THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 43, Issue of October 25, pp. 26783–26793, 1996
Printed in U.S.A.

Retinoic Acid-induced Transcriptional Modulation of the Human Interferon-γ Promoter*

(Received for publication, July 29, 1996)

Marco Cippitelli‡, Jianping Ye§, Vincenzo Viggiano§, Antonio Sica§, Paritosh Ghosh§, Alberto Gulino¶, Angela Santoni‖, and Howard A. Young§‡‡**

*From the ‡Intramural Research Support Program, Scientific Application International Corporation Frederick, and the §Laboratory of Experimental Immunology, Division of Basic Science, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201, the ¶Department of Experimental Medicine, University of L'Aquila, 67100 L'Aquila, Italy, the ‖Department of Experimental Medicine and Pathology, University "La Sapienza," Roma 00158, Italy, and the **Laboratory of Pathophysiology, Regina Elena Cancer Institute, Roma 00158, Italy*

Disregulation of vitamin A metabolism is able to generate different immunological effects, including altered response to infection, reduced IgG production, and differential regulation of cytokine gene expression (including interleukin-2 and -4 and interferon-γ (IFN-γ)). In particular, IFN-γ gene expression is significantly affected by vitamin A and/or its derivatives (*e.g.* retinoic acid (RA)). Here, we analyze the effect of retinoic acid on IFN-γ transcription. Transient transfection assays in the human T lymphoblastoid cell line Jurkat demonstrated that the activation of the IFN-γ promoter was significantly down-regulated in the presence of RA. Surprisingly, two different AP-1/CREB-ATF-binding elements situated in the initial 108 base pairs of the IFN-γ promoter and previously shown to be critical for transcriptional activity were unaffected by RA. Utilizing promoter deletions and electrophoretic mobility shift analysis, we identified a USF/EGR-1-binding element cooperating in the modulation of IFN-γ promoter activity by RA. This element was found to be situated in a position of the IFN-γ promoter close to a silencer element previously identified in our laboratory. These results suggest that direct modulation of IFN-γ promoter activity is one of the possible mechanisms involved in the inhibitory effect of retinoids on IFN-γ gene expression.

Retinoic acid (RA)[1] and other vitamin A derivatives are known to have different important roles in cellular differentiation, proliferation, and homeostasis (1–5). The heterogeneity of these responses suggests the existence of complex signaling pathways to account for the diverse effects of retinoids, and the molecular events involved in retinoid-mediated regulation of gene expression are not yet fully characterized (6–11). Retinoids act pharmacologically to restore the regulation of differentiation and growth in certain premalignant and malignant cells *in vitro* and *in vivo* (2, 13–15) and exert a profound influence on immune cells and immunological responses (16–34). Consequently, these compounds are currently being evaluated clinically for cancer prevention and therapy (2, 13–15). Retinoids have also been shown to regulate the development of the immune system since prenatal exposure to retinoic acid in humans and in animal models is able to generate thymic hypoplasia and impairment of T cell function (16, 17). Previous reports have already demonstrated the ability of retinoic acid to modulate the expression of the IL-2 gene through a direct interference with promoter activation (34, 35). Furthermore, recent reports demonstrated the relevant role of retinoids in the priming environment leading to $CD4^+$ TH1 or TH2 development in animal models (26–33). For example, in experimental allergic encephalomyelitis, treatment with all-*trans*-retinoic acid showed an improved clinical course correlating with the development of a TH2-like response, with increased production of IL-4 and a significant reduction in IL-2 and IFN-γ production (31). Moreover, vitamin A deficiency in animal models results in a strong regulatory T cell imbalance with excessive TH1-type cytokine synthesis and insufficient TH2 development and function (27–30, 33). In this context, the addition of all-*trans*-retinoic acid *in vitro* has been shown to significantly decrease the transcriptional level and secretion of IFN-γ both in $CD4^+$ and $CD8^+$ T cells and in NK cells (27–30, 33).

Our laboratory has recently described the negative transcriptional interference of the IFN-γ promoter activation pathway, mediated by the glucocorticoid receptor (GR), a member of the steroid/thyroid nuclear receptor superfamily that is able to exert strong inhibitory effects on the immune system and cytokine production (36). In this report, the effect of RA on the transcriptional activation of the IFN-γ promoter has been investigated using electrophoretic mobility shift assay (EMSA) and transient DNA transfection assays. Our data indicate that in Jurkat cells, the PMA/ionomycin-stimulated IFN-γ promoter activity is significantly down-regulated by retinoic acid after cotransfection with a human retinoic acid receptor-α (RARα) expression vector. Additionally, a novel promoter element situated in a position close to a silencer region previously identified in this laboratory (37) is involved in this inhibition. Moreover, an "E box"-like binding sequence present in this IFN-γ promoter element appears to be a critical site for this effect, and mutation of this sequence is able to eliminate the inhibitory action exerted by RA/RARα on the IFN-γ promoter. These data suggest that inhibition of the IFN-γ promoter is one of the possible mechanisms operating in the retinoid-mediated negative regulation of the IFN-γ gene and show the potential relevance of regulatory interaction(s) between RARs and E box-related binding factors in the modulation of the IFN-γ promoter in T lymphocytes.

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‡‡ To whom correspondence should be addressed: NCI-FCRDC, Bldg. 560, Rm. 31-93, Frederick, MD 21702-1201. Tel.: 301-846-5700; Fax: 301-846-1673.

[1] The abbreviations used are: RA, retinoic acid; IL, interleukin; TH, T helper; IFN, interferon; GR, glucocorticoid receptor; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; RARα, retinoic acid receptor-α; RXRα, retinoid X receptor-α; RARE, retinoic acid-responsive element; bp, base pair(s); kbp, kilobase pairs.

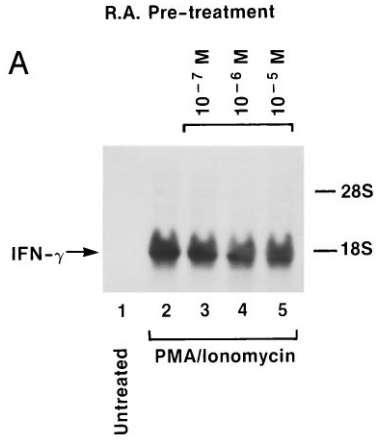
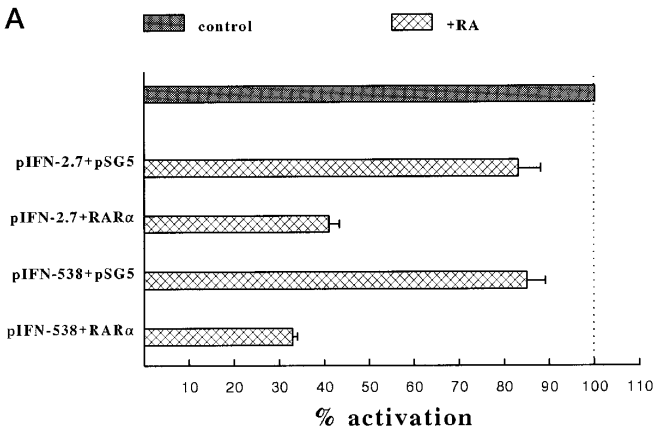


FIG. 1. **RA effect on IFN-γ mRNA.** Fresh purified human peripheral blood T cells were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin in the presence or absence of the indicated amount of retinoic acid for 2 h. Isolated RNA was analyzed by Northern blotting and hybridized with a random-primed ^{32}P-labeled human IFN-γ cDNA probe (*A*), followed by removal of the probe by boiling the membrane in 0.01 × SSC, 0.01% SDS for 20 min and rehybridization with a chicken β-actin cDNA probe (*B*). *Lane 1*, unstimulated cells; *lane 2*, PMA/ionomycin-stimulated cells; *lane 3*, PMA/ionomycin-treated cells pretreated (30 min) with 10^{-7} M RA; *lane 4*, PMA/ionomycin-treated cells pretreated with 10^{-6} M RA; *lane 5*, PMA/ionomycin-treated cells pretreated with 10^{-5} M RA.



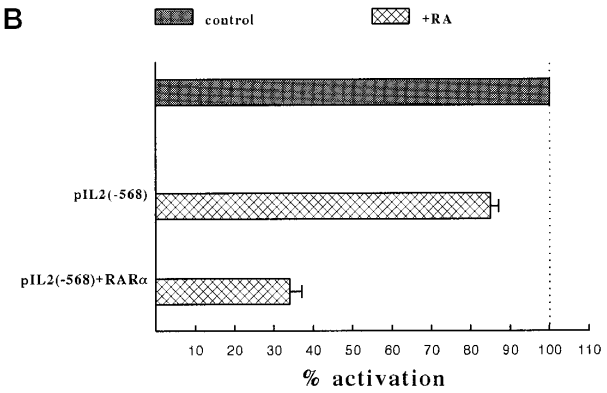


FIG. 2. **Effect of RA on IFN-γ promoter activity.** *A*, effect on the IFN-γ promoter; *B*, effect on the IL-2 promoter. 5 × 10^6 Jurkat T cells were cotransfected with 10 μg of the indicated reporter gene vector plus 2 μg of RARα expression vector (or pSG5 empty vector) as described under "Experimental Procedures." 24 h after transfection, cells were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin in the presence or absence of 1 μM retinoic acid. After a further 24 h, cells were harvested, and protein extracts were prepared for the β-galactosidase assay. The percentage of activation relative to the individual controls in the absence of retinoic acid is considered here as 100% (*control bar*) and represents the mean ± S.E. from at least four individual experiments. β-Galactosidase activities (units/microgram of protein) with PMA/ionomycin treatment for each DNA construct were as follows: (0.2 ± 0.019) × 10^{-4} (pIFN−2.7 kbp + pSG5), (0.27 ± 0.01) × 10^{-4} (pIFN−2.7 kbp + RARα), (0.3 ± 0.06) × 10^{-4} (pIFN−538 bp + pSG5), (0.26 ± 0.016) × 10^{-4} (pIFN−538 bp + RARα), (0.385 ± 0.05) × 10^{-4} (pIL2−568 bp + pSG5), and (0.39 ± 0.04) × 10^{-4} (pIL2−568 bp + RARα).

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Jurkat cells ($CD4^+$ human T lymphoblastoid cell line) were cultured in complete RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. Antibodies against transcription factors EGR-1, USF (upstream stimulatory factor), c-Myc, Max, and RARα were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PMA and all-*trans*-retinoic acid were purchased from Sigma, and ionomycin was purchased from Calbiochem. Purified human peripheral blood T cells were kindly provided by Dr. John Ortaldo (National Cancer Institute-FCRDC).

Nuclear Extraction—Nuclear proteins were prepared as follows (38). 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/ml aprotinin, and 100 μM dithiothreitol) and subsequently incubated for 4 min on ice. Cells were collected by centrifugation at 2500 rpm, and the supernatant was decanted. The nuclei were washed in buffer A without Nonidet P-40, collected at 2500 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 dithiothreitol for 5 min on ice. The samples were subsequently frozen and thawed (twice) utilizing dry ice and a 37 °C water bath, rotated for 30 min at 4 °C, and centrifuged at 14,000 rpm for 20 min. The clear supernatant was collected, and the proteins were dialyzed for 2 h (4 °C) 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 dithiothreitol. The amount of nuclear proteins obtained was quantified utilizing a commercial reagent (BCA, Pierce).

EMSA—The nuclear proteins (5 μg) 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 dithiothreitol, 0.5–2 μg of poly(dI-dC), and 4% Ficoll. In some cases, the indicated amount of double-strand oligomer was added as an unlabeled competitor, and the mixture was incubated at room temperature for 10 min prior to adding the DNA probe. For *in vitro* translated receptor binding assay, 5 μg of Jurkat nuclear extract and/or 3 μl of *in vitro* synthesized RARα and RXRα were preincubated in the binding buffer described above for 20 min on ice to allow heterodimerization. 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 (Eastman Kodak Co.) at −70 °C with intensifying screens. Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA synthesizer (Applied Biosystems Model 394). Complementary strands were denatured at 85 °C for 5 min and annealed at room temperature.

The double-strand probes were end-labeled using Klenow fragment (Life Technologies, Inc.) and [α-^{32}P]dCTP (Amersham Corp.); ~1 ng of labeled DNA was used in a standard EMSA reaction.

In supershift analysis, the antisera were added to the binding reaction, and the mixture was incubated for 30 min at room temperature prior to adding the labeled DNA probe.

The following double-strand oligomers were used as labeled probes or unlabeled competitors: IFN-γ-(−242 to −191), 5′-agctGTGCCTCAAAGAATCCCACCAGAATGGCACAGGTGGGCATAATGGGTCTGTC-3′; IFN-γ-(−242 to −219), 5′-agctGTGCCTCAAAGAATCCCACCAGAA-3′; IFN-γ-(−225 to −201), 5′-agctACCAGAATGGCACAGGTGGGCATAA-3′; IFN-γ-(−218 to −201), 5′-agctGGCACAGGTGGGCATAA-3′; ΔUSF, 5′-agctGGCAgAGcaGGGCATAA-3′; EGR, 5′-agctCGCCCTCGCCCCCGCGCCGGG-3′; IFN-γ-(−187 to −166), 5′-agctCGTCAAAGGACCCA-

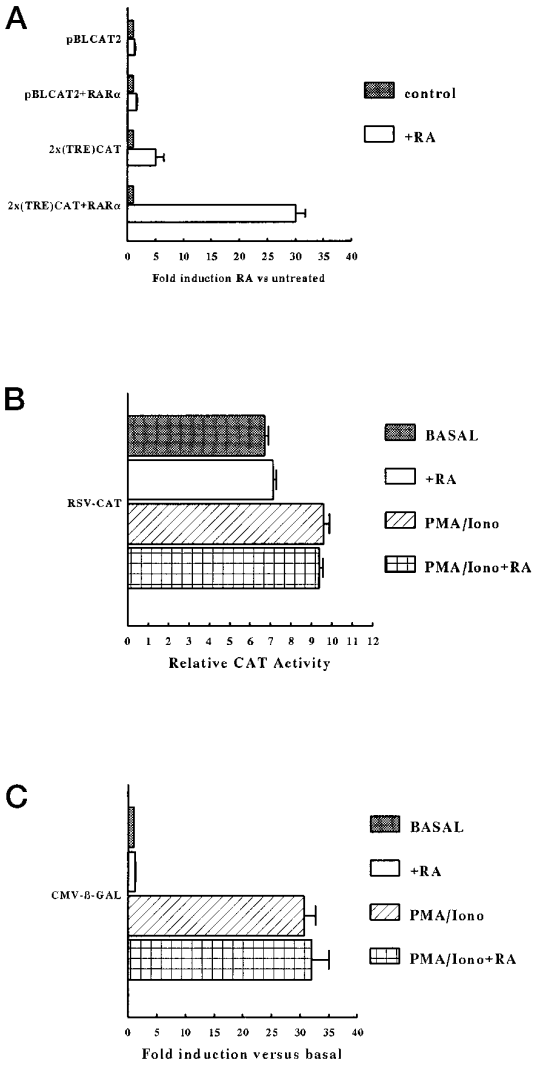


FIG. 3. **Effect of RA on the activity of 2x(TRE)-tkCAT (*A*), RSV-CAT (*B*), and CMV-β-GAL (*C*).** 5×10^6 Jurkat T cells were cotransfected with 10 μg of the indicated reporter gene vector plus 2 μg of RARα expression vector (or pSG5 empty vector) as described under "Experimental Procedures." 24 h after transfection, cells were stimulated with 0.1 μM retinoic acid (*A*) or with 10 ng/ml PMA and 1 μg/ml ionomycin (*Iono*) in the presence or absence of 1 μM retinoic acid (*B* and *C*). After a further 24 h, cells were harvested, and protein extracts were prepared for the β-galactosidase assay or chloramphenicol acetyltransferase assay.

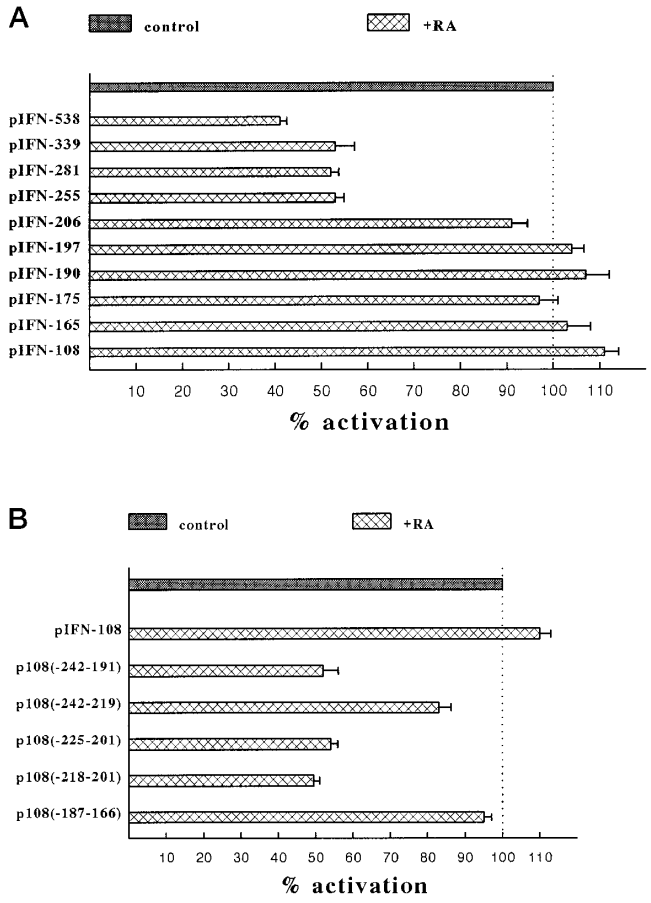


FIG. 4. **Effect of RA on different IFN-γ promoter deletions.** *A*, 5×10^6 Jurkat T cells were cotransfected with 10 μg of the indicated reporter gene vector plus 2 μg of RARα expression vector as described under "Experimental Procedures." 24 h after transfection, cells were stimulated with 10 ng/ml PMA and 1 μg/ml ionomycin in the presence or absence of 1 μM RA. After a further 24 h, cells were harvested, and protein extracts were prepared for the β-galactosidase assay. The percentage of activation relative to the individual controls in the absence of retinoic acid, considered here as 100% (*control bar*), represents the mean ± S.E. from at least four individual experiments. β-Galactosidase activities (units/microgram of protein) with PMA/ionomycin treatment for each construct were as follows: $(0.25 \pm 0.016) \times 10^{-4}$ (pIFN−538), $(0.31 \pm 0.034) \times 10^{-4}$ (pIFN−339), $(0.35 \pm 0.013) \times 10^{-4}$ (pIFN−281), $(0.21 \pm 0.02) \times 10^{-4}$ (pIFN−255), $(0.253 \pm 0.012) \times 10^{-4}$ (pIFN−206), $(0.3 \pm 0.025) \times 10^{-4}$ (pIFN−197), 0.229×10^{-4} (pIFN−190), $(0.234 \pm 0.018) \times 10^{-4}$ (pIFN−175), $(0.22 \pm 0.018) \times 10^{-4}$ (pIFN−165), and $(0.26 \pm 0.015) \times 10^{-4}$ (pIFN−108). *B*, the percentage of activation relative to the individual controls in the absence of retinoic acid, considered here as 100% (*control bar*), represents the mean ± S.E. from at least four individual experiments. β-Galactosidase activities (units/microgram of protein) with PMA/ionomycin treatment for each construct were as follows: $(0.27 \pm 0.019) \times 10^{-4}$ (pIFN−108), $(0.18 \pm 0.02) \times 10^{-4}$ (p108-(−242 to −191)), $(0.17 \pm 0.008) \times 10^{-4}$ (p108-(−242 to −219)), $(0.197 \pm 0.024) \times 10^{-4}$ (p108-(−225 to −201)), $(0.159 \pm 0.012) \times 10^{-4}$ (p108-(−218 to −201)), and $(0.18 \pm 0.016) \times 10^{-4}$ (p108-(−187 to −166)).

AGGAGTC-3′; Myc, 5′-gatcCCCCCACCACGTGGTGCCTGA-3′; and RAREβ, 5′-gatcCGGGTAGGGTTCACCGAAAGTTCACTCGA-3′.

Plasmid Constructions—The different deletions of the IFN-γ promoter, pIFN−2.7Kb, pIFN−538, pIFN−339, and pIFN−108 (39), and the human IL-2 promoter β-galactosidase reporter pIL2−568 (39) were kindly provided by Dr. Christopher B. Wilson (Department of Pediatrics and Immunology, University of Washington, Seattle, WA). The human RARα expression vector pSG5-RARα was kindly provided by Dr. Joseph Grippo (Hoffmann-La Roche, Nutley, NJ). The human RXRα expression vector pCMX-RXRα and the different deletion mutants of RARα (40) were kindly provided by Dr. R. M. Evans (The Salk Institute, La Jolla, CA). The pCMV-β-GAL expression vector was purchased from CLONTECH, and the pSG5 expression vector was purchased from Stratagene (La Jolla, CA).

To prepare 2x(TRE)-tkCAT, two copies of a palindromic thyroid hormone/retinoic acid-responsive element (41) were subcloned into the *Hin*dIII-*Bam*HI sites upstream of the thymidine kinase promoter in the pBLCAT2 parental vector.

The plasmids p108-(−242 to −191), p108-(−225 to −201), p108-(−218 to −201), p108-(−242 to −219), p108-(−187 to −166), p108-(−218 to −201Δ), and p108(EGR) contain one copy of the indicated regions of the IFN-γ promoter or the canonical EGR-binding sequence (42–44) subcloned into the *Hin*dIII-*Bgl*II sites of the pIFN−108 vector (upstream of the promoter fragment). Plasmids pIFN−281, pIFN−255, pIFN−206, pIFN−197, pIFN−190, pIFN−175, and pIFN−165, containing the progressive deletions of the IFN-γ promoter, were constructed by polymerase chain reaction amplification of the indicated fragment as described (39) with primers that generated a *Xba*I site at the variable 5′-terminus and a *Bam*HI site at the common 3′-terminus (+64 bp) using the pIFN−538 construct (39) as template. These fragments were subcloned in the *Xba*I-*Bgl*II sites of the promoterless pEQ3 parental vector, generating the indicated deletions (39).

DNA Transfections—Transfections of Jurkat cells were carried out by the DEAE-dextran method (46). For each treatment, 5×10^6 cells (harvested in log phase of growth) were incubated with the indicated amounts of plasmid DNA in the presence of 400 μg/ml DEAE-dextran in

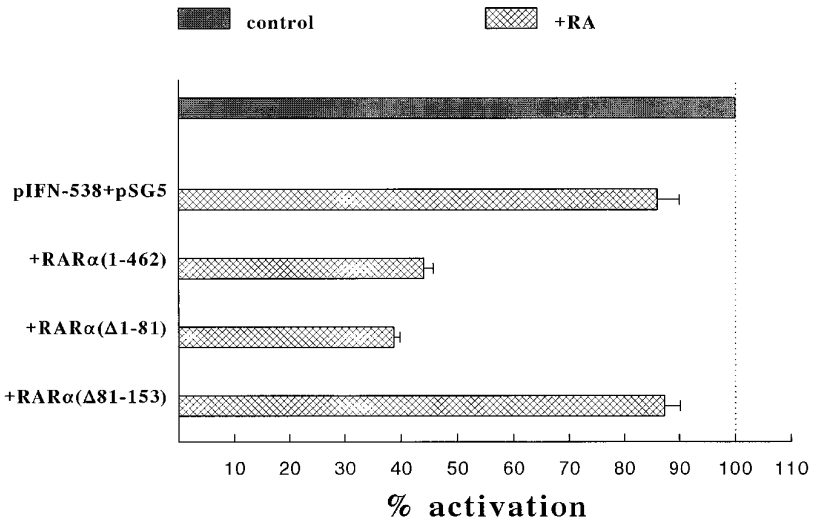


FIG. 5. **The RARα DNA-binding domain is required for the RA-mediated inhibition of the IFN-γ promoter.** 5×10^6 Jurkat T cells were cotransfected with 10 μg of the pIFN−538 reporter gene vector plus 2 μg of the indicated RARα expression vector (or pSG5 empty vector) as described under "Experimental Procedures." Cells were treated 24 h later with 10 ng/ml PMA and 1 μg/ml ionomycin, and protein extracts were prepared for the β-galactosidase assay. RARα-(1–462) indicates the wild-type receptor; RARα-(Δ1–81) and RARα-(Δ81–153) indicate the amino terminus and DNA-binding domain deletions, respectively. The percentage of activation relative to the controls in the absence of retinoic acid (considered as 100%) represents the mean ± S.E. from at least three individual experiments. β-Galactosidase activities (units/micrograms of protein) with PMA/ionomycin treatment for each construct were as follows: $(0.21 \pm 0.018) \times 10^{-4}$ (pIFN−538 + pSG5), $(0.2 \pm 0.033) \times 10^{-4}$ (pIFN−538 + RARα-(1–462)), $(0.175 \pm 0.034) \times 10^{-4}$ (pIFN−538 + RARα-(Δ1–81)), and $(0.15 \pm 0.036) \times 10^{-4}$ (pIFN−538 + RARα-(Δ81–153)).

RPMI 1640 medium, 50 mM Tris-Cl (pH 7.5) for 70 min at 37 °C. To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups, and empty expression vector DNA (pSG5) was added as needed to maintain a constant total DNA amount in each cotransfection series. Cells were then washed with RPMI 1640 medium, 50 mM Tris-Cl (pH 7.5) and replated in duplicate in complete medium. After 24 h, cells were treated with different combinations of stimuli, and after an additional 24 h, cells were harvested and washed in phosphate-buffered saline. Protein extracts were prepared for the β-galactosidase assay and/or chloramphenicol acetyltransferase assay by three cycles of rapid freezing and thawing, followed by centrifugation at 14,000 rpm (4 °C) for 15 min. Protein concentration was quantified utilizing a commercial reagent (BCA, Pierce).

β-Galactosidase Assay—The β-galactosidase assay was carried out according to the published procedure (47). Enzyme activity was determined spectrophotometrically at 570 nm by the hydrolysis of chlorophenol red/β-D-galactopyranoside. Duplicate β-galactosidase assays were normalized based on protein amount loaded at each point and generally had variations of <10%. Results are expressed as percent of activity relative to the control PMA/ionomycin-activable β-galactosidase expression in each cotransfection series, without RA in the case of the RARα addition or cotransfected with the empty vector in the control.

Chloramphenicol Acetyltransferase Assay—Chloramphenicol acetyltransferase assay was carried out according to the published procedure (48) by incubating different amounts of cell lysate protein for 12 h at 37 °C so that the assay was within the linear range. Acetylated and unacetylated [^{14}C]chloramphenicol were separated by TLC and quantified by a radioactivity scanner (AMBIS, Inc., San Diego CA).

In Vitro Translation—RNA templates for *in vitro* translation were generated from the plasmids pSG5-RARα and pCMX-RXRα by T7 polymerase (Promega, Madison, WI) and translated *in vitro* with rabbit reticulocyte lysate according to the manufacturer's recommendation.

mRNA Analysis—Total cellular RNA was isolated from 1×10^7 cells by using a single-step phenol/chloroform extraction procedure (RNAsol, Cinna Biotecx, Friendswood, TX). 10 μg of total cytoplasmic RNA were size-fractionated on a denaturing formaldehyde- agarose (0.8%) gel and transferred to Magnabond (Micron Separations, Inc., Westborough, MA). After UV cross-linking, blots were hybridized in Fasthyb (Digene, Silver Spring, MD) to ^{32}P-labeled cDNA probes prepared utilizing a random priming kit (Stratagene). All cDNA probes had a specific activity of at least $2–8 \times 10^8$ cpm/μg, and all hybridizations were performed with 1×10^6 cpm/ml. Blots were exposed to Kodak X-Omat x-ray film for 10 min (IFN-γ) or 6 h (β-actin) at −70 °C.

RESULTS

RA Inhibition of IFN-γ Gene Expression in Human Peripheral Blood T Cells—We examined the effects of various concentrations of RA on IFN-γ mRNA expression in fresh purified human peripheral blood T cells stimulated with PMA/ionomycin. As shown in Fig. 1 (*lanes 3–5*), RA treatment decreases the expression of the IFN-γ mRNA (10–17% as measured by densitometry analysis) relative to the expression of the actin mRNA levels under the same conditions. This inhibition, although low, was consistently observed and led us to investigate whether one of the possible mechanisms of RA-mediated inhibition could be the direct interference with the transcriptional activity of the IFN-γ promoter.

PMA/Ionomycin Activation of the IFN-γ Promoter Is Down-regulated by RA in Jurkat Cells—The IFN-γ promoter has been shown to contain both positive and negative regulatory regions, which are responsible for its activation and modulation in T cells (36, 37, 49–54). In a previous study, our laboratory investigated the negative transcriptional regulation of the IFN-γ promoter mediated by glucocorticoids and characterized the regions involved in this negative interference with the GR, a member of the steroid receptor superfamily (36). As RA has been shown to negatively interfere with IL-2 gene transcription in T cells (34, 35) and to play an important role in the immune system (16–35), we tested the sensitivity of IFN-γ promoter activity to RA using a transient transfection assay in Jurkat T cells.

As shown in Fig. 2*A*, the β-galactosidase activity driven by the promoter fragments −2.7 kbp to +64 bp (pIFN−2.7Kb) and −538 to +64 (pIFN−538) was significantly inhibited by treatment with RA. As a comparison for IFN-γ promoter activity, a β-galactosidase reporter gene driven by the human IL-2 promoter (nucleotides −568 to +50) was used in parallel and showed a comparable level of down-regulation in this system. These data indicate that the sensitivity of the IFN-γ promoter to RA was equivalent to that of the IL-2 promoter. The inhibition was dependent on the cotransfection of a functional RARα expression vector, as treatment with RA alone was able to exert only a weak effect on IFN-γ promoter activity in the Jurkat cell

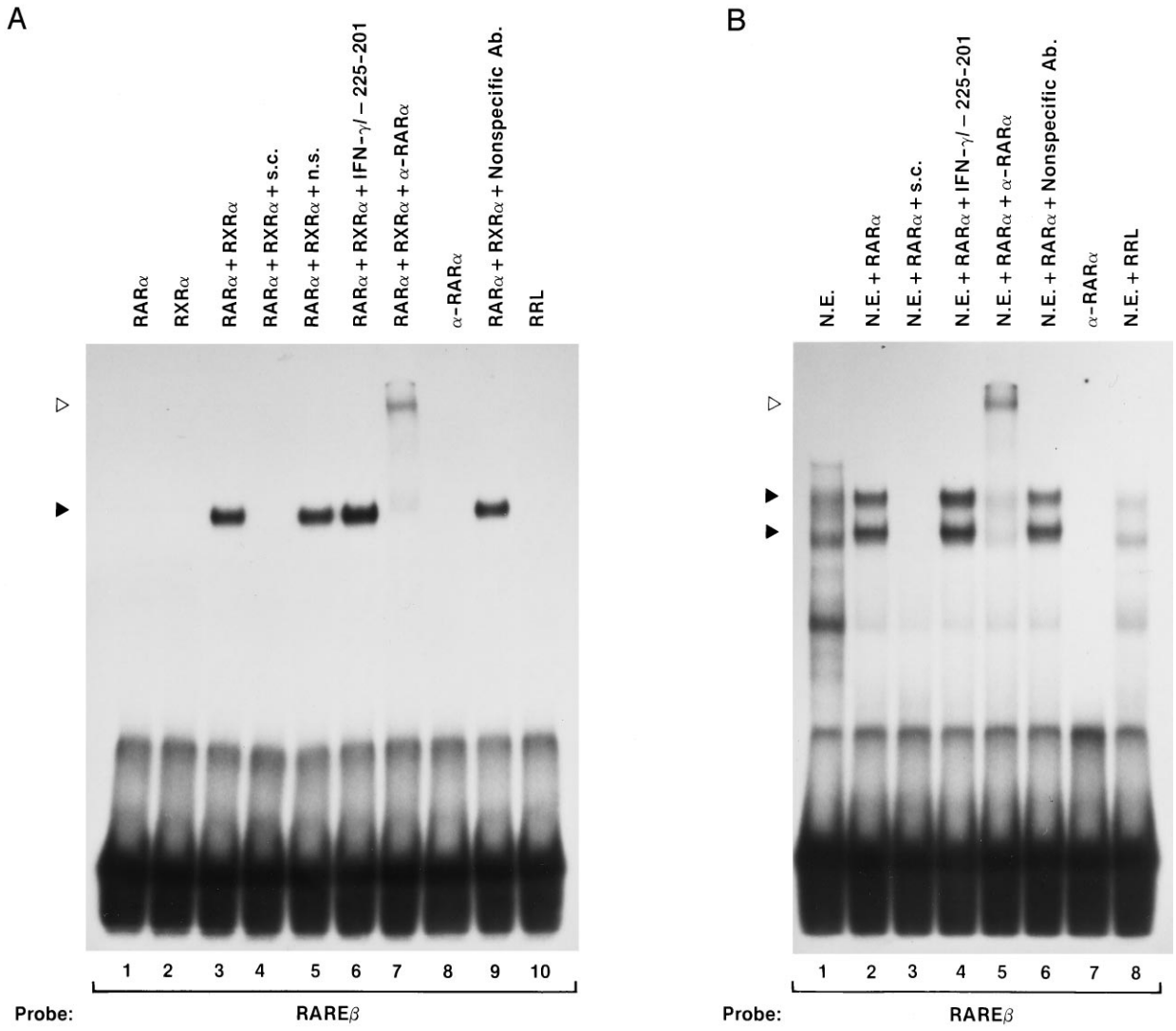


FIG. 6. **RARα does not bind the IFN-γ-(−218 to −201) promoter region.** EMSA was performed in the presence of *in vitro* synthesized RARα alone or complemented with either RXRα (*A*) or nuclear extracts (*N.E.*) from Jurkat cells (*B*). Purified anti-RARα or nonspecific antibody (*Ab.*) was added to the reaction mixture where indicated, as described under "Experimental Procedures." Binding reactions were carried out using a RAREβ-labeled oligonucleotide as a probe. The binding of RARα and RXRα alone or unprogrammed rabbit reticulocyte lysate (*RRL*) in the presence or absence of nuclear extracts from Jurkat cells is also shown. *Solid arrowheads* represent the DNA binding activity of *in vitro* synthesized RARα alone and complemented with RXRα or nuclear extracts. *Open arrowheads* represent the supershift in the presence of anti-RARα antibody. *s.c.*, specific competition; *n.s.*, nonspecific.

TABLE I
Sequence homology between the IFN-γ promoter region (−218 to −201 bp) and canonical consensus sequences for USF and EGR
S = C or G, and RO = reverse orientation.

Consensus USF	.....CACGTG
IFN-γ-(−218 to −201)	...GGCACAGGTGGGCATAA..
Consensus EGR	.....GCGSGGGCG.....RO

line used in this study (Fig. 2*A*). In this context, as shown in Fig. 3*A*, a chloramphenicol acetyltransferase reporter driven by two copies of a thyroid-responsive element (already shown to respond also to RA/RARs) (41) was strongly activated after RA treatment only in the presence of the cotransfected RARα expression vector. These experiments indicate that the Jurkat cells used in this study are partially resistant to RA, and the cotransfection of a RARα expression vector is required for the optimal RA-mediated inhibition of the IFN-γ promoter. These observations are in agreement with previous reports in which the RA-mediated inhibition of the human IL-2 promoter in Jurkat cells was dependent upon the presence of a functional cotransfected RARα (34, 35). As a further control for the specificity of the RA/RARα effects on IFN-γ promoter activation, a chloramphenicol acetyltransferase reporter gene driven by the Rous sarcoma virus long terminal repeat and a β-galactosidase reporter gene driven by the cytomegalovirus promoter were used in the same system. As shown in Fig. 3 (*B* and *C*), the basal activity or the PMA/ionomycin inducibility of these reporters was not modified by the presence of the RARα expression vector and RA treatment. These data are in agreement with previous observations by Felli *et al.* (34), where the PMA/ionomycin-mediated activation of different promoters (SV40 early promoter and thymidine kinase promoter) was not significantly affected by RA/RARα in Jurkat cells.

Serial Deletions of the IFN-γ Promoter Indicate the Presence of a Negative RA-responsive Region—RARs have been shown to differently activate or repress a number of genes through several mechanisms, including negative interference with different transcription factors (34, 35, 40, 55–58). To investigate the possible presence of RA-responsive IFN-γ promoter regions, we analyzed by transfection the activity of progressive deletions of the IFN-γ promoter in the presence of a RARα expression vector. Surprisingly, the promoter fragment spanning nucleo-

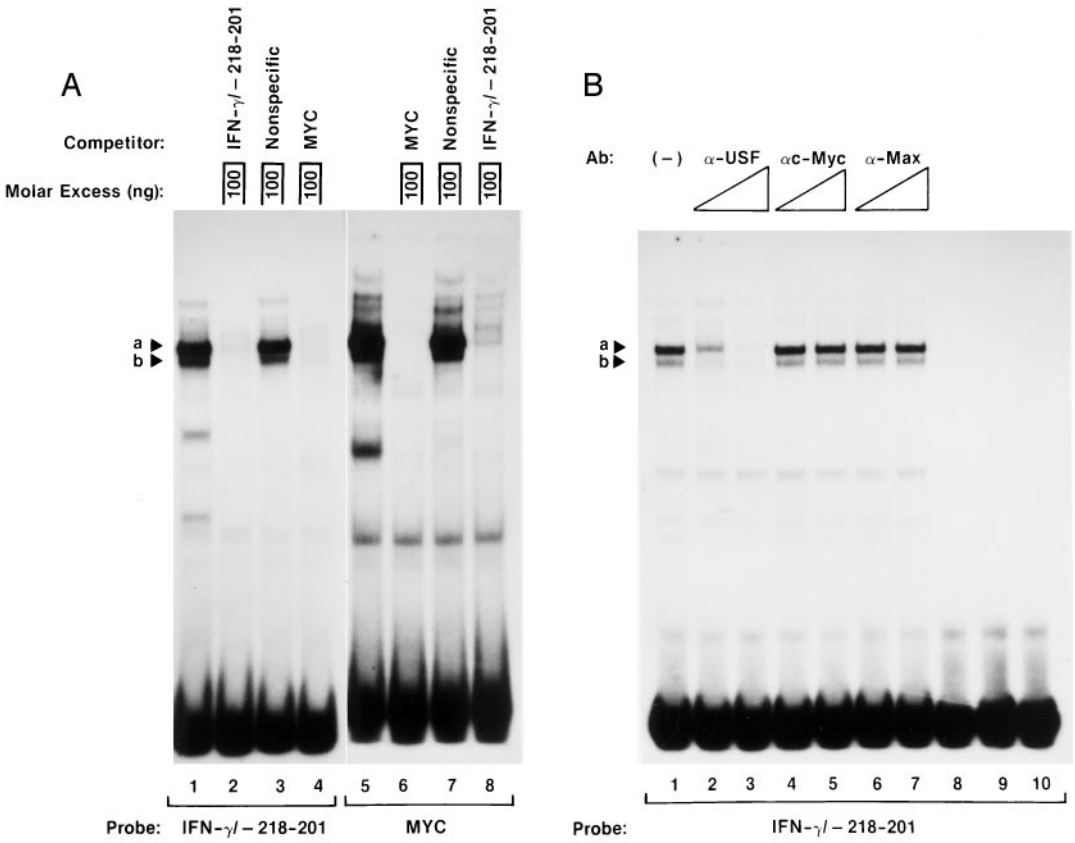


FIG. 7. **Electrophoretic mobility shift assay of the IFN-γ-(−218 to −201) promoter region.** *A*, EMSA was performed using the indicated ^{32}P-labeled oligonucleotides as probes in the presence of nuclear extracts from unstimulated Jurkat cells. *B*, shown is the supershift analysis of the DNA-protein complexes binding to the IFN-γ-(−218 to −201) promoter region. EMSA was performed using the indicated ^{32}P-labeled oligonucleotides as probes in the presence of nuclear extracts from unstimulated Jurkat cells. Purified anti-USF, anti-c-Myc, or anti-c-Max antibody (*Ab*) was added to the reaction mixture where indicated, as described under "Experimental Procedures." *Lanes 8–10* contain the probe indicated above plus the antibody for USF, c-Myc, or c-Max, respectively, without nuclear extracts as a control.

tides −108 to +64, already shown to be negatively modulated by glucocorticoids in T cells (36), was insensitive to the RA treatment, while the promoter constructs containing nucleotides −538 to −255 were all significantly down-regulated by RA treatment (Fig. 4*A*). These data suggested that a negative RA-responsive element (negative RARE) or a promoter region cooperating in this modulation might be present in the promoter segment spanning nucleotides −255 to −206. Interestingly, this region overlaps with a silencer element, previously identified by our laboratory, that is able to specifically interfere with the transactivating capability of the IFN-γ-(−108 to +64) promoter fragment in T cells (37). To better define the sequence(s) involved in the down-regulation observed with RA, different promoter fragments spanning the −255 to −206-bp region were subcloned 5′ to the IFN-γ-(−108 to +64) "core" promoter element. As shown in Fig. 4*B*, subcloning of the promoter fragment spanning nucleotides −218 to −201 conferred a RA-induced down-regulation to the IFN-γ-(−108 to +64) promoter element, while two different DNA promoter fragments upstream (−242 to −219 bp) and downstream (−187 to −166 bp) of this element were either not able or only partially able to elicit the same effect. Interestingly, the −218 to −201-bp minimal element was unable to modulate a heterologous promoter in the same manner as, when subcloned upstream of the thymidine kinase promoter in the pBLCAT2 parental reporter, treatment with RA did not further modify the thymidine kinase promoter activity in Jurkat cells (data not shown). This observation suggests the presence of a promoter-specific mechanism(s) involved in the RA/RARα-mediated negative modulation of the IFN-γ promoter and identifies an "IFN-γ negative RARE" in the region (−218 to −201 bp) that is likely involved or cooperating in this activity.

Lack of RAR Binding to the IFN-γ Negative RARE—To test the possibility of direct DNA binding of RARα to IFN-γ promoter sequences during the negative modulation mediated by RA, two different deletion mutants of this nuclear receptor were used in cotransfection assays. Truncation of the amino acids encompassing the NH_2 terminus of the receptor (RARα-(Δ1–81)) did not affect the down-regulation observed on the IFN-γ-(−538 to +64) promoter fragment, while deletion of the DNA-binding domain (RARα-(Δ81–153)) significantly reduced the negative effect observed with the wild-type receptor (RARα-(1–462)) (Fig. 5). These data suggest that direct binding to the IFN-γ promoter might be necessary for the negative effect observed here. However, sequence analysis of the IFN-γ promoter did not reveal an obvious retinoic acid-responsive consensus element on the basis of the sequences normally recognized by RARs on other genes (2). Nevertheless, the receptor can act through rather degenerate sequences in different systems (2, 9). Thus, to establish whether RARα was able to specifically bind to the IFN-γ-(−218 to −201) sequence, we utilized electrophoretic mobility shift analysis. As shown in Fig. 6*A*, *in vitro* translated nuclear RARα and RXRα are able to heterodimerize and specifically bind to a typical consensus RAREβ in EMSA (35, 59). The presence of RARα in the complex was confirmed by supershift with a specific antibody (Fig. 6*A*, *lane 7*). The addition of a 100-fold excess of unlabeled IFN-γ-(−225 to −201) oligonucleotide or IFN-γ-(−218 to −201) oligonucleotide did not affect the binding capability, suggesting that the RARα·RXRα complex has at least 100-fold lower affinity for

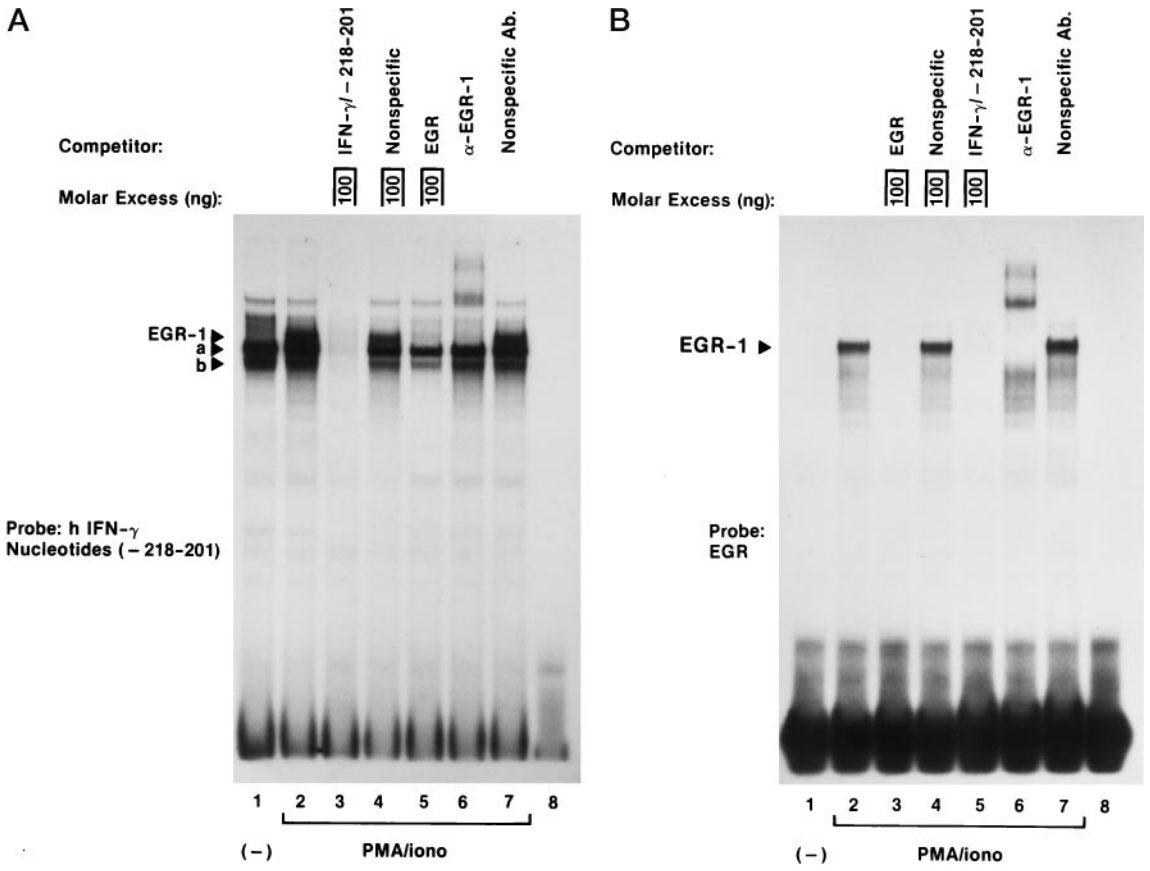


FIG. 8. **The IFN-γ-(−218 to −201) promoter region binds the EGR-1 factor in nuclear extracts from PMA/ionomycin-activated Jurkat cells.** EMSA was performed using the indicated ^{32}P-labeled oligonucleotides as probes in the presence of nuclear extracts from unstimulated or PMA/ionomycin (*iono*)-treated Jurkat cells. *Lane 1*, untreated cells; *lanes 2–7*, 4 h of PMA/ionomycin treatment. Purified anti-EGR-1 or nonspecific antibody (*Ab.*) was added to the reaction mixture where indicated, as described under "Experimental Procedures." *Lane 8* contains the probe indicated above plus the antibody for EGR-1, without nuclear extracts, as a control. *h*, human.

the oligonucleotides containing the identified IFN-γ negative RARE (data not shown). One possible explanation for the absence of binding activity observed using the *in vitro* translated receptors could be that RARα might require a specific cellular cofactor other than RXRα for optimal binding. To test this possibility, a nuclear extract from Jurkat cells was added to RARα, and the mixture was then tested by EMSA. The binding pattern obtained using *in vitro* translated RARα in the presence of a Jurkat cell nuclear extract using a consensus RAREβ as a radiolabeled probe is shown in Fig. 6*B*. In agreement with previous observations (35), a different pattern of RAREβ binding activity was obtained (Fig. 6*B*, *lanes 2–6*), probably due to the presence of different cofactors cooperating with RARα for DNA binding, and the complexes were specifically supershifted by an anti-RARα antibody (*lane 5*). A 100-fold excess of unlabeled IFN-γ-(−225 to −201) (Fig. 6*B*, *lane 4*) or IFN-γ-(−218 to −201) (data not shown) oligonucleotide did not affect the binding activity, in agreement with the lack of competition observed with *in vitro* translated receptors. The direct binding of RARα complexes to the labeled IFN-γ-(−225 to −201) or IFN-γ-(−218 to −201) oligonucleotide was also checked by EMSA using *in vitro* translated receptors or receptors complemented with nuclear extracts from Jurkat cells. However, we did not observe any specific binding of RARα or RARα + RXRα under these experimental conditions (data not shown). Taken together, these data suggest that the negative modulation of the IFN-γ promoter mediated by RA/RARα is not due to direct binding of RARα to this promoter region.

Band-shift Analysis of the Nuclear Factor(s) Binding to the IFN-γ Negative RA-responsive Region—To characterize the nuclear protein(s) specifically interacting with the minimal negative RARE identified by the deletion studies described above, we performed EMSA using nuclear extracts prepared from Jurkat T cells. A sequence homology search for the known nuclear factor-binding motifs indicated the presence of two overlapping sequences specific for the E box family- and EGR family-related DNA-binding proteins (Table I). Figs. 7 and 8 show the DNA binding pattern obtained using nuclear extracts from unstimulated and PMA/ionomycin-treated Jurkat cells, in the presence of a labeled probe spanning nucleotides −218 to −201 of the IFN-γ promoter. Two specific and constitutively expressed DNA-protein complexes were detected and are designated here as complexes a and b. Interestingly, this DNA binding activity was totally and specifically competed by a molar excess of an unlabeled oligonucleotide containing a typical consensus E box sequence (Fig. 7, *lane 4*), designated here as Myc (60). In comparison, the binding pattern obtained in the presence of a labeled oligonucleotide probe containing a typical consensus E box sequence is shown in Fig. 7*A* (*lanes 5–8*). The unlabeled oligonucleotide (IFN-γ-(−218 to −201)) was able to significantly compete for the binding (*lane 8*), confirming the capability of the IFN-γ-(−218 to −201) element to bind E box-related factors in EMSA. Different families of transcription factors are able to bind DNA sequences characterized by the presence of a central "..CANNTG.." core (E box motif), including Myc, Max/Myn, Mad/Mxi, USF, and TFE3/TFEB (60–68). To determine if any of these DNA-binding proteins were specifically interacting with the IFN-γ promoter region, a supershift analysis in the presence of increasing amounts of anti-c-Myc, anti-Max, and anti-USF antibodies is shown in Fig. 7*B*.

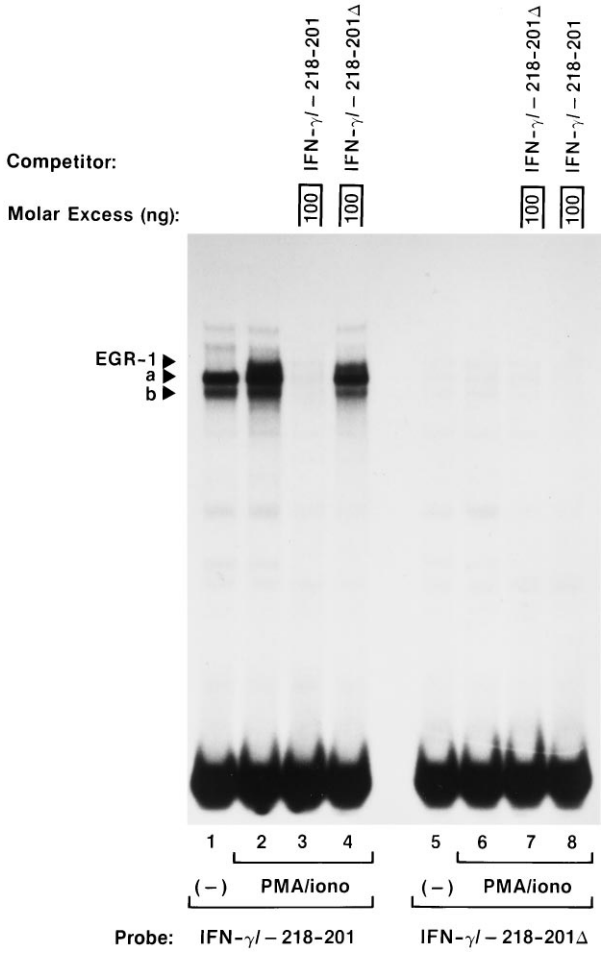


FIG. 9. **Identification of critical nucleotides in the IFN-γ-(−218 to −201) promoter region.** EMSA was performed as described under "Experimental Procedures" using the wild-type IFN-γ region (−218 to −201 bp) or the Δ-mutant oligonucleotide as labeled probe in the presence of nuclear extracts from unstimulated or PMA/ionomycin (*iono*)-treated Jurkat cells. *Lanes 1* and *5*, untreated cells; *lanes 2–4* and *6–8*, 4 h of PMA/ionomycin treatment.

Only the antibody specific for the USF transcription factor was able to inhibit the DNA binding of complexes a and b when used in the presence of nuclear extracts from Jurkat cells (*lanes 2* and *3*), indicating the presence of USF in these complexes.

A slower migrating complex, designated here as EGR-1, was induced after 4 h of PMA/ionomycin stimulation (Fig. 8*A*, *lanes 2–7*) and was specifically competed by a molar excess of an unlabeled competitor specific for EGR-binding factors (42–45), but not by an unrelated unlabeled competitor (*lanes 4* and *5*). Since both the sequence homology search and the EMSAs strongly suggested that EGR-related protein(s) were specific components of the induced complex described above, we wanted to determine if EGR protein(s) are present in this PMA/ionomycin-induced band. In Fig. 8*A*, an EMSA in the presence of an anti-EGR-1 antibody shows a complete supershift of the induced complex when used in the presence of nuclear extracts from PMA/ionomycin-stimulated Jurkat cells, while an unrelated antibody was not able to modify the binding capability or the migration of this complex in EMSA. Fig. 8 also shows a comparison between the band patterns obtained with a canonical EGR-binding sequence (44) and the identified IFN-γ negative RARE. The band indicated as *EGR-1* shows the induced complex, specifically competed by the unlabeled EGR and IFN-γ-(−218 to −201) oligonucleotides (Fig. 8*B*, *lanes 3* and *5*) and supershifted by the anti-EGR-1 antibody (*lane 6*).

TABLE II
Mutation of the IFN-γ promoter region (−218 to −201 bp)

IFN-γ-(−218 to −201)	5′-....GGCACAGGTGGGCATAA....-3′
IFN-γ-(−218 to −201)ΔUSF	5′-....GGCAgAGcaGGGCATAA....-3′

Mutation of the Identified IFN-γ E box/EGR Element Interferes with the Negative Modulation Mediated by RA—The role of USF/EGR-1 binding to the IFN-γ-(−218 to −201) region in the inhibition by RA/RARα of IFN-γ promoter activity was investigated by using a sequence mutation able to selectively abolish the DNA binding activity of the identified protein complexes. Fig. 9 and Table II show the mutation that was able to eliminate the DNA binding at this level in EMSA. When this mutant oligonucleotide was subcloned 5′ to the RA-insensitive IFN-γ-(−108 to +64) promoter element, the transcriptional activity of the β-galactosidase reporter plasmid p108-(−218 to −201Δ) after PMA/ionomycin treatment was not significantly affected by treatment with RA (Fig. 10), indicating that impairment of USF/EGR-1 binding abrogated RA inhibition of the IFN-γ promoter. Interestingly, a DNA-binding sequence specific for EGR family proteins was able to bind only the PMA/ionomycin-induced EGR-1 factor and not USF in Jurkat cells (Fig. 8*B*). In contrast to what was observed with the IFN-γ-(−218 to −201) region, when this binding element was subcloned 5′ to the IFN-γ-(−108 to +64) promoter element, although the transcriptional activity of the p108(EGR) β-galactosidase reporter after stimulation was enhanced (data not shown), the resulting activity was not significantly modulated by RA/RARα (Fig. 10). This observation suggests that EGR-1 and RARα do not cooperate or interact to inhibit transcription. Taken together, these data indicate that the IFN-γ-(−218 to −201) promoter region represents a sensitive element for the RA/RARα-mediated down-regulation of the IFN-γ promoter, possibly involving a direct or indirect negative interaction/cooperation of RARα with the USF DNA-binding factor.

DISCUSSION

In this report, we focused our interest on the inhibition of IFN-γ promoter activity mediated by retinoic acid in T cells. An increasing number of recent observations suggest that disregulation of vitamin A metabolism results in different effects on the immune system, including altered resistance to infections, reduced IgG production, and differential regulation of cytokine levels (IL-2, IL-4, and IFN-γ), leading to a regulatory T helper cell imbalance, with a predominance of $CD4^+$ TH1 cells and insufficient TH2-mediated functions (24–33). In particular, IFN-γ gene activity is significantly affected by vitamin A levels and/or its derivatives (*e.g.* retinoic acid). This phenomenon appears to involve a modulation of the IFN-γ gene at the transcriptional level in $CD4^+$ and $CD8^+$ T cells and in NK cells, suggesting the presence of an inhibitory mechanism acting on a control point common between these cell types (31).

IFN-γ is an immunoregulatory cytokine of crucial importance in nearly all phases of immune and inflammatory responses (49, 69, 70). Furthermore, this cytokine is relevant as a therapeutic agent for immunodeficiency states, infections, and neoplastic disease. IFN-γ expression seems to be restricted to activated T cells and large granular lymphocytes (49, 69, 70), and inhibition of IFN-γ production has been reported to be caused by different agents, including cyclosporin A, corticosteroids, and prostaglandins (49). Our laboratory has recently described the molecular mechanisms responsible for the negative transcriptional regulation of the IFN-γ promoter mediated by the GR (36). Negative gene regulation by steroid/retinoid hormone receptors appears to be an emerging theme, and it seems probable that different members of this superfamily may act by similar mechanisms. For example, transcriptional re-

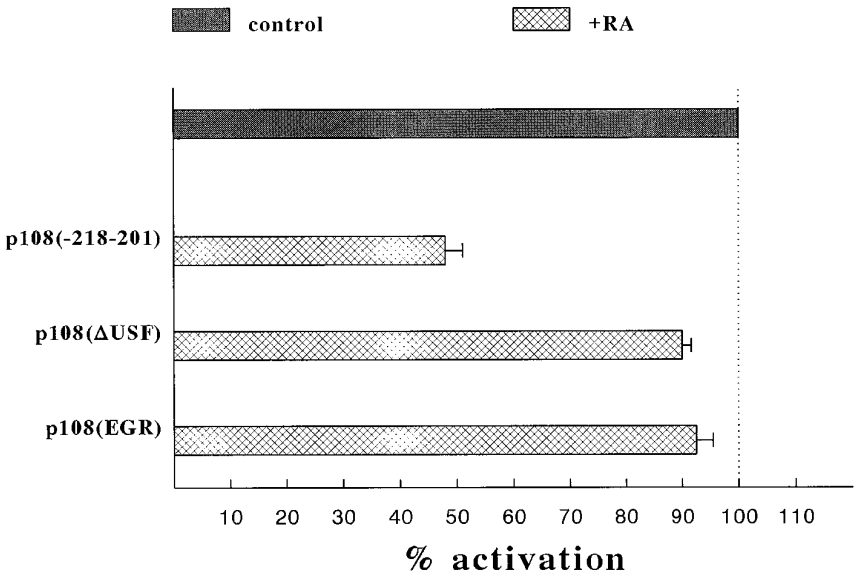


FIG. 10. **Mutation of the IFN-γ-(−218 to −201) region significantly eliminates the inhibition mediated by RA/RARα in transfection assays.** The percentage of activation relative to the individual controls in the absence of retinoic acid is considered here as 100% (*control bar*) and represents the mean ± S.E. from at least four individual experiments. β-Galactosidase activities (units/microgram of protein) with PMA/ionomycin treatment for each construct were as follows: $(0.159 \pm 0.012) \times 10^{-4}$ (p108(−218 to −201)), $(0.145 \pm 0.022) \times 10^{-4}$ (p108(ΔUSF)), and $(0.38 \pm 0.011) \times 10^{-4}$ (p108(EGR)).

pression may act either by receptor competition with positive transactivating factors for DNA binding to overlapping sequences or by interference with their transactivating action through direct protein-protein interaction (34, 35, 40, 55–58, 71–73). In this regard, the GR has been shown to down-regulate a number of different gene promoters through negative interaction with several transcription factors, including AP-1 family members, RelA, CREB family members, OCT-2A, and GATA-1 (74–81). Another family of nuclear receptors showing similar properties is RARs. Recently, negative modulation of the IL-2 promoter by RARs has been demonstrated through a selective impairment of the AP-1/OAP element function (34, 35). In this model, although an intact DNA-binding domain was required for the RARα-mediated negative regulation, direct binding of this receptor to the IL-2 OCT/OAP sequence was not demonstrated *in vitro*. However, the receptor has been shown to directly inhibit the functional synergism between AP-1 and OCT factors by interfering with the binding of Jun and Fos proteins to the OAP-binding site (35).

Our results demonstrate that in transient transfection assays, the IFN-γ gene promoter is significantly down-regulated in activated Jurkat T cells by retinoic acid at levels comparable to those observed with the IL-2 promoter. Surprisingly, the two different AP-1/CREB-ATF-binding elements situated in positions of the IFN-γ gene promoter previously shown to be critical for its full transcriptional activity (nucleotides −66 to −47 and −96 to −75) (36, 39) and sensitive to the GR-mediated transcriptional interference (36) were unaffected by RA/RARα (Fig. 4*A*). This result might be due to different capabilities of these nuclear receptors to directly interfere with several transactivating factors and/or the presence of a diverse genetic context. An example of this phenomenon is the negative interference mediated by RA/RARα on the AP-1 complex associated with the OCT-1 factor in the IL-2 promoter OCT/OAP element (35, 82). In this model, RA/RARα is not able to exert the same inhibitory action on the AP-1 complex associated with the NFAT element (34, 83). Recently, Cantorna *et al.* (33) have reported that RA specifically inhibits IFN-γ mRNA expression via the CD28 activation pathway and not the T cell receptor pathway in murine TH1 clones. These investigators have hypothesized that a CD28-responsive element (murine IFN-γ-(−170 to −160) promoter and human IFN-γ-(−163 to −153) promoter) may be involved in this response. While their results may highlight differences in RA responsiveness in murine T helper clones when compared with human Jurkat cells, it is quite possible that other regions of the IFN-γ promoter, as hypothesized by Cantorna *et al.*, are involved in the RA inhibitory effects on IFN-γ transcription. Furthermore, the extent of inhibition in the murine T helper clones is greater than what we observed in total peripheral blood T cells, reflecting the differences seen when utilizing a pure T cell population compared with total peripheral blood cells. Alternatively, the differences in inhibition may reflect differences in the RARs expressed in these different populations.

The promoter deletion analysis used in this study utilizing the human T lymphoblastoid cell line Jurkat as a model system has identified a negative retinoic acid-responsive element situated in a position close to a silencer region previously shown to interfere with the activation of the IFN-γ promoter in T cells (37). EMSA and sequence homology analysis have shown that the identified promoter element contains partially overlapping noncanonical binding sites for EGR-1 (42–45) and E box-related USF (65, 84–87). When subcloned 5′ to the RA/RARα-insensitive IFN-γ-(−108 to +64) promoter fragment, this region was able to significantly interfere with the activation triggered by PMA/ionomycin in the presence of RA/RARα, suggesting a possible involvement of this promoter region in the negative modulation exerted by RA. It is noteworthy that a consensus binding sequence for EGR transcription factors, when subcloned in the same position, was not down-regulated in the presence of RA, suggesting the absence of a direct effect through the EGR-1 proteins. Moreover, a mutation of the identified IFN-γ USF/EGR-1 sequence that eliminated the protein binding activity was no longer sensitive to the inhibitory action of RA/RARα in cotransfection assays. These observations suggest a possible involvement or cooperation of USF in the RA/RARα-mediated IFN-γ promoter inhibition described here.

The cellular transcription factor USF belongs to the class of basic helix-loop-helix leucine zipper proteins (67, 84–89) and appears to be composed of two distinct polypeptides with apparent molecular masses of 43 (USF1) and 44 (USF2) kDa (84, 86). Band-shift analysis has shown that USF is able to specifically bind DNA sequences characterized by the presence of a central CANNTG core (E box), also recognized by other families of transcription factors such as Myc, Max/Myn, Mad/Mxi, and TFE3/TFEB (60–68). Both USF1 and USF2 are ubiquitous proteins able to form homo- and heterodimers in different ratios (84–86). The biological role of USF has been investigated utilizing both *in vivo* transfection and *in vitro* transcription studies. Bacterially expressed recombinant USF1 has been

shown to stimulate transcription via an adenovirus major late promoter USF motif in a reconstituted system *in vitro* (85, 90), and recombinant USF1 was also shown to stimulate promoters by interacting with initiator elements, in cooperation with the TFII-I transcription factor (91). On the other hand, DNA-binding regions for USF (*e.g.* nucleotides −174 to −152 present in the negative regulatory element of the human immunodeficiency virus type 1 long terminal repeat) are able to act as negative regulators of transcription, in both the presence or absence of TAT-mediated transactivation and in different cell lines (92, 93). In another experimental system, the chicken αA-crystallin gene promoter, the USF proteins cooperate in the formation of positive and negative elements regulating the transcriptional activity (12). These observations suggest a possible negative transcriptional role for this factor in a particular genetic context. Our data demonstrate that RARα is not able to directly bind *in vitro* to the IFN-γ-(−218 to −201) element, suggesting the absence of a direct effect mediated by DNA binding of the receptor, and raise the possibility of indirect negative cooperation between DNA-binding proteins *in vivo*, possibly involving the USF factor. Proof of this hypothesis awaits studies with USF expression vectors and/or recombinant USF protein.

In conclusion, the data presented here add new insight regarding the effect of retinoids on IFN-γ gene regulation, and we propose the direct modulation of IFN-γ promoter activity by RA as one of the possible mechanisms involved in this effect.

Acknowledgments—We thank Dr. Christopher B. Wilson for providing plasmids pIFN−2.7Kb, pIFN−538, pIFN−339, pIFN−108, and pIL2−568; Dr. Ronald M. Evans for providing the different mutant forms of human RARα and the human RXRα expression vector pCMX-RXRα; Dr. Joseph Grippo and Dr. Keiko Ozato for providing the human RARα expression vector pSG5-RARα; William Bere for purification of human peripheral blood T cells; Dr. John Ortaldo for helpful comments; and Susan Charbonneau for secretarial assistance.

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J. Biol. Chem. 1996, 271:26783-26793.
doi: 10.1074/jbc.271.43.26783