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Regulation of a Cell Type-specific Silencer in the Human Interleukin-3 Gene Promoter by the Transcription Factor YY1 and an AP2 Sequence-recognizing Factor*

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Negative regulation of cytokine gene transcription is an important mechanism in maintaining homeostasis of immune function. In this study, we characterized a silencer element in the human interleukin-3 gene promoter that is responsible for the cell-specific expression of interleukin-3. This silencer activity was proposed to be mediated by an unidentified nuclear inhibitory protein (NIP). In this study, we have identified two nuclear factors that are responsible for the silencer activity in T cells. The NIP element forms four specific DNA-protein complexes (designated as complexes A–D) with the Jurkat nuclear proteins. Complex A contains a nuclear protein that shares DNA-binding specificity with the transcription factor AP2 (designated as an AP2 sequence-recognizing factor (ASRF)). Formation of this ASRF complex is required for the NIP silencer function, as mutation of the ASRF-binding site abrogated the silencer activity. Complex B contains the nuclear factor YY1 (Yin-Yang 1), whose function is to down-regulate ASRF activity in the silencer. YY1 activity is supported by data from mutation and cotransfection analyses. Complexes C and D are formed by nonspecific binding proteins and do not express any regulatory activity in the NIP element. These data indicate that a cell type-specific silencer activity might be determined by a unique profile of ubiquitous transcription factors.

Interleukin (IL)[1]-3 is a potent growth factor that is involved in the regulation of hematopoiesis (1, 2). Like the cytokine interferon-γ (IFN-γ) (3, 4), IL-3 is produced by activated T cells and natural killer cells (5, 6), and its expression is primarily controlled at the transcriptional level (7–9). Promoter deletion analysis revealed that enhancer and silencer elements located within 300 base pairs of the transcription start site control tissue-specific expression of the human IL-3 gene (7–9). Interestingly, there is a cell type-specific silencer element at positions −267 to −242 that binds a protein complex designated as the nuclear inhibitory protein (NIP) (8). Although this silencer element was partially characterized (10), the identification of the proteins bound to this element and the mechanisms of the silencer function remain unclear. In this study, we focused on the characterization of transcription factors associated with this silencer element.

Negative regulation of cytokine transcription plays an important role in regulating the response of the immune system to challenge (11). Although there are several possibilities for down-regulating gene transcription, silencer elements in the cytokine gene promoters constitute an important part of the gene regulation mechanism. A silencer function has been observed in the IL-2 (12), IL-3 (8), IL-4 (13), TNF-α (14, 15), IFN-α (16, 17), IFN-β (18, 19), and IFN-γ (20, 21) gene promoters. A silencer element (BE element) in the human IFN-γ gene promoter has been carefully investigated (21, 22). Two transcription factors interact with the IFN-γ silencer: 1) YY1 (Yin-Yang 1), a ubiquitous nuclear factor involved in the negative regulation of many mammalian genes (23); and 2) an AP2-like protein that shares DNA-binding specificity, but not antigenicity, with the transcription factor AP2. Simultaneous binding of both nuclear factors is required for the inhibitory activity of the IFN-γ BE element (22).

In this study, we observed that the NIP element shares a striking similarity with the IFN-γ BE silencer in protein-binding activities. Both YY1 and an AP2-like protein are found in DNA-protein complexes formed by NIP and T cell nuclear protein. However, the function of YY1 is totally different in NIP compared with the BE element. In the NIP element, the AP2 sequence-recognizing factor (ASRF) is required for the silencer function, and interestingly, YY1 acts an inhibitor of ASRF activity. This finding provides evidence that YY1 may act as a positive regulator in the IL-3 gene promoter, and more important, this extends the functional role of YY1 in regulating cytokine gene expression.

MATERIALS AND METHODS

Oligonucleotides and Antibodies—Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems Model 392 DNA/RNA synthesizer. The synthesized oligonucleotides were deprotected at 50 °C overnight. Complementary strands were denatured at 80 °C for 5 min and annealed at room temperature. The double-stranded probe was labeled with [^{32}P]dCTP (Amersham Pharmacia Biotech) using Klenow fragment (Life Technologies, Inc.). The sequences of oligonucleotides used in this study are as follow: a YY1-binding site in the Moloney murine leukemia virus gene (4), 5′-TGCCTTGCAAAATGGCGTTACTGCAG-3′; and an AP2-binding site in the SV40 virus (24), 5′-GGTGTGGAAAGTCCCCAGGCTCCCCAGCAC-3′. Antibodies against nuclear proteins YY1 and AP2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cells—Jurkat cells (human CD4$^+$ T lymphoblast cell line) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin (complete

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[1] The abbreviations used are: IL, interleukin; IFN, interferon; NIP, nuclear inhibitory protein; TNF, tumor necrosis factor; BE, bifunctional element; ASRF, AP2 sequence-recognizing factor; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol transferase.

medium). Fresh human peripheral blood total T cells were purified by the nylon wool method, and purity was monitored by CD3 staining ($CD3^+ > 95\%$). The cells were cultured in RPMI 1640 medium supplemented with 2% fetal calf serum, 2 mM glutamine, and antibiotics.

mRNA Assay—Jurkat cells (2×10^6) were treated with PMA (10 ng/ml) plus ionomycin (1 μg/ml) for different times as indicated in the figure legends. Total mRNA was extracted using Trizol (Life Technologies, Inc.) according to the manufacturer's instruction. IL-3 mRNA levels were determined by the ribonuclease protection assay using a RiboQuant multiprobe ribonuclease protection assay system (human cytokine/chemokine-4, Pharmingen, San Diego, CA).

Nuclear Extraction Procedure—The nuclear extracts were prepared as described before (22). Cells (5×10^7) were treated with 500 μl of lysis buffer (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) on ice for 4 min. After 1 min of centrifugation at 14,000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclei were washed once with the same volume of buffer without Nonidet P-40, put into a 300-μl volume of extraction buffer (500 mM KCl and 10% glycerol with the same concentrations of Hepes, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and dithiothreitol as lysis buffer), and pipetted several times. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70 °C. The protein concentration was determined with the BCA protein assay reagent (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—The DNA-protein binding reaction was conducted in a 24-μl reaction mixture that included 1 μg of poly(dI·dC) (Sigma), 3 μg of nuclear protein extract, 3 μg of bovine serum albumin, 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe, and 12 μl of 2× reaction buffer (40 mM Tris (pH 7.4), 8% Ficoll 400, 4 mM EDTA, and 1 mM dithiothreitol). In some cases, the indicated amount of double-stranded oligonucleotide was added as an unlabeled competitor. This mixture was incubated on ice for 10 min without or for 20 min with antibody in the absence of radiolabeled probe and then for 20 min at room temperature in the presence of radiolabeled probe. After incubation, the reaction mixture was resolved on a 5% acrylamide gel (National Diagnostics, Inc., Atlanta, GA) that had been prerun at 110 V for 1 h with 0.5× Tris borate/EDTA buffer. The loaded gel was run at 210 V for 90 min, dried, and placed on X-Omat film (Eastman Kodak Co.). The film was developed after overnight exposure at −70 °C.

Plasmid Vectors—Five plasmid vectors were used in this study: 1) The IL-3/pCAT plasmid (a generous gift from Dr. L. R. Gottschalk, Department of Medicine, University of Chicago, Chicago, IL (7); in this reporter vector, the chloramphenicol acetyltransferase (CAT) gene is controlled by the human IL-3 gene promoter (−175)); 2) the IFN-γ/β-galactosidase expression vector (25), in which the β-galactosidase gene is controlled by the human IFN-γ gene promoter (−108); 3) the pBCTKp/CAT reporter vector (26), in which the CAT gene is controlled by the herpes simplex virus thymidine kinase gene promoter (−105)); 4) the pGL2 control vector, a luciferase expression vector containing the SV40 promoter (Promega, Madison, WI; this vector was used for monitoring transfection efficiency in transfection experiments); and 5) the CMV-YY1 expression vector and its control pCEP vector (gifts from Dr. Keiko Ozato, Laboratory of Developmental and Molecular Immunity, NICHD, National Institutes of Health, Bethesda, MD (27)).

Transfection Assays—Jurkat T cells were grown in complete medium as described above. Cells (5×10^6) were transiently transfected with 5–10 μg of the reporter plasmid DNA with DEAE-dextran (22). The pGL2 control luciferase expression vector (0.5 μg) was used as an internal control. After transfection, the cells were washed once in phosphate-buffered saline solution and cultured in 10 ml of complete medium at 37 °C for 24 h. The cells were then stimulated with PMA (10 ng/ml) plus ionomycin (1 μg/ml) for 12 h, harvested, and disrupted by freezing-thawing three times. The cell lysate was used for the reporter gene assay. The CAT, β-galactosidase, and luciferase assays were carried out as described previously (21, 22). The CAT and β-galactosidase activities were normalized by protein concentration and luciferase activity for each transfection, and a mean value from three individual experiments was analyzed by Student's *t* test with a confidence level of $p < 0.05$–0.001.

RESULTS

Functional Characterization of NIP—In this study, Jurkat cells (a human $CD4^+$ T lymphoma) were used for characterization of the IL-3 silencer NIP. This cell line is able to express IL-3 mRNA upon activation by a combined stimulation of PMA plus ionomycin, which mimics signals of the activated T cell receptor. As shown in Fig. 1*A*, the induced IL-3 mRNA was observed at 2 h following stimulation (*lane 4*), and the mRNA level kept increasing up to 8 h (*lanes 5* and *6*). In the absence of stimulation, the IL-3 mRNA was not detectable (*lane 1*). This indicates that the IL-3 gene promoter was induced by a stimulation of PMA plus ionomycin in Jurkat cells. In the rest of study when the IL-3 promoter was assayed, the stimulation of PMA plus ionomycin was used to induce promoter activity.

Generally, silencer activities are dependent on promoter type and DNA orientation. To examine whether NIP has these features, the function of NIP was tested in different gene promoters and in the reverse orientation. Three promoters were employed to test the promoter specificity. They are the human IL-3 gene promoter (−175), the human IFN-γ gene promoter (−108), and the thymidine kinase promoter. Both the IL-3 (10-fold) and IFN-γ (6-fold) promoters were induced by stimulation (Fig. 1*B*). Without stimulation, the percent acetylation from the IL-3/pCAT reporter was 5%; with stimulation, the percent acetylation was increased to 55%. Similarly, the IFN-γ/β-galactosidase reporter was induced from 64- to 384-fold following stimulation. In contrast, the thymidine kinase/CAT reporter was not induced upon stimulation. Its percent acetylation was ~15% before or after stimulation. To analyze NIP activity, NIP was inserted into the promoters. The results show that in the IL-3 promoter (*IL-3p*), NIP reduced promoter activity by 60% (Fig. 1*C*), confirming the silencer function of NIP in the context of the IL-3 promoter. This also demonstrates that this IL-3 promoter construct is sufficient for characterization of the NIP function. In contrast, NIP did not show a silencer activity in either the IFN-γ promoter (*IFNp*) or the thymidine kinase promoter (*TKp*) (Fig. 1*C*). These results demonstrate that NIP activity appears to be specific for the human IL-3 promoter.

The reporters with a reversed NIP element were used to study DNA orientation dependence. The results show that NIP functioned only in the proper orientation (Fig. 1*D*, *NIPp*). In the reverse orientation (*NIPr*), NIP failed to inhibit the IL-3 promoter activity (Fig. 1*D*). This indicates that NIP is also orientation-specific in its function. In the absence of stimulation, the IL-3 gene promoter activity is too weak to be used for studying NIP activity; thus, only the induced promoter activity was employed in this study.

Position effect on NIP activity in the IFN-γ promoter was also examined. This was done by inserting a small DNA fragment of different lengths at the junction point between the NIP silencer and the IFN-γ promoter. These inserts can make the silencer fragment into a half-turn or full turn in the DNA α-helices. We also tested the NIP silencer activity in a longer IFN-γ promoter (−560). All the results indicate that the NIP silencer cannot repress a heterologous gene (IFN-γ) promoter (data not shown).

DNA-Protein Complexes Formed by NIP and Nuclear Proteins—To explore the mechanism of NIP function, the DNA-protein interaction between NIP and the nuclear proteins of Jurkat cells was investigated with EMSA. A radiolabeled double-stranded NIP oligonucleotide was used as a probe in the assay, and unlabeled NIP or Sp1 oligonucleotides (in micrograms) were used as competitors. The results are shown in Fig. 2. The NIP DNA formed four major complexes with a Jurkat nuclear extract (complexes A–D) (*lane 1*). All four complexes were reduced by the specific competitor (unlabeled NIP) in a dose-dependent pattern (*lanes 2–5*). Complexes C and D were reduced significantly by the nonspecific competitor Sp1 probe (*lane 6*). These results suggest that among the four complexes, A and B result from a specific interaction between the NIP probe and the Jurkat nuclear proteins. Complexes C and D

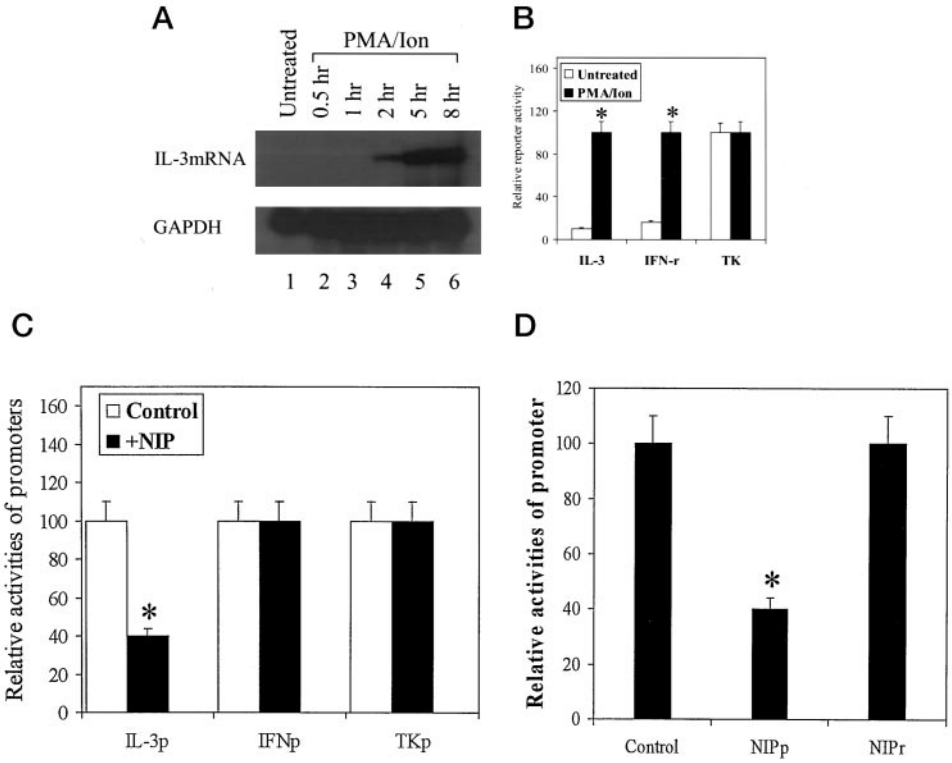

FIG. 1. **Functional characterization of the NIP silencer.** *A*, shown is the induction of IL-3 mRNA by PMA and ionomycin (*Ion*). Total cellular RNA was extracted from Jurkat cells stimulated by PMA (10 ng/ml) and ionomycin (1 μg/ml). The IL-3 mRNA level was determined by the ribonuclease protection assay as described under "Materials and Methods." Time points of the treatment are indicated at the top of each lane. *B*, shown is the inducibility of the IL-3 and IFN-γ gene promoters. The IL-3 (-175), IFN-γ (-108), and thymidine kinase (*TK*) promoters were examined in Jurkat cells the in transient transfection assay (as indicated). Inducibility of the promoters was tested following stimulation with PMA and ionomycin. The relative activities of the reporters were used to represent responses of the promoters. The *asterisks* indicate a significant increase from the control ($p < 0.001$). *C*, the activity of the NIP element was tested in the three promoters (*IL-3p*, *IFNp*, and *TKp*, IL-3, IFN-γ, and thymidine kinase promoters, respectively). One copy of NIP was inserted at the *Hin*dIII site in the promoters. In the IL-3 reporter plasmid, the *Hin*dIII site is 16 base pairs upstream of the IL-3 promoter (-175). *+NIP* means a promoter with one copy of NIP. The parental promoter serves as a control. The *asterisk* indicates a significant decrease from the control ($p < 0.001$). *D*, the activity of the NIP element was investigated in reverse orientation in the IL-3 gene promoter. *NIPp*, proper orientation; *NIPr*, reverse orientation. The parental IL-3 promoter was utilized as a control. The *asterisk* indicates a significant decrease from the control ($p < 0.001$). *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.

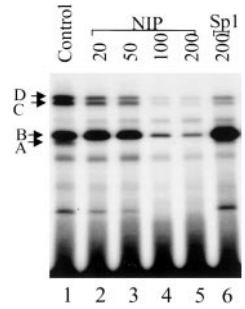

FIG. 2. **Specific DNA-protein complexes formed by the NIP oligonucleotide probe and Jurkat nuclear protein.** The gel shift assay was conducted as described under "Materials and Methods." Unlabeled oligonucleotides were used as specific or nonspecific competitors as indicated at the tops of the lanes. The *numbers* at the top of each lane refer to amount (in micrograms) of the competitor oligonucleotide.

result from a nonspecific interaction. It is interesting to note that when the level of complex A was reduced by 20 μg of unlabeled NIP (*lane 2*), the level of complex B was enhanced. When complexes C and D were reduced by the Sp1 probe, complex B was also enhanced (*lane 6*). There was a fast migrating band close to the free probe. This band also exhibited specific binding activity. Our later analysis suggests that this band is a derivative of complex B since it can be removed with unlabeled YY1 probe or antibody (Fig. 6). Complexes C and D appear to be nonspecific complexes, and mutation analysis of complexes C and D did not indicate any function of these two complexes (data not shown). Therefore, the rest of this study was focused on complexes A and B.

Protein Composition of Complex A—A search into the DNA-binding sequences of known transcription factors revealed that a binding site for the nuclear factor AP2 was present in the NIP oligonucleotide (*boxed* in Fig. 4*A*). To investigate if any of the complexes contains AP2, we carried out both an oligonucleotide competition assay and an antibody supershift assay (Fig. 3). In this experiment, an authentic SV40 AP2-binding oligonucleotide was used as a competitor against the NIP probe. The formation of complex A was inhibited by the AP2 oligonucleotide (Fig. 3*A*, *lane 3*). In the assay, the AP2 oligonucleotide competed with the NIP probe as efficiently as unlabeled NIP in complex A (*lane 2*). The AP2 oligonucleotide did not compete with the NIP probe in complexes B–D (compare *lanes 2* and *3*). This suggests that the protein in complex A, but not in complex B, shares DNA-binding specificity with the nuclear factor AP2. However, complex A was not supershifted or removed by the anti-AP2 antibody (Fig. 3*B*, *lane 2*). This antibody was able to remove an authentic AP2 complex formed by the AP2 oligonucleotide probe and Jurkat nuclear protein (Fig. 3*C*, *lanes 1* and *2*). These results imply that the protein in complex A is not AP2 or that the AP2 epitopes are masked by protein-protein interactions. Additionally, the mobilities of complex A and the authentic AP2 complex are totally different. Since the protein in complex A resembles AP2 in its DNA-binding specificity, but does not appear to be AP2, we designated it as ASRF.

Requirement of ASRF for the Silencer Function—The DNA-

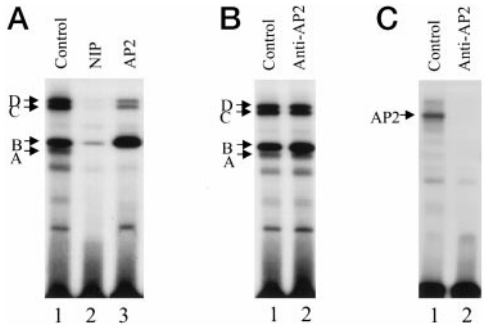

FIG. 3. **Complex A is formed by an AP2-like nuclear protein.** Oligonucleotide competition and antibody supershift experiments were conducted in gel shift assays to characterize complex A. *A*, complex A is specifically competed by an authentic AP2-binding oligonucleotide. 100 μg of competitor oligonucleotide was used in the reaction. *B*, supershift analysis with an antibody against the AP2 protein (sc-184, Santa Cruz Biotechnology, Inc.). 4 μg of antibody was used in the reaction. *C*, authentic AP2 complex formed by the radiolabeled AP2-binding oligonucleotide and the PMA/ionomycin-activated Jurkat nuclear proteins. The same anti-AP2 antibody (4 μg) was used to remove the AP2 complex as in described for *B*.

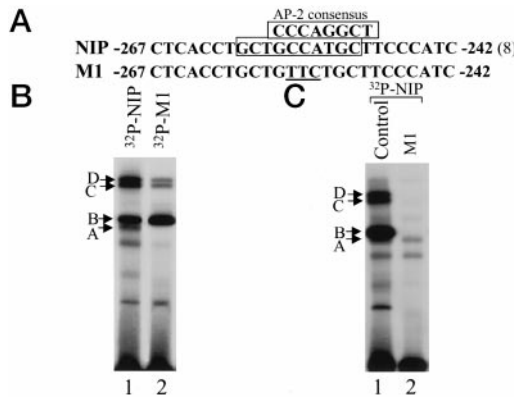

FIG. 4. **Mutation analysis of AP2-like binding activity.** *A*, DNA sequences of the NIP element and oligonucleotide M1. The *boxed* sequence in the NIP element is the binding sequence for the NIP protein as originally identified. The *box* above the NIP element represents a consensus DNA-binding sequence for the AP2 protein. The mutated sequence for the generation of M1 is *underlined*. *B*, comparison of the protein-binding patterns between NIP and M1. Radiolabeled NIP and M1 oligonucleotides were used as probes with the Jurkat nuclear protein in the gel shift assay. *C*, competition analysis of the M1 complexes. 100 μg of oligonucleotide M1 was used as a competitor against the NIP probe in *lane 2*.

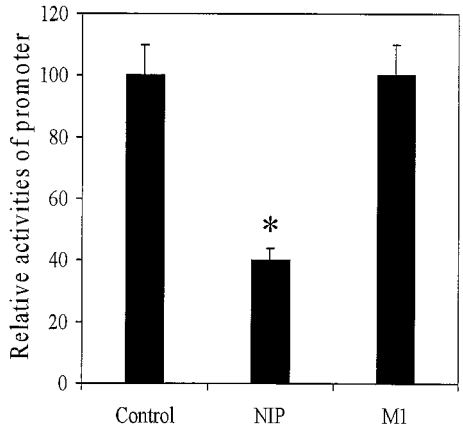

FIG. 5. **Functional analysis of AP2-like binding activity.** The function of the M1 mutation that was generated by deletion of the AP2-like binding activity from the NIP element was examined in the IL-3 promoter. One copy of M1 was inserted at a *Hin*dIII site 16 base pairs upstream of the IL-3 promoter (−175) in the reporter plasmid. Transient transfection of Jurkat cells and the reporter assay were carried out as described under "Materials and Methods." *Control*, IL-3 parental vector; *NIP*, IL-3 vector with the NIP element; *M1*, IL-3 promoter with one copy of the M1 element. The *asterisk* indicates a significant decrease from the control ($p < 0.001$).

binding sequence of ASRF was mapped out by a series of base substitutions in the NIP sequence (data not shown). A 3-base pair substitution in the AP2-like binding site was sufficient to delete ASRF-binding activity in the NIP element (oligonucleotide M1) (Fig. 4*A*). The protein-binding activity of M1 was tested in the gel shift assay directly using radiolabeled M1 as the oligonucleotide probe (Fig. 4*B*) or indirectly using unlabeled M1 as a competitor (Fig. 4*C*). The results show that M1 does not form complex A, but does form complexes B–D (Fig. 4*B*, *lane 2*). In the absence of complex A, the intensity of complex B was enhanced compared with the control (*lane 2 versus lane 1*). These data again support the hypothesis that there may be a competition between ASRF and complex B in the NIP element. Complexes C and D were reduced after mutation of the ASRF-binding site (Fig. 4*B*, *lane 2*). This suggests that formation of complexes C and D may somehow be modulated by the formation of the ASRF complex. In the competition experiment (Fig. 4*C*), M1 did not compete with the NIP probe in forming complex A, confirming that M1 cannot bind to ASRF (Fig. 4*C*, compare *lanes 1* and *2*).

The function of M1 in the IL-3 promoter was examined by inserting one copy of M1 into the IL-3/CAT reporter vector (Fig. 5, *M1*). In this experiment, the IL-3/CAT vector and the NIP vector (containing one copy of wild-type NIP in the IL-3/CAT reporter vector) were used as controls (Fig. 5, *Control* and *NIP*, respectively). The results demonstrate that in contrast to the NIP element, M1 has no silencer function at all. Since the only difference between M1 and NIP is the binding activity of ASRF, these data indicate that ASRF mediates the silencer function of NIP.

Complex B Contains the Nuclear Factor YY1—The search of transcription factor-binding sequences in the NIP element also revealed that there are two potential binding sites for the transcription factor YY1. One site overlaps with the AP2-like binding site, and the other is located at the 3′-end of NIP (under the YY1 consensus sequence in Fig. 7*A*). To investigate if YY1 is involved in the formation of any NIP complexes, an authentic YY1-binding oligonucleotide and an anti-YY1 antibody were utilized in the gel shift assay with the NIP probe (Fig. 6). The results from the oligonucleotide competition experiment show that complex B may contain YY1 because the authentic YY1 oligonucleotide efficiently competed the formation of complex B (Fig. 6*A*, *lane 3 versus lane 2*). In the antibody supershift assay, part of complex B was supershifted by the anti-YY1 antibody (Fig. 6*B*, *lane 2*), but was not affected by a control antibody (*lane 3*). To confirm the activity of the anti-YY1 antibody, a supershift experiment was performed with a YY1 complex formed by the radiolabeled YY1 oligonucleotide probe and Jurkat nuclear protein (*lanes 4–6*). The results show that the authentic YY1 complex migrated at the same position as complex B, and more important, it was supershifted to an identical level (labeled as *Shifted Bands*) as complex B by the anti-YY1 antibody (*lane 5*). These data strongly suggest that the transcription factor YY1 is involved in the formation of complex B.

Functional Antagonism of YY1 and ASRF—We next analyzed the function of the YY1 complex by mutation and cotransfection in Jurkat cells. Oligonucleotide M2 contains a 3-base pair substitution at the second YY1 homology site located at the 3′-end of the NIP oligonucleotide (Fig. 7*A*). The protein-binding activity of M2 was examined in the same way as described for M1. A radiolabeled M2 oligonucleotide was used

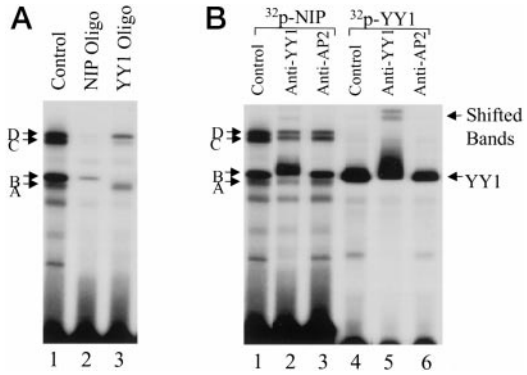

FIG. 6. **Complex B contains the nuclear protein YY1.** Oligonucleotide competition and antibody supershift experiments were conducted as described under "Materials and Methods." *A*, complex B is specifically competed by an authentic YY1-binding oligonucleotide. 100 μg of competitor oligonucleotide was used in the EMSA. *B*, supershift analysis with anti-YY1 antibody (sc-281, Santa Cruz Biotechnology, Inc.). Complex B was formed by incubating the NIP probe and Jurkat nuclear protein (*lanes 1–3*). 4 μg of anti-YY1 antibody or anti-AP2 antibody (as a control) was used as indicated at the top of each lane. Authentic YY1 complex was formed by the radiolabeled YY1-binding oligonucleotide and the Jurkat nuclear proteins (*lanes 4* and *5*). The supershifted bands are marked by an *arrow* in *lanes 2* and *5*.

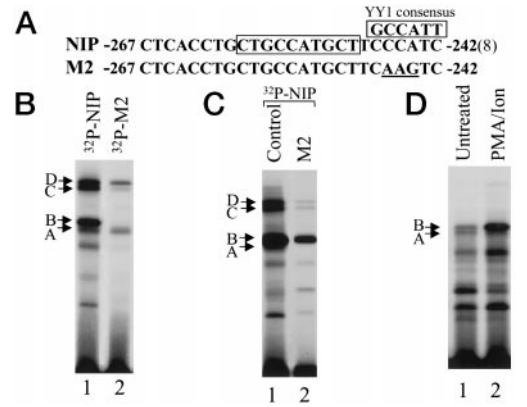

FIG. 7. **Modulation of YY1-binding activity.** *A*, DNA sequences of the NIP element and oligonucleotide M2. The *boxed* sequence in the NIP element is the binding sequence for the NIP protein as originally identified. The *box* above the NIP element identifies a consensus DNA-binding sequence for the YY1 protein. The mutated sequence for the generation of M2 is *underlined*. *B*, comparison of protein-binding patterns between NIP and M2. Radiolabeled NIP and M2 oligonucleotides were used as probes with the Jurkat nuclear protein in EMSA. *C*, competition analysis of the M2 DNA-protein complexes. 100 μg of oligonucleotide M1 was used as a competitor against the NIP probe in *lane 2*. *D*, induction of YY1 activity in fresh human T cells. The nuclear protein was extracted from fresh T cells after stimulation with PMA plus ionomycin (*Ion*) for 2 h. The DNA-binding activity of the nuclear protein was examined with the radiolabeled NIP probe.

as a probe in EMSA with Jurkat nuclear protein. The results of this experiment show that M2 lost YY1-binding activity (complex B) (Fig. 7*B*, *lane 2*), but retained the ability to form the other three complexes. Complexes C and D were reduced after mutation of the YY1-binding site (*lane 2*), suggesting there are certain interactions among these three complexes. However, this result indicates that the second YY1 homology sequence (mutated in M2) is responsible for the formation of the YY1 complex (complex B). The first YY1 homology sequence, which overlaps with the ASRF site, is not involved in the formation of the YY1 complex because M2 failed to demonstrate YY1-binding activity. The same conclusion can be drawn from the competition assay, in which unlabeled M2 was used as a competitor against the NIP probe (Fig. 7*C*).

In Jurkat cells, modulation of YY1-binding activity by PMA plus ionomycin is not significant. This might be due to a high basal level of YY1 in Jurkat cells. To explore the question of inducibility of YY1, purified human peripheral T cells were employed. The total T cells were separated from the human peripheral blood by the nylon wool method and treated with PMA plus ionomycin. This stimulation is sufficient to induce activation of the IL-3 gene in the T cells. After a 2-h treatment, the nuclear protein was extracted and examined in the gel shift assay. The results show that the basal level of YY1 (band B) in the T cells is low (Fig. 7*C*, *lane 1*), but its binding activity is inducible by stimulation (*lane 2*). There are four major complexes formed by the nuclear protein of unstimulated fresh T cells (*lane 1*). After PMA/ionomycin stimulation, three of the four complexes were induced, and one of them was reduced. If the reduced band was used to normalize the induced bands, the induction of the YY1 band would become even stronger. This suggests that activation of the DNA-binding activity of YY1 correlates with activation of IL-3 gene transcription in the T cells.

To test the functional activity of a change in the 3′-YY1 site, one copy of M2 was linked to the IL-3 promoter, and the reporter vector was transfected into Jurkat cells. The results of this experiment demonstrate that mutation of the YY1 site resulted in a silencer activity even stronger than that of wild-type NIP in the IL-3 promoter (Fig. 8*A*). Instead of the 60% inhibition of IL-3 promoter activity caused by NIP, >80% of the IL-3 promoter activity was lost in the presence of M2. This result suggests that the YY1 protein may play an opposite role to ASRF when binding to the NIP element. To test this hypothesis, a YY1 expression vector was cotransfected with the IL-3 promoter constructs. Overexpression of YY1 had no effect on the IL-3 promoter in the absence of the NIP element. In the presence of NIP, overexpression of YY1 enhanced promoter activity by 100%. The relative promoter activity was increased from 40% (NIP) to 80% (NIP + YY1) (Fig. 8*B*). These results provide functional evidence that YY1 inhibits ASRF in the IL-3 gene promoter.

DISCUSSION

In previous studies, we identified a regulatory activity of the transcription factor YY1 in the human IFN-γ gene promoter (21, 22) and the granulocyte/macrophage colony-stimulating factor gene promoter (28, 29). In the IFN-γ promoter, YY1 acts as a repressor protein through two mechanisms. In the silencer (BE) region, YY1 cooperates with another protein, which also shares DNA-binding specificity with AP2, to mediate the silencer function (21). In this case, binding of both YY1 and the AP2-like protein is required for the silencer function. We also observed that YY1 inhibited AP1 activity at an AP1-YY1-binding site in the IFN-γ promoter. YY1 competed with AP1 for the overlapping region in an AP1-YY1-binding site, leading to inhibition of the AP1 enhancer function (22). In the human granulocyte/macrophage colony-stimulating factor gene promoter, YY1 repressed AP1 activity and an Sp1-like activity through a similar competition in the CAT enhancer element (28, 29). Thus, in the IFN-γ and granulocyte/macrophage colony-stimulating factor gene promoters, competition with an activator is the common mechanism by which YY1 inhibits cytokine gene promoter activity.

The results from this study indicate that the NIP silencer is a promoter-specific *cis*-acting element (Fig. 1). This conclusion is different from a previous report in which that NIP activity is claimed as a nonspecific silencer for the IL-3 promoter (10). This difference might be due to the experimental systems. In that study, the human IL-3 silencer activity was characterized in the gibbon T cell line MLA 144. The IL-3 cDNA was used as a reporter of the IL-3 promoter, and RNase protection assay

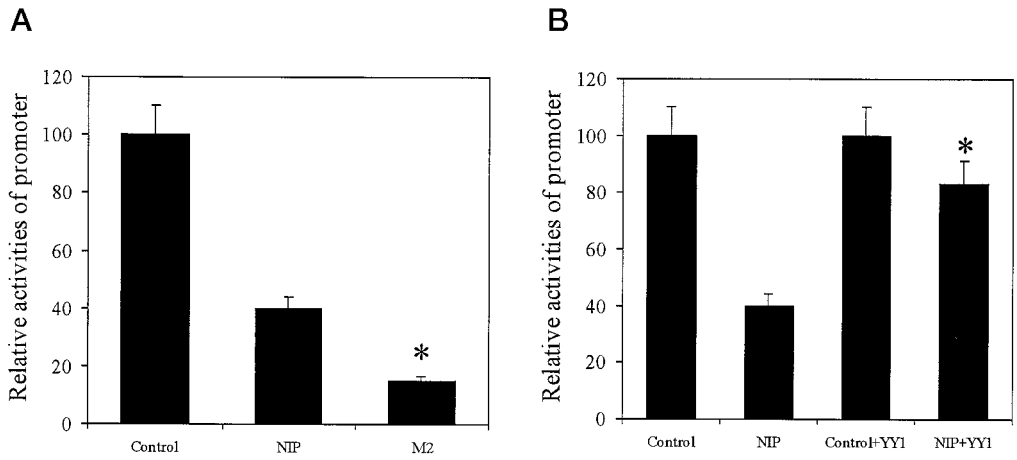

FIG. 8. **Functional analysis of YY1-binding activity.** *A*, the function of the M2 mutation was examined in the context of the IL-3 promoter. One copy of M2 was inserted at the *Hin*dIII site 16 base pairs upstream of the IL-3 promoter (−175) in the reporter plasmid. Transient transfection of Jurkat cells and the reporter assay were carried out as described under "Materials and Methods." *Control*, IL-3 parental vector; *NIP*, IL-3 vector with the NIP element; *M2*, IL-3 promoter with one copy of the M2 element. The *asterisk* indicates a significant decrease from NIP ($p < 0.001$). *B*, shown are the effects of overexpressed YY1 on the NIP function. A YY1 expression vector was utilized in the cotransfection analysis of the NIP function. The ratio of the YY1 expression vector to the IL-3 reporter vector was 1:1. The parental vector of the YY1 expression plasmid was utilized as a control DNA in the cotransfection. The control reporter (*Control*) contains the IL-3 promoter only. The experimental reporter vector contains one copy of NIP in the IL-3 promoter (*NIP*). The *asterisk* indicates a significant increase from NIP ($p < 0.001$).

was used to monitor reporter activity. The limitations of that system include the following. (*a*) Gibbon T cells may be different from human T cells, and this may result in a different activity of the NIP silencer. (*b*) In the reporter assay, reporter activity was not normalized by an internal control. It is widely accepted that a difference in transfection efficiency could result in a totally distinct conclusion. (*c*) Additionally, a result from only one reporter assay, not a mean value of multiple experiments, was shown in that report. In our study, the IL-3 silencer activity was characterized in the human Jurkat T cell line, and all the reporter (CAT or β-galactosidase) activities were normalized by an internal control. Moreover, a mean value of three experiments was used to show reporter activity. Position effect was examined in the IFN-γ promoter by adjusting the distance between the NIP element and the IFN-γ promoter. The results did not support a possibility that position plays a role in the NIP silencer activity in the heterologous promoter.

This study demonstrates that YY1 regulates the human IL-3 gene promoter through competition with ASRF activity in the NIP silencer element. We confirmed that Jurkat cells expresses IL-3 mRNA upon stimulation of PMA/ionomycin. Under the same condition, the IL-3 gene promoter exhibited a dramatic inducible activity, indicating that our experimental system is closely relevant to the physiological condition under which IL-3 expression is regulated. In the four specific DNA-protein complexes formed by the NIP oligonucleotide and Jurkat nuclear extracts (Fig. 2), only complexes A and B appear to be functional. Complex A is responsible for the silencer activity, and its activity is regulated by complex B. Protein analysis revealed that complex A contains a nuclear protein that shares DNA-binding specificity with AP2 (ASRF) (Fig. 3), and complex B contains the transcription factor YY1 (Fig. 6). It has been documented that a consensus YY1-binding sequence contains 9 base pairs ((C/G/A)(G/T)(D/T/A)CATN(T/A)(T/G/C)) (23). In our study, we found that the core sequence for YY1 binding can be reduced to 5 base pairs of CCATT. This has been verified by mutation analysis of the YY1-binding sites in the promoters of human IFN-γ (21, 22) and the granulocyte/macrophage colony-stimulating factor (28, 29). An extra flanking sequence is not required for YY1 protein binding. In the NIP element, this was again verified. In the IL-3 gene promoter, YY1 functions by regulating ASRF activity through competition for DNA binding. This conclusion is supported by the following evidence. (*a*) In the competition assay, when the binding of ASRF was reduced by the competition of an AP2 oligonucleotide, YY1 binding was enhanced (Fig. 3*A*). (*b*) In the mutation analysis, when ASRF-binding activity was abrogated in the NIP element by mutation, YY1-binding activity was increased (Fig. 4*B*). (*c*) In the functional analysis, loss of YY1 activity was associated with an increased silencer activity, indicating that ASRF activity was increased in the absence of YY1 (Fig. 8*A*). (*d*) In a cotransfection assay, overexpression of YY1 resulted in reduction of the silencer activity (Fig. 8*B*), suggesting that YY1 inhibited the AP2-like activity.

This study provides evidence for a new model of cytokine gene regulation by YY1. On the basis of previous data, two models by which YY1 regulates cytokine gene transcription have been suggested. In the first model, YY1 cooperates with an AP2-like protein in mediating silencer activity (21, 22). In the second model, YY1 inhibits the activity of an activator protein through competition for DNA binding (22). This model has also been proposed for regulation of non-cytokine genes. In the α-actin gene, YY1 inhibits the activity of the serum response factor through competition (30). In the rat serum amyloid A1 gene, YY1 antagonizes NF-κB activity by competition (31). In these two models, YY1 plays a negative role in gene regulation. Since the DNA-binding activity of YY1 preexists in the resting cells, these two models can explain the tight control of gene transcription of some cytokines such as IFN-γ (22). Here, we suggest a third model in which YY1 antagonizes the activity of a repressor protein through competition for DNA binding. In this model, YY1 plays a positive role in cytokine gene transcription. This finding may explain why the Jurkat cells used in this study can produce IL-3, but not IFN-γ. Jurkat T cells have a strong endogenous YY1 activity compared with natural killer cell lines (YT or NK3.3). In the IFN-γ promoter, YY1 acts as a repressor, whereas in the IL-3 promoter, YY1 acts as a derepressor. It is reasonable to suggest that in the Jurkat cells, the endogenous YY1 activity prevents activation of the IFN-γ promoter and facilitates activation of the IL-3 gene promoter.

The AP2-like activity in the IFN-γ silencer element (BE) and the ASRF activity in the IL-3 silencer element (NIP) might be different members of a novel transcription factor family. The

common features of the two AP2-like activities are as follows. (*a*) Both proteins recognize a similar DNA sequence. (*b*) Both proteins mediate the silencer activities in the respective promoters. (*c*) Their activities are promoter-dependent, and the DNA is orientation-dependent. The differences in the two proteins include the following. (*a*) The ASRF complex migrates slower than the IFN-γ AP2-like protein-DNA complex (data not shown). (*b*) ASRF is sufficient to mediate the silencer activity, whereas the IFN-γ AP2-like protein needs cooperation with YY1 to mediate the silencer activity. Interestingly, a protein with a similar DNA-binding specificity may also play a role in the TNF-α silencer. A silencer element (-254 to -230) in the TNF-α promoter has been identified (14), and it represses TNF-α transcription in U937 cells. This 25-base pair TNF-α repressor site contains a 10-base pair sequence homologous to the binding site of nuclear factor AP2, but it does not bind the AP2 protein (32). Taken together, this information suggests that these proteins may represent a novel family of transcription factors that function as repressors in the transcriptional regulation of cytokine gene expression.

In summary, we report here that the transcription factor YY1 and ASRF regulate the activity of the IL-3 NIP silencer element. ASRF activity is required for the silencer function, including promoter specificity and orientation dependence. In contrast, YY1 plays a role in the positive regulation of the human IL-3 gene promoter. This activity may be mediated by a direct competition with ASRF activity for DNA binding in the NIP element. These data support that a cell type-specific silencer activity might be determined by a unique profile of ubiquitous transcription factors.

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Regulation of a Cell Type-specific Silencer in the Human Interleukin-3 Gene Promoter by the Transcription Factor YY1 and an AP2 Sequence-recognizing Factor

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