexes in extracts from cells treated with TNFα alone or TNFα plus IFNγ (lanes 6, 11 and 16).

These results independently confirm that the NFκB and STAT-1 complexes that formed with extracts made after cells were treated with both cytokines are equivalent to the corresponding complexes that formed with extracts from cells treated with TNFα or IFNγ. Furthermore, with extracts from cells treated with TNFα and IFNγ, the NFκB and STAT-1 complexes still form independently, since excess unlabeled GAS/κB 3M mutant oligonucleotide competed against the former but not the latter, consistent with the location of the base changes within the GAS consensus but outside the non-consensus NFκB site.

DISCUSSION

This report presents data showing that IRF-1 DNA-binding activity is synergistically induced by TNFα plus IFNγ, and establishing that transcriptional regulation is the mechanism that underlies this example of synergism between TNFα and IFNγ. These results and the studies of MHC class I, ICAM-1 and IP-10 regulation (9,10,13) strongly support the idea that synergistic biological effects of TNFα and IFNγ result from synergistic transcriptional activation of genes that are also regulated by each cytokine alone.

A composite GAS/κB promoter element mediates the synergistic induction of IRF-1 transcription. Both NFκB and STAT-1 from cells treated with both cytokines bind to the element, but the binding is mutually exclusive and independent. The details of the protein–protein and protein–DNA interactions that result in synergistic induction of the IRF-1 gene remain to be determined. The regulation of IRF-1 transcription may be related to the synergistic induction by TNFα and IFNγ of the ICAM-1 or IP-10 genes (9,13), but is clearly distinct since those promoters include separate NFκB and STAT-1 binding sites that can be simultaneously occupied.

The function of IRF-1 in protein-synthesis dependent transcriptional activation of TNFα- or IFN-induced gene expression is most consistent with and has been predicted best from experiments that characterized induction of IRF-1 DNA-binding activity (25,27). It seems likely that the outcome of interactions between IRF-1 and other transcription factors including ICSBP, HMG I(Y), or ATF-2 (21,27,49) will be affected by this synergistic induction. This possibility should be considered in particular for these or other activities that are themselves modulated by either TNFα or IFNγ. The data shown here lead to the conclusion that synergistic induction of IRF-1 constitutes a step in a regulatory network. As shown in Figure 6, the regulation of IRF-1 gene expression and the function of newly synthesized

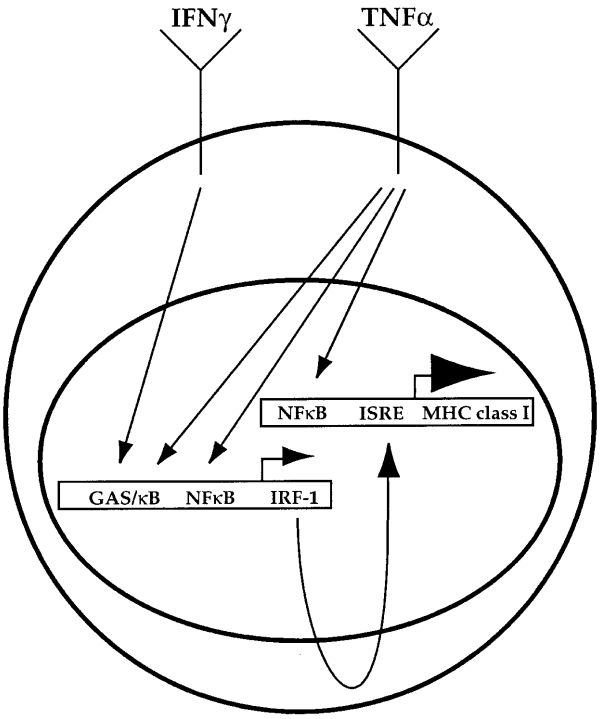

Figure 6. Schematic representation of a preamplifier model for regulation and function of IRF-1. Signals from occupied TNFα and IFNγ receptors lead to synergistic activation of IRF-1 gene expression. IRF-1 and NFκB then act to synergistically induce other genes, for example MHC class I, as a secondary protein-synthesis-dependent response to TNFα and IFNγ.

IRF-1 as a transcription factor establish a link between synergistic primary and secondary responses. Synergistic induction of IRF-1 would serve as a preamplifier for the subsequent interaction of NFκB with IRF-1 in the synergistic induction of MHC class I genes as a protein-synthesis dependent response to TNFα plus IFNγ (10). A similar network effect would pertain to synergistic induction of the iNOS gene (35,40). Thus, the synergistic induction of IRF-1 by TNFα plus IFNγ can clearly contribute to the role of this factor in inflammation and immunity. The potential impact of synergistic induction of IRF-1 on apoptotic pathways is not as clear, since pathways that lead to activation of NFκB or apoptosis in response to TNFα are distinct (50,51).

Regulation of IRF-1 expression

The synergistic induction of IRF-1 transcription by TNFα plus IFNγ was a primary response that did not require protein synthesis, since it occurred within 0.5 h and was not blocked by cycloheximide. Transcriptional regulation as the mechanism of synergistic induction is consistent with the conclusion that IRF-1 expression is generally regulated transcriptionally. Previous studies that examined transcription of the IRF-1 gene found induction by interferons, interleukin 6, prolactin and retinoic acid (25,28,52–54). TNFα plus IFNα synergistically induced DNA-binding activity, but not transcription, of IRF-1 (unpublished observations). This exception to regulation of IRF-1 expression primarily at the level of transcription could be peculiar to HepG2 cells, which exhibited unusually little induction of IRF-1 transcription by IFNα alone. The mechanisms that regulate the amount of IRF-1 DNA binding activity induced in response to TNFα plus IFNα remain to be determined. Nonetheless, this is the first clear example in which IRF-1 DNA binding activity is regulated without a corresponding change in the rate at which the IRF-1 gene is transcribed. In contrast, the synergistic activation of IRF-1 transcription by TNFα plus IFNγ was clearly reflected in synergistic induction of IRF-1 DNA binding activity.

Functional properties of the composite GAS/κB element

The regulatory element that mediated transcriptional synergism of IRF-1 induction in response to TNFα plus IFNγ was found to be a composite GAS/κB element. The role of this sequence in the response to IFNγ, through binding of tyrosine phosphorylated STAT-1, has been previously described (28). In contrast, it was unexpected that the GAS/κB element would be a functional binding site for NFκB that could mediate a response to TNFα, despite a previous report that had marked this non-consensus sequence as a putative NFκB site (48). However, removal of the proximal consensus NFκB site found at –49/–40 in the IRF-1 promoter lowered but did not eliminate the response to TNFα alone, and did not change the extent of synergism, i.e., the ratio of the synergistic induction to the sum of the IFNγ response plus the reduced TNFα response. These results left the GAS/κB element as the best candidate for both the ability to mediate the residual response to TNFα, and synergism of TNFα plus IFNγ. This function was confirmed when the GAS/κB oligonucleotide was tested directly in transfection experiments. Furthermore, the IRF-1 composite GAS/κB site was bound by both the p50 and p65 subunits of NFκB, though the relative contributions of the conserved half-site and the novel half-site are unknown.

This promoter design may be unique to the regulation of IRF-1 expression, and a contribution to the synergistic response of the GAS/κB element from specific flanking sequences has not been ruled out. However, it is quite likely that composite GAS/κB elements will be important regulatory sequences that allow integration of TNFα and IFNγ signalling in many different genes. Any GAS consensus with the sequence TTCCCNGAA will include the highly conserved NFκB half-site, which is the reverse complement of the underlined bases. Figure 7 shows two possible alignments of the NFκB consensus sequence with the IRF-1 GAS/κB element, and the single alignments with the GAS elements from the FcγR1 and ICAM-1 genes. Either alignment of the IRF-1 GAS/κB element deviates from highly conserved bases of the NFκB consensus at two positions. The same is true for the potential non-consensus NFκB site that overlaps the FcγR1 GAS homology. In contrast, the ICAM-1 GAS homology deviates from the NFκB consensus at three positions outside the conserved half-site, and this GAS element will not by itself mediate a synergistic response to TNFα plus IFNγ (9). It will be of interest to determine if the IRF-1 or other GAS/κB elements also will mediate synergism for any transcriptional regulation that involves NFκB and STAT family members, perhaps in response to inducers other than TNFα and IFNγ.

The precise mechanism by which the GAS/κB element mediates a synergistic response to TNFα and IFNγ remains to be determined. Although the results obtained with cell extracts did not reveal any interaction between STAT-1 and NFκB, recombinant STAT-1 and NFκB (p50/p65) have been found to interact in EMSA performed with the GAS/κB oligonucleotide (unpublished observations). It is likely that the interaction of these proteins will be an important aspect of this synergism. Since no

IRF-1 GAS/κB	T T T C G G G G A A A T C A G
NFκB consensus	G G G R H W Y Y C C
	G G G R H W Y Y C C
FCγRI GAS	T T T C T G G G A A A T A C A
NFκB consensus	G G G R H W Y Y C C
ICAM-1 GAS	T T T C C G G G A A A G C A G
NFκB consensus	G G G R H W Y Y C C

Figure 7. Alignment of IRF-1 GAS/κB with the FcγR1 and ICAM-1 GAS, and comparison of possible non-consensus NFκB sites to the NFκB consensus sequence. The GAS homologies and flanking sequences are taken from the literature (55,56). The NFκB consensus sequence, based on 40 sites, is adapted from reference 57. The International Union of Biochemists' single letter code for degenerate nucleic acid sequences is used to indicate any base present at a particular position in four or more sites.

other known promoter architecture or individual regulatory elements are fully comparable to those of the IRF-1 gene, further study of IRF-1 induction is likely to reveal biological cross-talk and mechanistic features of NFκB and STAT-1 function that have not been recognized previously.

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