Synergy between Interferon-γ and Tumor Necrosis Factor-α in Transcriptional Activation Is Mediated by Cooperation between Signal Transducer and Activator of Transcription 1 and Nuclear Factor κB*

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Interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) cooperate to induce the expression of many gene products during inflammation. The present report demonstrates that a portion of this cooperativity is mediated by synergism between two distinct transcription factors: signal transducer and activator of transcription 1 (STAT1) and nuclear factor κB (NF-κB). IFNγ and TNFα synergistically induce expression of mRNAs encoding interferon regulatory factor-1 (IRF-1), intercellular adhesion molecule-1, Mig (monokine induced by γ-interferon), and RANTES (regulated on activation normal T cell expressed and secreted) in normal but not STAT1-deficient mouse fibroblasts, indicating a requirement for STAT1. Transient transfection assays in fibroblasts using site-directed mutants of a 1.3-kilobase pair sequence of the IRF-1 gene promoter revealed that the synergy was dependent upon two sequence elements: a STAT binding element and a κB motif. Artificial constructs containing a single copy of both a STAT binding element and a κB motif linked to the herpes virus thymidine kinase promoter were able to mediate synergistic response to IFNγ and TNFα; such response varied with both the relative spacing and the specific sequence of the regions between these two sites. Cooperatively responsive sequence constructs bound both STAT1α and NF-κB in nuclear extracts prepared from IFNγ- and/or TNFα-stimulated fibroblasts, although binding of individual factors was not cooperative. Thus, the frequently observed synergy between IFNγ and TNFα in promoting inflammatory response depends in part upon cooperation between STAT1α and NF-κB, which is most likely mediated by their independent interaction with one or more components of the basal transcription complex.

Interactions of the JAK-STAT and the κB signaling pathways appear to be indispensable for stimulus-dependent, transcriptional activation of many inflammatory genes. Furthermore, SBE and κB motifs are found in the promoter regions of many inflammatory genes. Many studies have reported functional synergy between TNFα and IFNγ in promoting inflammatory function and gene expression, some of which could involve an interplay between STAT1 and κB binding factors that play often critical roles in this process (1, 2). Although both cytokines independently exert a number of biological activities in a cell type-specific fashion, they have been shown in many circumstances to function cooperatively or antagonistically in controlling expression of a variety of cytokines and cell surface molecules (3–7).

Much recent work on cytokine-mediated intracellular signaling pathways has provided a general paradigm for the molecular mechanisms by which extracellular signals induce transcription of target genes (8–11). A variety of cytokines, growth factors, and hormones trigger phosphorylation of latent cytoplasmic transcription factors termed signal transducers and activators of transcription (STATs) via one or more members of the Janus (Jak) family of protein tyrosine kinases. Tyrosine-phosphorylated STATs assemble in dimeric or oligomeric form, translocate to the nucleus, and bind to specific DNA sequence motifs or STAT binding elements (SBEs) (12). IFNγ has been shown to induce tyrosine phosphorylation of STAT1α, and a homodimeric form of STAT1α binds to the IFNγ-activation sequence (13), an SBE that has been identified as a critical sequence motif involved in the transcriptional activation of many IFN-inducible genes including the IRF-1 and ICAM-1 genes (14–17).

The κB sequence motif has been shown to be an essential cis-acting regulatory element for mediating the TNFα-, interleukin-1-, and lipopolysaccharide-induced transcriptional activation of multiple cytokines and cell surface molecules (18–20). Although this sequence motif is recognized by members of the Rel homology family, including NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA, c-Rel, and RelB, various forms of the κB sequence motif have been shown to exhibit differential affinity for and functional response to different dimeric combinations of Rel family proteins. Cell type-specific expression of the Rel family members also mediates specificity for κB-dependent gene expression. Furthermore, members of the Rel family have been shown to physically and functionally interact with members of other transcription factor families (21–23). The combination of these variables generates high potential for diversity in the control of gene expression during inflammation.

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(3–6). The present study was undertaken to determine whether IFNγ-activated STAT1 can cooperate with TNFα-activated NF-κB to promote enhanced transcription. The results show that IFNγ and TNFα synergize to induce expression of several genes that contain both SBE and κB motifs. The findings indicate that both the SBE and κB motifs are required for cooperativity and that the synergistic function of STAT1α and NF-κB appear to result from independent activation and recognition of cognate nucleotide sequence motifs.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco's modified Eagle's medium, minimum essential medium nonessential amino acid solution, sodium pyruvate, and antibiotic-antimycotic were obtained from Life Technologies, Inc. Fetal bovine serum was purchased from Bio Whittaker (Walkersville, MA). DEAE-dextran and polyethyleneoxycetyldicytidylic acid (poly(dI-dC)) were purchased from Pharmacia LKB Ltd. (Uppsala, Sweden). MAGNA MNNA transfer membrane was obtained from Micron Separations Inc. (Westboro, MA). Restriction enzymes, Klenow fragment of Escherichia coli DNA polymerase I, T4 kinase, and bovine serum albumin were purchased from Boehringer Mannheim. Ultra DNA polymerase was obtained from Perkin-Elmer. Dupont NEN was the source of [α-32P]CTP and [γ-32P]ATP. 1-Deoxy-dichloroacetlyl-1-[14C]chloramphenicol was obtained from Amesher Corp. Thin layer chromatography (TLC) plates (Silica Gel 60) were obtained from Merck (Darmstadt, Germany). Protein assay reagents were obtained from Bio-Rad. Site-directed mutagenesis kits, the luciferase reporter plasmid (pGL2-Basic), and luciferase enzyme assay reagents were purchased from Promega Corp. (Madison, WI). Recombinant mouse IFNγ (specific activity, 6.8 × 108 units/mg) was obtained from Life Technologies, Inc. Recombinant mouse TNFα (specific activity, 2.6 × 107 units/mg) was a generous gift from Genentech Inc. (South San Francisco, CA). Anti sera to mouse p50 (NF-κB1), p65 (RelA), c-Rel, STAT3, andhumanSp1 were obtained from Santa Cruz Biotechnology (Hercules, CA). Mouse preprolactin antibody (STAT1 p91/p84) was obtained from Transduction laboratories (Lexington, KY). Dithiothreitol, HEPES, normal rabbit IgG, chloroquine diphosphate, dimethyl sulfoxide, leupeptin, antipain, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride, were obtained from Sigma. Other reagents were purchased from Mallinkrodt, Inc. (Paris, KY).

Cell Culture—Fibroblasts from STAT1-deficient and wild type mice were prepared as described previously (24). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mM nonessential amino acid solution, 10 mM sodium pyruvate, 20 mM of L-glutamine. NIH3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin (complete medium) and subcultured twice weekly. In experiments, the cells were grown to confluence in 100- or 150-mm diameter culture dishes and washed with phosphate-buffered saline, replenished with fresh culture medium, and cultured for 2 h. To standardize transfaction efficiencies, the transfected cells were then harvested in trypsin-EDTA solution, pooled, and seeded in four 100-mm diameter Petri dishes. The cells were cultured in medium containing 0.2% fetal bovine serum for 24 h to deprive growth factors and then stimulated with IFNγ and/or TNFα for 16 h for the CAT reporter gene and for 8 h for the luciferase reporter gene, respectively. After stimulation, the cells were washed and extracted in lysis buffer (Promega), and luciferase activity was assayed using reagents provided by Promega according to the manufacturer's instructions. Twenty μg of extract protein were utilized in each assay. CAT activity was assessed by determination of the conversion of 14C-chloramphenicol into acetylated forms detected by thin layer chromatography as described previously (35). The acetylated products were quantified using a phosphorescence detection system (Molecular Dynamics, Sunnyvale, CA).

Preparation of Plasmid DNA—A DNA encoding mouse IFN-1 (25) was cloned from a mouse macrophage cDNA library (26) using a reverse transcriptase-PCR fragment as a probe as described previously.2 The plasmid encoding the cDNA for mouse ICAM-1 was obtained from the American Type Culture Collection (Rockville, MD) (27). cDNA fragments for mouse Mig and RANTES were prepared by reverse transcriptase-PCR using a set of primers corresponding to the mouse Mig and RANTES cDNA sequences obtained from the GenBank2 data base (28–30) and cloned into pBluescript (Stratagene, La Jolla, CA). The nucleotide sequences were independently confirmed. The plasmid encoding GAPDH was obtained from Dr. David Stern (Columbia University, New York, NY). Methods for plasmid DNA preparations were as described in Sambrook et al. (31). One μg of plasmid DNA or 100 ng of PCR products were radiolabeled by random priming with [α-32P]dCTP. The resultant specific activity was approximately 107 cpm/μg, which was measured at 10% unlabelled.

Preparation of RNA and Northern Hybridization Analysis—Each assay utilized confluent monolayer of fibroblasts cultured in 100-mm diameter plastic Petri dishes for preparation of total RNA. After treatment of the cells with the indicated stimuli, total cellular RNA was extracted by the guanidine isothiocyanate-cecum chloride method (32). Samples of total RNA (5 μg) were separated on a 1% agarose, 2.2 M formaldehyde-agarose gel and subsequently transferred onto MAGNA membranes with 20 × SSC by capillary transfer according to previously published methods (31). The RNA was cross-linked to the membrane with a UV cross-linker (Stratagene). The blots were prehybridized for 8–12 h at 42 °C in 50% formamide, 1% SDS, 5 × SSC, 1 × Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured salmon sperm DNA, and 50 mM Na phosphate (pH 6.5) and then hybridized at 1 × 106 cpm/ml of radiolabeled cDNA plasmid probe at 42 °C for 16–24 h. After hybridization, blots were washed with 0.1% SDS, 2 × SSC for 30 min at room temperature followed by two washes at 55 °C. The blots were then exposed using XAR-5 x-ray film with intensifying screens at −70 °C.

Preparation of Reporter Gene Plasmid DNA—The luciferase reporter constructs containing the 1.3-kb IRF-1 promoter was kindly provided by Dr. Bryan Williams (Department of Cancer Biology, Cleveland Clinic Foundation). The SBE site at positions −123 to −113 and the κB site at positions −49 to −40 of the IRF-1 promoter (14) were respectively mutated in the 1.3-kb 5′-flanking sequence of the IRF-1 gene by oligonucleotide-directed, site-specific mutagenesis as described previously (33). The mutant sequence utilized for the SBE and the κB were TTCCTCCG and GGGAATCC, respectively. Lowercase letters represent the mutant nucleotides.

One or two copies of the IRF-1 SBE or the IP-10 κB2 were placed in front of the −105 or the −81 base pair herpes simplex virus-thymidine kinase (Tk) promoter (34) linked to the chloramphenicol acetyl transferase (CAT) gene (pTK-105 CAT) (35) or the luciferase gene (pTK-105 Luc or pTK-161 Luc) (36). Site-directed mutagenesis of these reporter genes into fibroblasts or NIH3T3 cells were as described previously (35). The resultant specific activity was approximately 107 cpm/ml, which was measured at 10% unlabelled.

Preparation of Oligonucleotides and PCR-amplified DNA—The following oligonucleotides were used in this study.

IRF-1 SBE

\[ 5'-\text{tca}G\text{CTCGAGTGT}3\text{C}G\text{G}G\text{ACCTATGGAGGCTTTGACTCGGc}3'-3\text{CCG}G\text{ACCTAAGGGGGGACTCGGc}3'-5\text{mutIRF-1 SBE (antisense)} \]

\[ 5'-\text{tca}G\text{CTCGAGTGT}3\text{C}G\text{G}G\text{ACCTATGGAGGCTTTGACTCGGc}3'-3\text{CCG}G\text{ACCTAAGGGGGGACTCGGc}3'-5\text{mutIRF-1 κB (antisense)} \]

IP-10 κB2

\[ 5'-\text{gc}G\text{ATGAGGAGGAATCTACATCGTTGATAC}3'-3\text{CC}C\text{TCTCTCTCCTTAAAGTGCAGAATCGGc}3'-5\text{mutIP-10 κB2 (antisense)} \]

\[ 5'-\text{gc}G\text{ATGAGGAGGAATCTACATCGTTGATAC}3'-3\text{CC}C\text{TCTCTCTCCTTAAAGTGCAGAATCGGc}3'-5\text{mutIP-10 κB2 (antisense)} \]

The nucleotide sequences of IRF-1 SBE and κB were taken from Sims et al. (14). The IP-10 κB2 sequence was taken from Ohmori and Hamilton (33, 37). Lowercase letters represent the bases included for creat...
ing restriction sites. Underlined sequences represent the consensus sequences for the SBE and xB elements, respectively. Boldface type indicates the substituted bases for mutation. Oligonucleotides were synthesized using an Applied Biosystem DNA synthesizer (model 381A) or obtained from Ransom Hill Bioscience Inc. (Ramona, CA). Double-stranded oligonucleotides were prepared by annealing the complementary single strands. A DNA fragment corresponding to the region between −129 and −37 of the IRF-1 promoter (14) was generated by PCR using a sense oligonucleotide of the IRF-1 SBE and an antisense oligonucleotide of the IRF-1 xB as primers, and the luciferase reporter plasmid containing the 1.3-kb IRF-1 promoter was used as a template. A mutant fragment was also generated by using a sense oligonucleotide of mut1 SBE and antisense oligonucleotide mut1IRF-1xB as described above. Double-stranded oligonucleotides were radiolabeled with the Klenow fragment of DNA polymerase I and [γ-32P]dCTP in a fill-in reaction for 5′ protruding ends. PCR-amplified DNA fragments were radiolabeled with T4 kinase and [γ-32P]ATP.

Preparation of Nuclear Extracts—Nuclear extracts were prepared using a modification of the method of Dignam et al. (38) as described previously (5, 37). After stimulation, the cells were washed with ice-cold phosphate-buffered saline three times, harvested, and resuspended in 300 μl of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin, antipain, aprotinin, and pepstatin) for 10 min on ice. The cells were then lysed in 0.6% Nonidet P-40 by vortexing for 10 s. Nuclei were separated from cytosol by centrifugation for 10 min. Protein concentration was measured in fibroblasts from wild-type mice or from mice in which the STAT1 gene has been deleted through homologous recombination (24). Serum-starved cultures were stimulated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 3 h in the presence or absence of cycloheximide (CHX; 5 μg/ml) prior to preparation of total RNA and analysis of specific mRNA levels by Northern hybridization as described under “Experimental Procedures.” Five μg of total RNA were analyzed in each lane. Blots were hybridized with the indicated radiolabeled cDNA probes. Similar results were obtained in three independent experiments.

**FIG. 1.** Schematic representation of the promoter region of the IRF-1, ICAM-1, Mig, and RANTES genes. Potential cis-regulatory elements and critical defined sequences are schematically shown based on gene sequences obtained from the GenBankTM data base and the following references; human IRF-1 (14, 15, 49); human ICAM-1 (16, 17, 44, 45, 68); mouse Mig (41, 42); mouse RANTES (30, 46). The numbers above the promoter regions represent the nucleotide position relative to the transcription start site.

**FIG. 2.** STAT1 is essential for optimal synergy between IFNγ and TNFα. Serum-starved confluent monolayers of fibroblasts from wild-type mice (A) or STAT1-deficient mice (B) were either untreated (UT) or stimulated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 3 h in the presence or absence of cycloheximide (CHX; 5 μg/ml) prior to preparation of total RNA and analysis of specific mRNA levels by Northern hybridization as described under “Experimental Procedures.” Five μg of total RNA were analyzed in each lane. Blots were hybridized with the indicated radiolabeled cDNA probes. Similar results were obtained in three independent experiments.

**RESULTS**

**STAT1 Is Essential for the Synergistic Induction of IFNγ and TNFα-mediated Gene Expression—**IFNγ and TNFα have been shown to cooperatively regulate transcription of many inflammatory genes (3–6, 40). Previous studies have demonstrated that the IFNγ-induced transcriptional activation of the IRF-1, ICAM-1, and Mig genes depends upon IFNγ response elements or SBEs in the promoter region of the genes (Fig. 1), which are recognized by STAT1 or STAT1-containing factor(s) (14–17, 41–43). The promoter regions of these IFNγ-inducible genes also contain one or more xB sequence motifs, and their transcriptional activation in response to TNFα is dependent upon activation of xB binding activities (44, 45). On the basis of these observations, we postulated that IFNγ-induced STAT1 and TNFα-induced NF-xB cooperatively regulate transcription of genes containing both SBE and xB motifs. Analysis of RANTES gene expression was also included, since IFNγ and TNFα can cooperatively induce expression of this chemokine gene, although no SBE has been identified in the promoter (6, 30, 46–48). Levels of endogenous mRNA expression were determined in fibroblasts from wild-type mice and from mice in which the STAT1 gene has been deletet through homologous recombination (24). Serum-starved cultures were stimulated with IFNγ and TNFα either alone or in combination for 3 h prior to isolation of RNA and Northern analysis. While the sensitivity of normal cells to IFNγ or TNFα alone varied with each gene, all four genes were strongly expressed in cells stimulated with both agents (Fig. 2A). IFNγ and TNFα cooperativity was markedly reduced (>90%) in STAT1-deficient fibroblasts without affecting the sensitivity to TNFα alone (Fig. 2B). These four responses were not mechanistically identical; the synergistic enhancement of RANTES mRNA expression was blocked in cells co-treated with cycloheximide (CHX), while expression of IRF-1, ICAM-1, and Mig was unaltered (Fig. 2C). Thus, the synergy between IFNγ and TNFα may involve protein synthesis-
The kB and SBE sites in the 1.3-kb promoter of the IRF-1 gene are required for optimal IFNγ and TNFα-induced transcriptional activity. The diagram at the left shows wild type and mutant 1.3-kb IRF-1 luciferase constructs. Potential enhancer elements are shown schematically. The numbers above the enhancer elements refer to the nucleotide position relative to the transcription start site of the IRF-1 gene (14). Fibroblasts from wild type mice (top part) or from STAT1-deficient mice (bottom part) were transfected with the indicated luciferase constructs as described under “Experimental Procedures.” Twenty-four hours after transfection, the cells were either left untreated or stimulated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 8 h prior to analysis of luciferase activity. The relative luciferase activity is expressed as -fold induction in stimulated as compared with unstimulated samples. Mean arbitrary luciferase units (units/μg) for different constructs in unstimulated cultures are as follows: pGL Basic, 0.2; pGL IRF-1, 1.3; mut SBE, 2.8; mut kB, 3.9, mut SBE + kB, 1.2. Each column and bar represents the mean ± S.E. from four independent experiments.

While the magnitude of cooperative response is markedly reduced in wild type cells transfected with mutations in individual motifs (either SBE or kB) and in STAT1-deficient cells, there is residual cooperativity evident in both circumstances. This apparent leakiness may derive from multiple sources. For example, the mutated motifs may retain some low affinity interaction with individual factors. Alternatively, there may be other sites that are able to participate, providing the lower magnitude cooperativity. Indeed, low but detectable cooperativity is evident using promoter constructs in which both the SBE and the proximal kB sites are mutated. The very low but reproducible response to IFNγ seen in STAT1-deficient cells (both in Fig. 1 and Fig. 3) may reflect minor compensatory action of IFNγ functioning through STAT1-independent systems. Indeed, in EMSA experiments using nuclear extracts from IFNγ-treated STAT1-deficient fibroblasts, we detected low but significant levels of STAT3 that were not seen in wild type cells (data not shown). Since STAT3 can act to modulate transcription through IFNγ activation sequence motifs, this could account for the leaky response to IFNγ. It should be emphasized that these low level responses may be detectable in our experimental system due to the very high sensitivity of the luciferase reporter gene.

Binding of IFNγ-induced STAT1 and TNFα-induced NF-κB Is Not Cooperative—IFNγ is well documented to stimulate the phosphorylation and nuclear localization of STAT1a homodimers (8–11). Similarly, TNFα is a potent stimulus of the nuclear translocation of various members of the Rel homology family (19–20). The functional cooperativity between IFNγ and TNFα might result from cooperative effects on DNA binding activities of the respective transcription factors. Thus, we next compared the binding activities of STAT1 and NF-κB to their respective sequence motifs using nuclear extracts prepared from cells stimulated with IFNγ and/or TNFα for 30 min by EMSA (Fig. 4). Although nuclear extracts from fibroblasts stimulated with IFNγ showed little or no inducible kB binding activity, cells stimulated with TNFα exhibited two inducible complexes designated as C1 and C2 in Fig. 4A. When cultures
NF-κB (p50) and RelA (p65) bind to the IRF-1 proximal κB site in TNFα-induced fibroblasts. A, fibroblasts from wild type mice were either untreated (UT) or treated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) as indicated for 30 min prior to the preparation of nuclear extracts. Five μg of each nuclear extract were analyzed for κB binding activity by EMSA using a radiolabeled oligonucleotide containing the IRF-1 κB sequence motif. Two major complexes are indicated as C1 and C2. B, nuclear extracts from IFNγ and TNFα-stimulated cells were incubated with the indicated antibodies (1 μg) before analysis of the κB binding activity as described above. C, competition analysis of IRF-1 κB binding activity. Specificity of binding was assessed by competition with a 50-fold molar excess of unlabeled wild type or mutant oligonucleotide corresponding to the κB or SBE motifs as shown at the bottom. Mutated nucleotides are indicated in italic type. Underlined sequences represent the κB and SBE motifs, respectively. The overlined sequence indicates a potential κB motif contained within the SBE site. An oligonucleotide fragment corresponding to the region between −129 and −37 (SBE-κB) or an oligonucleotide fragment in which both the SBE and the κB sites have been mutated (mSBE-mκB) was also used as a competitor. Nuclear extracts (5 μg) from wild type fibroblasts treated with IFNγ (100 units/ml) and TNFα (10 ng/ml) for 30 min were analyzed. Similar results were obtained from three independent experiments.

were co-stimulated with IFNγ and TNFα, the magnitude of the binding activity and pattern of complex formation were essentially the same as seen in cells stimulated with TNFα alone. As shown in Fig. 4B, the C1 complex was fully reactive with antisera specific for NF-κB1, while the C2 complex showed partial reactivity with anti-NF-κB1 and full reactivity with antisera specific for RelA. Antibodies specific for c-Rel and STAT1 did not recognize any of the IFNγ- and/or TNFα-induced κB binding activities. These results suggest that the binding activity induced by TNFα that recognizes the IRF-1 κB site is composed of NF-κB1/RelA heterodimers and RelA homodimers. Specificity for the Rel protein binding was further assessed by oligonucleotide competition assays (Fig. 4C). Oligonucleotides containing the wild type IRF-1 κB motif competed effectively for the binding of these Rel proteins, while a mutant oligonucleotide was inactive (lanes 2 and 3). Interestingly, oligonucleotides containing a wild type IRF-1 SBE or a mutant SBE in which two adenine residues in the 3′ half of the inverted repeat were changed (m1 SBE) also partially competed for the binding of NF-κB1 and RelA (lanes 4 and 5). Another mutant SBE (m2 SBE), in which the intervening sequence between the inverted repeats was also altered, could not compete for binding to the κB motif. Since the adenine residues in the inverted repeat have been previously shown to be critical for recognition by STAT1 (14), sequence preferences for STAT1 and NF-κB appear to be distinct. This ability of SBE to compete for NF-κB recognition may result from the κB-like site in the 5′ portion of the IRF-1 SBE motif. In addition it may reflect the low affinity recognition of SBEs by NF-κB as previously reported (51).

Consistent with previous reports, nuclear extracts from IFNγ-treated fibroblasts contained a prominent stimulus-dependent DNA binding activity specific for the IRF-1 SBE (Fig. 5A), and this complex is fully reactive with antibody to STAT1 (data not shown). Interestingly, TNFα also induced a DNA binding activity that recognized the IRF-1 SBE forming a complex that migrated at a slightly different mobility. This complex was reactive with antibodies specific for NF-κB1 and RelA (Fig. 5B, lanes 3 and 4). These findings are also consistent with the results in Fig. 4C showing competition between the SBE and κB sites for the IRF-1 κB site. When nuclear extracts from cells stimulated with both IFNγ and TNFα were analyzed, a single broad band was observed, consistent with the presence of both the STAT1 and NF-κB complexes seen with IFNγ or TNFα stimulation alone. The most prominent component in this complex was STAT1, as indicated by immunoreactivity with anti-STAT1 (Fig. 5B, lane 6). The more slowly migrating complex, which was not reactive with anti-STAT1, was reactive with anti-NF-κB1 and anti-RelA (lanes 8 and 9). Competition assays showed that an oligonucleotide containing a wild type SBE effectively competed for the binding of all complexes (Fig. 5C, lane 4), while the wild type κB motif either did not compete or did so poorly (lane 2). The m1 SBE did not compete, indicating that most of the binding activity present was STAT1, since this mutation appears to affect primarily the formation of STAT1 complexes and not NF-κB. A large DNA fragment containing both the SBE and the κB sites was also able to compete complex formation in response to treatment with IFNγ and TNFα. When this larger fragment (spanning positions −129 and −37 of the IRF-1 promoter) was used as a probe in EMSA, each complex was formed independently, and no evidence was obtained for cooperativity in binding between factors activated.
Transcriptional Synergism by STAT1 and NF-κB

**Fig. 5.** SBE binding activity in fibroblasts treated with IFNγ and TNFα. A, fibroblasts from a wild type mouse were either untreated (UT) or treated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) as indicated for 30 min prior to the preparation of nuclear extracts. Five μg of each nuclear extract were analyzed for DNA binding activity by EMSA using radiolabeled oligonucleotides containing the IRF-1 SBE sequence motif. B, nuclear extracts (5 μg) from wild type fibroblasts treated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 30 min were incubated with the indicated antibodies before analysis of the SBE binding activity. C, competition analysis of IRF-1 SBE binding activity. Specificity of binding was assessed by competition with a 50-fold molar excess of unlabeled wild type or mutant oligonucleotide corresponding to the xB or SBE motifs as described under "Experimental Procedures" and as shown in Fig. 4. A double-stranded oligonucleotide corresponding to the region between −129 and −37 (SBE-xB) or an oligonucleotide to which the SBE and the xB sites have been mutated (mSBE-mxB) was also used as a competitor. Nuclear extracts (5 μg) from wild-type fibroblasts treated with IFNγ (100 units/ml) and TNFα (10 ng/ml) for 30 min were analyzed. Similar results were obtained from three independent experiments.

**SBE and xB Sequences Cooperate in an Artificial Promoter**—To explore the generality of the xB motif and SBE functional cooperativity, we asked whether transcriptional synergy could be reconstituted using isolated sequence elements placed in a heterologous promoter. Initially, one copy of the IRF-1 SBE and/or the xB2 motif from the mouse IP-10 gene (33, 37) were placed in front of the TK promoter (TK-105) linked to the CAT reporter gene and tested for sensitivity to IFNγ and/or TNFα following transient transfection in NIH3T3 cells (Fig. 6). Although one copy of the xB2 motif exhibited little sensitivity to IFNγ or TNFα either alone or in combination with the SBE, one copy of the IRF-1 SBE motif was sensitive to IFNγ or IFNγ and TNFα. When a construct containing one copy each of the xB2 and the SBE was analyzed, a strong synergistic response was seen in cells stimulated with the combination of agents. Mutation of the SBE site abolished all stimulatory sensitivity of the combination construct and was essentially identical to that of a construct containing only a single xB site. Thus cooperativity was not due to creation of fortuitous binding sites in the region where inserted sequences are coupled. Interestingly, cooperativity between IFNγ and TNFα was also seen using the construct containing only the IRF-1 SBE and using the construct containing wild type SBE and mutant xB. The synergistic response of such constructs to IFNγ and TNFα appears to depend upon the distal portion of the TK promoter, which contains a GC box and a CCAAT box; no cooperativity was seen in a truncated form of the TK promoter in which the distal GC box and CCAAT box have been deleted (pTK-81, see Fig. 7).

The spacial relationship between the two cooperating sites may be an important determinant of their synergistic interaction. To examine this possibility, reporter constructs in which the sequence motif orientation and the nucleotide spacing between motifs were varied were prepared and examined in transient assays (Fig. 7). For these experiments, a truncated form of the TK-luciferase vector (pTK-81) was utilized in which both a GC box (Sp1 binding site) and a CCAAT box have been deleted. When a single copy of either a xB site or the IRF-1 SBE was linked to this reporter plasmid, no cooperative response was obtained. As mentioned above, this result suggests that the cooperative response seen with constructs containing a single SBE site (see Fig. 6) requires one or both of the sites deleted from the TK promoter. When a construct containing a single copy each of the SBE and the xB motif was examined, a strong synergistic response was obtained. The synergy was not dependent upon the relative order of sites. Although constructs containing the SBE in either a distal or proximal relationship to the TK promoter exhibited variable response to IFNγ alone, cooperative responses were comparable (6–7-fold). The variability in sensitivity to IFNγ is also observed in Fig. 7 when comparing the response of pTK SBE and pTK mxB2 + SBE, where the spacing of the SBE relative to the TK promoter is comparably altered. When the spacing between the two sites was incrementally increased, sensitivity to individual and combination stimulation was reduced. An increase of 5 nucleotides only modestly reduced the cooperativity, indicating that the orientation of bound factors relative to each other and the turn of the helix was not a limiting feature of the response. As the spacing interval was increased, the response was much more dramatically reduced. Interestingly, when the sites were separated by 64 nucleotides, a distance equivalent to that separating the SBE and xB sites in the endogenous IRF-1 promoter, sensitivity to stimulation was lost entirely. These results indicate that while spacing may influence the magnitude of cooperativity, other features of the sequence between sites are probably of more critical importance.
DISCUSSION

IFNγ and TNFα utilize distinct signaling pathways leading to altered gene transcription (8–11, 52). When these cytokines have been used in combination, both cooperative and antagonistic effects on gene transcription have been observed (3–7, 40). The present study was undertaken to define the mechanisms involved in such a synergistic response. The results demonstrate that STAT1 activation by IFNγ and NF-κB activation by TNFα are the principle events necessary for cooperative induction of genes containing appropriate SBE and κB sequence motifs. Independent interaction of STAT1 and NF-κB with their cognate binding sites is sufficient for mediating the cooperativity. These conclusions are based on the following observations. 1) IFNγ and TNFα synergized strongly to promote expression of multiple genes that contain at least one copy of an SBE and a κB site, including the IRF-1, ICAM-1, and Mig genes. 2) This activity was abolished in fibroblasts prepared from mice in which the STAT1 gene has been deleted by ho-

**Fig. 6.** κB and SBE motifs confer functional cooperativity on a heterologous promoter in response to IFNγ and TNFα. One copy of an oligonucleotide corresponding to the wild type or mutant form of the IRF-1 SBE or the IP-10 κB2 site (see “Experimental Procedures”) was linked upstream of the TK-105 promoter (pTK-105) containing the CAT gene. The nucleotide length between the SBE and the κB sites was 25 bases. The combination of the SBE and the κB sequences are schematically indicated. These CAT constructs were transiently transfected into NIH3T3 cells as described under “Experimental Procedures.” Twenty-four hours after transfection, the cells were either untreated or stimulated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 16 h and assayed for CAT activity. The relative CAT activity is shown as -fold induction of stimulated versus unstimulated samples. Mean percentage of acetylation for different constructs in unstimulated cultures ranged from 0.2 to 0.4%. The maximum CAT activity was 27% acetylation, which was obtained in cultures transfected with pTK κB2 + SBE and stimulated with IFNγ and TNFα. Each column and bar represents the mean ± S.E. from three independent experiments.

**Fig. 7.** Effect of motif order and spacing on IFNγ and TNFα-induced cooperativity. One copy of an oligonucleotide corresponding to the IRF-1 SBE or the κB motif from the IP-10 gene (see “Experimental Procedures”) was placed in front of the TK-81 promoter linked to the luciferase gene (pTK-81 Luc) as indicated schematically. Constructs with increasing nucleotide spacing between the SBE and the κB site were prepared as described under “Experimental Procedures.” These constructs were transiently transfected into NIH3T3 cells, and following a 24-h rest, the cells were either untreated or stimulated with IFNγ (100 units/ml) and/or TNFα (10 ng/ml) for 8 h prior to harvest and determination of luciferase activity. The relative luciferase activity is presented as a percentage of activity obtained in cells transfected with the pTK-81 SP25 κB1 SBE plasmid stimulated with IFNγ and TNFα. The -fold induction of stimulated versus unstimulated samples is also indicated. Each column and bar represents the mean ± S.E. from three independent experiments.
mologous recombination. 3) Synergistic transcription induced by IFNγ and TNFα was observed in normal fibroblasts transfected with a reporter gene under control of a 1.3-kb fragment of the IRF-1 gene promoter. 4) The synergistic induction of the IRF-1 promoter activity was nearly abolished in a STAT1-deficient cell line. 5) Site-directed mutagenesis of the SBE and the proximal kB site in the IRF-1 gene promoter significantly reduced the magnitude of the synergistic response. 6) IFNγ and TNFα independently activated STAT1 and NF-kB (NF-κB1/RelA), respectively, as measured by binding to their cognate sequence motifs. 7) No cooperative effects on DNA binding activities were observed. 8) The SBE and kB motifs could confer transcriptional synergy in response to IFNγ and TNFα when examined in a heterologous promoter.

IFNγ-induced transcriptional synergy appears to be mediated by multiple pathways involving both protein synthesis-dependent and -independent mechanisms (5, 53). The results presented in this study indicate that cooperative effects involving IFNγ and TNFα exhibit similar behavior (Fig. 2). An important observation is that both protein synthesis-dependent and -independent cooperativity still depends largely on STAT1, consistent with the recent reports showing STAT1 to be obligatory for IFN-mediated biological activities (24, 54). The requirement for protein synthesis during IFNγ/TNFα-mediated RANTES gene expression suggests that some IFNγ-induced protein(s) (e.g. IRF-1) might be necessary for cooperativity in this circumstance, consistent with such roles for other genes (24, 53, 55). Inspection of the RANTES promoter sequence suggests the presence of IRF-binding motifs (56). Furthermore, functional kB motifs have been identified in the promoter (Fig. 1) (46) and cooperative regulation of transcription by IFN-γ and NF-κB has been previously reported (57, 58). In contrast, direct activation of STAT1, which may include the formation of STAT1α homodimers, heterodimers, or other oligomeric interactions, appears to be involved in the cooperative induction of IRF-1, ICAM-1, and Mig gene expression. IFNγ-dependent transcription of the IRF-1, ICAM-1, and Mig genes has been shown to depend upon SBE motifs that bind STAT1 in homo- or heterodimeric forms (14–17, 41–43). Furthermore, synergistic induction of IP-10 gene transcription by IFNγ and TNFα also depends on an IFNγ-inducible factor that contains STAT1 and binds the IFN-stimulated response element found in the IP-10 promoter (5).

TNFα is well documented as a potent inducer of NF-κB and has been reported elsewhere to cooperate functionally with other transcription factors (5, 21, 23, 57, 58). The results in the present study indicate that NF-κB (NF-κB1/RelA) can cooperate with STAT1 to promote synergistic transcriptional activity. The proximal kB site in the IRF-1 promoter is a functional kB motif, which is recognized by a combination of NF-κB1 and RelA in fibroblasts. TNFα-mediated ICAM-1 gene transcription has been shown to depend upon a kB motif recognized by Rel family members (44, 45). Interestingly, despite the fact that the Mig gene is not independently induced by stimuli that activate NF-κB (e.g. TNFα and lipopolysaccharide) (28), the cooperative induction of this gene by IFNγ and TNFα suggests that the kB motifs found in the Mig promoter are functional when STAT1 is also available.

Interestingly, we noted that the IRF-1 SBE appeared able to mediate a synergistic response to stimulation with IFNγ and TNFα independently of the proximal kB site (see Figs. 3 and 6). Since the SBE site was also recognized by STAT1 and NF-κB, this dual recognition might contribute to the functional synergy. While this possibility cannot be ruled out, several considerations suggest that the cooperativity observed derives from other sources. For example, in Fig. 3, the constructs containing mutations in the SBE, in the kB site, and in the double mutant all showed some synergistic response to the stimulus combination. Because this fragment is large (1.3 kb), there are apparently other independent sites that can cooperate with the SBE, the kB site, or each other. Second, although the cooperative behavior of the artificial construct utilized in Fig. 6 (pTK SBE) appears to depend solely upon the SBE, data shown in Fig. 7 illustrate that such cooperativity is dependent upon a 25-base pair fragment of the TK promoter between positions −105 and −81. When the pTK-81 promoter was used with the isolated SBE, no cooperativity was evident. While we do not understand the mechanism(s) through which cooperativity occurs in this setting, the results suggest that the SBE is not independently capable of mediating cooperative response to IFNγ and TNFα.

The molecular mechanisms involved in functional synergy between distinct transcription factors appear to be multifactorial (23, 59–63). In some cases, direct protein-protein interaction between activator proteins has been observed (23, 59). The physical interaction may result in cooperative DNA binding, more stable protein-DNA interactions, and/or increased affinity of one or both activator proteins, ultimately creating a highly stable multiprotein complex that has markedly enhanced functional properties (23, 63). In this regard, members of the NF-κB and the STAT families have been observed to interact with members of other distinct factor families (21–23, 64, 65) although not with each other. Both NF-κB and STAT1 formed complexes on the IRF-1 SBE, but these appeared to be independent interactions between individual factors and DNA, since each complex exhibited a distinct mobility in EMSA. Furthermore, the presence of one factor did not alter the interaction of the other with its cognate site, nor did the presence of both factors promote the formation of any unique complexes not detected in cells treated with either stimulus alone. Nevertheless, we cannot completely rule out the possibility that a weak interaction between STAT1 and NF-κB in vivo might produce the observed functional cooperativity, since in vitro study of protein-protein interaction will only detect relatively high affinity interactions. Furthermore, analysis of nucleotide spacing between these sequence motifs indicated that, although spacial distances may quantitatively modify the response, the specific intervening nucleotide sequences were more important. This latter observation may suggest a role for other factors or an influence of flanking sequence on the functional behavior of transacting factors bound to DNA. This possibility is also supported by the finding that a single SBE motif could mediate moderate cooperative response to IFNγ and TNFα when other stimulus-insensitive sites are present.

An alternative mechanism for transcriptional synergy might involve independent interaction of the activation domains of individual factors with components of the general transcription machinery such as the TATA-binding protein, TATA-binding protein-associated factors, TFIIA, and TFIIIB (61, 62). The same activator domain may interact with more than one component of the RNA polymerase complex. These multiprotein interactions could facilitate assembly of a preinitiation complex, stabilize the complex on promoter DNA, and thus promote the frequency of transcriptional initiation and elongation. Members of the Rel family have been reported to interact directly with TATA-binding protein and TFIIIB (66, 67), and thus it is conceivable that the activation domains of these factors and of STAT1 may differentially interact with basal transcription components.

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Synergy between Interferon-γ and Tumor Necrosis Factor-α in Transcriptional Activation Is Mediated by Cooperation between Signal Transducer and Activator of Transcriptional α and Tumor Necrosis Factor-γ 

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