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Identification of *cis*-Acting Regulatory Elements Controlling Interleukin-4 Gene Expression in T Cells: Roles for NF-Y and NF-AT$_c$

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Activity of the murine interleukin-4 (IL-4) promoter was localized to several *cis*-acting elements present within the first 300 bp from the transcriptional initiation site. Five repeated elements, P0 to P4, that share the common consensus ATTTTCCNNT were located between -40 and -250, and each was shown to interact with the T-cell-specific factor NF(P). These distinct P sites appear functionally interchangeable and cooperatively confer cyclosporin A-sensitive and ionomycin-inducible promoter activity. NF(P) may be closely related to the cytoplasmic component of NF-AT (nuclear factor of activated T cells), a T-cell-specific factor essential for IL-2 gene transcription, as judged from indistinguishable molecular weights and protease fragmentation patterns of UV-photolabeled factors. Also, we identified an element in the IL-4 promoter with homology to the Y box common to all major histocompatibility complex class II gene promoters. Our data show that the IL-4 promoter Y box $^{-114}$CTGATTGG^{-107} significantly enhances overall promoter activity, since point mutations within this element diminish promoter activity by 85%. The factor binding this region is indistinguishable from the cloned nuclear factor NF-Y, as judged from interactions with specific anti-NF-Y monoclonal and polyclonal antibodies. Last, we point out the presence of two sites that share sequence identity to the OAP region of the ARRE-1 site within the IL-2 promoter (K. S. Ullman, W. M. Flanagan, C. A. Edwards, and G. R. Crabtree, Science 254:558–562, 1991). These regions, $^{-85}$GTGTAATA^{-78} and $^{-245}$GTGTAATT^{-238}, reside adjacent to the NF(P) binding sites P1 and P4 and bind a distinct nuclear factor.

The T helper 1 (Th1) and Th2 T-cell subsets are fully differentiated CD4$^+$ T cells that promote predominantly cellular or humoral immune responses through secretion of distinct patterns of cytokines (7, 37). Th1 cells produce interleukin-2 (IL-2), gamma interferon, and lymphotoxin and promote cell-mediated immunity for elimination of viruses and intracellular parasites (38, 43, 47). In contrast, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 and promote humoral immunity against extracellular pathogens through activation of B cells and regulation of isotype switching (16, 38). Several exogenous factors, including IL-12, transforming growth factor β (TGF-β), and IL-4, can influence the development of undifferentiated CD4$^+$ T cells toward either the Th1 or Th2 phenotype (26, 27, 44, 50). IL-4 appears essential for induction of Th2 differentiation. First, IL-4 directly promotes Th2 development from naive CD4$^+$ T cells by stimulating the production of IL-4 and other Th2 cytokines while suppressing production of IL-2 and gamma interferon (26, 44, 50). Second, neutralization of IL-4 during primary stimulation of naive CD4$^+$ T cells promotes Th1 phenotype development in vitro (26). Third, mice with germ line disruption of the IL-4 gene fail to generate Th2 cells in vivo (31). The mechanisms by which the absence or presence of IL-4 induces Th1- or Th2-specific cytokine production remain undefined. Since cytokine secretion is controlled at the level of transcription (9), understanding Th1 and Th2 differentiation requires thorough identification of the *trans*-acting factors controlling cytokine gene expression.

The promoter/enhancer region of the IL-2 gene provides a well-characterized model for the regulation of cytokine gene expression. Sites for several transcription factors, including NF-AT (nuclear factor of activated T cells), OAP, Oct-1, Oct-2, AP-1, CD28RC, and NF-κB, reside within a region extending approximately 300 bp upstream of the transcriptional start site (14, 19, 25, 29, 30, 45, 46). NF-AT is composed of a phorbol 12-myristate 13-acetate (PMA)-inducible nuclear component and a preexisting T-cell-specific cytoplasmic component, whose nuclear translocation is inhibited by cyclosporin A (CsA) treatment (18). This factor along with the other transcription factors of the IL-2 promoter explains many characteristics of the IL-2 gene, such as the T-cell-restricted and activation-dependent expression, yet does not account for its exclusive expression by Th1-type T cells.

By comparison, characterization of the transcriptional regulation of other cytokine genes is less complete. IL-4 has a limited range of expression, being produced only by mast cells, basophils, and the Th2 subset of CD4$^+$ T cells. A potential enhancer region located within the first intron of the IL-4 gene appears to be mast cell specific (23). Two studies have examined IL-4 gene regulation in T cells by using the human T-cell leukemia line Jurkat (1, 32). Jurkat cells produce abundant IL-2 message, but their expression of IL-4 is minimal in comparison with that of Th2 cells (26, 32), potentially limiting their use in examining the transcriptional regulation of IL-4. Nonetheless, one study identified a potential negative regulatory element (NRE) in the human IL-4 promoter that interacts with a T-cell-specific and a ubiquitous factor (32). NRE represses a positive regulatory element (PRE) contained within a 70-bp region located downstream of the NRE site. The sequences directly responsible for transcriptional activity of the PRE were not identified. A second study identified a separate *cis*-acting element, the P sequence, and showed that this sequence mediated PMA-ionomycin-induced transcriptional activation via the

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nuclear factor NF(P) (1). The contribution of these sites to Th1- and Th2-specific IL-4 gene regulation is not known.

In a separate study (22), using an IL-4-producing subline of the murine thymoma EL4, we extended the characterization of the P sequence and NF(P). We demonstrated that NF(P)-dependent transcription is activated by ionomycin treatment alone and does not require PMA. NF(P)-dependent transcriptional activity is inhibited by CsA. NF(P) expression is T cell specific, being absent in B cells and fibroblasts, in contrast to other factors that interact with *cis*-acting sites within the IL-4 promoter. In this report, we show that in addition to the one previously identified P sequence, four other NF(P) binding sites reside within the proximal 300-bp promoter region. Each of these newly identified sites can interact with NF(P), and as a group, these five NF(P) binding sites share a consensus sequence, ATTT TCCNNT, that we show is sufficient for NF(P) binding. The presence of at least two of these sites is needed for ionomycin-inducible promoter activity. Our photo-cross-linking data suggest that NF(P) may be closely related to the cytoplasmic component of NF-AT (35, 40), which has been shown recently to bind directly to a specific DNA sequence independently of the nuclear component (35). Additionally, we identify two previously unrecognized *cis*-acting regulatory elements within the IL-4 promoter. First, the Y box, $^{-114}$CTGATTGG^{-107}, also present in all major histocompatibility complex (MHC) class II gene promoters (4, 20), interacts with the ubiquitous nuclear factor NF-Y (55) and contributes significantly to promoter activity. Finally, we point out the presence of two sites that share sequence identity to the OAP region of the IL-2 promoter (53) that may interact with c-Jun dimers (54). These two regions, $^{-85}$GT GTAATA^{-78} and $^{-245}$GTGTAATT^{-238}, reside adjacent to the NF(P) binding sites P1 and P4 and bind a distinct factor.

MATERIALS AND METHODS

Selection and characterization of cell lines. IL-2 and IL-4 production by 11 sublines of the murine thymoma EL4 (gifts from O. Kanagawa, St. Louis, Mo.) at 5×10^5 cells per ml after stimulation with 50 ng of PMA per ml and 1 μM ionomycin was measured by enzyme-linked immunosorbent assay as previously described (26). One subline, EL4.8, produced 100 to 200 U of IL-4 per ml, whereas the remaining sublines produced undetectable levels of IL-4 (<1 to 3 U/ml). One of these, EL4.OVA, was selected for use as a non-IL-4-secreting control cell line for transfection studies. All cell lines were maintained in Iscove's modified Dulbecco's Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 0.1 mM minimal essential medium nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 mM β-mercaptoethanol.

Plasmid constructs. To create a high-copy-number luciferase reporter plasmid, the 3.8-kb *Pst*I-*Nde*I fragment from PXP-2 (39) was blunted and cloned into the blunted *Sac*I and *Kpn*I sites of Bluescript II (SK−) (Stratagene, La Jolla, Calif.). The resulting construct, pBS-LUC, maintains the tandem upstream transcriptional terminators, multiple cloning site, luciferase coding sequence, and downstream simian virus 40 splice and polyadenylation sites present in the original PXP-2 vector.

For controls, CMV-LUC was made by cloning the 760-bp *Bgl*II fragment, containing the immediate-early promoter region from cytomegalovirus, from pCMV-IE (gift of S. J. Korsmeyer, St. Louis, Mo.) (48) into the unique *Bgl*II site of pBS-LUC. CMV-CAT was made by placing the same 760-bp fragment into the *Bam*HI site of pBS-CAT (gift of D. Y. Loh, St. Louis, Mo.) (33). IL2-LUC was made by placing a 2.1-kb *Pst*I fragment from P407 (gift of O. Kanagawa, St. Louis, Mo.), containing nucleotides −2060 to +40 of the murine IL-2 promoter and 5′ flanking region (13), into the *Bam*HI-*Xho*I sites in pBS-LUC.

IL-4 genomic cloning and promoter constructs. The DNA fragment extending from −741 to +60 relative to the start site of transcription of the murine IL-4 gene (2, 41) was generated by using the polymerase chain reaction (PCR) with BALB/c genomic liver DNA as the template and two *Bam*HI-tailed oligonucleotides, 4-3 and 4-4. Oligonucleotide 4-3 extends from −741 to −722 and oligonucleotide 4-4 extends from +60 to +41 relative to the start site of transcription of the murine IL-4 promoter. The 800-bp PCR fragment was digested with *Bam*HI and cloned into the *Bgl*II site of pBS-LUC to create IL4-LUC.

An EMBL-3 phage library of BALB/c genomic DNA (Clontech Laboratories, Palo Alto, Calif.) was screened by using the 800-bp IL-4 promoter DNA fragment as a probe to identify two phage clones containing 12- and 15-kb inserts. From one phage clone, a 6-kb *Bam*HI fragment was identified to lie closely upstream of the 800-bp promoter region by restriction mapping. This 6-kb *Bam*HI fragment was cloned into the *Bam*HI site of IL4-LUC to produce the plasmid pB6-LUC.

For 5′ promoter deletions, DNA fragments were produced by PCR using 5′ oligonucleotides, tailed with *Sal*I sites, and annealing over 20 bp of the IL-4 promoter, beginning at −455 (DM1), −353 (DM2), −262 (DM3), −189 (DM4), −140 (DM5), −115 (DM6), −79 (DM7, P1-0), −58 (DM8), and −48 (DM9) relative to the start site of transcription. PCR reaction products, made by using each 5′ DM oligonucleotide with the 3′ oligonucleotide 4-4 (located at +60) described above and an IL-4 genomic template, were *Sal*I-*Bam*HI digested and cloned into the *Sal*I-*Bgl*II sites of pBS-LUC to produce constructs DM1 through DM9 (see Fig. 2A).

For internal promoter deletions, DNA fragments were produced by PCR using 3′ oligonucleotides, tailed with *Xho*I sites, and annealing over 20 bp to the IL-4 promoter, located at −80 (ID15), −129 (ID16), −217 (ID17), −288 (ID18), or −407 (ID19). PCR reaction products, made by using each of these 3′ oligonucleotides together with the 5′ oligonucleotide 4-3 (located at −741) and an IL-4 genomic template, were digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI-*Sal*I sites of DM8 to produce constructs ID15 through ID19 (see Fig. 2B). Various regions of the IL-4 promoter, generated as PCR products by using 5′ oligonucleotides with *Sal*I tails and 3′ oligonucleotides with *Xho*I tails, were cloned into the *Sal*I site of DM8; the region from −262 to −129 was used to produce p3.5-LUC (Fig. 6, construct c); the region from −189 to −80 to produce p5-LUC (Fig. 6, construct d); the region between −140 and −80 to produce p7-LUC (Fig. 6, construct e).

To replace site P1 in P1-0 with the sequences of sites P2, P3, and P4, synthetic double-stranded oligonucleotides CS2 (TCGACGTAAATTTTCCTGTGC), CS3 (TCGACTTTCAT TTTCCAATTC), and CS4 (TCGACGTGTAATTTCCTA TGC), each containing *Sal*I-*Xho*I overhanging ends, were cloned into the *Sal*I site of DM8 to produce constructs P2-0, P3-0, and P4-0, respectively (Fig. 7, constructs b to d). To selectively mutate site P0 in each of these constructs, the synthetic double-stranded oligonucleotides P1M (CATAAA ATTTTCCAATGTAAACTCAGGC), P2M (CGGTAAATT TTCCTGTGAAACTAGAGGC), P3M (CGGTTTCATTT

TCCAATTGGTCTGAAGGC), and P4M (CGGTGTAAT TTCCTATGCTGAAACAGGC), containing *Bam*HI-*Xho*I overhangs (not shown), were cloned into the *Bam*HI-*Sal*I site of DM9 to produce P1-X, P2-X, P3-X, and P4-X (Fig. 7, constructs e to h), in which the consensus of the P0 site is disrupted while the spacing of the upstream P site relative to the TATA box is maintained. Each oligonucleotide contains the native flanking sequences around the respective P sites, with underlined bases representing where changes in sequence have been introduced to alter the P0 site in the final construct (Fig. 7).

For promoters containing 8- to 10-bp clustered point mutations, DNA fragments were produced by PCR using 5′ oligonucleotides with *Sal*I tails and one or two mutated bases and annealing over 19 bp of the IL-4 promoter beginning at −77 (DM-OAP), −87 (DM-F2), −96 (DM-B), −104 (DM-Y), and −114 (DM-A) relative to the start site of transcription. PCR products made by using each of these 5′ DM oligonucleotides with the 3′ oligonucleotide 4-4 (located at +41) and an IL-4 genomic template were cloned into the *Sal*I-*Bgl*II sites of pBS-LUC to produce plasmids DMOAP, DMF2, DMB, DMY, and DMA. Next, DNA fragments produced by PCR using 3′ oligonucleotides with *Xho*I tails and one or two mutated bases and annealing over 19 bp of the IL-4 promoter beginning at −88, −97, −105, −115, and −125 and the *Bam*HI-tailed 5′ oligonucleotide 4-3 (located at −741) were cloned into the *Bam*HI-*Sal*I sites of plasmids DMOAP, DMF2, DMB, DMY, and DMA, respectively, to produce the internal mutant IL-4 promoter constructs mOAP-LUC, mF2-LUC, mB-LUC, mY-LUC, and mA-LUC (Fig. 10, constructs e to a, respectively).

All plasmid constructs were twice banded in cesium chloride and verified by restriction mapping and by dideoxynucleotide sequencing, using Sequenase (U.S. Biochemical, Cleveland, Ohio).

Transient transfections. EL4.8 or EL4.OVA cells (10×10^6) were washed once and resuspended in 1 ml of complete Iscove's medium containing 20% fetal calf serum with 20 μg of the luciferase expression plasmid as indicated in the figure legends and 20 μg of CMV-CAT and electroporated in triplicate in 0.33-ml aliquots, using 0.4-cm cuvettes in a Bio-Rad gene pulser (Bio-Rad, Richmond, Calif.) at 280 V and 960 μF. After electroporation, cells were divided equally into separate wells as indicated by the conditions specified in the figure legends and after 12 h were treated with either medium alone, 1 μM ionomycin, 50 ng of PMA per ml, or both as indicated in the figure legends. CsA (100 ng/ml) was added 10 min before PMA-ionomycin stimulation when indicated. After 5 h of stimulation, cells were harvested and lysed in 100 μl of 250 mM Tris (pH 7.4) by three freeze-thaw cycles. Following centrifugation, the cell extracts were assayed for luciferase and chloramphenicol acetyltransferase (CAT) activity.

Luciferase and CAT assays. Luciferase activity was determined from 20 μl of cell extract in duplicate, using the luciferase assay substrate (Promega Corp., Madison, Wis.) with an Opticomp II automated luminometer (MGM Instruments, Hamden, Conn.), reading relative light activity for 20 s. CAT activity was assayed in duplicate by using [^{3}H]chloramphenicol during a 2-h incubation at 37°C as described previously (3). Butyrylated [^{3}H]chloramphenicol was extracted with pristane-xylene (2:1) and measured by liquid scintillation. All transfections were internally controlled by cotransfection with the CMV-CAT construct. After normalization to this control, the values for the experimental constructs were compared with the stimulated expression of IL4-LUC.

Preparation of nuclear extracts. Cells were either not activated or activated with 50 ng of PMA per ml and 1 μM ionomycin for 4 h prior to harvesting as indicated in the figure legends. Nuclear extracts were prepared as described by Go and Miller (21). Briefly, 5×10^7 cells were washed once with phosphate-buffered saline and once with cold buffer A and lysed in cold buffer A containing 0.2% Nonidet P-40. Nuclei were pelleted and resuspended in cold buffer B, and $(NH_4)_2SO_4$ was added to a final concentration of 0.3 M. Tubes were rotated at 4°C for 30 min, and the nuclear membranes were pelleted. The nuclear proteins from the supernatant were precipitated on ice with the addition of $(NH_4)_2SO_4$ to 1.5 M. Following centrifugation, the pellet was resuspended in 50 μl of buffer B. Protein concentrations were determined by using the Bio-Rad protein assay kit, and aliquots were frozen at −70°C.

Oligonucleotides. Complementary double-stranded oligonucleotides contained overhanging *Sal*I-*Xho*I ends and were synthesized by using an Applied Biosystems PCRmate (Applied Biosystems, Foster City, Calif.) or a Beckman Oligosynthesizer 1000 (Beckman Instruments, Fullerton, Calif.). These oligonucleotides (and their sequences, shown 5′ to 3′) were P0 (TCGACGTAAACTCATTTTCCCTTGGC), P1 (T CGACCTGGTGTAATAAAATTTTCCAATGTC), P2 (TCG ACCAATTGGTCTGATTTCACAGGAAAATTTACC), P3 (TCGACGTGTTTCATTTTCCAATTGGTC), P4 (TCGAC ATTATGGTGTAATTTCCTATGCC), SP (TCGACTAATA AAATTTTC), 4-5 (TCGACGAAAATTTACCTGTTTCTC TTTTTTCTCCTC), 5-1 (TCGACTTTTTCTCCTGGAAGA GAGGTGCTGATTGGC), 5-2 (TCGACGGAAGAGAGGT GCTGATTGGCCCAGAATAAC), 5-3 (TCGACTGCTGAT TGGCCCAGAATAACTGACAATCTC), 5-4 (TCGACCCC AGAATAACTGACAATCTGGTGTAATAAC), 5-5 (TCGA CGGAAGAGAGGTGCTGATTGGC), 5-6 (TCGACCCCA GAATAACTGACAATCTC), NFAT-1 (TCGACCAAAGA GGAAAATTTGTTTCATACAGAAGGC), AP-1 (TCGACA ATTCCAGAGAGTCATCAGAC), ARRE-1 (TCGACGAA AATATGTGTAATATGTAAAACATCGTC), TCF (TCGA CTAGATCTTCAAAGGAAGGC), OAP-4 (TCGACCTG GTGTAATAAAATC), IL4-Y (TCGACAGGTGCTGATTG GCCCAGC), Eα-Y (TCGACATTTTTCTGATTGGTTAAA AGTC), and MP (GATCATTTTCCGAT TAGTAAAAGGCTAG).

Electrophoretic mobility shift assays (EMSAs). Complementary double-stranded oligonucleotides containing overhanging *Sal*I-*Xho*I ends were radiolabeled with [^{32}P]dCTP, using the large fragment of DNA polymerase I. A total of 2.5×10^4 cpm (0.1 ng) of radiolabeled oligonucleotide probe was incubated in the binding reaction buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol) with 5 μg of nuclear extract, 2 to 4 μg of poly(dI-dC), and a 200-fold molar excess (20 ng) of unlabeled competitor oligonucleotides (as indicated in the figure legends) for 40 min on ice. Following binding, the 20-μl reaction mixture was electrophoresed through a nondenaturing 5% polyacrylamide gel at 150 V for 1.5 h at 4°C in 0.4× TBE buffer (1× TBE is 89 mM Tris [pH 8.2], 89 mM boric acid, and 2 mM EDTA).

For antibody supershifts in EMSAs (Fig. 9C), we used the following reagents: 7 μg of Y-A7 (mouse monoclonal antibody against NF-Y_A), 2 μg of Y-A1a (mouse monoclonal antibody against NF-Y_A), 2 μg of Y-A/C (rabbit polyclonal antiserum against the C-terminal peptide of NF-Y_A), or 2 μg

of Y-B (rabbit polyclonal antiserum against NF-Y_B). All antibodies are gifts of D. Mathis, Strasbourg, France.

UV cross-linking using photoreactive arylazide-labeled oligonucleotide probes. Oligonucleotides used in cross-linking studies were hP1 (TCGACCTAAAATTTTCCAATGTAAA CTCATCCGGTC), hexP (XGACCGG), hNFAT1 (TCGAC CAAAGAGGAAAATTTGTTTCATACAGAAGGC), and hexNF (XGCCTTCTG). The X position indicates an amine group introduced at the 5′ end by using Aminolink 2 (Applied Biosystems).

To introduce the photoreactive arylazide group (57) 4-azidosalicylic acid (ASA) into the 5′-amino-labeled oligomers, hexP and hexNF were resuspended in 50 mM $NaHCO_3$-$NaCO_3$ buffer (pH 9) at a concentration of 10 μg/ml. *N*-hydroxysuccinimidyl-ASA (Pierce, Rockford, Ill.) resuspended in dimethlformamide (DMF) was added in a 50-fold molar excess to the oligomer and allowed to react in the dark at room temperature for 4 h. After ethanol precipitation, each ASA-labeled oligomer was used to anneal to the corresponding oligonucleotide template (hP1 or hNFAT1), and radiolabeled probe was synthesized by using Klenow enzyme, [^{32}P]dCTP, and unlabeled dGTP, dATP, and dTTP (11). This reaction produced double-stranded oligonucleotide probes with a photoreactive ASA group covalently attached to the [^{32}P]dCTP-labeled DNA strand. Binding reactions to PMA-ionomycin-stimulated EL4.8 nuclear extracts with use of these probes were carried out in the dark as described above except that 7.5×10^4 rather than 2.5×10^4 cpm of probe was used for each reaction. Samples were irradiated with a 366-nm UV source at a 5-cm distance for 15 min on ice, boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer for 5 min, and electrophoresed through a standard Laemmli SDS-polyacrylamide gel (5 or 8% as indicated) at 50 mA for 2 to 5 h.

RESULTS

Identification and derivation of IL-4-secreting T-cell lines. As a starting point for understanding the basis of phenotype selective cytokine expression, this study identifies *cis*-acting elements regulating the IL-4 gene in T-cell tumor lines. To obtain transfectable IL-4-producing cell lines, we examined the inducible cytokine production from a number of EL4 sublines. EL4.8 produced 100 to 200 U of IL-4 per ml upon PMA-ionomycin stimulation, in contrast to 10 other EL4 sublines that produced less than 3 U/ml upon stimulation. EL4.OVA produced levels of IL-2 similar to those produced by EL4.8 and provided a convenient control for comparisons with EL4.8 in transfection studies.

To evaluate EL4.8 as a valid choice for transient transfections, we examined the relative expression of IL-2 promoter and IL-4 promoter constructs in EL4.8 and EL4.OVA cells. After normalization for transfection efficiency, the relative levels of IL-2 expression were similar between EL4.8 and EL4.OVA, whereas IL-4 reporter constructs were significantly more active in EL4.8 than in EL4.OVA (Fig. 1). Thus, the higher expression of endogenous IL-4 in EL4.8 than in EL4.OVA is accompanied by a higher expression of IL-4 promoter constructs during transient transfections.

Sequences within the IL-4 promoter required for inducible expression. To determine whether an 800-bp promoter region was useful for initial examination of the IL-4 promoter, we compared the activity of this 800-bp region (IL4-LUC) with that of a construct containing an additional 6 kb of upstream DNA (pB6-LUC). After normalization for transfection efficiency and copy number, these constructs showed no significant difference in activity (data not shown). Thus, we examined a series of successive 5′ deletions of the 800-bp promoter region for constitutive and inducible transcriptional activity in transient transfection of EL4.8 cells. Deletion of the region between −741 and −455 produced no significant change in activity compared with the full 800-bp region (Fig. 2A). However, deletion of the region between −455 and −262 reduced activity by approximately 60% (Fig. 2A). Notably, successive deletion of more proximal regions produced only slight variations, with a consistent increase in activity to approximately 50 to 70% of full activity expressed by construct DM7, which contains only 79 bp upstream of the initiation site (Fig. 2A). Markedly, the subsequent deletion of the region between −79 and −58 produced a dramatic loss of virtually all constitutive and inducible activity (Fig. 2A, DM7 and DM8).

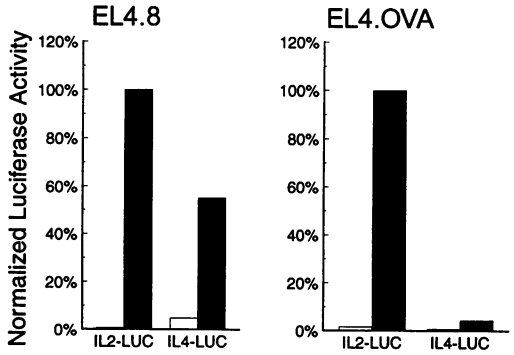

FIG. 1. Transient expression of IL-2 and IL-4 reporter constructs parallels endogenous IL-2 and IL-4 production by the EL4 sublines EL4.8 and EL4.OVA. EL4.8 or EL4.OVA cells were cotransfected with 20 μg of either IL2-LUC or IL4-LUC and 20 μg of CMV-CAT as described in Materials and Methods. Relative luciferase activity from unactivated (open bars) or PMA-ionomycin-activated (closed bars) cells is presented as the percentage of IL2-LUC activity after normalization for transfection efficiency. The experiment was repeated twice with similar results.

The region between −79 and −58 contains a region previously identified as the P sequence by Abe et al. (1). The P sequence interacts with the nuclear factor NF(P) and was implied to be the only site within the IL-4 promoter required for inducible expression. To determine whether any additional elements contributed to promoter activity, we produced a series of internal deletions that eliminated the P sequence (Fig. 2B). ID15, an internal deletion construct which specifically removes the P sequence (nucleotides −79 to −58), showed significant activity compared with the full 800-bp region. In fact, selective deletion of the P sequence reduced activity by only 30% (Fig. 2B, ID15). Therefore, the P sequence was not essential for maintaining inducible promoter activity, implying that additional functional *cis*-acting elements resided within this 800-bp region. Next, we extended these internal deletions further into the 5′ upstream region to localize the limits of this activity. Deletion of increasingly larger internal regions produced a consistent and gradual decrease in promoter activity, with less than 5 to 10% of full activity remaining when 350 bp (−407 to −58) of the promoter had been deleted (Fig. 2B, ID15 to ID19). These results suggested that the majority of the promoter activity resides in *cis*-acting elements between −407 and −58 and was not exclusively located within the previously identified P sequence.

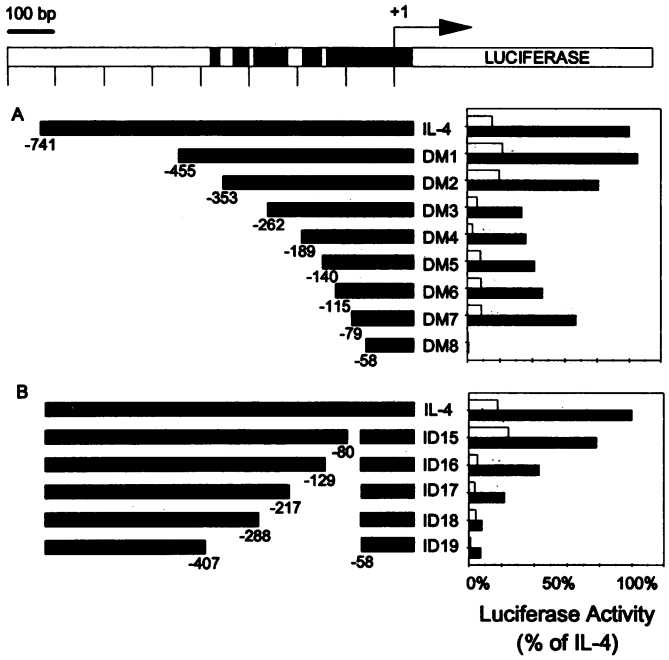

FIG. 2. Localization of regulatory elements between −407 and −58 relative to the start site of transcription for the murine IL-4 promoter. EL4.8 cells were cotransfected with 20 μg of the indicated luciferase plasmid and 20 μg of CMV-CAT and left unstimulated (open bars) or stimulated with PMA and ionomycin (closed bars). Activity of 5′ deletion constructs (A) or internal deletion constructs (B) is presented as a percentage of IL4-LUC (IL-4) activity after normalization for transfection efficiency. The region of the IL-4 promoter contained in each construct is indicated by the shaded bars at the left, with the number under each indicating the 5′ limit of the region. The diagram at the top represents the murine IL-4 promoter from −800 to +60, with +1 indicating the start site of transcription. The black boxes above the promoter region indicate areas with strong homology between human and mouse sequences, and the open box at the right indicates the luciferase coding sequence. The scale is shown by the black line (100 bp). All constructs are identical from −58 to +60 and contain the natural TATA box, initiation site, and 5′ untranslated regions from the murine IL-4 promoter. Similar results were obtained from four independent transfection experiments.

Multiple *cis*-acting elements within the IL-4 promoter interact with NF(P). Since substantial promoter activity remained when the P sequence was deleted from the IL-4 promoter (Fig. 2B, ID15), we examined the characteristics of this remaining activity. As previously shown, both the full length IL-4 promoter and P-sequence-dependent activity are primarily stimulated by ionomycin but not by PMA and are inhibited by CsA (22) (Table 1, IL4-LUC and DM7). Deletion of the P sequence from the full-length IL-4 promoter in ID15 left activity that also was stimulated by ionomycin and inhibited by CsA (Table 1). Constructs that eliminate the P sequence with increasingly larger internal deletions also showed these same characteristics (Table 1, ID16 to ID18). From this result, we inferred that the *cis*-acting elements located upstream of the P sequence have activation characteristics similar to those of the P sequence. In contrast, cytomegalovirus promoter activity was constitutive, being unaffected by treatment with PMA, ionomycin, or CsA. IL-2 promoter activity required both PMA and ionomycin treatment for optimal activity and was inhibited by CsA (Table 1).

TABLE 1. Ionomycin-inducible activity in IL-4 promoter fragments

Plasmid	Normalized luciferase activity (relative light units)[a]				
	−	+	−	+	+
	−	−	+	+	+
	−	−	−	−	+
IL4-LUC	4,467	10,745	20,763	28,442	3,582
DM7	1,564	2,554	4,985	7,171	1,178
ID15	3,325	6,999	11,050	10,875	4,509
ID16	769	2,800	7,682	8,524	1,124
ID17	670	1,832	3,102	4,539	778
ID18	726	985	1,054	1,130	698
IL2-LUC	1,760	27,777	7,348	125,103	2,200
CMV-LUC	598,070	715,196	642,603	694,115	722,535

[a] Twenty micrograms of the indicated luciferase plasmid and 20 μg of CMV-CAT were cotransfected into 16 × 10^6 EL4.8 cells as described in Materials and Methods. The transfected cells were divided equally into five wells and treated after 12 h with medium alone; 50 ng of PMA per ml; 1 μM ionomycin; both PMA and ionomycin; or PMA, ionomycin, and 100 ng of CsA per ml. CsA was added 10 min before PMA-ionomycin activation. After 5 h, cells from each condition were harvested, and luciferase and CAT activities were determined; luciferase activity was normalized as described in Materials and Methods. Data presented are means of duplicate determinations which varied less than 10%. The experiment was repeated three times with similar results. − and + indicate the absence and presence, respectively, of PMA (top), ionomycin (middle), and CsA (bottom) at the concentrations specified above.

These results suggested the possibility of additional sites related to the P sequence. Thus, we sought to identify other regions of the IL-4 promoter that might interact with NF(P). Various DNA fragments, spanning the region of the IL-4 promoter from −300 to −50, were tested for the ability to interact with NF(P) in EMSA (Fig. 3). Several regions distinct from the P sequence inhibited NF(P) binding to the radiolabeled P-sequence oligonucleotide P1. Three distinct upstream regions and one downstream region competed with the P-sequence oligonucleotide probe for NF(P) binding (Fig. 3). Several other regions of the IL-4 promoter as well as an AP-1 oligonucleotide (29) were inactive for NF(P) competition (Fig. 3), confirming the specificity of the observed inhibition. Thus, additional NF(P) binding sites that are distinct from the previously identified P sequence located at −79 to −58 may be present within the IL-4 promoter. To identify these sites, we aligned the sequences of the regions that were able to compete for NF(P) binding (Fig. 4). A common sequence motif, ATTTTCCNNT, was found within these regions, suggesting a consensus sequence for NF(P) binding that is contained within the previously identified P sequence (1). We refer to these sites as P0 through P4, with P0 being the most proximal element relative to the initiation site. P1 refers to the originally identified P sequence. Four of these asymmetric P sites are in the same orientation, while the P2 site is in the reverse orientation. No functional data for any of these new potential NF(P) binding sites have previously been reported.

To test whether this minimal consensus motif, ATTTTC CNNT, was sufficient for NF(P) binding, we used a 15-bp double-stranded oligonucleotide probe spanning the consensus sequence for analysis in EMSA. This probe, MP, bound a nuclear factor similar in mobility and sequence specificity to NF(P) (Fig. 5A). This complex formed by MP was completely inhibited by the competitor oligonucleotides P0, P1, P2, and P3, each containing the potential NF(P) binding site. Importantly, an oligonucleotide, SP, lacking the 3′ end of the P consensus was unable to compete for complex

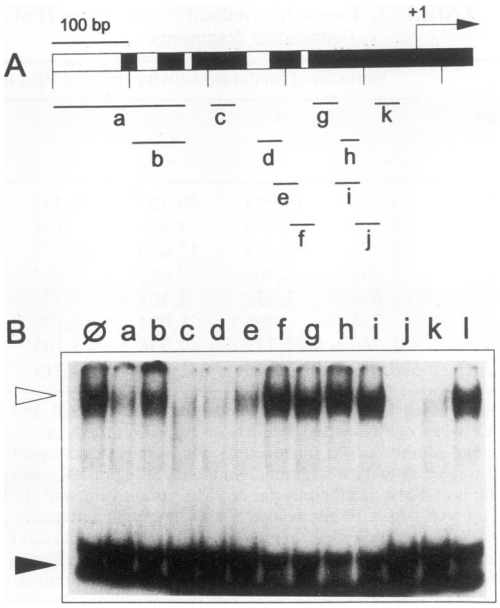

FIG. 3. Multiple regions of the IL-4 promoter interact with NF(P). (A) The location of each competitor is indicated beneath the diagram of the murine IL-4 promoter. The black boxes indicate regions of strong sequence conservation between human and mouse. The limits of each competitor region are −453 to −288 (a), −340 to −288 (b), −251 to −230 (c), −189 to −168 (d), −177 to −146 (e), −156 to −127 (f), −136 to −107 (g), −106 to −87 (h), −106 to −77 (i), −88 to −64 (j), and −65 to −46 (k). Oligonucleotides a and b were generated by PCR, while oligonucleotides c through k were synthesized as complementary double-stranded oligonucleotides. (B) Nuclear extracts from PMA-ionomycin-stimulated EL4.8 cells were incubated with 0.1 ng (2.5×10^4 cpm) of a ^{32}P-labeled P1 oligonucleotide probe in the presence of a 200-fold molar excess (20 ng) of various competitor oligonucleotides from the IL-4 promoter. Lanes: ϕ, absence of competitor; a to k, unlabeled oligonucleotides described for panel A; l, unlabeled AP-1 oligonucleotide. Specific NF(P) complex is indicated by the open arrowhead; free P1 probe is indicated by the closed arrowhead.

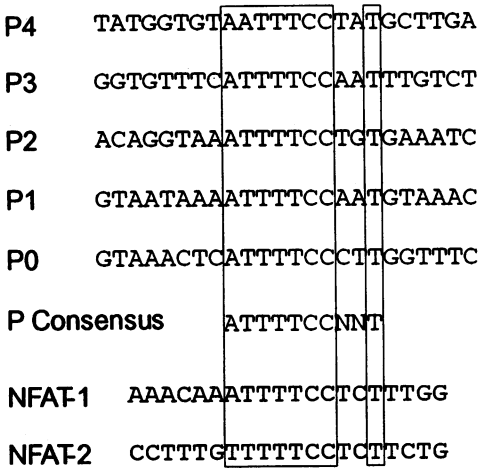

FIG. 4. A consensus sequence for NF(P) binding is repeated five times within the IL-4 promoter. Alignment of sequences from the regions that competed for NF(P) binding (Fig. 3) identifies a repeated motif, indicated by the boxed nucleotides. We refer to these sites within the IL-4 promoter as P4 for the most distal site through P0 for the most proximal site. P2 is positioned in the reverse orientation relative to the other P sites. The locations of these sites are as follows: P0, −57 to −48; P1, −75 to −66; P2, −151 to −160; P3, −182 to −173; and P4, −241 to −232. Homologous regions from the distal and proximal NF-AT sites (noncoding strand) of the murine IL-2 promoter are shown.

formation, implying that the full consensus was necessary for interactions with NF(P) (Fig. 5A). While TCF (58) and AP-1 oligonucleotides showed no inhibition of NF(P) complex formation with MP, the NFAT-1 (46) oligonucleotide completely blocked this complex (Fig. 5A), similar to its reported inhibition of NF(P) binding (22). Thus, the consensus sequence, ATTTTCCNNT, appeared sufficient for binding NF(P).

Not only did each of these newly identified P sites compete for NF(P) binding, but each had sufficiently high affinity for NF(P) to form stable complexes. Each of the ^{32}P-labeled probes P0 through P4 bound a complex with mobility similar to that of NF(P) binding to P1 (Fig. 5B). The sequence specificity of each of these complexes was similar to that of NF(P) labeled by P1, as shown by the similar patterns of inhibition by various oligonucleotide competitors (Fig. 5B). Furthermore, the MP oligonucleotide blocked complex formation by each P-site probe, whereas the SP oligonucleotide did not. Thus, these data strongly suggested that each of these newly identified P sites interacts with a complex identical or very similar to NF(P).

The P sites are functionally interchangeable and operate cooperatively. We next addressed the functional activity of these other NF(P) binding sites. Promoter regions containing various P sites in their natural arrangement were placed into construct DM8, which contains the P0 site (Fig. 6). P3.5-LUC, containing P4, P3, and P2, or P5-LUC, containing P3 and P2, restored 15 to 20% of full-length promoter activity (Fig. 6, constructs c and d), while P7-LUC, containing no additional P sites, had minimal activity (Fig. 6, construct e). For comparison, both DM7 and ID15 each retained only approximately 40% of full-length promoter activity (Fig. 6, constructs a and b). This result suggested that regions containing the P4, P3, and P2 sites in their natural arrangement are transcriptionally active but cannot completely restore full promoter activity.

To examine the transcriptional activity of each P site individually, we replaced P1 in construct P1-0 with each of the upstream P sites while maintaining the natural spacing of the P1 and P0 sites (Fig. 7). Construct DM8, containing only one P site (P0), has lost virtually all inducible activity (Fig. 7, construct i). Each of the P sites, P2, P3, and P4, could restore inducible activity to levels comparable to that produced with the P1 site (Fig. 7, constructs a to d). In each case, the activity was inducible by ionomycin treatment alone and was not enhanced by additional PMA treatment (data not shown). Thus, the upstream P2, P3, and P4 sites, within the context of an adjacent P0 site, can confer activity that is similar to that of the P1 site. Next we examined whether each upstream P site could function independently of the downstream P site (P0). To test this, we mutated the NF(P) binding consensus of the P0 site in each of the constructs P1-X through P4-X (Fig. 7, constructs e to h). This resulted in a near complete loss of ionomycin-inducible activity. Thus, the activity of constructs P1-0 to P4-0 requires two intact P sites, suggesting some cooperative interaction.

Potential relationship between NF(P) and NF-AT$_c$. The sequence similarity between the NF(P) binding consensus and NF-AT binding sites from the IL-2 promoter (Fig. 4) and

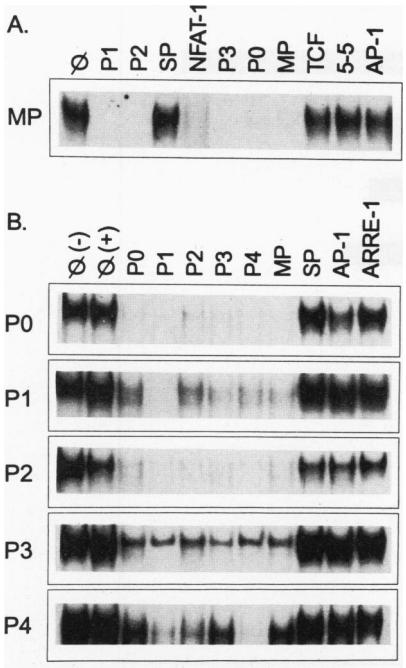

FIG. 5. NF(P) binds to a minimal consensus motif (MP) and to each of the repeated P sites. (A) Nuclear extracts from PMA-ionomycin-activated EL4.8 cells were incubated with a ^{32}P-labeled MP oligonucleotide probe (GATCATTTTCCGATC) in the absence (ϕ) or presence of a 200-fold molar excess of the indicated competitor oligonucleotides. (B) Nuclear extracts from unstimulated (leftmost lane) or PMA-ionomycin-stimulated (remaining lanes) EL4.8 cells were incubated with a ^{32}P-labeled P0, P1, P2, P3, or P4 oligonucleotide probe in the absence (ϕ) or presence of a 200-fold molar excess of the indicated competitor oligonucleotides. Specific complex formed with each probe is indicated at the left by probe name.

the ability of NF-AT oligonucleotides to compete for NF(P) binding (Fig. 5A) prompted us to test whether these factors might be related. To directly compare the molecular masses of NF(P) and NF-AT, we synthesized ASA-derivatized oligonucleotides (57) containing either NF(P) (ASA-hP1) or NF-AT (ASA-hNFAT1) binding sites which can be covalently attached to bound proteins by exposure to UV irradiation (Fig. 8). Each ASA-derivatized probe behaved similarly to nonderivatized probes, as assessed by EMSA (data not shown). The appearance of radiolabeled proteins is dependent on UV irradiation (Fig. 8A, lane 2) and can be completely blocked by specific competitor oligonucleotides (Fig. 8A). Thus, incorporation of ^{32}P label into proteins produced by ASA-hP1 photo-cross-linking to EL4.8 nuclear extracts is inhibited by P1 and NF-AT but not by IL4-Y and AP-1 oligonucleotides (Fig. 8A). The ^{32}P label is incorporated into two specific proteins with molecular masses of approximately 110 and 140 kDa (Fig. 8A and B). These apparent molecular masses reflect the native protein with the attached oligonucleotide and so overestimate the true mass by about 10 kDa. Surprisingly, proteins of identical molecular masses were radiolabeled by using either the ASA-hP1 or ASA-hNFAT1 probe, which are of the same length (Fig. 8B). The estimated molecular masses of these proteins agrees with the recently reported size of NF-AT$_c$ (35, 40). Proteins of these molecular masses are specific to the ASA-hP1 and ASA-hNFAT probes, since probes specific for Oct-1 and Oct-2 produced radiolabeled proteins of different molecular masses (data not shown). Further, the fragmentation patterns produced by V-8 protease digestion of these radiolabeled proteins, using ASA-hP1 and ASA-hNFAT1, were also identical (Fig. 8C). These results suggest that NF(P) and NF-AT$_c$ have subunits of very similar molecular masses and appear closely related on the basis of V-8 protease fragmentation.

The IL-4 promoter contains a functional NF-Y binding site that strongly enhances overall activity. Multiple NF(P) binding sites in the IL-4 promoter may account for its ionomycin-inducible transcriptional activity. However, one promoter region lacking NF(P) binding sites also enhances activity. Deleting the region between −129 and −80 from ID15 to produce ID16 caused a corresponding decrease in activity (Fig. 2B; compare ID15 to ID16). Placing this same region into DM8 upstream of the P0 site failed to produce significant ionomycin-inducible activity (Fig. 6, construct e). While

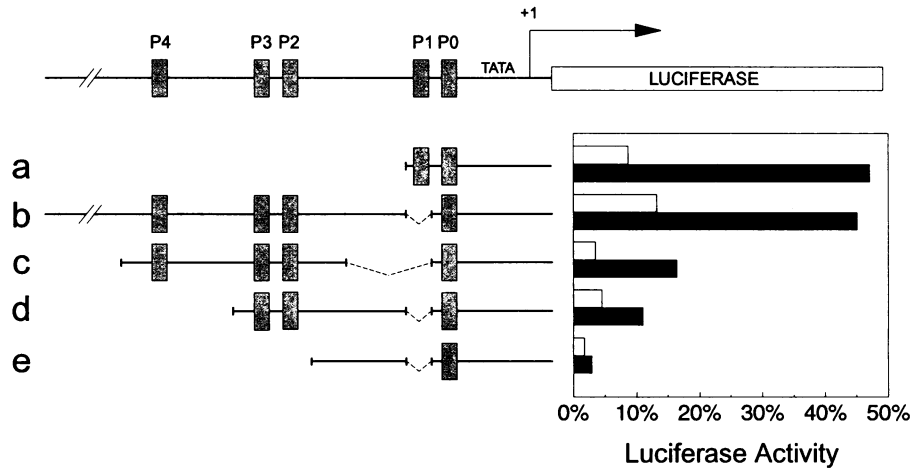

FIG. 6. Promoter regions containing P2, P3, and P4 sites confer inducible activity. The constitutive (open bars) and PMA-ionomycin-inducible (closed bars) promoter activity in EL4.8 cells is expressed as a percentage of IL4-LUC activity after normalization for transfection efficiency for the following constructs: DM7 (a), ID15 (b), p3.5-LUC (c), p5-LUC (d), and p7-LUC (e). The promoter region contained within each construct is shown by the diagram at the left of the bar graph, with deletions as indicated. The location of each P site is indicated by shaded boxes. The experiment was repeated three times with similar results.

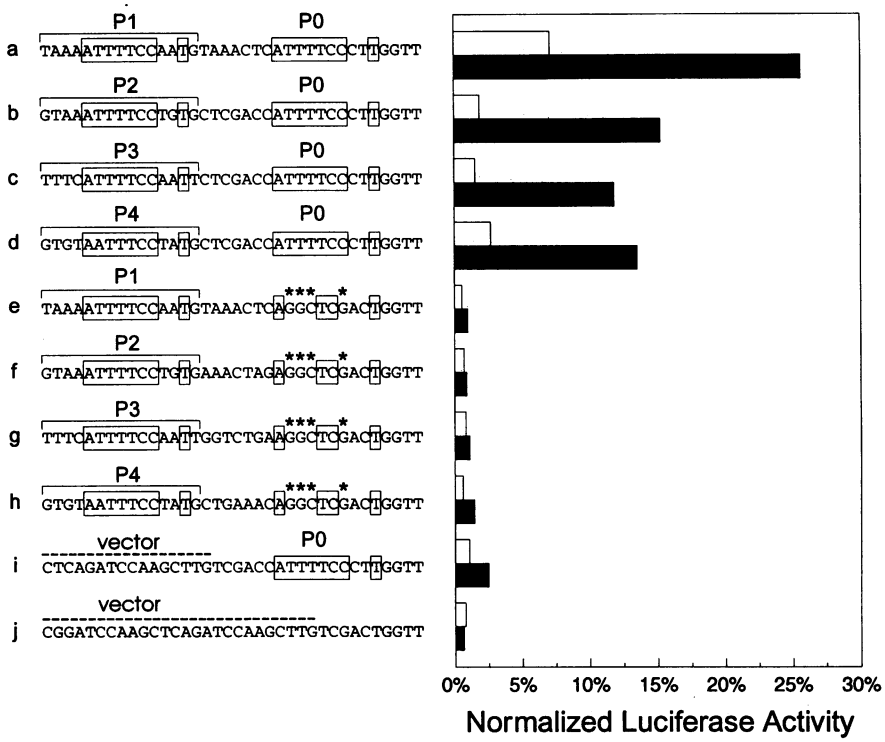

FIG. 7. NF(P)-dependent transcriptional activity involves cooperative interactions between adjacent P sites. EL4.8 cells were cotransfected with 20 μg of the indicated plasmid and 20 μg of CMV-CAT and left untreated (open bars) or stimulated with ionomycin (closed bars). The normalized luciferase activity of each construct, presented as the percentage of IL4-LUC activity, is the average from three separate experiments. Constructs used are P1-0 (a), P2-0 (b), P3-0 (c), P4-0 (d), P1-X (e), P2-X (f), P3-X (g), P4-X (h), DM8 (i), and DM9 (j).

consistent with the region lacking P sites, this result does not explain the loss of activity seen in ID16 (Fig. 2B). Thus, we further examined the region between −129 and −80 for other potential *cis*-acting elements.

We initially used six overlapping oligonucleotides spanning this entire region as probes for EL4.8 nuclear extracts (Fig. 9A). Two distinct complexes were formed with these probes (data not shown). The slower-migrating complex bound probes 5-1, 5-2, and 5-5, which overlap in the region between −125 and −116 (data not shown). This region contains an 8-bp identity with the Y-box element common to all MHC class II genes (4, 20, 24). The specific probe IL4-Y, centered on the Y box, was tested by EMSA for factor binding (Fig. 9A and B). A single complex formed with the IL4-Y probe that was inhibited only by oligonucleotides containing the Y-box consensus (Fig. 9B) and not by P1, ARRE-1, 5-4, or 5-6. Since the Y box in the MHC class II gene promoters binds the protein NF-Y (55), we examined whether this same nuclear factor was interacting with the Y box in the IL-4 promoter. To test this possibility, we compared the interactions of an Eα promoter Y-box probe (Eα-Y) (56) with competitors and specific antibody reagents to those of the IL-4 Y-box probe (Fig. 9C). First, the complex formed with use of the Eα-Y probe is inhibited by the IL4-Y oligonucleotide, and conversely, Eα-Y inhibits the complex formed with use of the IL4-Y probe (Fig. 9C). Moreover, a panel of anti-NF-Y monoclonal and polyclonal antibodies produces identical supershift patterns with each of these probes (Fig. 9C). These inhibitors and panel of antibodies fail to alter the binding or mobility of the NF(P) complex formed with the P1 probe, confirming the specificity of these supershifts. Thus, the IL-4 promoter Y box binds a single complex that appears identical to NF-Y.

We next examined the functional role of the IL-4 promoter Y box by using a series of linker-scanning mutations throughout the −124 to −77 region (Fig. 10). A 10-bp mutation of the Y box within the context of the full 800-bp promoter caused an 85% reduction in overall promoter activity (Fig. 10, construct b). By contrast, 10- and 8-bp mutations immediately flanking the Y box produced only 15 to 20% change in activity (Fig. 10, constructs a and c). The activity in the Y-box mutant remains ionomycin inducible. In fact, the ratio of ionomycin inducibility of the mutant Y construct (Fig. 10, construct b) actually increases twofold over that of the full-length and the flanking mutant constructs, suggesting that the activity lost is of a constitutive nature. This result is consistent with the constitutive expression of NF-Y. These data strongly suggest the IL-4 promoter Y box binds to NF-Y and contributes significantly to overall IL-4 promoter activity.

A second, faster-migrating complex bound to probe 5-4 but did not bind to probe 5-1, 5-2, or 5-3 (data not shown). The competitor inhibition pattern of this complex suggested that binding involved the region between −88 and −76 (data not shown). Inspection of this region showed an 8-bp identity to the OAP region described for the IL-2 promoter (53). Thus, a specific probe, OAP-4, centered on this region was examined in EMSA for factor binding (Fig. 9A and D). A single complex formed with this probe was inhibited by itself and by the ARRE-1 oligonucleotide, which contains the OAP region from the IL-2 promoter. In addition, the P1 and P4 oligonucleotides, but not the MP oligonucleotide, inhib-

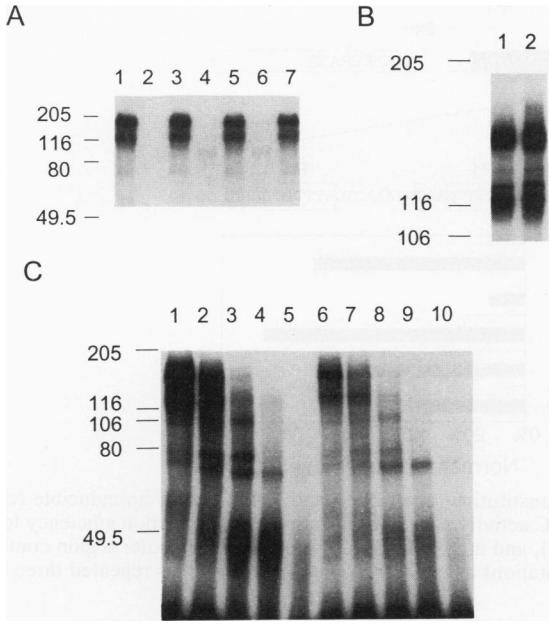

FIG. 8. NF(P) interacts with a protein potentially related to the cytoplasmic component of NF-AT. (A) Nuclear extracts from PMA-ionomycin-activated EL4.8 cells were incubated with a ^{32}P-labeled ASA-hP1 oligonucleotide probe and then UV irradiated as described in Materials and Methods (lanes 1 and 3 to 7) or not UV irradiated (lane 2). The binding reactions occurred in the absence (lanes 1 to 3) or presence of the following competitor oligonucleotides: P1 (lane 4), AP-1 (lane 5), NFAT-1 (lane 6), and IL4-Y (lane 7). Samples were denatured and analyzed by SDS-PAGE (8% gel). (B) Nuclear extracts from PMA-ionomycin-activated EL4.8 cells were incubated with a ^{32}P-labeled ASA-hP1 (lane 1) or ^{32}P-labeled ASA-hNFAT1 (lane 2) oligonucleotide probe and then UV irradiated. Samples were denatured and analyzed by SDS-PAGE (5% gel). (C) Nuclear extracts from PMA-ionomycin-activated EL4.8 cells were incubated with a ^{32}P-labeled ASA-hP1 (lanes 1 to 5) or ASA-hNFAT1 (lanes 6 to 10) oligonucleotide probe, UV irradiated, and incubated at 37°C for 20 min with the following concentrations of V-8 protease: 0 ng (lanes 1 and 6), 6 ng (lanes 2 and 7); 20 ng (lanes 3 and 8); 60 ng (lanes 4 and 9), and 200 ng (lanes 5 and 10). Samples were denatured and analyzed by SDS-PAGE (8% polyacrylamide gel). Sizes are indicated in kilodaltons.

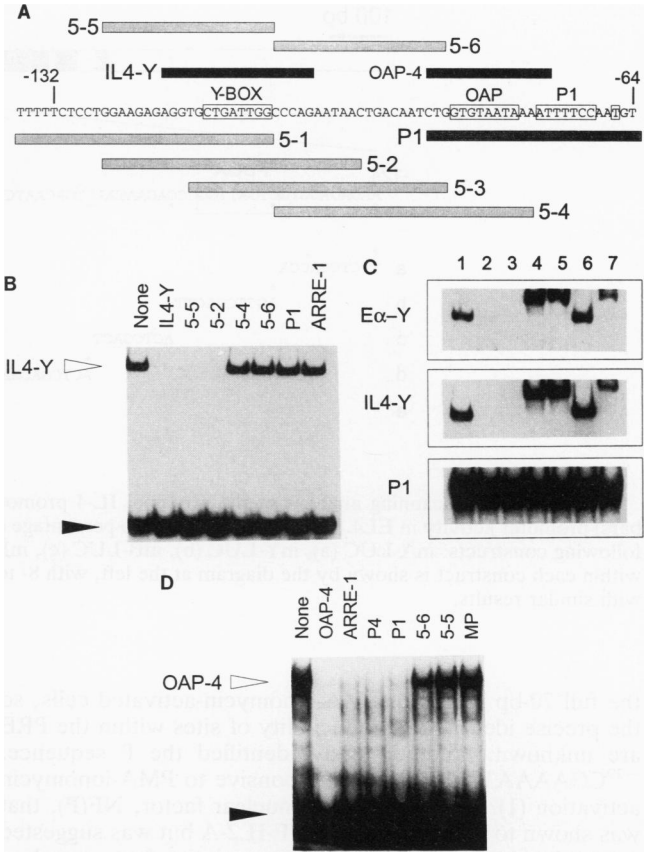

FIG. 9. NF-Y binds to the IL-4 Y box probe, and a distinct nuclear factor binds to the OAP region of the IL-4 promoter. (A) Locations of oligonucleotide probes and competitors within the murine IL-4 promoter region −136 to −64. (B and D) Nuclear extracts from PMA-ionomycin-stimulated EL4.8 cells were incubated with a ^{32}P-labeled IL4-Y (B) or OAP-4 (D) oligonucleotide probe in the absence (None) or presence of the indicated competitor oligonucleotides. Specific complex is indicated by the open arrowhead; free probe is indicated by the closed arrowhead. (C) Nuclear extracts from PMA-ionomycin-stimulated EL4.8 cells were incubated with a ^{32}P-labeled Eα-Y, IL4-Y, or P1 oligonucleotide probe in the absence (lane 1) or presence of competitor oligonucleotide IL4-Y (lane 2) or Eα-Y (lane 3) or in the presence of the following antibody against NF-Y: monoclonal Y-A1a (lane 4), monoclonal Y-A7 (lane 5), polyclonal Y-A/C (lane 6), or polyclonal Y-B (lane 7). Specific complex formed with each probe is indicated at the left by probe name.

ited complex formation. Thus, this complex is distinct from NF(P), but the inhibition by oligonucleotide P4 suggests that the related sequence, $^{-245}$GTGTAATT^{-238}, found overlapping the P4 site may also interact with the same factor. Destruction of the OAP site by clustered point mutations within the context of the full-length IL-4 promoter led to a 60% reduction in activity (Fig. 10, construct e). Although not as dramatic as the Y-box mutation, this result suggests that the factor binding in this region plays a role in IL-4 promoter activity.

DISCUSSION

Understanding the molecular basis of restricted patterns of cytokine production by specialized Th1 and Th2 CD4$^+$ T cells requires that the mechanisms governing individual cytokine genes be defined. Because we and others have shown that IL-4 expression is critical for the development of naive CD4$^+$ T cells toward the Th2 phenotype (26, 44, 51), the aim of this study was identification of *cis*-acting elements that regulate IL-4 gene expression in T cells. An important aspect of our approach was the initial selection of transfectable T-cell lines having a high-IL-4-producing phenotype. This selection provided for a relatively greater expression of the IL-4 promoter constructs than in related sublines producing much less endogenous IL-4 (Fig. 1).

Previous studies of the human IL-4 gene identified several potential regulatory elements. The NRE region contained binding sites for two *trans*-acting factors (32). The NRE-1 site, $^{-311}$CTCCCTCT^{-303}, interacts with a T-cell-specific factor, Neg-1, while the adjacent NRE-2 site, $^{-288}$CTTTT TGCTTTGC^{-300}, binds a ubiquitous factor, Neg-2. These sites exerted transcriptional repression only upon a downstream region (−270 to −200) which contains a ubiquitously functional PRE (32). However, that study did not define the minimal elements within the PRE region responsible for conferring activity. Rather, it examined only the activity of

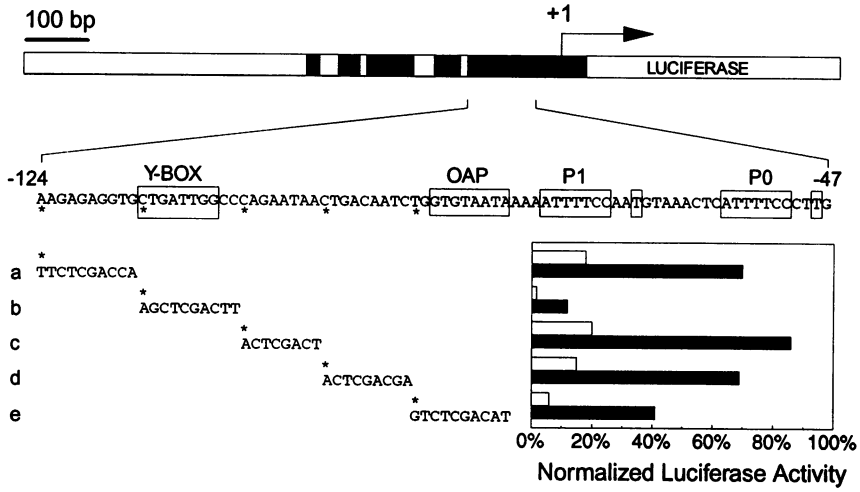

FIG. 10. Linker-scanning analysis of the proximal IL-4 promoter. The constitutive (open bars) and PMA-ionomycin-inducible (closed bars) promoter activity in EL4.8 cells is expressed as a percentage of IL4-LUC activity after normalization for transfection efficiency for the following constructs: mA-LUC (a), mY-LUC (b), mB-LUC (c), mF2-LUC (d), and mOAP-LUC (e). The 800-bp promoter region contained within each construct is shown by the diagram at the left, with 8- to 10-bp mutations as indicated. The experiment was repeated three times with similar results.

the full 70-bp region in PMA-ionomycin-activated cells, so the precise identity and inducibility of sites within the PRE are unknown. Another study identified the P sequence, $^{-79}$CGAAAATTTCC^{-69}, as responsive to PMA-ionomycin activation (1). This site binds a nuclear factor, NF(P), that was shown to be distinct from NF-IL2-A but was suggested to be related to the Rel family of transcription factors such as NF-κB (1).

Our data imply that *cis*-acting elements besides those described above are also important in IL-4 gene regulation. In a series of 5′ truncations of the IL-4 promoter, we verified that the P sequence between −79 and −58 was active but did not find deletion of the NRE to cause an apparent increase in promoter activity (Fig. 2A). NRE activity might be minimal in EL4.8, since we selected this cell line as a high IL-4 producer. Jurkat cells, used in two previous studies (1, 32), make relatively little IL-4 by comparison and might express more NRE activity. The effects of the NRE were also evident in the study by Abe et al. (1) but only as a threefold reduction in activity occurring between constructs extending to −297 and −439 upstream of the transcription start site. To identify other potential sites, we began by testing promoter constructs in which the P sequence was specifically deleted and, surprisingly, found significant residual inducible activity. Thus, we concluded that multiple positive *cis*-acting elements are present within the proximal 5′ flanking region distinct from the P sequence.

In this study, we identify several previously unrecognized *cis*-acting elements within the proximal 250 bp of the murine IL-4 promoter. First, we show that four NF(P) binding sites are present within the region −241 to −48 in addition to the previously identified P sequence. Each of these NF(P) binding sites can function cooperatively to confer ionomycin-inducible transcriptional activity (Fig. 7). Each identified P site can restore activity to a construct containing a single P site, P0, (Fig. 7, constructs a to d), yet each appears unable to confer such activity when in complete isolation (Fig. 7, constructs e to h). Inducible activity of the promoter does not completely reside within the first P site identified (P1) (1), since deletion of this site leaves significant residual activity (Fig. 2B, ID15). We propose that these P sites may interact cooperatively to provide inducible IL-4 promoter activity that is responsive to a Ca^{2+}-dependent signaling pathway, a feature consistent with the properties of IL-4 expression by Th2 T cells.

NF-AT$_c$ has several characteristics similar to those of NF(P). The complete NF-AT complex contains an inducible nuclear factor and the T-cell-specific CsA-sensitive cytoplasmic factor, NF-AT$_c$ (35, 40, 46). The nuclear component interacts with a noncanonical AP-1 site in the 3′ NF-AT binding region and may consist of Fra-1 and JunB (6, 10, 28, 35, 40). NF-AT$_c$ interacts with the 5′ NF-AT binding region, but its identity has not been unambiguously determined (35, 40, 52). Recent biochemical characterization of NF-AT$_c$ suggests that it is a phosphoprotein with a molecular mass of approximately 90 to 125 kDa (35, 40). We noticed that the NF(P) consensus sequence was contained within the 5′ region of the NF-AT binding site (Fig. 4). Also we demonstrated that the NF-AT oligonucleotide competed for NF(P) binding (Fig. 5A). In addition, NF(P) is T cell specific (19), inhibited by CsA, and activated by ionomycin, similar to properties of NF-AT$_c$ (15, 34, 46). Last, the induction of IL-4 gene transcription in T cells can occur without new protein synthesis (5), consistent with NF-AT$_c$ being present in T cells prior to activation. Therefore, we decided to directly compare the protein factors binding to the P and NF-AT sites by using arylazide-derivatized UV-activatable photoprobes (57) (Fig. 8). Using this approach, we were able to covalently attach radiolabeled probes to bound proteins. The incorporation of ^{32}P label was specific, since it could be prevented by competition with a specific P or NF-AT oligonucleotide but not by other inhibitors (Fig. 8). The similarity in molecular masses and the similar V-8 protease degradation patterns of proteins radiolabeled with the two photoprobes suggest a close relationship between these two factors. Moreover, our findings with use of the ASA-hNFAT probe agree with the recent biochemical analysis of NF-AT$_c$ molecular mass carried out by using proteins renatured from SDS-polyacrylamide gels (35, 40), lending support to this approach. The selective photolabeling of the cytoplasmic

rather than the AP-1 components of NF-AT might have resulted from steric restrictions to arylazide attachment, as described for this technique by Wang et al. (57). Final determination of the relationship between NF(P) and NF-AT_c will require their molecular cloning.

Several additional features of these multiple NF(P) binding sites are noteworthy. First, each P site is functional, and each site, as well as the minimal consensus probe, can bind NF(P) in vitro. These data define the nucleotides relevant for functional NF(P) recognition and suggest that 5'-ATTTTC CNNT-3' may be the minimal sequence required for binding. Second, the locations and sequences of each P site are highly conserved between mouse and human. The murine P0, P3, and P4 sites are identical to their human counterparts. P2 occurs in the reverse orientation relative to the other P sites in both mouse and human. The human P2 site, ATGTTC CNNT, has one base pair difference from the P consensus sequence. Human P1, AATTTCCNNT, differs from the murine P1 at one nucleotide but is identical to both human and murine P4; therefore, this difference does not disrupt the NF(P) binding site. Third, P0 may be related to the CLEO element identified in the granulocyte-macrophage colony-stimulating factor promoter (36), which binds two closely migrating complexes, NF-CLEOa and NF-CLEOb. Formation of the higher-mobility CLEOa complex is sensitive to bases between −49 and −42, an area homologous to P0. Furthermore, the region from −69 to −40 of the murine IL-4 promoter, which contains the P0 site, competed for NF-CLEO complex formation and bound a factor similar in mobility and specificity to NF-CLE0 (36). These findings suggest a potential relationship between NF-CLEO and NF(P), but more detailed examination of fine sequence specificity is required. Last, the location of P4 site, at −241 to −232, places it in the center of the PRE region identified by Li-Weber et al. (32). Potentially, the activity of the PRE region could have resulted from NF(P).

Our data suggest a cooperative mode of transcriptional activation for NF(P). First, two P sites appear to act cooperatively to support activation. While the P0 site alone appears inactive, it contributes significantly to the activity of constructs P1-0 through P4-0. Thus, one P site is inactive in isolation, whereas a dramatic increase (10- to 20-fold) in activity occurs upon the addition of a second P site (Fig. 6), but a much less dramatic increase (2- to 3-fold) occurs with addition of a third (unpublished data). These features are similar to the mode of transcriptional activation exemplified by Sp1 (42, 49). A single Sp1 binding site activates transcription only 2- to 3-fold, whereas two Sp1 binding sites confers up to an 80-fold induction (42), suggesting that the two sites interact synergistically. The addition of more Sp1 binding sites does not result in further synergistic activation (42). For Sp1, synergistic activation is not at the level of cooperative binding to DNA but rather appears due to the formation of higher-order complexes of Sp1 involving non-DNA-binding protein domains (49). In general, the synergistic actions between multiple DNA binding sites can involve either formation of higher-order complexes, cooperative recruitment of coactivators, or DNA bending dependent upon multiple site interactions. Therefore, the cloning of NF(P) and perhaps related members will be necessary for determining which form of transactivation is responsible for the inducible activity demonstrated by the multiple P sites within the IL-4 promoter.

In addition, this study identifies activity within two previously unrecognized regions of the IL-4 promoter. An NF-Y binding consensus (55), $^{-114}$CTGATTGG^{-107}, is active and confers up to fivefold enhancement of promoter activity (Fig. 10). While multiple factors, including CBP, CTF/NF-1, and NF-Y, interact with the CCAAT motif (12), our data strongly support NF-Y as the factor interacting with this region (Fig. 9C). NF-Y is constitutive and ubiquitous yet participates in other promoters that control expression in a lineage- and activation-specific manner. For example, MHC class II proteins are expressed on specific antigen-presenting cells, such as macrophages, B cells, and dendritic cells, and are induced under the effects of certain cytokines, such as gamma interferon and IL-4 (4, 17, 20). Currently, it is not understood whether NF-Y might contribute in any way to this restricted pattern of expression (4, 17, 20). In the MHC class II promoters, the Y box is found at a fixed spacing (20 bp) from the adjacent X and X_2 regions. The X_2 region may represent a site for AP-1 interaction (20, 59). Interestingly, the OAP site, a potential c-Jun binding site (54), is found at this same distance from the Y box in the IL-4 promoter.

This study identified a third factor binding site within the IL-4 promoter (Fig. 9D). The regions $^{-85}$GTGTAATA^{-78} and $^{-245}$GTGTAATT^{-238} share sequence similarity with the OAP region within the ARRE-1 site of the IL-2 promoter (53). This sequence within the IL-2 promoter does not directly interact with the Oct-1 protein but binds a distinct factor, OAP40 (53). Mutation of this region to TTTTAATA within the full IL-2 promoter produced 75% reduction in activity (53). Likewise, our mutation of the OAP site within the IL-4 promoter produced a 50 to 60% loss in activity (Fig. 10). The OAP site within the ARRE-1 was recently shown to be involved in TGF-β's inhibition of IL-2 gene transcription (8). Mutations in the upstream promoter sequence, within either the OAP site or the Oct-1 consensus, abrogated TGF-β's repression of transcription, suggesting that both sites are required for this effect. TGF-β also inhibits IL-4 production and may be a critical determinant of Th phenotype development during early T-cell activation (50).

One possibility regarding the role of these sites in Th differentiation is worth mentioning. IL-4 is expressed by Th2 but not Th1 T cells. Since NF(P) and NF-Y are expressed by both Th1 and Th2 cells, these factors may not be responsible for phenotype-restricted expression of IL-4. The diversity in transcription factors making up the AP-1 family may provide a means for regulating the activity of NF(P) or NF-Y through actions at adjacent sites such as OAP.

In summary, this study localized IL-4 promoter activity to several distinct *cis*-acting elements. Five distinct NF(P) binding sites confer ionomycin-inducible activity that depends on the cooperative interaction between adjacent P sites. These sites bind a T-cell-specific, CsA-sensitive factor that may be closely related to NF-AT_c. In addition, there is a Y-box element within the IL-4 promoter that binds to the ubiquitous and constitutive NF-Y protein and contributes significantly to overall activity. Determining the roles for the two potential OAP sites and identifying the mechanism of Th phenotype-restricted IL-4 expression remain for future studies.

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