Yin-Yang 1 Activates Interleukin-4 Gene Expression in T Cells*

Jia Guo‡, Vincenzo Casolaro§, Edward Seto¶, Wen-Meng Yang†, Cindy Chang‡,
Maria-Cristina Seminario§§, Judith Keen¶, and Steve N. Georass**

From the Divisions of §Pulmonary and Critical Care Medicine and ¶Allergy and Clinical Immunology, The Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland 21224 and the ¶¶H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Interleukin-4 (IL-4) is a multifunctional cytokine that plays an important role in immune and inflammatory responses. Expression of the IL-4 gene is tightly controlled at the level of gene transcription by both positive and negative regulatory elements in the IL-4 promoter. Several constitutive nuclear factors have been identified that can interact with IL-4 promoter elements in DNA binding assays. Here we report that the zinc-finger protein YY-1 (Yin-Yang 1) can bind to multiple elements within the human IL-4 promoter. Cotransfection of Jurkat T cells with different IL-4 promoter/reporter constructs together with expression vectors encoding antisense, wild-type, or zinc finger-deleted mutant YY-1 suggested that YY-1 enhanced IL-4 promoter activity in a DNA-binding domain-dependent manner. Site-directed mutagenesis revealed that a proximal YY-1-binding site, termed Y0 (−59TCAATTTT−53), was essential for YY-1-driven IL-4 promoter activity. In addition, cotransfected YY-1 enhanced both IL-4 promoter activity and endogenous IL-4 gene expression in nontransformed peripheral blood T cells. Thus, YY-1 positively regulates IL-4 gene expression in lymphocytes.

Interleukin-4 (IL-4),1 a pleiotropic cytokine produced by activated T cells and basophils, plays a critical role in cellular and humoral immune responses (1). Dysregulated expression of IL-4 has been linked with autoimmune and allergic diseases (2, 3). In T cells, IL-4 gene expression is regulated at the transcriptional level by both ubiquitous and cell type-restricted factors (4–11). These factors interact with a proximal promoter region composed of multiple regulatory elements and can both positively and negatively influence transcriptional activation (see Fig. 1). Other regions have been identified outside of the proximal IL-4 promoter that can regulate IL-4 gene expression, including the IL-4/VIL-13 intergenic region (12), the IL-4 second intron (13), and downstream of the IL-4 gene (14). Some of these elements appear to coordinately regulate IL-4 gene expression at the chromatin level (15).

Major insights into the regulation of IL-4 gene expression came from studies using transgenic or knockout approaches to investigate the molecular basis of Th2 differentiation in mice (reviewed in Ref. 16). Using these approaches, several transcription factors have been identified that are critical for this process, including Stat6 (17), NFATc (18), c-Maf (19), GATA-3 (20), and JunB (21). The mechanisms by which these factors influence T cell differentiation are under active investigation. To date, direct binding to and/or activation of the IL-4 promoter has been demonstrated for NFATc (22), c-Maf (19), and JunB (21), but not for Stat6 (23) or GATA-3 (24). A two-step model has recently been proposed to explain IL-4 gene expression in Th2 cells (25). Based on the appearance of DNase-hypersensitive sites in the IL-4 gene locus (26, 27), this is thought to involve an initial chromatin remodeling step followed by cytokine gene transcription in response to T cell receptor-activated transcription factors. Although chromatin remodeling is likely an important regulatory step during Th2 differentiation, the proximal IL-4 promoter confers a high degree of tissue specificity when linked to reporter genes in transgenic mice (15, 28, 29). Thus, an analysis of the factors that regulate the activity of the proximal IL-4 promoter will enhance our understanding of IL-4 gene expression.

We recently performed a detailed deletional analysis of the human proximal IL-4 promoter and discovered novel binding sites for several transcription factors, including NFAT (nuclear factor of activated T cells) (9), CP-2 (30), and an uncharacterized repressor factor (10). This analysis also uncovered binding sites for several other constitutive nuclear factors, in keeping with previous studies on the IL-4 promoter in other systems (4, 5, 8, 31). Unlike the inducible IL-4 promoter-binding factors that have been intensively studied, only a few of these constitutive factors have been well characterized. Here we report that YY-1 (Yin-Yang 1), a constitutive nuclear member of the GLI-Krüppel family of zinc-finger transcription factors, can interact with four binding sites in the human proximal IL-4 promoter. We use site-directed mutagenesis together with cotransfection assays to define the role of YY-1 in regulating the transcriptional activation of the IL-4 promoter.

EXPERIMENTAL PROCEDURES

Plasmid Construction—A luciferase-based human IL-4 promoter construct containing 270 bp upstream from the transcription start site and ending at +65 (termed 270loc) was synthesized as described (9). A construct containing 235 bp upstream from the transcription start site was amplified by PCR from 270loc and ligated into the pCR-2.1-TOPO vector (Invitrogen), followed by ligation into the XhoI site of pGL3-Basic (Promega). A construct containing 145 bp upstream from the transcription start site was amplified by PCR from genomic DNA and ligated into the KpnI and SacI sites of pGL3. Mutations were introduced into the Y0 YY-1-binding site within 270loc using site-directed mutagenesis with...
the QuikChange kit (Stratagene) to construct control 270ucY0mut. The following mutations specifically disrupted the Y0 site (see Fig. 4): wild-type 270uc, −358CTATTTTTTTTTTT−353 to 270ucY0mut, −358TGTTTTTTTTTTT−353 (mutations in lowercase). All products were sequenced to confirm accurate replication. An SV40 promoter-driven YY-1 expression vector was synthesized by Custom DNA (Bio-Rad). The YY-1 expression vector (pSEAP2-Control) was inserted into the EcoRI sites of pcSG5 (Stratagene). An expression vector encoding a YY-1 mutant with a deletion of amino acids 333−408 within the zinc-finger domain (ZFDmut) was synthesized by restricting pSG5-YY-1 with HindIII and BamHI and religation using the following linkers: HindIII, 5′-AGCTTCCACAACTGATGGA-3′; EcoRI, 5′-GATCCTCTTGGAATTCTGGA-3′. The antisense YY-1 vector was a kind gift of Dr. Michael Atkinson (University of Pennsylvania) and has been described (32). Expression vectors encoding full-length NFATp (pREP4-NFATp) and NFATc (pSH107-NFATc) were gifts of Dr. Timothy Hoey (Tularik Inc.) and Dr. Gerald Crabtree (Stanford University), respectively.

**Cell Lines and Transfections**—Jurkat T cells (courtesy of Dr. Jack Strominger, Harvard University) were maintained and transfected using 2.5 × 10^6 cells, 1 μg of reporter, and 3 μl of Superfect® (QIAGEN Inc.) per μg of plasmid DNA as previously reported (22). COS-7 cells were obtained from American Type Culture Collection (Manassas, VA). Jurkat cells and a Jurkat subline stably transfected with the SV40 large T antigen (JTAg cells, kindly provided by Dr. Ron Wange, NIA, National Institutes of Health) were transfected using electroporation as described in Refs. 34 and 35) into the EcoRI sites of pSG5 (Stratagene). Cotransfections were performed with 1 μg of reporter plasmid and then electroporated at 300 V and 960 microfarads (Bio-Rad Gene-Pulser II). These expression vector or empty vector to keep total DNA constant. Cells were transfected with pSEAP2-Control (1 μg per well) using 2.5 μl of reporter DNA containing 5% bovine serum albumin and 0.1% Tween 20 for 1 h, membranes were probed with mouse anti-YY-1 monoclonal antibody H-10 (1:500 dilution; Santa Cruz Biotechnology) for 10 h at 4 °C, followed by incubation for 1 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3000 dilution; Amersham Biosciences, Inc.). After a final washing step, immunoreactive bands were revealed by enhanced chemiluminescence and autoradiography using the ECL Western blotting detection kit (Amersham Biosciences, Inc.) according to the manufacturer's directions.

**Electrophoretic Mobility Shift Assays (EMSAs)**—The following 30-bp oligonucleotides and their complements were synthesized: 5′-ATTGCCGAGGAGGGATGTCTAGTTG-3′ (P0, −38 to −69); 5′-TGAGTTTCATCCTGGAAATTTCTTGAAC-3′ (P1, −57 to −92); 5′-TGCTGTTCTCAGGAATTTACCTGGTTTT-3′ (P2, −175 to −146); 5′-AATCCAGACCAATGGAATTTTATTTTTTTATTTTTT-3′ (P3, −169 to −201), 5′-AGGTTCGATGGAAATTTACATTACATTTGCTG-3′ (P4, −216 to −248), and 5′-CCGATCCTCCTGGGAAATAGTACATTACA-3′ (P5, −340 to −372). Mutations were introduced into the P0 and P2 consensus YY-1 binding sites (underlined) as indicated in Fig. 2A. A recombinant fragment of murine NFATp (encoding 298 amino acids of the DNA-binding domain, highly conserved among different NFAT family members) was expressed as a hexahistidine-tagged protein and extracted as described (34). Recombinant YY-1 was extracted from bacteria transformed with a histidine-tagged YY-1 expression vector. Nuclear extracts were obtained from 5 × 10^6 Jurkat cells using the method of Schreiber et al. (35). EMSAs were performed using 5 μg of nuclear protein and a 32P-labeled probe with 0.5 μg of poly(G:dC) (Amersham Biosciences, Inc.) in a final volume of 10 μl. Experiments with recombinant proteins were performed with 1-μl aliquots of the recombinant NFATp DNA-binding domain with or without 1 μl of YY-1, and the final buffer composition in all samples was adjusted to contain 84 mM KCl, 34 mM NaCl, 7% glycerol, 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, and 0.1% Nonidet P-40. Free probes and protein-DNA complexes were resolved by 5% PAGE with 0.5× Tris borate/EDTA. In some experiments, EMSAs were incubated with 1 μl of the following antisera for 30 min at 4 °C prior to addition of probe: rabbit anti-NFATp polyclonal antibody (Upstate Biotechnology, Inc.), rabbit anti-YY-1 polyclonal antibody C-20 (Santa Cruz Biotechnology), rabbit anti-Sti6 polyclonal antibody 8-20 (Santa Cruz Biotechnology), and isotype- and species-matched control IgG (Sigma).

**RESULTS**

**Multiple YY-1-binding Sites in the Human IL-4 Promoter**—The IL-4 promoter contains multiple binding sites for members of the NFAT family of transcription factors, termed the P elements P0−P5 (4, 5, 9) (Fig. 1). During a detailed deletional analysis of the human IL-4 promoter, we identified a factor (termed complex IV) that bound downstream of NFAT to the P2 element (10). As shown in Fig. 2 (A−C), multiple nuclear factors from Jurkat cells recognized this element in EMSAs. These included a slowly migrating factor with apparent repressor properties (termed Rep-1 (10)), a Ca^2+−induced factor containing NFATp, and the constitutive complexes III−V. We previously reported that complex IV binds in a sequence-specific manner and that its formation is not competed for by a panel of consensus oligonucleotide controls, including those containing high affinity NFAT and AP-1 sites (10). Mutational analysis revealed that the formation of complex IV required sequences located just downstream of the NFAT consensus sequence −163GGAACA−158 (Fig. 2, A and B). Inspection of this region revealed a sequence (−160ACATTTT−154) highly homologous to a consensus YY-1-binding site, which we termed Y2 (Fig. 2B). The most conserved nucleotide in the YY-1 consensus sequence is the second cytosine (−5′-CAGTNTT−3′), which appears to be required for high affinity DNA binding (36). When we mutated this nucleotide within the P2 element, we did not detect complex IV binding in EMSAs using Jurkat nuclear extracts (data not shown). When we mutated this nucleotide within the P2 element, we did not detect complex IV binding in EMSAs using Jurkat nuclear extracts (data not shown).
YY-1 Enhances IL-4 Gene Expression

Fig. 1. Diagram of the IL-4 promoter. Regulatory elements are boxed, and the major factors shown to bind them are indicated to the right. YY-1-binding sites described in this report are indicated to the left. The diagram is not drawn to scale and is a compilation of studies of the human and mouse IL-4 promoters. NRE, negative regulatory element; ISRE, interferon-stimulated response element; CPRE, CP-2 response element; MARE, c-Maf response element; C/EBP, CCAAT/enhancer-binding protein; IRF-2, interferon-regulation factor-2; NF-Y, nuclear factor Y. Not shown). Using anti-transcription factor antibodies and nuclear extracts from Jurkat T cells in EMSA, we found that complex IV indeed contained immunoreactive YY-1 (Fig. 2C, lanes 3 and 6). Fig. 2D also shows that factors previously shown to bind to the P2 region, such as NFATp and Stat6 (23), did not affect complex IV. YY-1 appeared to bind in a competitive manner with complex III, which bound just upstream of the NFAT site (Fig. 2A, compare lanes 1 and 7). The identities of the constitutive complexes III and V are currently unknown.

Because the IL-4 promoter P elements are homologous even outside of the NFAT core consensus sequence, we speculated that YY-1 would interact with additional sequences within the IL-4 promoter. Fig. 3 shows that a YY-1-immunoreactive complex formed on oligonucleotide probes encompassing the P3 and P4 (but not P1 and P5) elements using Jurkat nuclear extracts in EMSA. Importantly, sequence inspection revealed five of six base pair matches for the consensus YY-1-binding sites in the P3 and P4 (but not P1 and P5) oligonucleotides (Fig. 3).

The most proximal IL-4 NFAT site (termed P0) has been shown to bind both constitutive and inducible nuclear factors and to contribute to promoter activity in T cell lines and Th2 cells (29, 37). Sequence inspection revealed a potential YY-1-binding site located immediately adjacent to the P0 NFAT consensus sequence (Fig. 4). Fig. 4A shows that a YY-1-immunoreactive complex formed on the P0 element using Jurkat nuclear extracts in EMSA. To precisely map the YY-1-binding site in this region, we introduced mutations into the YY-1 consensus sequence and studied the ability of recombinant YY-1 to bind to wild-type and mutant oligonucleotides. As shown in Fig. 4B, mutation of two nucleotides within the YY-1 consensus sequence (including the second cytosine, C$^{59}$) drastically impaired the ability of YY-1 to bind to this region (compare lanes 4 and 6). As a control, we also found that recombinant YY-1 readily bound to the P2 oligonucleotide. These experiments mapped the proximal IL-4 promoter YY-1-binding site to sequence $5'$-CTTGCATT'TT-3'$, which we termed Y0. The introduced mutations also inhibited the binding of native YY-1 to the Y0 element (Fig. 4C). Note that a constitutive complex

**Fig. 2.** Multiple nuclear factors, including YY-1, bind the P2 element. A, Jurkat nuclear extracts from cells activated with (even-numbered lanes) or without (odd-numbered lanes) the calcium ionophore A23187 were analyzed by EMSAs with wild-type (wt) and mutant oligonucleotide (Oligo) probes. Mutational analysis localized the binding site for complex IV just downstream of the NFAT site. Note that ionophore-induced NFATp (see D) bound to the wild-type and m5 probes, but not to the m3 and m4 oligonucleotides, which contain disrupted NFAT sites. In contrast, complex IV did not recognize the m4 and m5 probes (lanes 3–6). Complex IV binding was constitutive and not appreciably affected by ionophore treatment. Only the portion of the gel showing the relevant complexes is shown for clarity. B, shown are the sequences of the oligonucleotides used in these experiments. The YY-1 site (defined by the m4 and m5 mutations) is indicated by the double overline. The cytosine at –159, which is critical for YY-1 binding, is underlined. C, complex IV contains immunoreactive YY-1. Note that YY-1 bound more strongly to the mutant oligonucleotide (m3) that did not support binding of complex III. A supershift (s.s.) was detectable only on the mutant probe (lane 6). D, complex II contains immunoreactive NFATp (lane 2) because its formation was inhibited by antibodies directed against this factor, but not by species-matched control (lane 1) or anti-Stat6 (lane 3) antibody. None of the antibodies affected the binding of YY-1.

**Fig. 3.** YY-1 interacts with the P3 and P4 NFAT elements. Nuclear extracts from Jurkat T cells were analyzed by EMSAs with wild-type (wt) and mutant oligonucleotide (Oligo) probes. Mutational analysis localized the binding site for complex IV just downstream of the NFAT site. Note that ionophore-induced NFATp (see D) bound to the wild-type and m5 probes, but not to the m3 and m4 oligonucleotides, which contain disrupted NFAT sites. In contrast, complex IV did not recognize the m4 and m5 probes (lanes 3–6). Complex IV binding was constitutive and not appreciably affected by ionophore treatment. Only the portion of the gel showing the relevant complexes is shown for clarity. B, shown are the sequences of the oligonucleotides used in these experiments. The YY-1 site (defined by the m4 and m5 mutations) is indicated by the double overline. The cytosine at –159, which is critical for YY-1 binding, is underlined. C, complex IV contains immunoreactive YY-1. Note that YY-1 bound more strongly to the mutant oligonucleotide (m3) that did not support binding of complex III. A supershift (s.s.) was detectable only on the mutant probe (lane 6). D, complex II contains immunoreactive NFATp (lane 2) because its formation was inhibited by antibodies directed against this factor, but not by species-matched control (lane 1) or anti-Stat6 (lane 3) antibody. None of the antibodies affected the binding of YY-1.
YY-1 Enhances IL-4 Gene Expression

FIG. 4. Definition of the P0 YY-1-binding site. The sequences of the wild-type (wt) and mutant P0 oligonucleotide probes used in EMSA (including consensus binding sites) are indicated at the top. A, Jurkat nuclear extracts were used in EMSA with a wild-type P0 oligonucleotide (Oligo) probe. The YY-1-immunoreactive complex is indicated by an asterisk. B, recombinant YY-1 was expressed in bacteria and extracted as described under “Experimental Procedures.” A strong complex formed on the P2 (lane 2) and wild-type P0 (lane 4) oligonucleotides. Mutation of two base pairs within the YY-1 consensus region drastically reduced YY-1 binding (lane 6). C, EMSA was carried out using Jurkat nuclear extracts with wild-type and mutant radiolabeled P0 oligonucleotide probes. The YY-1-specific complex formed on the wild-type probe (lane 1), but not on the mutant probe (mut; lane 2). A constitutive complex (asterisk) formed equally well on both oligonucleotides (see “Results” for discussion).

that was partially inhibited by the anti-YY-1 antibody (Fig. 4A, asterisk) bound equally well to the mutant probe (Fig. 4C). The identity of this complex, which does not represent sequence-specific binding by YY-1, is currently unknown. We did not detect NFATp binding to the P0 element in these experiments (data not shown), in keeping with prior studies showing that the P0 element binds NFAT with lower affinity than other P elements (37).

An Antisense YY-1 Vector Inhibits Basal IL-4 Promoter Activity in Jurkat T Cells—Our biochemical data showed that YY-1 can interact with four binding sites in the proximal IL-4 promoter. Sequence inspection and computer analysis did not detect additional YY-1-binding sites within ~1000 bp surrounding the IL-4 transcription start site. To test the functional significance of YY-1 in regulating IL-4 promoter activity, we next studied the effect of cotransfecting an antisense YY-1 expression vector with different promoter constructs into Jurkat T cells. These experiments were prompted by the observations that (i) the IL-4 promoter is constitutively active in transiently transfected Jurkat cells (30) and (ii) YY-1 is a constitutive nuclear protein in these cells (Fig. 2). We used both a full-length promoter construct (270 luc) and a minimal construct containing only the Y0 site (145 luc; see “Experimental Procedures”) together with an antisense YY-1 vector that has been shown to inhibit glucocorticoid receptor expression by ~50% in other systems (32). Interestingly, cotransfection of antisense YY-1 inhibited transcription driven by the full-length IL-4 promoter by ~80% (Fig. 5). The activity of the minimal construct 145 luc was also reduced by antisense YY-1, although this result did not reach statistical significance (p = 0.06). Transfection of antisense YY-1 did not appear to affect cell growth (see “Experimental Procedures”). These results suggest that YY-1 contributes to constitutive IL-4 promoter activity in Jurkat cells.

FIG. 5. Antisense YY-1 inhibits constitutive IL-4 promoter activity in Jurkat T cells. An antisense YY-1 expression vector (YY1 a.s.; gray bars) or empty vector (white bars) was cotransfected with the indicated reporter constructs, followed 18 h later by cell lysis and analysis of reporter gene activity by luminesmetry. Results are expressed relative to the constitutive activity of each construct and are the means ± S.E. of three experiments. *, p < 0.05. Antisense YY-1 did not affect cell growth as determined by counting viable cells using trypan blue exclusion and comparing total protein content in lysates of control and antisense-transfected cells. wt, wild-type.

YY-1 Activates the IL-4 Promoter in Two Different Jurkat Sublines, but Not in COS-7 Cells—We next overexpressed YY-1 and reasoned that increasing the cellular concentration of this factor would further enhance IL-4 promoter activity. In these experiments, Jurkat cells were cotransfected by electroporation with a series of IL-4 promoter constructs and a wild-type YY-1 expression vector. Cotransfection of YY-1 resulted in a concentration-dependent enhancement of transcription driven by the full-length IL-4 promoter (data not shown) as well as of transcription driven by 145 luc (Fig. 6A). Western blot analysis confirmed that total cellular YY-1 content was also increased in a concentration-dependent manner in these cells (Fig. 6A). Overexpressed YY-1 also enhanced IL-4 promoter activity in transiently transfected JTAg cells, a subline of Jurkat cells stably transfected with the 5V40 T antigen (Fig. 6B; see “Experimental Procedures”). In both JTAg and standard Jurkat cells, the expression of YY-1 was confined largely to the cell nucleus, indicating that the subcellular localization of overexpressed YY-1 was faithfully regulated (Fig. 6B and data not shown). The detection of some YY-1 within the cytoplasmic fraction likely reflects ongoing expression of YY-1 from the transfected construct. In parallel experiments, we examined the effects of overexpressing YY-1 on IL-4 promoter activity in non-lymphoid COS-7 cells. IL-4 145 luc was constitutively active in these cells, as was reported for the human IL-4 promoter in non-lymphoid HeLa cells (38). Interestingly, cotransfection of YY-1 sufficient to increase total cellular YY-1 expression as determined by Western blotting down-regulated the IL-4 promoter in COS-7 cells (~70% inhibition; n = 4) (data not shown). Thus, IL-4 promoter enhancement by YY-1 appears to be cell type-specific.

The Y0 Element Is Critical for YY-1-dependent IL-4 Promoter Activity in Jurkat T Cells—The observation that 145 luc, which contains only the Y0 element, can be transactivated by overexpressed YY-1 suggests that the Y0 element is critical for YY-1-dependent IL-4 promoter activity. To test this hypothesis, we mutated Y0 in the context of the full-length promoter to generate the construct 270 luc Y0mut and studied promoter activity under a variety of experimental conditions. Strikingly, the constitutive activity of 270 luc Y0mut was significantly reduced compared with that of the wild-type construct (Fig. 7A). In addition, whereas overexpression of YY-1 transactivated the wild-type IL-4 promoter, the activity of 270 luc Y0mut was not enhanced under similar conditions (Fig. 7A). To ensure that these observations were not due to subtle differences in transfection efficiency, we normalized the activity of 270 luc Y0mut and the wild-type reporter to an internal control plasmid co-transfected in parallel. When analyzed in this fashion, the constitutive activity of the Y0 mutant construct was still significantly reduced and was not enhanced by overexpressed
YY-1 Enhances IL-4 Gene Expression in Nontransformed Human T Cells—We next studied the ability of YY-1 to regulate IL-4 promoter activity in nontransformed human peripheral blood T cells. IL-4 promoter activity was undetectable in unstimulated cells, but was inducible following activation of Ca<sup>2+</sup> and protein kinase C-sensitive signaling pathways (Fig. 8). Interestingly, cotransfection of YY-1 resulted in a concentration-dependent, 2–8-fold enhancement of promoter activity (Fig. 8A). Similar results were obtained when normalizing luciferase activity to a cotransfected internal control vector (−5-fold enhancement) (data not shown). We compared the transactivating ability of YY-1 with that of two factors known to enhance IL-4 gene expression in T cells, viz. NFATp and NFATc, by cotransfecting expression vectors encoding each of these factors (see “Experimental Procedures”). YY-1 activated the IL-4 promoter to a similar degree compared with the NFAT proteins. Importantly, the secretion of IL-4 protein from YY-1-cotransfected peripheral blood T cells was significantly increased (Fig. 8B), indicating that overexpressed YY-1 enhances endogenous IL-4 gene expression.

**Experimental Procedures**

**Fig. 6.** Cotransfection of YY-1 results in concentration-dependent enhancement of IL-4 promoter activity in Jurkat cells. A, Jurkat cells were cotransfected by electroporation with 145luc (5 µg) and the indicated amount of an SV40-driven YY-1 expression vector (pSG5-YY-1; see “Experimental Procedures”) or empty vector to keep total DNA constant. Eighteen hours later, cells were lysed, and reporter gene activity was analyzed using luminometry. Twenty-microgram aliquots of whole cell lysates from cells cotransfected in duplicate with 5 µg (lanes 2 and 3), 10 µg (lanes 4 and 5), and 20 µg (lanes 6 and 7) of expression vector were analyzed for YY-1 expression by Western blotting (lower panel). Results are from one experiment performed in duplicate and are representative of three.

B, YY-1 also strongly enhanced the IL-4 promoter in a subline of Jurkat cells expressing the SV40 T antigen (JTAg (JTAg) cells). Transfections were carried out with 10 µg of pSG5-YY-1 and analyzed as described for A. Results are the means of two independent experiments. Twenty-micrograms aliquots of cytoplasmic (Cyt; lanes 1 and 3) and nuclear (lanes 2 and 4) lysates from JTAg cells cotransfected with empty vector or the wild-type (wt) YY-1 vector were analyzed by Western blotting (lower panel). A faint cytoplasmic YY-1-specific band was visible upon longer exposure in lane 1.

YY-1 (Fig. 7C). This suggests that in the context of the full-length promoter, the Y0 site is critical for constitutive YY-1-dependent promoter activity. To exclude the possibility that disruption of the Y0 site affected the adjacent NFAT-binding site, we used a recombinant fragment of the NFATp DNA-binding domain in EMSA. In these experiments, we found that NFAT bound equally well to the wild-type and Y0 mutant oligonucleotide probes (data not shown). This is supported by the observation that calcium inducibility of 270lucY0mut was not appreciably hindered compared with that of wild-type 270luc (Fig. 7C). In addition, to exclude the possibility that the TgtaTTC−59 mutation used in 270lucY0mut generated a new protein-binding site, we used an oligonucleotide encompassing this sequence in additional EMSAs with Jurkat nuclear extracts. In these experiments, only the YY-1-specific band was found to be inhibited, and no new binding activities were observed (data not shown). Therefore, the reduced activity of the Y0 mutant is due to specific inhibition of YY-1 binding to its cognate site in this region.

**An Intact YY-1 DNA-binding Domain Is Required for IL-4 Promoter Transactivation**—To determine whether DNA binding by YY-1 was required for IL-4 promoter activity, we synthesized a mutant YY-1 expression vector disrupting three of the four zinc-finger domains (ZFDmut; see “Experimental Procedures”) known to be required for DNA binding. We studied the ability of both the wild-type and ZFDmut constructs to transactivate different IL-4 promoter constructs in transient transfection assays. Unlike wild-type YY-1, which enhanced the activity of each construct examined, cotransfection of ZFDmut did not transactivate any of the reporter constructs (Fig. 7B). Similar results were obtained using transiently transfected JTAg cells (data not shown) and when normalizing data to an internal control reporter plasmid (Fig. 7D). Taken together with the results obtained from the site-directed mutagenesis experiments, these data suggest that YY-1 transactivates the IL-4 promoter by directly binding to the proximal promoter.
the P5 promoter of adeno-associated virus (46), the number of other defects (45). Since its original isolation as a repressor of results in peri-implantation lethality secondary to neural and protein secreted likely reflect cell toxicity following electroporation.

**Experimental Procedures**

Cotransfection of YY-1 (but not empty expression vector) significantly enhanced IL-4 protein secretion. Values are means ± S.E. of four experiments. *, p < 0.05. The low levels of IL-4 protein secreted likely reflect cell toxicity following electroporation.

**DISCUSSION**

Because of its importance in allergic and inflammatory immune reactions, the molecular regulation of IL-4 gene expression has come under intense scrutiny (15, 16, 39). In activated T cells, IL-4 expression is controlled at the transcriptional level by the coordinated actions of multiple transcription factors interacting with a proximal promoter region (40). Here we report the novel observations that the transcription factor YY-1 bound to multiple elements within the IL-4 promoter and that YY-1 enhanced IL-4 gene expression in T cells. Using site-directed mutagenesis, we found that a proximal YY-1 element (termed Y0) was critical for both constitutive and YY-1-dependent IL-4 promoter transactivation in Jurkat cells. Support for an enhancing role for the Y0 element is provided by our recent observation that CP-2 is a critical transactivator of the IL-4 promoter (30) and is supported by the detection of direct interactions between YY-1 and CP-2 in Jurkat cells (50). Studies aimed at identifying the role of cofactors in YY-1-dependent IL-4 gene expression are underway.

IL-4 gene expression is regulated at the transcriptional level by a proximal promoter and other regulatory elements. Although essential for tissue-restricted gene expression (29), the IL-4 promoter alone is a relatively weak activator of transcription. We (61) and others (62) previously showed that the promoter is inducible ~3-fold with a calcium signal alone, reflecting the need for NFAT activation. These observations underscore the importance of carefully controlling for the efficiency of transient transfections, as was done throughout this work. Based on cotransfection experiments and site-directed mutagenesis of the IL-4 promoter, we have shown that the Y0 element is required for YY-1-dependent promoter activation. Support for an enhancing role for the Y0 element is provided by a previous study of the mouse IL-4 promoter by Hodge et al. (37), who found that mutation of nucleotides within the murine counterpart of the Y0 sequence (~50TCATTT~54 to ~59caagTTT~54) in the context of a 157-bp reporter construct abrogates anti-CD3 antibody-induced IL-4 promoter activity in D10 cells. Although not addressed in that report, our finding that D10 cells constitutively express nuclear YY-1 suggests a role for YY-1 in mouse IL-4 promoter activity in D10 cells. Because the P0 NFAT and Y0 YY-1 sites are intimately associated, precise nucleotide substitutions were needed to distinguish between the cognate sites in each element (Fig. 4). Thus, previous mutational analyses in which the ability of YY-1 to

---

**Figure 8.** Cotransfected YY-1 enhances IL-4 promoter activity and protein expression in primary T cells. A, peripheral blood T cells were transfected with a 235-bp IL-4 promoter construct (235luc, 5 μg) together with expression vectors encoding YY-1, NFATc, and NFATp (see “Experimental Procedures”). Cells were incubated with or without A23187 (A2; 1 μM) plus phorbol 12-myristate 13-acetate (PMA; 20 ng/ml) for 18 h, followed by cell lysis and analysis of reporter gene activity using luminometry. Note that cotransfection of YY-1 enhanced IL-4 promoter-driven transcription in a concentration-dependent manner. B, IL-4 protein secretion was measured in cell supernatants using a sensitive enzyme-linked immunosorbent assay (see “Experimental Procedures”). Cotransfection of YY-1 (but not empty expression vector) significantly enhanced IL-4 protein secretion. Values are means ± S.E. of four experiments. *, p < 0.05. The low levels of IL-4 protein secreted likely reflect cell toxicity following electroporation.
bind to the IL-4 promoter was not accounted for (see, for example, P0 mutants in Refs. 11 and 63) need to be reevaluated. The functional role(s) of the other YY-1-binding sites identified in this study remain to be determined. However, the observations that (i) mutation of Y0 alone in the context of the full-length promoter abrogated YY-1-dependent transactivation and (ii) overexpressed YY-1 was able to induce transcription in a construct containing only Y0 (1451uc) suggest that this proximal element alone plays a critical role in regulating IL-4 transcriptional activation.

YY-1 has recently been shown to regulate the expression of several T cell cytokines. The interferon-γ promoter contains at least two YY-1-binding sites, and YY-1 was initially shown to inhibit IFN-γ promoter activity in Jurkat cells in part by competing for AP-1 binding (64). However, in a more recent report, Sweetser et al. (65) concluded that YY-1 acts in a more complex fashion to regulate the IFN-γ promoter. They found that YY-1 cooperates with NFAT to activate the IFN-γ promoter in primary mouse splenocytes, but not in Jurkat cells. YY-1 was also shown to down-regulate the expression of the IL-5 promoter in a specific human T cell line (66). Repressor activity appears to involve YY-1 together with Oct-1 and octamer-like proteins (66). These results underscore the complex nature of transcriptional regulation by YY-1 and suggest that the effect of YY-1 on cytokine gene expression will be highly dependent on the promoter context.

Taken together with our findings that YY-1 enhances IL-4 promoter-driven transcription, the apparent ability of YY-1 to differentially regulate IFN-γ and IL-5 promoter activities (65, 66) suggests that YY-1 does not play a dominant role in the polarization of cytokine gene expression in Th cells. In support of this notion, when we analyzed nuclear extracts from established Th1 (AE7) and Th2 (D10) clones by Western blotting, we were unable to detect YY-1. Therefore, YY-1 expression will be highly dependent on the protein that is involved in the specific function of YY-1 and suggest that the effect of YY-1 on cytokine gene expression will be highly dependent on the promoter context.
62. Paliogianni, F., Hama, N., Mavrothalassitis, G. J., Thyphronitis, G., and Boumpas, D. T. (1996) Cell. Immunol. 168, 33–38
63. Takemoto, N., Koyano-Nakagawa, N., Arai, N., Arai, K., and Yokota, T. (1997) Int. Immunol. 9, 1329–1338
64. Ye, J., Cippitelli, M., Dorman, L., Ortaal, J., and Young, H. (1996) Mol. Cell. Biol. 16, 4744–4753
65. Sweetser, M. T., Hoej, T., Sun, Y. L., Weaver, W. M., Price, G. A., and Wilson, C. B. (1998) J. Biol. Chem. 273, 34775–34783
66. Mordvinov, V. A., Schwenger, G. T., Fournier, R., De Boer, M. L., Peroni, S. E., Singh, A. D., Karlen, S., Holland, J. W., and Sanderson, C. J. (1999) J. Allergy Clin. Immunol. 103, 1125–1135
67. Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Pathan, C. G., and Glimcher, L. H. (2000) Cell 100, 655–669
68. Kelso, A., Groves, P., Ramm, L., and Doyle, A. G. (1999) Int. Immunol. 11, 617–621
69. Walowitz, J. L., Bradley, M. E., Chen, S., and Lee, T. (1998) J. Biol. Chem. 273, 6656–6661
70. Bovolenta, C., Camorali, L., Lorini, A. L., Vallant, G., Ghezzi, S., Tambussi, G., Lazzarin, A., and Poli, G. (1999) J. Immunol. 163, 6892–6897

YY-1 Enhances IL-4 Gene Expression
Yin-Yang 1 Activates Interleukin-4 Gene Expression in T Cells
Jia Guo, Vincenzo Casolaro, Edward Seto, Wen-Ming Yang, Cindy Chang, Maria-Cristina Seminario, Judith Keen and Steve N. Georas

J. Biol. Chem. 2001, 276:48871-48878.
doi: 10.1074/jbc.M101592200 originally published online October 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101592200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 70 references, 37 of which can be accessed free at http://www.jbc.org/content/276/52/48871.full.html#ref-list-1