Interaction of NF-κB and NFAT with the Interferon-γ Promoter*

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Interferon-γ (IFN-γ) is a pleiotropic lymphokine whose production is restricted to activated T cells and NK cells. Along with other cytokines, IFN-γ gene expression is inhibited by the immunosuppressant cyclosporin A. We have previously identified an intronic enhancer region (C3) of the IFN-γ gene that binds the NF-κB protein c-Rel and that shows partial DNA sequence homology with the cyclosporin A-sensitive NFAT binding site and the 3′-half of the NF-κB consensus site. Sequence analysis of the IFN-γ promoter revealed the presence of two additional C3-related elements (C3-1P and C3-3P). In addition, an NF-κB site (IFN-γ κB) was identified within the promoter region. Based on this observation, we have analyzed the potential role of NF-κB and NFAT family members in regulating IFN-γ transcription. Electrophoretic mobility shift assay analysis demonstrated that after T cell activation, the p50 and p65 NF-κB subunits bind specifically to the newly identified IFN-γ κB and C3-related sites. In addition, we identified the NFAT proteins as a component of the inducible complexes that bind to the C3-3P site. Site-directed mutagenesis and transfection studies demonstrate that calcineurin-inducible transcriptional factors enhance the transcriptional activity of the IFN-γ promoter through the cyclosporin-sensitive C3-3P site, whereas NF-κB proteins functionally interact with the C3-related sites. In addition, when located downstream to the β-galactosidase gene driven by the IFN-γ promoter, the intronic C3 site worked in concert with both the IFN-γ κB and the C3-3P site to enhance gene transcription.

These results demonstrate that the coordinate activities of NFAT and NF-κB proteins are involved in the molecular mechanisms controlling IFN-γ gene transcription.

Interferon-γ (IFN-γ)[1] is an important immunoregulatory

protein responsible for several immunological effects (1), including induction of the Fc receptor, major histocompatibility complex class I and II expression (2), regulation of cytokine gene expression (IL-1, IL-6, and tumor necrosis factor) (3), and promotion of activation of immune effector cells, including B lymphocytes and monocytes. IFN-γ production *in vivo* is largely restricted to activated T cells and large granular lymphocytes (1, 4–6). Although the immunological properties of IFN-γ have been widely investigated (for reviews see Refs. 1, 7, and 8), the molecular mechanisms by which IFN-γ gene transcription is regulated have been shown to involve a wide variety of transcription factors (9–18). Interestingly, the IFN-γ gene belongs to a group of lymphokines whose expression is inhibited by the immunosuppressant cyclosporin A (1, 19), and in activated T cells, its transcription parallels that of other lymphokine genes (*e.g.* IL-2) whose promoter activity is enhanced by both NF-κB and NFAT proteins (19). This laboratory has identified a c-Rel binding site (C3) (20), located in the first intron of the human IFN-γ gene, that may have a role in the control of IFN-γ transcription. This site, which lies in a previously described IFN-γ enhancer region (9), has a strong homology with the 3′-half of NF-κB consensus sequence and the NFAT binding sites. In this regard, whereas NF-κB is a ubiquitous factor controlling the transcription of a number of cytokines (21–25), its role in the IFN-γ gene expression has been suggested (20) despite the lack of identification of canonical NF-κB binding sites within its genomic DNA. Similarly with NF-κB, a role for the cyclosporin A-sensitive NFAT proteins as activators of IFN-γ gene transcription has also been proposed (19, 26, 27) and a potential NFAT binding site in the IFN-γ promoter has been identified (15).

Cloning of the p50 and p65 NF-κB subunits (21, 22, 23) revealed a significant homology between the amino-terminal portion of these proteins, which contains the binding and dimerization domain (Rel homology domain), with the Rel family of oncoproteins and with the *Drosophila* developmental morphogen dorsal (22, 24, 25). Interestingly, the cloning of members of the NFAT family of transcriptional factors demonstrated structural similarity between the Rel homology domain, the carboxyl terminus of the NFATc, and the middle portion of NFATp proteins (28–30). These findings were also paralleled by the observation that NFAT and NF-κB proteins may recognize similar DNA sequences (31, 32). As NF-κB and NFAT transcriptional factors play a pivotal role in coordinating induction of many cytokine genes in activated T cells, the identification of additional C3-related sites located in the IFN-γ promoter prompted us to investigate the possible role of NF-κB and NFAT proteins in enhancing IFN-γ gene transcription.

Here, we provide evidence that cooperation between NFAT and NF-κB proteins induces maximal transcription of the

IFN-γ gene, resulting from a synergistic activity between promoter and intronic enhancers.

MATERIALS AND METHODS

Cell Culture—Fresh human peripheral blood T lymphocytes were incubated in RPMI 1640 medium containing 2% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. For growth of the human T cell line, Jurkat, the concentration of fetal calf serum was 10%. For the different treatments (as stated in the text), we used the following concentrations: phorbol 12-myristate 13-acetate (PMA), 10 ng/ml; phytohemagglutinin (PHA), 1 µg/ml; and cyclosporin A (CsA), 100 ng/ml.

EMSA Analysis—For nuclear extract preparation, the cells were cultured for 4 h in the presence or absence of the indicated treatments, and nuclear proteins were prepared as follows. The cellular pellet was resuspended in 10–20 times its volume in buffer A (lysis buffer) containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM Hepes (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg of aprotinin, 100 µM DTT and was subsequently incubated 5 min in ice. Cells were collected by centrifugation at 2000 rpm, and the supernatant was decanted. The nuclei were washed in buffer A without Nonidet P-40, collected at 2000 rpm and resuspended in buffer B (extraction buffer) containing 500 mM KCl, 25 mM Hepes pH (7.8), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, and 100 µM DTT for 5 min in ice. The samples were subsequently frozen and thawed (twice) utilizing dry ice and a 37 °C water bath, rotated 20 min at 4 °C, and centrifuged at 14,000 rpm for 20 min. The clear supernatant was collected, and the proteins were dialyzed for 4 h against buffer C (dialysis buffer) containing 50 mM KCl, 25 mM Hepes (pH 7.8), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, and 100 µM DTT. The nuclear proteins were incubated with radiolabeled DNA probes in a 20-µl reaction mixture containing 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 0.5 mM DTT, 1 µg of poly(dI-dC), and 4% Ficoll. Nucleoprotein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels in 0.5 × Tris borate/EDTA buffer at 12 V/cm for 2 h at room temperature. Dried gels were exposed to Kodak XAR-5 film at −70 °C with intensifying screens. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems, model 392) and were end-labeled using Klenow enzyme and [α-^{32}P]dCTP; approximately 1 ng of labeled DNA was used in a standard electrophoretic mobility shift assay (EMSA) reaction. The following antisera were used in supershift analysis: 1141, raised against a peptide containing residues 2–16 of human p50 (33); 1226, raised against a peptide containing the carboxyl-terminal 14 residues of human p65 (33); anti-NFATc antiserum (7A6) (30); anti-NFATc antiserum (796) recognizes all members of the NFAT family (34).

Transfection and CAT Assay—The CD4+ human T lymphoblastoid cell line Jurkat (clone J. LEI) was transiently transfected by the DEAE-dextran method (35). The cells were harvested 48 h later and assayed as described previously for CAT (36) and β-galactosidase (13) activities.

Plasmid Constructions—The p50 expression vector was constructed as follows: the *Hin*dIII-*Not*I fragment of a CMV construct expressing the p105 precursor of p50 NF-κB subunit (37) was subcloned into a K-RSPA vector (36). The *Xba*I-*Xba*I fragment (amino acids 505–966) was deleted to obtain the RSVp50 (amino acids 1–504) expression vector. The RSV-based p65 and RSV control expression vectors were kindly provided by Dr. Gary Nabel (38). To prepare the C3-3PCAT ((C3-3P)3TKCAT) construct, three copies of the IFN-γ C3-3P sequence 3 (GGA GGT ACA AAA AAA TTT CCA GTC C) were subcloned upstream to the thymidine kinase (TK) promoter in the pBLCAT2 parental vector (39). The constitutively active mutant of calcineurin expression vector was kindly provided by Dr. Neil A. Clipstone and Dr. Gerard Crabtree (27). The wild type −796 to +64 IFN-γ promoter DNA and the different mutants (m1, m2, m3, m4, m5, m6, and m7) represented in Fig. 6 (*panel A*) were generated by polymerase chain reaction amplification from a promoterless LacZ plasmid, pEQ3, containing the human IFN-γ promoter region nucleotide −2700 to + 64 (13). The amplified products were then subcloned in the *Hin*dIII-*Bgl*II restriction sites of the pEQ3 parental vector. To prepare the IFN-γ −796 to −761 TKGal construct (*plasmid B*, Fig. 5), the IFN-γ promoter region (nucleotides −796 to −761) was sub-

TABLE I
Sequence homology between the IFN-γ κB and IFN-γ C3-related sites with NF-κB (51, 52) and NFAT (19, 32, 50, 54) consensus sequences

The underlined nucleotides in the human and murine NFAT sites represent regions important for the interaction with the AP-1 transcriptional factor (55). N, any nucleotide; W, A or T; Y, C or T; R, A or G. (R.O.), reverse orientation.

	Sequence
Consensus p50 NF-κB subunit	G G G G A **T Y C C C**
Consensus p65 NF-κB subunit	G G G R N **T T T C C**
Consensus c-Rel	N G G N N **W T T C C**
IFN-γ κB site (−786 to −776)	G G G G **A** G **T T C C** (R.O.)
hNFAT (IL-2)	T A T G <u>A A A C A</u> G **T T T T T C C** (R.O.)
mNFAT (IL-2)	T A T G <u>A A A C A</u> **A A T T T T C C** (R.O.)
NF(p) (IL-4) (−78 to −69)	G **A A A A T T T C C**
C3 1st intron (459–470)	T A T G **A A T T T T C C**
C3–1P (−772 to −763)	A **A A A T A T T C C** (R.O.)
C3–3P (−278 to −268)	A **A A A A T T C C**

cloned upstream to the thymidine kinase promoter in the *Sma*I-*Hin*dIII restriction sites of the TKGal parental vector (plasmid A, Fig. 5). This vector was derived by subcloning the *Hin*dIII-*Xho*I fragment of the pBLCAT2 plasmid, containing the thymidine kinase promoter, in the *Hin*dIII-*Xho*I restriction sites of the pEQ3 plasmid.

RESULTS

NF-κB Family Members Together with the NFATc Protein Are Components of the PMA/PHA-inducible C3-3P Complexes—Sequence analysis of the IFN-γ promoter region of the human gene has identified two DNA sequences, C3-1P and C3-3P, highly homologous to the c-Rel binding site (C3) present in the first intron of this gene (20). As shown in Table I, these C3-related elements also share sequence similarity with NFAT and NF-κB binding sites. In addition, a putative IFN-γ NF-κB binding site was also identified in the IFN-γ promoter. These observations prompted us to investigate the potential interaction of NFAT and NF-κB proteins with these DNA elements.

Based on previous reports indicating the promoter region (nucleotide −284 to −260) of the IFN-γ gene, which contains the C3-3P element (AAAATTTCC), as a regulatory DNA sequence necessary for its inducible transcriptional activity (11–15) and on the sequence homology (Table I), we performed EMSA analysis utilizing a ^{32}P-labeled C3-3P containing oligonucleotide (Fig. 1, *panels A–C*). In the presence of nuclear extract from untreated T lymphocytes, only a weak complex formation was observed (*panel B, lane 1*). However, PMA/PHA treatment enhanced the protein complex binding to this DNA element (*lane 2*). Fig. 1 (*panel A*) shows a competition study of the C3-3P DNA-protein complex. Binding of the complexes to the DNA probe was sequence-specific since it was blocked by excess of unlabeled C3-3P oligonucleotide (*lane 2*) but not by excess of nonspecific unlabeled oligonucleotide (*lane 8*). Since we previously described the C3 site as a c-Rel binding site (20), we also used an oligonucleotide containing the IL-2Rα NF-κB binding site as an unlabeled competitor. This oligonucleotide (*lane 3*) was able to compete the C3-3P complex, suggesting a possible involvement of Rel family members in the assembly of the C3-3P complexes. As shown in Table I and as reported elsewhere (15) while this work was in progress, the C3-3P site displays sequence similarity also with NFAT binding sites. We therefore used as competitors cold oligonucleotides containing the human IL-4 NF(P) (*panel A, lane 4*) and the human distal
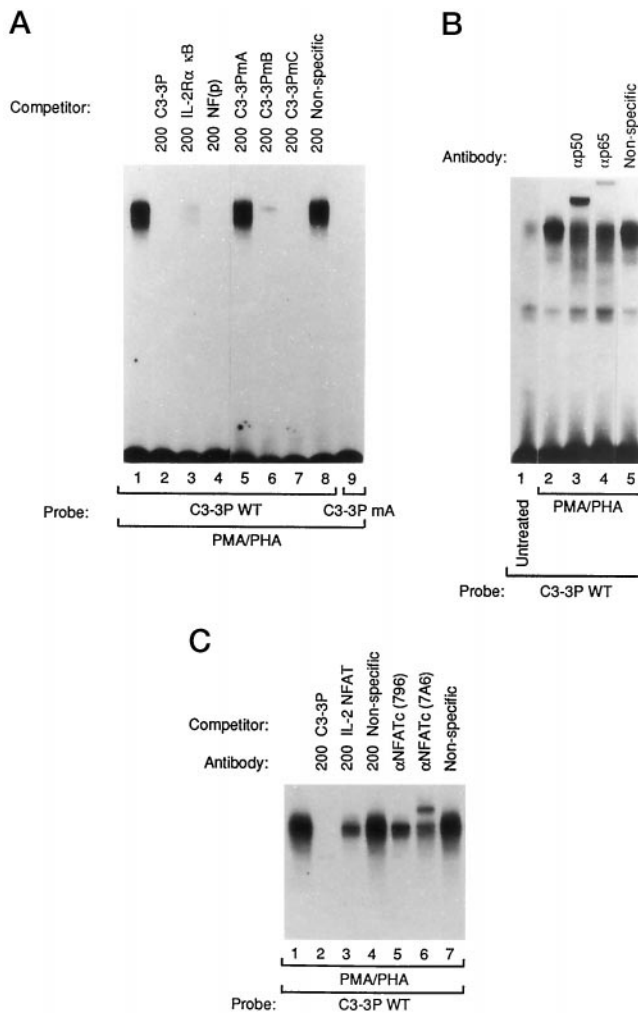
TABLE II
Mutagenesis study of the C3–3P binding site

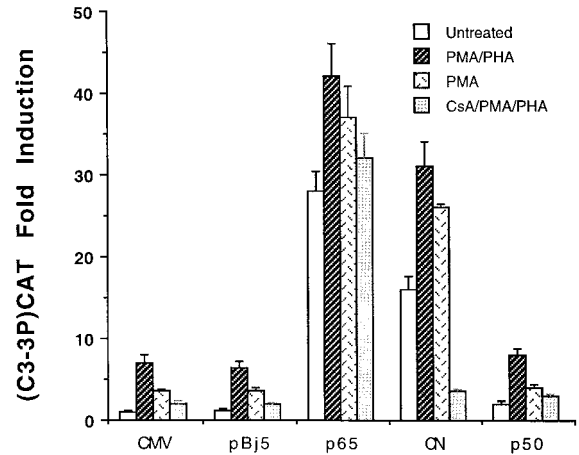|		Competition of the C3–3P DNA-protein complex
C3–3P wild type	5′-GGTACAAAAAAATTTCCAGT-3′	+
C3–3PmA	5′-GGTACAAAAAAATTTttAGT-3′	−
C3–3PmB	5′-GGTACAAAAggTTTCCAGT-3′	+
C3–3PmC	5′-GGTACAggAAATTTCCAGT-3′	+

FIG. 2. **Transfection analysis of the IFN-γ C3-3P site in the Jurkat T cell line.** Transient transfections were performed using the (C3-3P)3TKCAT2 reporter construct. Four μg of the CAT constructs were co-transfected with 4 μg of expression vectors as indicated. Fold of induction refers to the level of CAT activity detected in unstimulated Jurkat cells transfected with the (C3-3P)3TKCAT2 construct together with the empty expression vector CMV (to which a value of 1.0 was assigned). Transfection efficiencies were normalized for the amount of plasmid uptake using a β-galactosidase reporter vector as a control and are expressed as the average ($X \pm$ S.E.) of four different experiments.

FIG. 1. **Gel shift analysis of the C3-3P DNA-protein complex.** *Panel A, lanes 1–9*, human T lymphocytes treated with PMA/PHA for 4 h; *lanes 2–8*, competition with 200 ng of unlabeled C3-3P, human IL-2Rα κB site, IL-4 NF(p), C3-3P mA, C3-3P mB, C3-3P mC, and nonspecific competitor (SP-1), respectively. In *lane 9* a ^{32}P-labeled C3-3P mA oligonucleotide was used as probe. *Panel B, lane 1*, untreated T lymphocytes; *lanes 2–5*, PMA/PHA-treated T cells; *lane 3*, anti-p50 (antiserum 1141); *lane 4*, anti-p65 (antiserum 1226); *lane 5*, nonspecific antiserum. *Panel C, lanes 2–4*, competition with 200 ng of unlabeled C3-3P, distal IL-2 NFAT, and nonspecific oligonucleotide, respectively; *lane 5*, anti-NFATc (antiserum 796); *lane 6*, anti-NFATc (antiserum 7A6); *lane 7*, nonspecific antiserum.

IL-2 NFAT (*panel C, lane 3*) sites. Both these oligonucleotides were able to compete the C3-3P DNA-protein complex, although with different affinities, suggesting that NFAT proteins may also be part of it. These results are in agreement with the recent observation that NF-κB and NFAT proteins may bind identical DNA sequences (31, 32). Next, mutagenesis analysis of the C3-3P site (AAAAATTTCC) was carried out by using as cold competitors (*panel A, lanes 5–7*) three oligonucleotides (C3-3PmA, C3-3PmB, and C3-3PmC) containing different mutations (Table II) within the C3-3P sequence. As shown, only oligonucleotide C3-3PmA (AAAAATTTTT) (*lane 5*) was unable to compete the formation of the C3-3P complex. These results indicated that the two cytosines located in the 3′-half of the C3-3P site are essential for its binding activity. In support of this observation, when oligonucleotide C3-3PmA was used as ^{32}P-labeled probe in the presence of a nuclear extract from PMA/PHA activated fresh human T lymphocytes, no binding activity was observed (*lane 9*).

To characterize the composition of the C3-3P complex, we

performed supershift analysis. Since the NF-κB and NFAT oligonucleotides were effective competitors for the C3-3P complex formation, we utilized antisera raised against peptides of the p50, p65 NF-κB subunits (*panel B*) and the NFATc protein (*panel C*). As visible in *panel B*, both the anti-p50 (*lane 3*) and anti-p65 (*lane 4*) antisera were able to supershift the middle portion of the C3-3P DNA-protein complex, whereas a nonspecific antiserum (*lane 5*) had no effect. Also, anti-NFAT antisera (*panel C*) were able to compete or supershift, respectively (*lanes 5 and 6*), a considerable part of the complex but not the NF-κB heterodimer. When we tested an anti-c-Rel antiserum, only a minor fraction of the C3-3P complexes were supershifted (data not shown). These results identified both NF-κB and NFAT proteins as the inducible binding factors interacting with the C3-3P site.

NF-κB p65 and a Constitutively Active Mutant of Calcineurin Functionally Activate the IFN-γ C3-3P Enhancer Activity—Our binding studies demonstrated the interaction of NF-κB and NFAT proteins with the C3-3P site. To define the functional significance of these DNA-protein complexes, we subcloned three copies of the C3-3P site upstream of the thymidine kinase (TK) promoter in the pBLCAT2 parental vector. As shown in Fig. 2, this construct was co-transfected into the human lymphoma T cell line, Jurkat, with either expression vectors for p65 NF-κB, p50 NF-κB, or a constitutively active mutant of the calcineurin phosphatase (27).

Co-transfection of either the p65 NF-κB or the calcineurin expression vector resulted in a significant transactivation of the C3-3P-CAT activity over the activities observed in the presence of either the CMV or pBJ5 parental vectors. In untreated cells the C3-3PCAT construct was activated about 28-

TABLE III
Mutagenesis study of the IFN-γ DNA region nucleotides −796 to −761

The table summarizes the capability of the indicated cold oligonucleotides to compete the proteins binding to the ^{32}P-labeled IFN-γ WT oligonucleotide.

	IFN-γ κB C3–1P	Competition
IFN-γ WT (−796 to −761)	5′-CACTGCGTCTGGAACTCCCCCTGGGAATATTTTCT-3′	++++
M1	5′-CACTGCGTCTttAACTCCCCCTGGGAATATTTTCT-3′	+++
M2	5′-CACTGCGTCTGGAACTCCCCCTtttAATATTTTCT-3′	++++
M4	5′-CACTGCGTCTGGAACTCttttTGGGAATATTTTCT-3′	+++
IFN-γ κB	5′-GTCTGGAACTCCCCCTG-3′	++++
IFN-γ κB M1	5′-GTCTttAACTCCCCCTG-3′	−
IFN-γ κB M4	5′-GTCTGGAACTCttttTG-3′	−
C3–1P	5′-CCTGGGAATATTTTCT-3′	+++
C3–1P M2	5′-CCTtttAATATTTTCT-3′	−

fold by p65 and 15-fold by calcineurin, whereas the p50 expression vector and the parental pRSPA and pBJ5 empty vectors did not significantly affect reporter activity. In cells treated with PMA or PMA/PHA, however, the effects of the p65 and calcineurin expression vectors were further increased. As expected, cyclosporin A prevented the calcineurin-induced increase in promoter activity but only slightly inhibited the up-regulation due to p65. The CAT activity of the pBLCAT2 parental vector, used as a control, was not significantly affected by these treatments (data not shown). Thus, we have identified a site (C3-3P) within the IFN-γ promoter region that is highly responsive to NF-κB p65 and to a factor, presumably NFATc, that is activated by calcineurin.

The IFN-γ κB and C3-1P Sites Have NF-κB Binding Activity—As the possible presence of additional NF-κB binding sites suggests an NF-κB dependent activation of IFN-γ gene transcription, we next characterized the nuclear protein binding activity of the IFN-γ κB and C3-1P sites. In EMSA analysis, we used as a ^{32}P-labeled probe, an oligonucleotide spanning the IFN-γ promoter region (nucleotides −796 to −761) (Table III) and containing both the IFN-γ κB and C3-1P elements (Fig. 3). As shown in Fig. 3, *panel B*, incubation of this probe with a nuclear extract from PMA/PHA-treated fresh human T lymphocytes resulted in the formation of an inducible DNA-protein complex (*lane 2*). While in the presence of nuclear extract from untreated T lymphocytes, only a weak binding was observed (*lane 1*). As shown in Fig. 3, *panel A*, the DNA-protein complex formation was specific since competition with a cold nonspecific oligonucleotide did not abolish the binding (*lane 12*), and the cold wild type oligonucleotide containing the IFN-γ promoter region nucleotides −796 to −761 was able to specifically block the complex formation (*lane 7*).

To investigate the binding activity of the C3-1P site, a cold oligonucleotide containing the C3-1P sequence (Table III) was used as competitor (Fig. 3, *panel A, lane 2*). This oligonucleotide could efficiently, but not completely, compete the DNA-protein formation, whereas a mutant of the C3-1P oligonucleotide, C3-1P M2 (*lane 3*), did not. We also tested the capability of a cold oligonucleotide containing the IFN-γ κB site to act as a competitor. This oligonucleotide (*lane 4*) completely blocked the formation of the DNA-protein complex, while two different mutants, the IFN-γ κB M1 (*lane 5*) and IFN-γ κB M4 (*lane 6*) oligonucleotides, were unable to block the complex formation. To evaluate better the individual contribution of the IFN-γ κB and C3-1P sites to this binding activity, we generated additional mutations within the wild type oligonucleotide (nucleotide −796 to −761). In agreement with results seen in *lanes 2–5*, the mutants M4 and M1 (*lanes 8* and *9*) could efficiently, but not totally, block the DNA-protein complex formation, whereas the mutant M2 (*lane 10*) completely abolished the binding. A cold oligonucleotide containing the IL-2 Rα NF-κB site also completely blocked the formation of the complex (*lane*
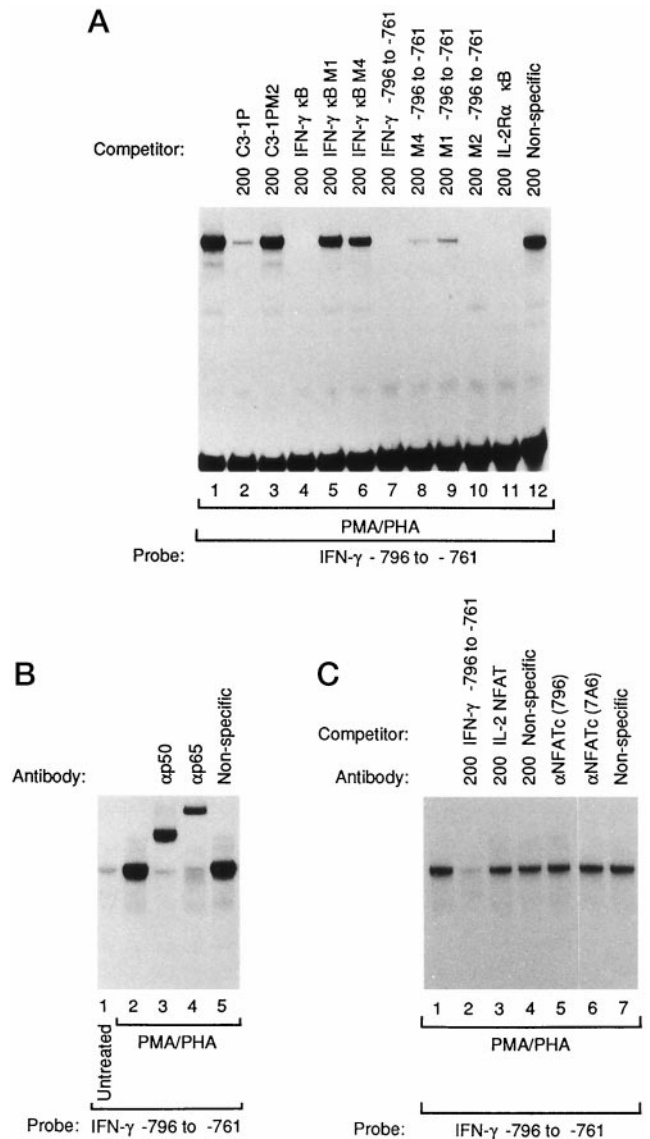

FIG. 3. **Supershift and competition analysis of the ^{32}P-labeled oligonucleotide containing the IFN-γ promoter DNA nucleotides −796 to −761.** *Panel A, lanes 1–12*, T cells treated with PMA/PHA for 4 h; *lanes 2–12*, competition with 200 ng of unlabeled C3-1P, C3-1P M2, IFN-γ κB, IFN-γ κB M1, IFN-γ κB M4, IFN-γ promoter DNA nucleotides −796 to −761, M4, M1, M2, human IL-2Rα κB site, and nonspecific competitor, respectively. *Panel B, lane 1*, untreated T cells; *lanes 2–5*, PMA/PHA; *lane 3*, anti-p50 (antiserum 1141); *lane 4*, anti-p65 (antiserum 1226); *lane 5*, nonspecific antiserum. *Panel C, lanes 1–7*, PMA/PHA; *lanes 2–4*, competition with 200 ng of unlabeled IFN-γ −796 to −761, distal IL-2 NFAT and nonspecific oligonucleotide, respectively; *lane 5*, anti-NFATc (antiserum 796); *lane 6*, anti-NFATc (antiserum 7A6); *lane 7*, nonspecific antiserum.

FIG. 4. **Gel shift and supershift analysis of the IFN-γ κB and C3-1P promoter sites.** ^{32}P-Labeled oligonucleotides containing the IFN-γ κB (*panels A* and *B*) or the C3-1P (*panels C* and *D*) site were used as indicated in the presence of a nuclear extract from fresh human T lymphocytes. *Panel A, lane 1*, untreated T cells; *lanes 2–8*, T cells treated with PMA/PHA for 4 h; *lanes 3–5*, competition with 200 ng of unlabeled IFN-γ κB, human IL-2Rα κB site, and nonspecific competitor, respectively; *lane 6*, anti-p50 (antiserum 1141); *lane 7*, anti-p65 (antiserum 1226); *lane 8*, nonspecific antiserum. *Panel B, lanes 1–7*, T cells treated with PMA/PHA for 4 h; *lanes 2–4*, competition with 200 ng of unlabeled IFN-γ κB, distal IL-2 NFAT, and nonspecific oligonucleotide, respectively; *lane 5*, anti-NFATc (antiserum 796); *lane 6*, anti-NFATc (antiserum 7A6); *lane 7*, nonspecific antiserum. *Panel C, lane 1* untreated T cells. *Lanes 2–9*, T cells treated with PMA/PHA for 4 h; *lanes 3–5*, competition with 200 ng of unlabeled C3-1P, human IL-2Rα κB site, and nonspecific oligonucleotide, respectively; *lane 6*, no antiserum; *lane 7*, anti-p50 (antiserum 1141); *lane 8*, anti-p65 (antiserum 1226); *lane 9*, nonspecific antiserum. *Panel D, lanes 1–7*, T cells treated with PMA/PHA for 4 h; *lanes 2–4*, competition with 200 ng of unlabeled C3-1P, distal IL-2 NFAT, and nonspecific oligonucleotide, respectively; *lane 5*, anti-NFATc (antiserum 796); *lane 6*, anti-NFATc (antiserum 7A6); *lane 7*, nonspecific antiserum.
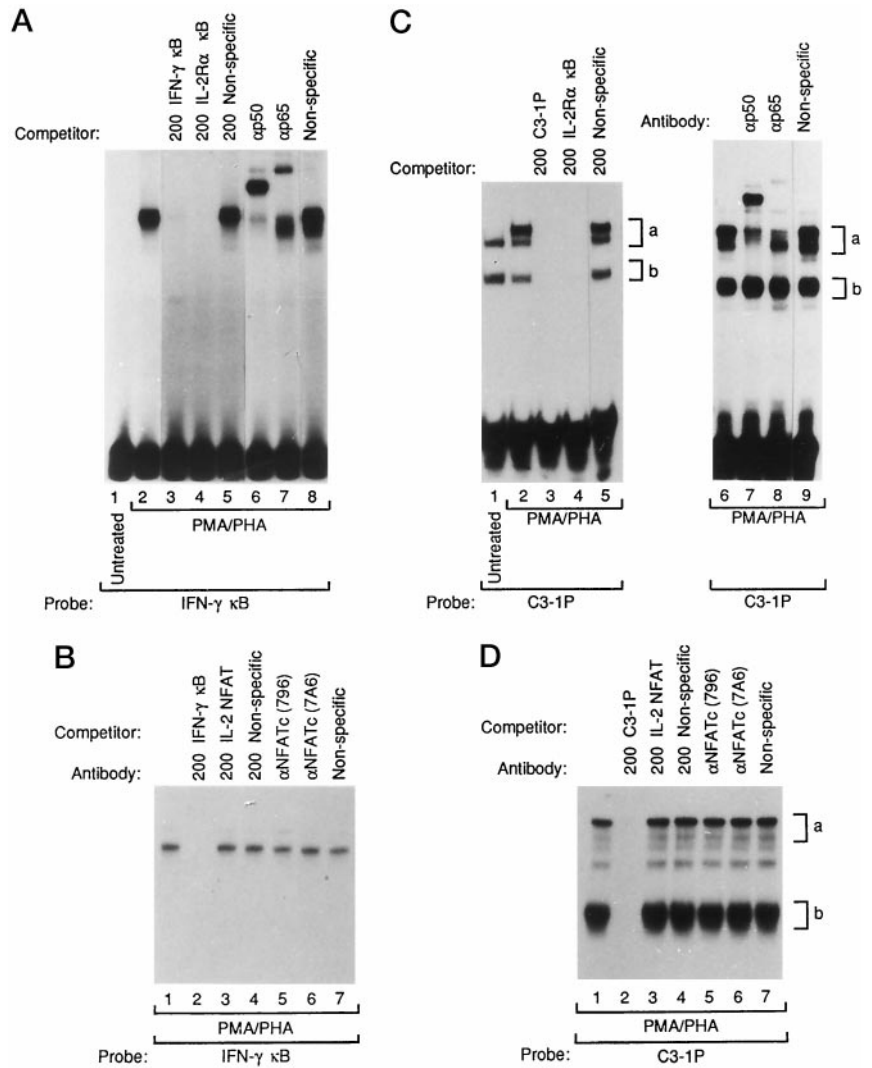
11). The results of this study are summarized in Table III and indicate that the sequence −796 to −761 of the IFN-γ promoter interacts with protein complexes whose binding is specifically competed by cold NF-κB oligonucleotides.

Based on this observation, we performed supershift analysis to investigate the possible interaction of NF-κB proteins with this IFN-γ promoter region (Fig. 3, *panel B*). This complex was completely shifted by anti-p50 and anti-p65 antisera (*lanes 3* and *4*), whereas a nonspecific antiserum (*lane 5*) did not affect complex formation. Due to the observation that NF-κB and NFAT proteins may bind similar DNA sequences (31, 32), we also investigated whether NFAT protein may share affinity for the oligonucleotide containing the IFN-γ region −796 to −761. As shown in Fig. 3, *panel C*, no competition was observed with a cold oligonucleotide containing the distal IL-2NFAT site (*lane 3*), and NFAT antisera (*lanes 5* and *6*) were not able to supershift or block DNA-protein complex formation.

As both the cold IFN-γ κB and C3-1P oligonucleotides were able to compete the NF-κB binding to the IFN-γ WT oligonucleotide, we also performed EMSA analysis utilizing these oligonucleotides (Table III) as ^{32}P-labeled oligonucleotide probes. Cold competition and supershift analysis (Fig. 4) confirmed that both the IFN-γ κB and the C3-1P sites specifically bind the inducible p50/p65 NF-κB heterodimer. In particular, the C3-1P oligonucleotide (Fig. 4, *panels C* and *D*) showed formation of multiple DNA-protein complexes. Interestingly, our data identify the slower C3-1P migrating complex of region a, supershifted either by anti-p50 or anti-p65 antisera (*panel C, lanes 7* and *8*), as the p50/p65 NF-κB heterodimer. In addition, a faster migrating C3-1P DNA-protein complex (region a) was completely eliminated by the anti-p50 antiserum, but not by the anti-p65 antiserum, suggesting that this complex represents the p50 NF-κB homodimer. A third C3-1P complex (region b) was also observed, but the protein composition of this complex was not characterized. These results confirmed the binding of NF-κB proteins but not NFAT proteins to both the IFN-γ κB and C3-1P sites. The formation of additional DNA-protein complexes that were detected using the short C3-1P oligonucleotide may indicate its partial affinity for other protein complexes.

The p65 Subunit Is a Functional Transactivator of the IFN-γ Promoter Region −796 to −761—To investigate the functional significance of NF-κB protein binding to the IFN-γ κB-C3-1P tandem promoter element, we subcloned one copy of this region (nucleotides −796 to −761) upstream of the TK promoter in the TK β-galactosidase parental vector. This construct (plasmid B) was then transfected in the Jurkat T cell line. As shown in Fig. 5, both PMA/PHA treatment and co-transfection with a p65 NF-κB expression vector were able to induce β-galactosidase activity in the presence of the IFN-γ κB-C3-1P tandem promoter element. Combination of these treatments resulted in a even stronger transactivation. In contrast a p50 NF-κB expression vector was not able to induce β-galactosidase activity. These results provide evidence for a functional interaction of NF-κB with the IFN-γ κB-C3-1P tandem promoter element.
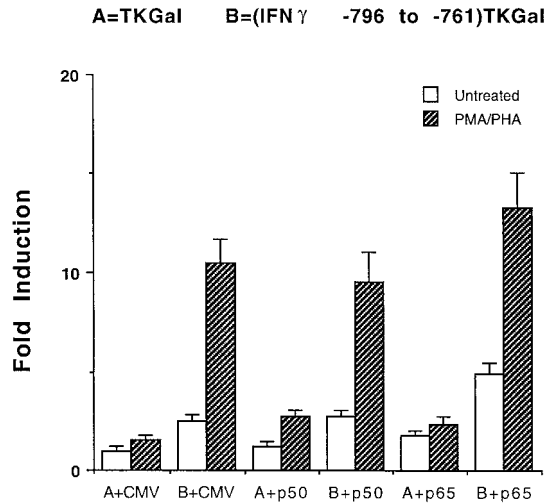
A=TKGal   B=(IFNγ   -796 to -761)TKGal

FIG. 5. **Functional analysis of the IFN-γ DNA promoter region nucleotides −796 to −761.** Jurkat T cells were transfected with either the (IFN-γ −796 to −761)TKGal construct (plasmid B) or the TKGal parental vector (plasmid A). Four μg of plasmids A or B were co-transfected with 4 μg of either the p50, p65, or parental CMV vectors as indicated. Fold of induction refers to the level of β-galactosidase activity detected in unstimulated Jurkat cells transfected with construct A together with the empty expression vector CMV (to which a value of 1.0 was assigned). Transfection efficiencies were normalized for the amount of plasmid uptake using a CAT reporter vector as a control, and the results are expressed as the average (X ± S.E.) of four different experiments.

The IFN-γ κB, C3-1P, and C3-3P Sites Are Regulatory DNA Elements Required for Maximal Transcriptional Activity of the IFN-γ Promoter—Although both the C3-3P site and the IFN-γ κB-C3-1P tandem promoter element showed significant enhancer activity when subcloned upstream of a heterologous promoter, we investigated their functional role in the context of the native IFN-γ promoter. Based on our mutagenesis studies (Tables II and III) we generated a number of mutations within the IFN-γ promoter region (nucleotide −796 to +64) that selectively abolished the binding activities of either the C3-3P, C3-1P, or IFN-γ κB sites (Fig. 6, *panel A*). The mutant constructs were subcloned upstream to the β-galactosidase reporter gene in the parental vector pEQ3 (13). As shown in Fig. 6 (*panel B*), all the mutated constructs displayed lower activity than the wild type construct, demonstrating the functional role of the mutated DNA elements IFN-γ κB, C3-1P, and C3-3P in the induction of the human IFN-γ promoter transcriptional activity. Furthermore, the combination of mutations m2 and m5 (plasmid m6) or m5 and m4 (plasmid m7) slightly increased the inhibitory effect observed with the single mutations.

The Calcineurin Phosphatase Induces Transcriptional Activity of the IFN-γ Promoter through the C3-3P DNA Element—The fact that the C3-3PCAT activity can be induced by either NF-κB or NFAT proteins raised the question of which proteins regulate the C3-3P enhancer activity in the context of the intact IFN-γ promoter. To provide insight into this issue, we compared the CsA sensitivity of the wild type and C3-3P mutant (m5) constructs (Fig. 7; *panel A*). In addition, we co-transfected both reporter constructs with either the calcineurin phosphatase (*panel A*) or the p65 NF-κB (*panel B*) expression vector. As shown in Fig. 7, the calcineurin expression vector was able to enhance the β-galactosidase activity of the WT construct in both untreated and PMA/PHA-treated cells, and CsA co-treatment resulted in the suppression of the calcineurin-induced activities. The calcineurin vector also potentiated to a minor extent the activity of the m5 mutant; while in the presence of CsA the PMA/PHA-induced activities of the WT
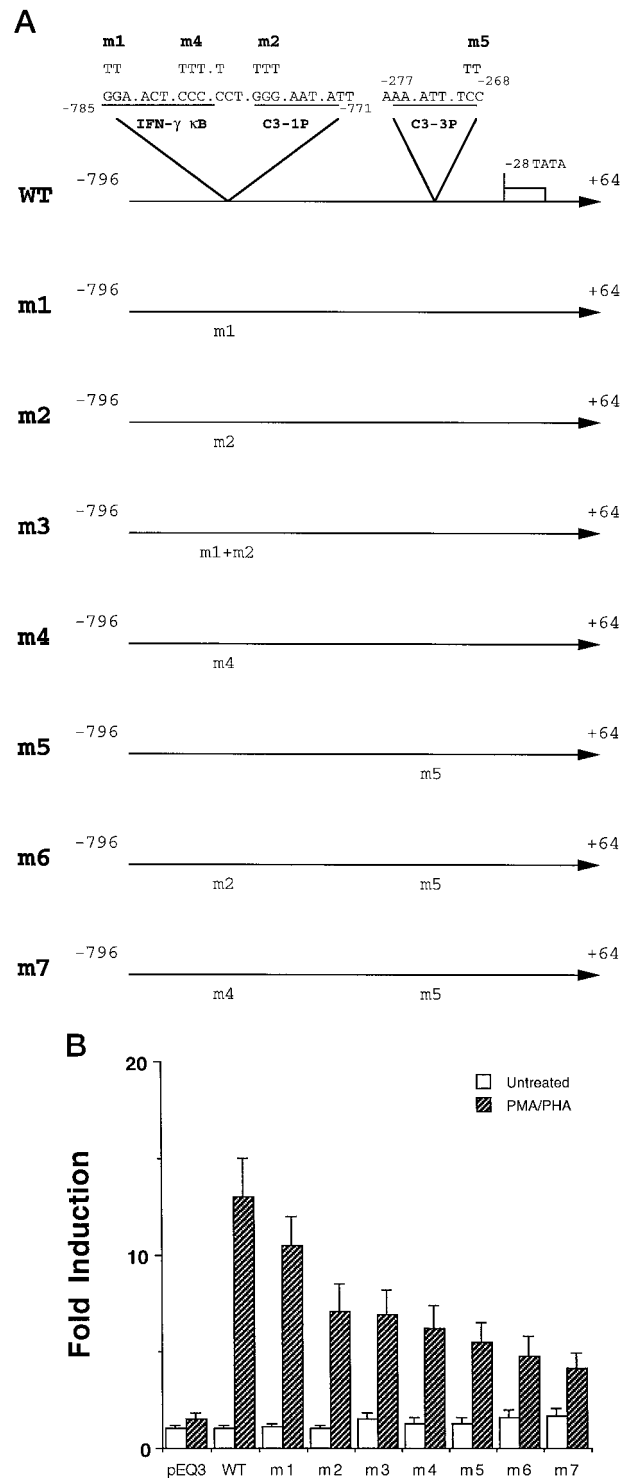

FIG. 6. *Panel A*, schematic representation of the IFN-γ promoter mutants transfected into the Jurkat T cell line. *Panel B*, site-directed mutagenesis of the IFN-γ κB; C3-1P and C3-3P sites inhibit the maximal transcriptional activity of the IFN-γ promoter nucleotides −796 to +64. Six μg of the indicated plasmids were transfected as indicated. Fold of induction refers to the level of β-galactosidase detected in unstimulated Jurkat cells transfected with the WT construct (to which a value of 1.0 was assigned). Results are expressed as the average (X ± S.E.) of four different experiments.

and m5 constructs were equivalent. While co-transfection of the p65 NF-κB expression vector (*panel B*) resulted in a lower activity of the m5 mutant in untreated and PMA/PHA-treated cells, in comparison with the WT construct, no appreciable differences were observed (as fold of induction) between these
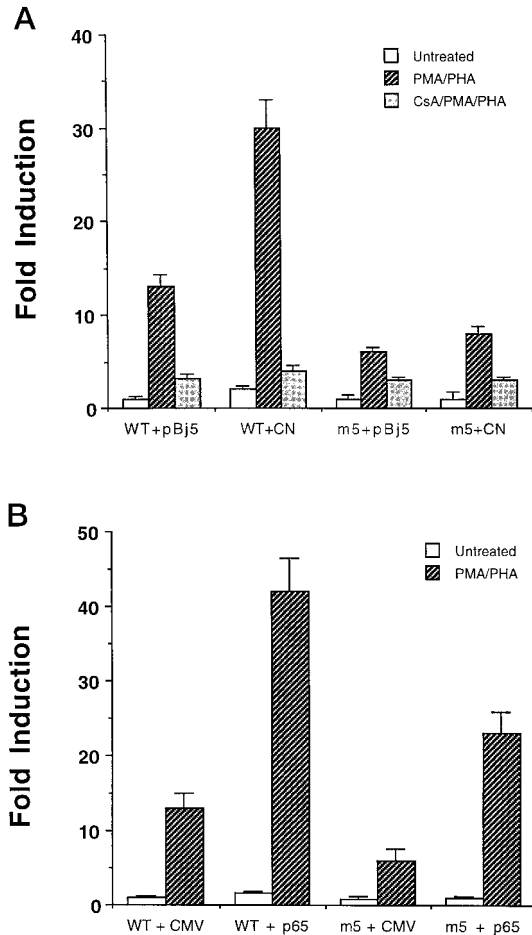
A

B

FIG. 7. **Effects of the calcineurin (CN) (panel A) and p65 NF-κB (panel B) on the β-galactosidase activity expressed by the WT and m5 reporter constructs.** Fold of induction refers to the level of β-galactosidase activity detected in unstimulated Jurkat cells transfected with the WT construct together with the empty expression vectors CMV or pBJ5, respectively (to which a value of 1.0 was assigned). Four μg of the indicated plasmids were transfected as indicated into the Jurkat T cell line. Results are expressed as the average ($X \pm$ S.E.) of three different experiments.

FIG. 8. **Effect of the c-Rel intronic site (C3) on IFN-γ promoter transcriptional activity.** Fold of induction refers to the respective levels of PMA/PHA-induced β-galactosidase activity expressed in Jurkat cells by the WT, m2, m4, or m5 reporter constructs. Values were normalized with the β-galactosidase activity expressed by the WT construct to which a value of 1.0 was assigned. Four μg of the indicated plasmids were transfected into the Jurkat T cell line. Results are expressed as the average ($X \pm$ S.E.) of three different experiments. *C3* represents one copy of the intronic C3 site; *2C3* represents two copies of the intronic C3 site.

ase activity. Noteworthy, mutations affecting either the C3-3P (m5) or the IFN-γ κB (m4) binding activities resulted in a lack of synergism with the C3 enhancer, whereas loss of C3-1P binding (m2) only partially decreased it. Thus, both the C3-3P and IFN-γ κB binding sites appear to cooperate with the intronic C3 enhancer to promote optimal activity of the IFN-γ promoter.

DISCUSSION

A number of reports have investigated the molecular mechanisms regulating the IFN-γ gene transcription. A consensus GATA motif and an NFIL-2-like element have been identified within the CsA-sensitive proximal promoter (nucleotide −108 to −40) of the human IFN-γ gene (13), and the binding of CREB/ATF, AP-1, and octamer families of transcription factors to this region of the promoter has been reported (16). This region of the promoter has been subdivided into two elements, a proximal element (−70 to −47) and a distal element (−98 to −72) (13), and the importance of these elements in regulating IFN-γ gene expression has been elegantly demonstrated through the use of transgenic mice by Aune and co-workers (17). In this report, the proximal and distal elements were shown to direct transcription in memory but not naive T cells in response to stimulation through the T cell receptor. Additionally, the CD4+ but not the CD8+ population of T cells expressed proximal activity. Thus these elements seem to be essential for IFN-γ expression in these cell types. More recently, Barbulescu and co-workers (18) have used *in vivo* footprinting to identify a new site (−196 to −183) that binds a PMA/PHA-inducible complex in Jurkat T cells and purified CD45RO peripheral blood T cells. These investigators have proposed a model where induction of the AP-1 proteins to this region of the promoter is involved in the increased transcription of the IFN-γ gene in response to stimulation. Although the promoter sites described in these recent publications are required for the transcription of the IFN-γ gene, it is also clear that additional negative (40) and positive (11) regulatory elements play a role in the control of IFN-γ mRNA initiation and that optimal conditions for IFN-γ gene expression likely require the cooperation between different regulatory elements. We have previously identified a novel c-Rel binding site (C3) (20) that is located within an IFN-γ intronic enhancer region and that shares sequence homology with either NFAT or

reporter constructs. As in the presence of the calcineurin expression vector, we observed a higher fold of induction with the WT construct in comparison with the m5 mutant construct. These results suggest that within the native IFN-γ promoter the C3-3P enhancer activity is mainly activated by calcineurin-inducible transcriptional factors consistent with a previous report (15), suggesting that the NF-κB-mediated induction of the IFN-γ promoter activity acts mainly through the IFN-γ κB-C3-1P tandem promoter element.

The Intronic c-Rel Binding Site (C3) Enhances IFN-γ Promoter Activity—To define better the relative significance of the NFAT and NF-κB binding sites identified within the IFN-γ promoter and to investigate possible functional cooperation with the intronic C3 enhancer region, one or two copies of the previously identified c-Rel intronic site C3 (20) were linked downstream of the β-galactosidase gene in the WT, m2, m4, and m5 constructs. Fig. 8 represents the β-galactosidase activity expressed by the constructs containing one or two copies, respectively, of the intronic C3 site over the activities expressed by the parental constructs. Values were normalized with the PMA/PHA-induced activity expressed by the WT construct, to which a value of 1.0 was assigned. Upon PMA/PHA treatment, the WT construct containing either one or two copies of the intronic C3 site displayed a strong increase of the β-galactosid-
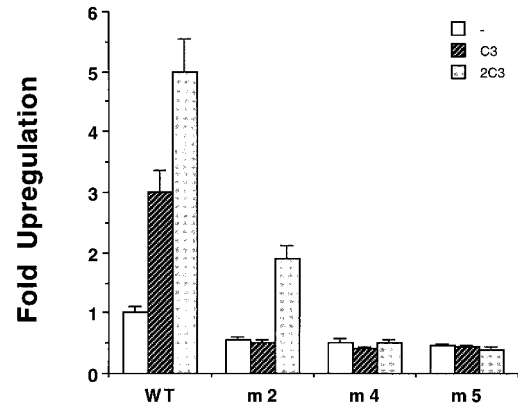
NF-κB binding sites (Table I). In this report, we have characterized the binding activity and functional properties of two newly identified C3-related sites (C3-1P and C3-3P) and a novel NF-κB (IFN-γ κB) site located in the promoter region and provide evidence for a functional cooperation between these newly identified enhancers and the intronic c-Rel site C3. Our data demonstrate the presence, within the human IFN-γ promoter, of multiple targets for the NF-κB binding activity. The importance of these sites in regulating IFN-γ expression likely depends upon the specific extracellular signal that triggers IFN-γ expression. We have previously demonstrated (41) that IL-2 but not IL-12 induces the nuclear appearance in the nucleus of NF-κB proteins in a human NK cell line, and the interaction of the NF-κB proteins within the regions of the IFN-γ promoter described here may serve to enhance the transcription initiation of the gene. Although the specific biochemical pathways triggering the activation of IFN-γ expression have not been completely defined, a recent report (42) has implicated Raf kinase as an important intracellular mediator for the induction of IFN-γ expression through the T cell receptor in human CD4+ Th 1 cells. Raf kinase has been demonstrated to be important for AP-1 activation (43), and Kanno and Siebenlist (44) have also reported a functional synergy between Raf and calcineurin that results in NF-κB activation. Thus the activation of numerous transcription factors, including NF-κB, may be critical for achieving a maximal activation of IFN-γ transcription. The activation may also be dependent upon cell type because in addition to T cells and NK cells, murine peritoneal macrophages (45) and mast cells (46), human eosinophils (47), human keratinocytes (48), and primary human B cells (49) have all been recently reported to express IFN-γ mRNA.

In addition to binding NF-κB p50/p65, the C3-3P site described in this article also binds the NFATc protein, emphasizing its similarity with the IL-4 NF(p) site (Table I), which also binds NF-κB and NFAT proteins (32). This site exactly corresponds to a region previously mapped by Brown and co-workers (11) that responds to mitogen stimulation. In addition, while our work was in progress, Campbell and co-workers (15) reported the same region as a potential NFAT binding site. In our experiments, both anti-NF-κB and NFAT antisera were able to supershift co-migrating but discernible portions of the C3-3P DNA-protein complexes, suggesting that NF-κB and NFAT proteins independently bind the C3-3P site. The ability of both NF-κB and NFAT proteins to bind the same site is not surprising given the recent discovery that the NFATc phosphoprotein is a distant relative of the Rel/NF-κB proteins (28, 30). In addition, a previous report has also described the binding of an NFAT-like factor to a classic κB site (31). By analogy with the IL-4 NF(P) site (32), the common capability of NF-κB and NFAT proteins to functionally interact with the C3-3P site highlight the possibility that NF-κB and NFAT proteins, although expressed through independent signaling pathways, may exercise their transcriptional activity through common enhancer elements. These results add IFN-γ to the family of genes that are regulated, at least in part, by the NF-κB and NFAT families of transcription factors.

We have previously reported (20) that a bacterially expressed p50 NF-κB protein could bind to the intronic C3 oligonucleotide. However, we could not detect binding of the p50 homodimer at the C3-3P site, thus supporting its selective binding by the p50/p65 heterodimer. Thus, although it is known that the NF-κB p50 homodimer binds preferentially the 5′-end Gs containing κB sites (51, 52), thus making possible the binding of the p50 homodimer to the 5′-end of the single C3-1P oligonucleotide, it is possible that occupancy of the IFN-γ κB

site by the p50/p65 heterodimer may impair the access of the p50 NF-κB homodimer at the 5′-end of the C3-1P site. The observation that p50 NF-κB does not significantly affect the activity of the C3-3P, IFN-γ κB-C3-1P DNA tandem element as well as the IFN-γ promoter activity (not shown) is in line with the observation that p50 is devoid of an activation domain and that p65 is necessary for transcriptional activity (25).

Understanding the role of the first intron in regulating IFN-γ gene expression is of special interest as Xu and co-workers (53) have identified STAT binding sites very near the intronic C3 element. As the enhanced promoter activity observed when the C3 site was placed downstream of the reporter gene was eliminated when either the IFN-γ κB or the C3-3P binding activity were selectively abolished by point mutation, optimal transcription of the IFN-γ gene results from synergism between promoter and intronic enhancers. Thus it may be possible that cooperation between STATs and NF-κB occurs, further enhancing IFN-γ transcription. This hypothesis will require more detailed mutational analysis of these sites in the context of the human IFN-γ genomic DNA structure.

In conclusion, this work provides evidence for the functional interaction between the NF-κB and NFAT family of DNA-binding proteins with regulatory regions of the IFN-γ promoter and defines the C3-3P site and the IFN-γ κB-C3-1P tandem element as enhancer elements required for maximal IFN-γ gene transcription. Our data establish the IFN-γ gene as an additional transcriptional model where the NF-κB and NFAT DNA-protein complexes play a role in enhancing transcriptional activity.

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Interaction of NF-κB and NFAT with the Interferon-γ Promoter

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