MOLECULAR AND CELLULAR BIOLOGY, Aug. 1994, p. 5300–5308
0270-7306/94/$04.00+0

Vol. 14, No. 8

Transcriptional Inhibition of the Interleukin-8 Gene by Interferon Is Mediated by the NF-κB Site

IGOR C. OLIVEIRA,[1] NAOFUMI MUKAIDA,[2] KOUJI MATSUSHIMA,[2] AND JAN VILČEK[1]*

Department of Microbiology and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016,[1] and Department of Pharmacology, Cancer Research Institute, Kanazawa University, Kanazawa 920, Japan[2]

Received 26 August 1993/Returned for modification 24 September 1993/Accepted 5 May 1994

The cytokine interleukin-8 (IL-8) is an important mediator of neutrophil, lymphocyte, and basophil chemotaxis and activation. Earlier we demonstrated that beta interferon (IFN-β) can inhibit tumor necrosis factor (TNF)-induced IL-8 gene expression at the transcriptional level, apparently by a novel mechanism. To define the *cis*-acting elements and *trans*-acting factors involved in this inhibition, DNA constructs containing portions of the 5′-flanking region of the IL-8 gene were linked to the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into human diploid FS-4 fibroblasts. The region spanning positions −98 to +44 was sufficient to confer both inducibility by TNF and inhibition by simultaneous treatment with IFN-β. Inhibition of TNF- or IL-1-induced CAT activity by IFN-β or IFN-α was also observed when a DNA fragment containing only the NF-IL-6 and NF-κB sites (positions −94 to −70) was placed upstream of the homologous or a heterologous minimal promoter. A construct containing three copies of the NF-κB element in front of the CAT gene also was inducible by TNF, and this stimulatory effect too was inhibited by IFN-β, indicating that the NF-κB element is sufficient to confer inhibition by IFN-β. This inhibitory effect was specific for the NF-κB site of the IL-8 gene since it was less marked with constructs containing three copies of the NF-κB site from the HLA-B7 gene. Gel shift assays with a probe containing the NF-κB and NF-IL-6 binding sites of the IL-8 gene (positions −101 to −63) showed that IFN-β treatment did not block the activation of NF-κB proteins or their ability to bind to the NF-κB site. However, nuclear extracts from cells treated with TNF in the presence of IFN-β gave rise to an additional band that appears to contain protein components from the NF-κB and NF-IL-6 families. NF-κB site-mediated suppression of IL-8 gene expression by IFN-β represents a hitherto unknown mechanism and target of IFN action.

Interleukin-8 (IL-8), a protein grouped within the C-X-C subfamily of chemotactic cytokines called chemokines (14), was initially described as a mediator of neutrophil chemotaxis and activation. IL-8 is now also known to be chemotactic for T lymphocytes and basophils, induce leukotriene B4 and histamine release in basophils, have mitogenic activity for keratinocytes (27, 38), promote angiogenesis (9), and mediate some other actions (27, 38). Different stimuli, such as IL-1, tumor necrosis factor (TNF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), viruses, bacterial lipopolysaccharide, and double-stranded RNA, are known to induce IL-8 production in many cell types, including monocytes, endothelial cells, and fibroblasts (36). Deregulated IL-8 synthesis is associated with some pathophysiological manifestations of diseases such as psoriasis (24) and rheumatoid arthritis (38). Analysis of the genomic structure of IL-8 revealed many potential targets for regulation, at both the transcriptional and posttranscriptional levels (22). Within its 5′-flanking region, the IL-8 gene contains potential binding sites for the transcription factors AP-1, AP-2, HNF-1, IRF-1, glucocorticoid response element, NF-κB, and NF-IL-6. In its 3′-flanking region, the IL-8 gene contains the repetitive ATTTA motif, implicated in the destabilization of some cytokine and oncogene mRNAs (22, 32). Analysis of the *cis*-acting elements involved in the transcriptional induction of the IL-8 gene by TNF, IL-1, and TPA (21) or by hepatitis B virus X protein (18) demonstrated that a short stretch of DNA spanning positions −94 to −70 was necessary and sufficient to confer inducibility. Sequence analysis of this fragment indicated that it contains NF-κB-like (positions −80 to −71) and NF-IL-6-like (positions −94 to −81) elements (18, 21, 22). An inhibitory effect of dexamethasone on IL-8 gene expression was found to involve the glucocorticoid response element (positions −330 to −120) along with the NF-κB-like and NF-IL-6-like sites (20).

Earlier we showed that beta or gamma interferon (IFN-β or IFN-γ) caused a selective inhibition of IL-8 mRNA accumulation induced by TNF and some other agents in normal fibroblasts (26). This IFN-mediated inhibition was also seen in the presence of inhibitors of protein synthesis, suggesting that unlike other known actions of IFN that depend on the activation of gene expression in the target cell, the inhibition of IL-8 mRNA synthesis was due to a direct modification of some preexisting factor(s). Treatment of cells with IFN-β inhibited IL-8 mRNA transcription in nuclear run-on assays, pointing to an inhibitory mechanism operating at least partly at the transcriptional level (26). In the present study, we sought to elucidate the molecular mechanisms responsible for the inhibition of IL-8 gene expression by IFN.

MATERIALS AND METHODS

Cytokines and antibodies. Recombinant human TNF-α (specific activity, 3×10^7 U/mg) was a gift from Masafumi Tsujimoto, Suntory Institute for Biomedical Research, Osaka, Japan. IL-1α (3×10^8 U/mg) was a gift from Peter Lomedico and Alvin Stern, Hoffmann La-Roche, Nutley, N.J. Recombinant human IFN-β derived from CHO cells (Betaferon; 3×10^8 U/mg) was generously provided by Wieland Wolf, Bioferon, Laupheim, Germany. Preparations of IFN-α-2b (Intron

* Corresponding author. Mailing address: Department of Microbiology, NYU Medical Center, 550 First Ave., New York, NY 10016. Phone: (212) 263-5315. Fax: (212) 263-7933.

A; Schering Corp., Kenilworth, N.J.) and IFN-γ-1b (Actimmune; Genentech, South San Francisco, Calif.) were standard pharmaceutical products. Polyclonal antibodies specific for the p50 (N terminus) and p65 (C terminus) NF-κB proteins were a generous gift from Nancy Rice, NCI-Frederick Cancer Research and Development Center, Frederick, Md.

Cell culture. Human diploid FS-4 fibroblasts were maintained essentially as described previously (26). In all experiments, the cells were seeded in 150-cm^2 dishes (2×10^6 cells per dish). Except when used for chloramphenicol acetyltransferase (CAT) assays, the cultures were switched to medium containing 0.25% fetal bovine serum and incubated for additional 3 to 5 days before use. All inducers were added to the cultures without prior medium change.

Plasmid construction and oligonucleotides. Plasmid pUXCAT 3xHLAκB contains three tandemly repeated copies of the NF-κB binding site (underlined) from the HLA-B7 gene (7), positions −189 to −177 (TCGAGTGGGGATTCCCCATGGGGATTCCCCATGGGGATTCCCCACCC). Plasmid pUXCAT 3xColAP-1 contains three copies of the AP-1 binding site (underlined) from the collagenase gene (3), positions −73 to −64 (TCGAGATGAGTCAGAATGAGTCAGAATGAGTCAGACCC). Plasmid pBL IL-8 contains a fragment spanning positions −94 to −71 (underlined) from the IL-8 gene (TCGACCAGTTGCAAATCGTGGAATTTCCTT). Oligonucleotides corresponding to these sequences were synthesized, purified, and annealed to their complementary strands following subcloning into the *Sal*I-*Xba*I sites of pBLCAT2 (17) for pBL IL-8 and into the *Xho*I-*Sma*I sites of pUXCAT −50 (21) for pUXCAT 3xHLAκB and pUXCAT 3xColAP-1. The construction of the remaining plasmids of the pUXCAT series has been described elsewhere (21). For electrophoretic mobility shift assays (EMSA), four oligonucleotides were used: NF-IL-6/NF-κB elements from the IL-8 gene, positions −101 to −63 (oligonucleotide 1 [O1], 39-mer, AGCTTGGGCCATCAGTTGCAAATCGTGGAATTTCCTCTGACATAG); the NF-κB element from the IL-8 gene, positions −84 to −68 (oligonucleotide 2 [O2], 17-mer, TCGTGGAATTTCCTCTG); the proximal NF-IL-6 half-site and distal NF-κB half-site from the IL-8 gene, positions −88 to −75 (oligonucleotide 3 [O3], 14-mer, CAAATCGTGGAATT); and the NF-IL-6 element from the IL-8 gene, positions −97 to −78 (oligonucleotide 4 [O4], 20-mer, CATCAGTTGCAAATCGTGGA). These oligonucleotides were synthesized, purified, and annealed to their complementary strands. The positions and sizes provided for all synthetic oligonucleotides refer only to the underlined nucleotides.

Transfections and CAT assays. Human FS-4 fibroblasts were transfected by the calcium coprecipitation method with the aid of a kit (5′ to 3′, Boulder, Colo.), using the protocol recommended by the manufacturer with minor modifications. Briefly, 1 day before the experiment, confluent cultures of FS-4 cells were trypsinized, and cells were seeded at a density of 3.5×10^6 to 4.0×10^6 cells per 150-cm^2 dish and incubated overnight at 37°C. The cultures were replenished with fresh medium and kept at 37°C for at least 4 h before transfection. The DNA-calcium precipitate containing 5 to 10 μg of the appropriate plasmid DNA and 15 μg of plasmid pCMV-β encoding β-galactosidase (Clontech, Palo Alto, Calif.) was added to the culture medium, and the cells were incubated for an additional 4 h. Each construct was transfected into two 150-cm^2 dishes. The cultures were then glycerol shocked and replenished with fresh medium. After 2 to 3 h, trypsin was added, the cells obtained from two 150-cm^2 dishes were subdivided into four 60-cm^2 dishes (1.8×10^6 to 2.0×10^6 cells per dish), and the resulting cultures were incubated overnight. This procedure eliminates differences in transfection efficiency seen when the same construct is used for transfection of separate cultures. (For the experiment shown in Fig. 3, cells in three 150-cm^2 dishes were transfected, pooled after trypsinization, and seeded in an appropriate number of smaller 60-cm^2 dishes.) The cultures were then treated with the appropriate cytokines for 5 h. Cell extracts were prepared by subjecting the cells to three cycles of freeze-thawing in 0.25 mM Tris-HCl (pH 7.8). Immediately thereafter, equal amounts of protein from the extracts were used for the CAT determinations (31). The β-galactosidase activity in the extracts was determined with the aid of a kit (Promega, Madison, Wis.) as instructed by the manufacturer. After thin-layer chromatography, radioactivity was measured with the aid of the AMBIS Radioanalytic System (Ambis, Inc., San Diego, Calif.), and the percent conversion to acetylated chloramphenicol was calculated. All transfection experiments were repeated at least five times.

EMSA. Nuclear protein extracts were prepared essentially as described previously (2). The binding reactions were performed with 4 to 8 μg of nuclear protein extract in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 8.0)–40 to 60 mM KCl–1 mM EDTA–1 mM β-mercaptoethanol–12% glycerol–2 μg of poly(dI-dC) in a final volume of 30 μl at room temperature for 20 min. A total of 100 ng of the oligonucleotides to be used as probes was labeled either by Klenow enzyme (O1) or T4 polynucleotide kinase (O2), and 1 ng (>10,000 cpm/μg) of the radiolabeled material was used in each binding reaction. For the competition assays, an excess of the appropriate unlabeled oligonucleotide was added to the binding reaction mixture, and the mixture was incubated for 10 min before the addition of the probe. After gentle mixing, the reaction was allowed to proceed for 20 min at room temperature. In the supershift experiments, the antibodies were added to the binding reaction mixtures before the probe and poly(dI-dC), and the mixtures were incubated at room temperature for 30 min. After addition of both poly(dI-dC) and the probe, the reaction mixture was gently mixed and further incubated for 20 min under the same conditions. The samples were electrophoresed at 80 to 100 V (in a buffer containing 6.6 mM Tris-HCl [pH 7.9], 3.3 mM sodium acetate [pH 7.9], and 1.0 mM EDTA [pH 8.0]) through a 4% native polyacrylamide gel. The gel was dried and exposed to Kodak XAR-5 film at −70°C.

RESULTS

A fragment spanning positions −98 to +44 of the IL-8 gene is sufficient to mediate induction by TNF and inhibition by IFN-β. Earlier we showed that treatment of FS-4 fibroblasts with IFN-β or IFN-γ inhibited IL-8 mRNA accumulation induced by TNF and some other agents (13, 26). Nuclear run-on assays indicated that IFN-β selectively inhibited TNF-induced expression of the IL-8 gene at the transcriptional level (26). To determine which *cis*-acting elements of the IL-8 enhancer region were involved in this transcriptional inhibition by IFN-β, we transfected FS-4 cells with plasmid constructs containing the CAT reporter gene driven by fragments generated by serial deletions of the 5′-flanking region of the IL-8 gene (Fig. 1A). In agreement with earlier data (18, 21), we observed that deletion of sequences upstream of position −272 did not reduce inducibility by TNF (Fig. 1B and C). A construct with a deletion to −98 retained significant inducibility by TNF, whereas little or no reproducible induction was seen with the remaining three shorter constructs. Treatment with IFN-β inhibited TNF-induced CAT activity in all con-

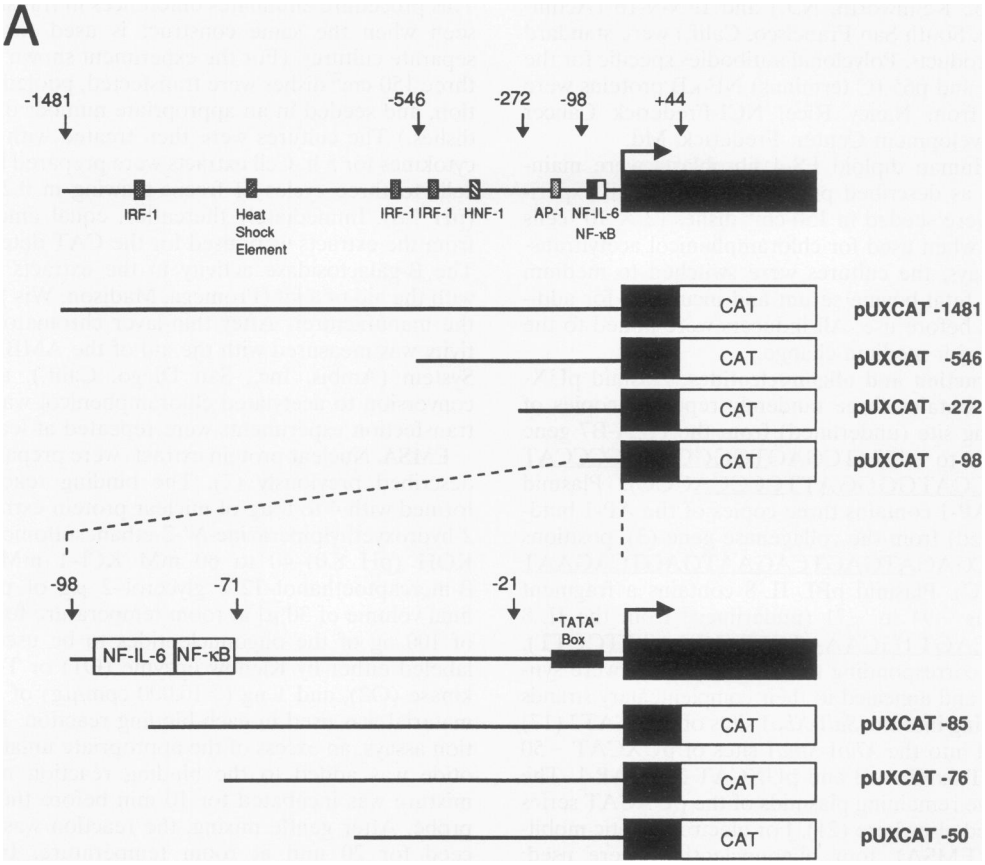

FIG. 1. Sequences downstream of position −98 in the 5′-flanking region of the IL-8 gene are sufficient for inhibition by IFN-β. (A) Schematic representation of the cloned 5′-flanking region of the IL-8 gene (positions −1481 to +44; the relative positions of the potential binding sites for transcription factors present in this region are approximate). Also shown are diagrams of the deletion constructs used, which were cloned in the pUXCAT plasmid as described in Materials and Methods. (B) After transfection with the appropriate plasmid (in two 150-cm^2 dishes), the cells were trypsinized, subcultured in four 60-cm^2 plates, and incubated overnight. The cultures were then left untreated (Con) or treated with TNF (20 ng/ml) in the presence or absence of IFN-β (1,000 U/ml), as indicated. The results were normalized for β-galactosidase expression and represent the means ± standard deviations of three independent experiments. Preparation of cell extracts and CAT assays were done as described in Materials and Methods. (C) Same as panel B, showing an autoradiogram of a representative experiment.

structs that responded to TNF, with the degree of inhibition produced by IFN-β ranging from about 40 to 70%.

On the basis of these results and in agreement with the results of earlier studies (18, 21), we conclude that IL-8 gene sequences downstream of position −98 are sufficient for induction by TNF in human FS-4 fibroblasts, although sequences further upstream apparently do contribute to inducibility. Furthermore, the −98 to +44 fragment was also sufficient to confer inhibition of TNF-stimulated CAT activity by IFN-β.

The NF-κB element in the 5′-flanking region of the IL-8 gene is sufficient to mediate the inhibitory effect of IFN-β on TNF-induced gene activation. The IL-8 gene fragment spanning positions −98 to +44 was shown to contain potential binding sites for at least four transcription factors: AP-3, Oct-1, NF-κB, and NF-IL-6 (21). Among these sites, only NF-κB and NF-IL-6 were shown to be essential for inducibility by TNF, IL-1, or TPA of CAT constructs transfected into a human fibrosarcoma cell line (21). To determine whether IFN-β was inhibiting IL-8 transcription through the same elements that are required for its activation, we used several constructs derived from pUXCAT −98 (Fig. 2A). The constructs used included one that contained intact NF-IL-6 and NF-κB sites of the IL-8 gene along with the homologous minimal promoter; this construct has an internal deletion from −71 to −50, which did not affect the response to TNF stimulation (data not shown). The other constructs used contained three tandemly repeated copies of the following elements: the NF-κB site from the IL-8 gene; the NF-IL-6 site from the IL-8 gene; the NF-κB element from the HLA-B7 gene; and the AP-1 binding site from the collagenase gene. In addition, we used a construct comprising the NF-IL-6 and NF-κB elements from the IL-8 gene linked to the minimal promoter from the herpes simplex virus thymidine kinase (TK) gene as well as constructs that contained the IL-8 or TK gene minimal promoters without any known enhancer elements. All of these constructs were used for the transfection of FS-4 cultures (Fig. 2B).

Constructs that contain either one copy each of the NF-IL-6 and NF-κB sites or three tandemly repeated copies of the NF-κB site from the IL-8 gene, linked to the homologous minimal promoter, showed a strong stimulation of CAT activity after TNF treatment; this stimulation by TNF was significantly reduced by simultaneous treatment with IFN-β. In agreement with earlier data (21), a construct comprising three copies of the NF-IL-6 site from the IL-8 gene was not inducible by TNF. A construct containing three copies of the NF-κB site

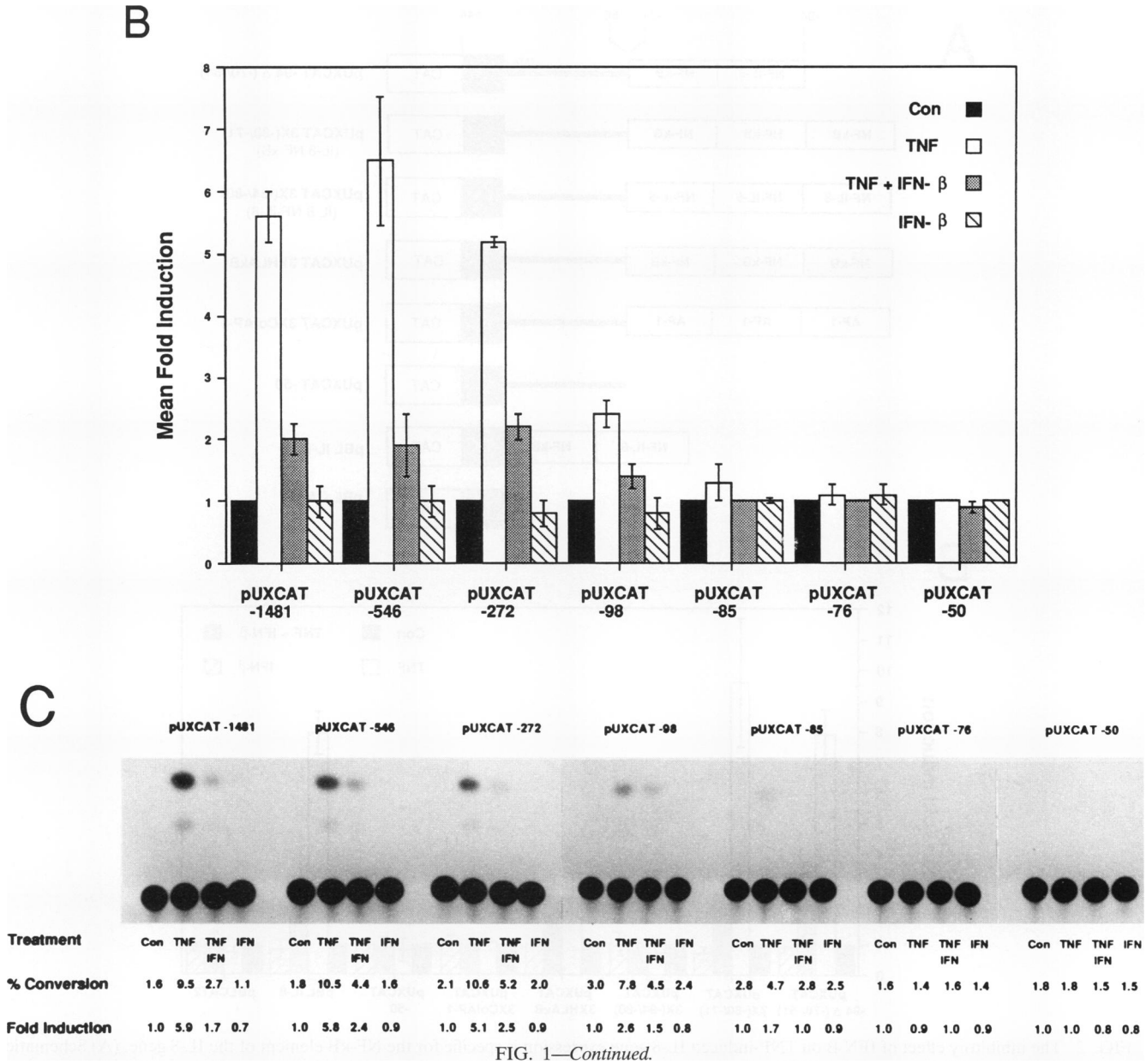

FIG. 1—*Continued.*

from the human HLA-B7 gene (pUXCAT 3xHLAκB) was transcriptionally activated by treatment with TNF, albeit less strongly than the construct containing three copies of the NF-κB site of the IL-8 gene. Simultaneous treatment with IFN-β did not significantly reduce the stimulatory effect of TNF in cells transfected with the pUXCAT 3xHLAκB construct. Cells transfected with the construct containing three tandemly repeated copies of the collagenase gene AP-1 element (pUXCAT 3xColAP-1) showed a high CAT activity (4.8 to 9.5% conversion; data not shown) that was neither further enhanced by treatment with TNF nor inhibited by IFN. A marked stimulation by TNF was seen in cells transfected with the pBL IL-8 construct, in which the NF-IL-6 and NF-κB elements of the IL-8 gene were linked to the TK gene minimal promoter, and simultaneous treatment with IFN led to a significant inhibitory effect in cells transfected with this construct.

These experiments show that the inhibitory effect of IFN can be seen with all constructs that contain the NF-κB element from the IL-8 gene. The results also show that the NF-IL-6 element is not essential for the inhibition by IFN-β and that it occurs irrespective of whether the homologous or a heterologous minimal promoter is present. Finally, the lack of a marked inhibitory effect of IFN-β on the construct comprising three NF-κB sites from the HLA-B7 gene suggests that the inhibitory effect of IFN-β is highly sequence specific. The IL-8 gene NF-κB binding site (TGGAATTTCC) differs from the HLA-B7 gene NF-κB binding site (TGGGGATTCC) in three positions (7, 21).

Inhibitory effect of IFN-β, IFN-α, and IFN-γ on the transcriptional activation induced by either TNF or IL-1. Earlier we demonstrated that IFN-β or IFN-γ inhibited IL-8 mRNA accumulation not only after induction with TNF but also upon stimulation with IL-1 or poly(I) · poly(C) (26). We addressed

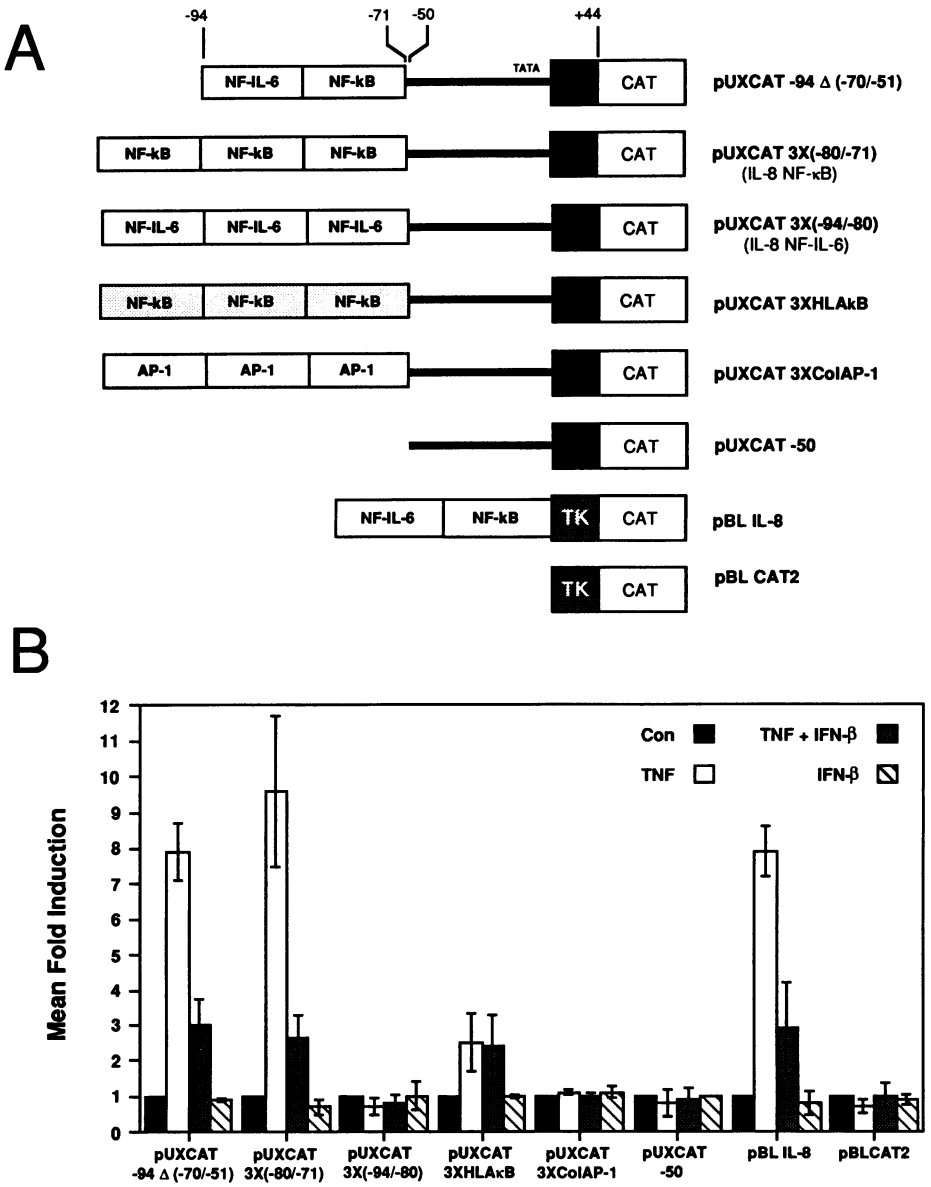

FIG. 2. The inhibitory effect of IFN-β on TNF-induced IL-8 gene expression is specific for the NF-κB element of the IL-8 gene. (A) Schematic representation of the constructs used. Plasmid pUXCAT −94Δ (−70/−51) represents a portion of the IL-8 gene (with an internal deletion that leaves the NF-IL-6 and NF-κB sites along with the minimal promoter intact), linked to the CAT gene. Three copies of the *cis*-acting elements corresponding to either the NF-κB or NF-IL-6 site from the IL-8 gene, NF-κB from HLA-B7 gene, and AP-1 from collagenase gene were ligated to the minimal promoter region of the IL-8 gene, resulting in plasmids pUXCAT 3x(−80/−71), pUXCAT 3x(−94/−80), pUXCAT 3xHLAκB, and pUXCAT 3xColAP-1, respectively. In addition, the NF-κB and NF-IL-6 elements of the IL-8 gene (positions −94 to −71) were ligated to the minimal promoter region of the TK gene (pBL IL-8). (B) Transfections, cytokine treatments, and CAT assays were done as described in the legend to Fig. 1B.

the question of whether the IL-8 promoter construct pUXCAT −94Δ (−70/−51), which contains the NF-IL-6 and NF-κB elements (Fig. 2A), would respond to IL-1 as well as to TNF. Furthermore, we compared IFN-β with IFN-α and IFN-γ with respect to the ability to downregulate TNF- and IL-1-induced CAT activity. Treatment of the transfected cultures with either TNF or IL-1 generated a strong activation of this construct (Fig. 3). In addition, all three IFNs inhibited TNF- or IL-1-induced CAT activity. However, the inhibitory effects of IFN-α and IFN-γ were consistently less marked than the inhibition by IFN-β.

Protein-DNA complex formation with nuclear extracts from FS-4 cells treated with TNF and IFN-β. To gain information on the *trans*-acting factors that might be involved in the inhibitory action of IFN-β, we performed EMSA, using as a probe a fragment spanning positions −101 to −63 of the IL-8 gene (Fig. 4A, O1). Incubation of nuclear extracts from unstimulated FS-4 cells with probe O1 gave rise to two faint complexes: a fast-migrating complex (C1) and a more slowly migrating complex (C2) (Fig. 4B, lanes 1 and 6). Treatment of FS-4 cells with TNF caused a dramatic increase in the formation of both C1 and C2 complexes and a weak induction of

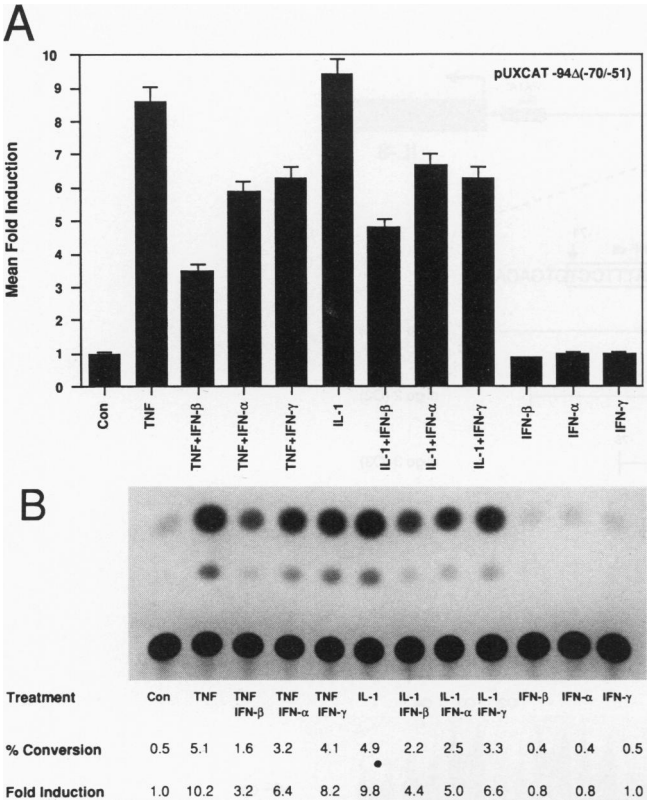

FIG. 3. The region spanning positions −94 to −71 is sufficient to mediate both induction by TNF or IL-1 and inhibition by IFN-β, IFN-α, or IFN-γ. FS-4 fibroblasts were transfected with pUXCAT −94Δ (−70/−51) (shown in Fig. 2A), treated with cytokines, and processed for CAT assays as described in the legend to Fig. 1B. IFN-β, IFN-α, and IFN-γ were used at 1,000 U/ml, and IL-1 was added at 1 ng/ml. Con, untreated control.

another complex, C3 (lanes 3 and 7). Nuclear extracts from cells treated simultaneously with TNF and IFN-β did not show a change in the C1 and C2 banding pattern but revealed a strong induction of complex C3 (lanes 4 and 8). All three complexes, C1, C2, and C3, could be competed for by unlabeled O2, containing the NF-κB element from the IL-8 gene (compare lanes 8 and 9). When unlabeled O4, containing the NF-IL-6 element, was used as a competitor, complexes C1 and C2 were not significantly affected but complex C3 was abolished (compare lanes 8 and 11). Finally, an unrelated sequence (O3) did not compete for any of the complexes (compare lanes 8 and 10).

To better characterize the components of complexes C1, C2, and C3, we performed supershift experiments with antibodies against the human NF-κB proteins, p50 and p65 (Fig. 5). Complexes obtained from nuclear extracts of cells treated either with TNF alone or simultaneously with TNF and IFN-β showed identical patterns of supershift for complexes C1 and C2 upon addition of anti-p50 and anti-p65 antibodies (lanes 3 to 5 and 7 to 9). Addition of anti-p50 antibody to the binding reactions resulted in the formation of a very slowly migrating complex with the concomitant decrease in the intensities of complexes C1 and C2. Similarly, addition of anti-p65 to the binding reactions also resulted in the formation of a heavier complex when incubated with both types of extracts. However, addition of anti-p65 to the binding reactions did not decrease the intensity of complex C1, although it could supershift complex C2. The complex induced in nuclear extracts from cells treated with TNF and IFN-β (C3) appeared to be reduced after the addition of anti-p50, whereas it became eliminated after treatment with anti-p65 antibodies (lanes 6 to 8). Addition of nonimmune serum to the binding reactions did not cause formation of any detectable supershifted complexes (lanes 5 and 9).

We conclude that NF-κB is involved in the formation of all three complexes observed, which is in agreement with the results of the competition experiments shown in Fig. 4 and with previously published data that report the essential role of NF-κB in the transcriptional induction of the IL-8 gene (18, 21). Treatment with IFN-β produced no quantitative or qualitative differences in the TNF-induced complexes C1 and C2, suggesting that IFN-β treatment does not directly affect the activation of NF-κB proteins or their ability to bind to the NF-κB site. From the competition and the supershift data, we conclude that complex C3, whose increased formation coincides with the transcriptional inhibition of the IL-8 gene by IFN-β, contains proteins of the NF-κB family of transcription factors, i.e., p65 and possibly p50. In addition, competition experiments suggest that a NF-IL-6-like factor also may play a role in the formation of complex C3.

DISCUSSION

Inhibitory action of IFN-β targets the NF-κB element of the IL-8 gene. The main goal of our study was to determine which *cis*-acting element(s) in the IL-8 promoter is responsible for the inhibitory action of IFN-β on the transcriptional activation of the IL-8 gene by TNF in human fibroblasts. The inhibition of IL-8 gene expression by IFN-β is unusual because, unlike other known IFN actions, it is apparently not mediated by de novo-synthesized IFN-induced proteins (26). In view of the rapid establishment of the inhibition and its resistance to blocking by agents that inhibit protein synthesis, we had concluded that this action of IFN is likely due to the modification of some preexisting factor(s). To be able to address the question of what factor(s) may be the target of IFN action, we first sought to identify the *cis*-acting elements involved. This task was aided by the earlier demonstration that an NF-κB-like element and an NF-IL-6-like element, located in close proximity to each other in the enhancer region of the IL-8 gene, are necessary and sufficient for the transcriptional activation of the IL-8 gene by TNF as well as by a variety of other inducers (18, 21). We confirmed the role of these two elements in the stimulatory actions of TNF on IL-8 gene constructs upon their transfection into FS-4 fibroblasts (Fig. 1). Moreover, we showed that all constructs of the 5′-flanking region of the IL-8 gene which responded to the stimulatory action of TNF were also inhibited by simultaneous treatment with IFN-β. We noted that in many experiments, IFN slightly reduced basal CAT activity in the absence of any inducing treatment (e.g., Fig. 1B and C). The inhibition of basal levels of CAT activity by IFN also may be mediated by the NF-κB site, possibly as a result of the presence of basal NF-κB activity in unstimulated cells (10).

Constructs containing three tandemly repeated copies of the NF-IL-6 element showed no basal activity and no inducibility by TNF upon transfection into FS-4 cells (Fig. 2). In contrast, constructs composed solely of three tandemly repeated copies of the NF-κB element from the IL-8 gene were activated by TNF and inhibited by simultaneous treatment with IFN-β. Since the inhibition by IFN-β of constructs containing three copies of the NF-κB element was at least as pronounced as the

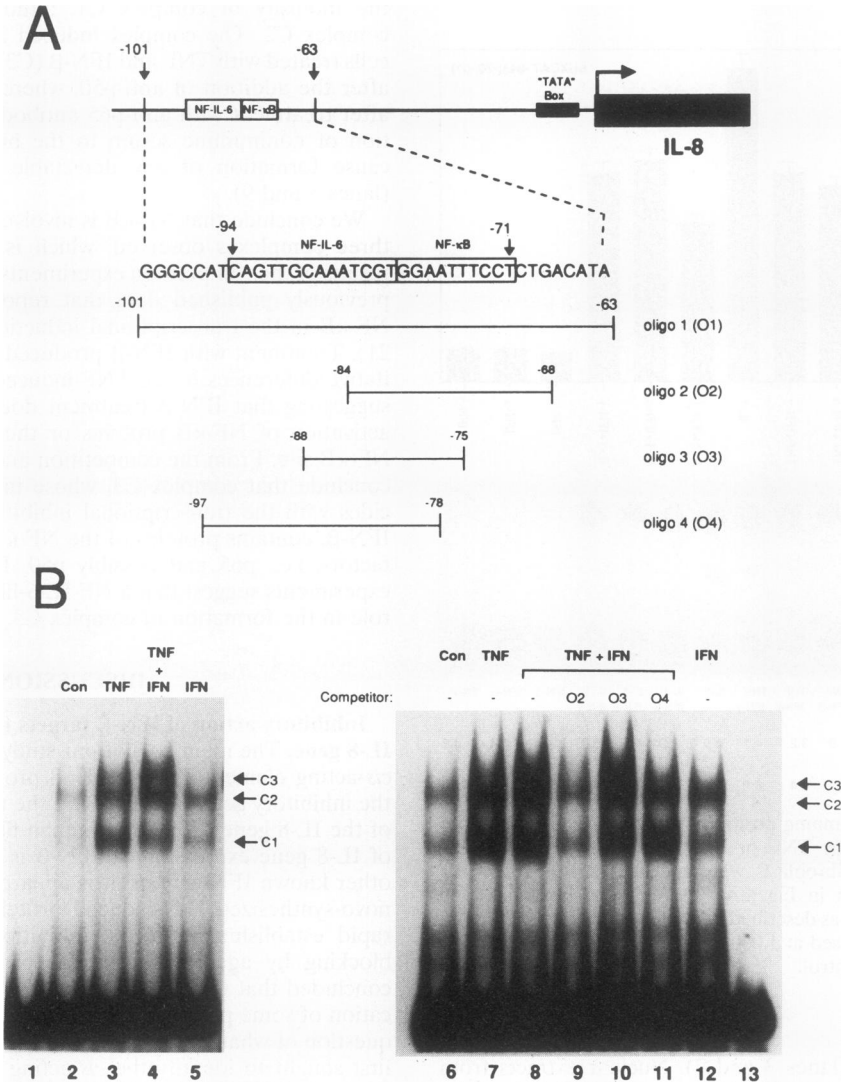

FIG. 4. Formation of protein-DNA complexes with nuclear extracts from FS-4 fibroblasts treated with TNF and/or IFN-β. (A) Schematic diagram of the synthetic oligonucleotides used in EMSA. For details, see Materials and Methods. (B) FS-4 cells were either left untreated (Con) or treated for 2 h with TNF (20 ng/ml) in the presence or absence of IFN-β (1,000 U/ml), as indicated. Nuclear extracts were prepared, and EMSA was done as described in Materials and Methods. For competition assays, a 100-fold excess of the specified unlabeled oligonucleotide was added to the binding reactions as described in Materials and Methods. The positions of complexes C1, C2, and C3 are indicated by arrows.

inhibition seen with the longest native IL-8 promoter construct (Fig. 1), we conclude that the NF-κB element in the enhancer region of the IL-8 gene is sufficient to mediate this inhibitory effect. Thus, whereas the NF-IL-6 element is required for the efficient activation of the IL-8 promoter, it appears to be dispensable for the inhibitory action of IFN-β. The specificity of the inhibitory effect of IFN-β is supported by the results obtained with two control constructs. First, no significant inhibitory effect could be observed with a construct containing three tandemly repeated copies of the collagenase AP-1 binding site, which showed a high basal level of CAT expression (4.8 to 9.5% conversion; data not shown) and no further stimulation by TNF. Second, IFN-β did not significantly inhibit either the constitutive or TNF-induced CAT activity elicited with a construct containing three tandemly repeated copies of a heterologous NF-κB binding site from the HLA-B7 gene. The NF-κB family of proteins consists of many different members, including RelA/p65, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52 (23). Our finding that the inhibitory effect of IFN-β on IL-8 gene is element and sequence specific is consistent with the view that the system of transcriptional regulation orchestrated by the NF-κB family of proteins involves several layers of complexity, including different subunit associations (29), half-site recognition, and sequence-specific subunit affinity (11, 16, 37).

It should be noted that the inhibition of IL-8 gene expression by IFN seems to operate only in some types of cells. Aman et al. (1) described an inhibitory effect of IFN-α on the induced expression of IL-8 in normal and transformed myelomonocytic cells that appears to be similar to the inhibition seen in FS-4 fibroblasts (26). On the other hand, we failed to detect an inhibition of IL-8 mRNA by IFN-β in some tumor cell lines (26). The fact that NF-κB-mediated inhibitory action of IFN on IL-8 gene expression is apparently restricted to some types of cells further illustrates the complexity of this phenomenon.

Simultaneous treatment of cells with TNF and IFN-β does

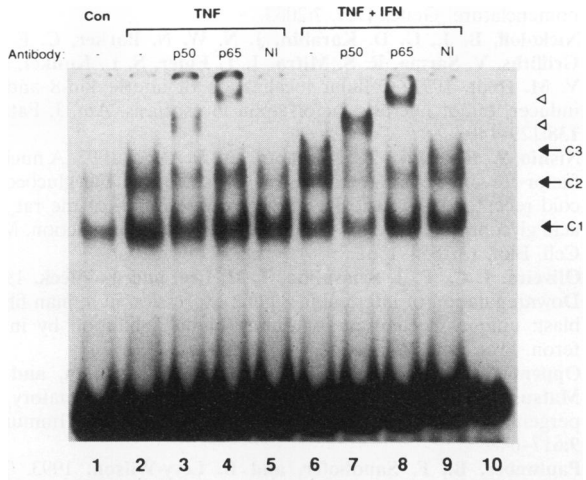

FIG. 5. Analysis of the components of complexes C1, C2, and C3 by supershift with antibodies to NF-κB p50 and p65. FS-4 cells were either left untreated (Con) or treated for 2 h with TNF (20 ng/ml) in the presence or absence of IFN-β (1,000 U/ml), as in the experiment shown in Fig. 4. Nuclear extracts were prepared, and EMSA was done with O1 (see Fig. 4A) as a probe in the presence of either anti-p50, anti-p65, or non-immune (NI) serum as described in Materials and Methods. Open arrowheads indicate supershifted complexes; the positions of complexes C1, C2, and C3 are also indicated.

not prevent NF-κB activation and leads to the induction of a distinct nuclear protein-DNA complex. EMSA with a DNA probe comprising the NF-IL-6 and NF-κB elements of the IL-8 gene revealed that TNF treatment led to the activation of nuclear proteins forming two distinct complexes, designated C1 and C2 (Fig. 4). Together, the competition experiments with an excess of unlabeled NF-κB oligonucleotide and the supershift experiments with antisera specific for the p50 and p65 NF-κB proteins showed that C1 contains p50 and C2 contains both p50 and p65 proteins (Fig. 5). Hence, C1 may represent p50 homodimers and C2 may be composed of p50-p65 heterodimers. Simultaneous treatment of cells with TNF and IFN-β did not alter the gel shift pattern and apparent composition of C1 and C2, suggesting that NF-κB activation, translocation to the nucleus, or ability to bind DNA was not affected by IFN-β treatment.

The nature of the proteins responsible for the formation of C3, induced in cells treated with TNF and IFN-β (but present at a low level in cells treated with TNF alone), has not been established with certainty. Supershift experiments suggest that NF-κB p65, and possibly p50, is part of C3 (Fig. 5). Interestingly, competition experiments showed a disappearance of C3 after the addition of an excess of unlabeled NF-IL-6 probe as well as of unlabeled NF-κB probe (Fig. 4). Together, these results suggest that C3 may represent a complex composed of members of the NF-κB and NF-IL-6 families. One interesting albeit unconfirmed possibility is that C3 contains a heterodimer of NF-κB (p65?) with a protein from the NF-IL-6 family.

Formation of heterodimers involving members of different families of transcription factors is quite commonly observed (6, 8, 15, 25, 28, 35). NF-IL-6 belongs to the C/EBP family of transcription factors, which contain a basic region adjacent to the leucine heptad repeat motif (bZIP) that facilitates the formation of homodimers and heterodimers with other leucine zipper-containing nuclear proteins. Formation of heterodimers between members of the C/EBP family and NF-κB p50 proteins has been recently demonstrated (12, 19, 34). Cross-coupling of NF-κB proteins with proteins of the C/EBP family was shown to occur through an interaction of the Rel homology domain, a region of about 300 amino acids present at the N terminus of NF-κB proteins, with the bZIP region in the C/EBP proteins (34). The notion that the C3 complex may contain members of the NF-κB and C/EBP-β/NF-IL-6 families is further supported by results reported recently by another group investigating the regulation of the IL-8 promoter (33). Upon stimulation of HeLa cells with TNF, these investigators demonstrated the simultaneous binding of p50, p65, and C/EBP-β/NF-IL-6 to the IL-8 promoter with the appearance of three distinct complexes, one of which was shown to be composed of proteins that belong to the NF-κB and C/EBP-β/NF-IL-6 families. Moreover, in experiments that used an IL-8 NF-κB promoter-CAT construct, cotransfection of increasing amounts of a C/EBP-β/NF-IL-6 expression vector resulted in a bimodal behavior in that C/EBP-β/NF-IL-6 was stimulatory at low concentrations and inhibitory at higher doses (33). The inhibition of promoters containing NF-κB binding sites by C/EBP-β/NF-IL-6 has also been confirmed by other investigators (34). In view of these recent studies, and the fact that induction of C3 by simultaneous treatment with TNF and IFN-β correlates with the inhibition of IL-8 gene expression, it is tempting to speculate that formation of the C3 complex is involved in the inhibitory action of IFN-β, although more work will be needed to demonstrate that the two events are causally related. Inhibitory effects on transcription through the NF-κB site have also been described in other systems (4, 5, 30). However, the inhibitory effect of IFN-β on the IL-8 gene is, to our knowledge, the first example of such an effect in a biologically relevant context, i.e., not dependent on the addition of exogenous *trans*-acting factors.

ACKNOWLEDGMENTS

We thank Rosana M. Oliveira, Mary L. Stephenson, Wlodzimierz Szer, and Nancy Rice for helpful advice, Evelyn Huang, Gene Lee, and Ryutaro Kamijo for critical reading of the manuscript, Angel Feliciano for technical assistance, and Ilene Totillo for preparation of the manuscript.

This work was supported by NCI grant R35CA49731. I.C.O. is supported by fellowship 1335189-4 from the Brazilian Ministry of Education (CAPES).

REFERENCES

1. **Aman, M. J., G. Rudolf, J. Goldschmitt, W. E. Aulitzky, C. Lam, C. Huber, and C. Peschel.** 1993. Type-I interferons are potent inhibitors of interleukin-8 production in hematopoietic and bone marrow stromal cells. Blood **82**:2371–2378.
2. **Andrews, N. C., and D. V. Faller.** 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19**:2499.
3. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729–739.
4. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-*rel* oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63**:803–814.
5. **Bressler, P., K. Brown, W. Timmer, V. Bours, U. Siebenlist, and A. S. Fauci.** 1993. Mutational analysis of the p50 subunit of NF-κB and inhibition of NF-κB activity by *trans*-dominant p50 mutants. J. Virol. **67**:288–293.
6. **Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg.** 1992. RXRα, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11**:1409–1418.
7. **Ganguly, S., H. A. Vasavada, and S. M. Weissman.** 1989. Multiple

enhancer-like sequences in the HLA-B7 gene. Proc. Natl. Acad. Sci. USA **86:**5247–5251.
8. **Jonat, C., H. J. Rahmsdorf, K.-K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62:**1189–1204.
9. **Koch, A. E., P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner, S. G. Elner, and R. M. Strieter.** 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science **258:**1798–1801.
10. **Kunsch, C., and C. A. Rosen.** 1993. NF-κB subunit-specific regulation of the interleukin-8 promoter. Mol. Cell. Biol. **13:**6137–6146.
11. **Kunsch, C., S. M. Ruben, and C. A. Rosen.** 1992. Selection of optimal κB/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. Mol. Cell. Biol. **12:**4412–4421.
12. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89:**8145–8149.
13. **Lee, T. H., G. W. Lee, E. B. Ziff, and J. Vilček.** 1990. Isolation and characterization of eight tumor necrosis factor-induced gene sequences from human fibroblasts. Mol. Cell. Biol. **10:**1982–1988.
14. **Lindley, I. J. D., J. Westwick, and S. L. Kunkel.** 1993. Nomenclature announcement—the chemokines. Immunol. Today **14:**24.
15. **Lopez, G., F. Schaufele, P. Webb, J. M. Holloway, J. D. Baxter, and P. J. Kushner.** 1993. Positive and negative modulation of Jun action by thyroid hormone receptor at a unique AP1 site. Mol. Cell. Biol. **13:**3042–3049.
16. **Lowenthal, J. W., D. W. Ballard, E. Böhnlein, and W. C. Greene.** 1989. Tumor necrosis factor α induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA **86:**2331–2335.
17. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15:**5490.
18. **Mahé, Y., N. Mukaida, K. Kuno, M. Akiyama, N. Ikeda, K. Matsushima, and S. Murakami.** 1991. Hepatitis B virus X protein transactivates human interleukin-8 gene through acting on nuclear factor κB and CCAAT/enhancer-binding protein-like *cis*-elements. J. Biol. Chem. **266:**13759–13763.
19. **Matsusaka, T., K. Fujikawa, Y. Nishio, N. Mukaida, K. Matsushima, T. Kishimoto, and S. Akira.** 1993. Transcriptional factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc. Natl. Acad. Sci. USA **90:**10193–10197.
20. **Mukaida, N., G. L. Gussella, T. Kasahara, Y. Ko, C. O. C. Zachariae, T. Kawai, and K. Matsushima.** 1992. Molecular analysis of the inhibition of interleukin-8 production by dexamethasone in a human fibrosarcoma cell line. Immunology **75:**674–679.
21. **Mukaida, N., Y. Mahe, and K. Matsushima.** 1990. Cooperative interaction of nuclear factor-κB and *cis*-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. J. Biol. Chem. **265:**21128–21133.
22. **Mukaida, N., M. Shiroo, and K. Matsushima.** 1989. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. J. Immunol. **143:**1366–1371.
23. **Nabel, G. J., and I. M. Verma.** 1993. Proposed NF-κB/IκB family nomenclature. Genes Dev. **7:**2063.
24. **Nickoloff, B. J., G. D. Karabin, J. N. W. N. Barker, C. E. M. Griffiths, V. Sarma, R. S. Mitra, J. T. Elder, S. L. Kunkel, and V. M. Dixit.** 1991. Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-alpha in psoriasis. Am. J. Pathol. **138:**129–140.
25. **Nishio, Y., H. Isshiki, T. Kishimoto, and S. Akira.** 1993. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat α1-acid glycoprotein gene via direct protein-protein interaction. Mol. Cell. Biol. **13:**1854–1862.
26. **Oliveira, I. C., P. J. Sciavolino, T. H. Lee, and J. Vilček.** 1992. Downregulation of interleukin 8 gene expression in human fibroblast: unique mechanism of transcriptional inhibition by interferon. Proc. Natl. Acad. Sci. USA **89:**9049–9053.
27. **Oppenheim, J. J., C. O. C. Zachariae, N. Mukaida, and K. Matsushima.** 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu. Rev. Immunol. **9:**617–648.
28. **Paulweber, B., F. Sandhofer, and B. Levy-Wilson.** 1993. The mechanism by which the human apolipoprotein B gene reducer operates involves blocking of transcriptional activation by hepatocyte nuclear factor 3. Mol. Cell. Biol. **13:**1534–1546.
29. **Perkins, N. D., R. M. Schmid, C. S. Duckett, K. Leung, N. R. Rice, and G. J. Nabel.** 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. Proc. Natl. Acad. Sci. USA **89:**1529–1533.
30. **Ruben, S. M., J. F. Klement, T. A. Coleman, M. Maher, C.-H. Chen, and C. A. Rosen.** 1992. I-Rel: a novel *rel*-related protein that inhibits NF-κB transcriptional activity. Genes Dev. **6:**745–760.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Shaw, G., and R. Kamen.** 1986. A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46:**659–667.
33. **Stein, B., and A. S. Baldwin, Jr.** 1993. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. Mol. Cell. Biol. **13:**7191–7198.
34. **Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr.** 1993. Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. Mol. Cell. Biol. **13:**3964–3974.
35. **Vallejo, M., D. Ron, C. P. Miller, and J. F. Habener.** 1993. C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. Proc. Natl. Acad. Sci. USA **90:**4679–4683.
36. **Van Damme, J.** 1991. Interleukin-8 and related molecules, p. 201–214. *In* A. W. Thomson (ed.), The cytokine handbook. Academic Press, London.
37. **Zabel, U., R. Schreck, and P. A. Baeuerle.** 1990. DNA binding of purified transcription factor NF-κB. Affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. J. Biol. Chem. **266:**252–260.
38. **Zachariae, C. O. C., and K. Matsushima.** 1992. Interleukin-8, p. 181–195. *In* B. B. Aggarwal and J. V. Gutterman (ed.), Human cytokines. Handbook for basic and clinical research. Blackwell Scientific Publications, Cambridge, Mass.