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Molecular Cloning and Characterization of NF-IL3A, a Transcriptional Activator of the Human Interleukin-3 Promoter

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To isolate transcription factors important in the regulation of the human interleukin-3 (IL-3) gene, we screened a λgt11 cDNA library, constructed from phytohemagglutinin-stimulated human T-cell RNA, with a probe containing regulatory sequences in the upstream region of the IL-3 gene (located from bp −165 to −128 and referred to as the DNase I footprint A region). We isolated a 0.96-kb cDNA that encoded a basic amino acid domain and a leucine zipper domain and used the "rapid amplification and cloning of 3′ ends" technique to isolate the 3′ half of the cDNA clone, generating a 1.9-kb full-length cDNA clone. Using in vitro-translated protein, which we call NF-IL3A, we defined the IL-3 promoter sequences bound by NF-IL3A in DNase I footprinting assays as TAATTACGTCTG and, using gel shift assays, defined ATTACG as the minimal sequence required for binding of NF-IL3A in vitro. Proteins that bind to the NF-IL3A binding site are found in both unstimulated and stimulated T-cell lines in similar amounts, although the level of NF-IL3A mRNA increases after T-cell activation in several mature T-cell lines. The NF-IL3A protein is nearly identical to a recently identified transcriptional repressor protein, E4BP4, and NF-IL3A binds specifically to regulatory sequences in both the adenovirus E4 promoter and the human gamma interferon promoter. Cotransfection experiments demonstrate that introduction of an expression vector containing the NF-IL3A cDNA into resting T cells transactivates IL-3 promoter-chloramphenicol acetyltransferase gene plasmids that contain the A region; this effect requires the presence of an intact NF-IL3A binding site. One or more copies of the A region also confer NF-IL3A responsiveness on a heterologous promoter in T cells. NF-IL3A appears to play an important role in the expression of IL-3 by T cells.

Interleukin-3 (IL-3) is a multilineage hematopoietic growth factor which stimulates the proliferation of hematopoietic progenitor cells and enhances the functional activity of mature effector cells (27, 36). The expression of IL-3 is restricted to activated, but not resting, T cells, natural killer (NK) cells, and mast cell lines (9, 27, 29). In order to examine the regulatory mechanisms controlling the expression of IL-3, several investigators, including ourselves, have identified regulatory elements in the 5′-flanking region of the IL-3 promoter (3, 10, 13, 19, 21, 22, 33, 35). These regulatory elements include a consensus AP-1 binding site, which can bind c-Fos/c-Jun heterodimers (13), several consensus binding sites for ets proteins (13), a CD28 response element (12, 35), and two regions identified by DNase I footprinting experiments as the A region and the B region (35). The A region contains regulatory sequences important in the inducible expression of IL-3 (22, 33, 35), and methylation interference experiments have identified several distinct regions within this A region that are involved in DNA-protein interactions (10, 37). Electrophoretic mobility shift assays (EMSAs) combined with UV cross-linking have identified several proteins of 56 to 65 kDa that are capable of binding to the IL-3 A region (37).

To identify and characterize IL-3 A region-binding proteins, we screened a phytohemagglutinin (PHA)-stimulated human T-cell cDNA λgt11 expression library with a ^{32}P-labeled, multimerized, double-stranded IL-3 A region synthetic oligonucleotide probe. Approximately one million recombinant clones were screened, and one clone that generated a β-galactosidase fusion product that bound to the IL-3 A region but not to unrelated control DNA was identified. DNA sequence analysis of this clone demonstrated the presence of a potential DNA-binding basic-amino-acid region and a leucine zipper repeat. The 3′ portion of the cDNA clone was not present, so rapid amplification and cloning of 3′ ends (3′ RACE) was performed to generate a full-length cDNA clone.

A 1.9-kb full-length cDNA that contains the entire coding region for a 58-kDa protein that we call NF-IL3A was generated. In vitro-translated NF-IL3A binds specifically to the TA ATTACGTCTG sequence in the A region of the IL-3 promoter, and cotransfection experiments in T cells demonstrate that NF-IL3A can transactivate IL-3 promoter-chloramphenicol acetyltransferase (CAT) reporter gene plasmids that contain these sequences. We observed constitutive and mitogen-inducible expression of NF-IL3A mRNA in T-cell lines but very limited expression in a variety of other normal human tissues by Northern (RNA) blot analysis. Our data suggest that NF-IL3A plays a regulatory role in the expression of IL-3 by T cells.

MATERIALS AND METHODS

Isolation of a full-length NF-IL3A cDNA clone. A λgt11 library containing cDNA from PHA-stimulated human T cells (purchased from Clontech) was screened with concatemerized double-stranded synthetic oligonucleotides by the protocol of Singh et al. (34). The oligonucleotides contained the IL-3 A region

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sequence, gatccAGAAAGTCATGGATGAATAATTACGTCTGTGGTTTTCTg, and its complement, which are the IL-3 sequences that extend from bp −165 to bp −128, with a *Bam*HI 5′ overhang. The oligonucleotide was concatemerized to ~400 bp in size by using T4 DNA ligase and labeled with [^{32}P]dCTP by random prime labeling. All positive plaques were picked, replated, and rescreened in secondary and tertiary screening procedures. λ DNA containing the 5′ half of the NF-IL3A cDNA clone was obtained from the positive clones by using the Pharmacia λ DNA isolation kit. The DNA was digested with *Eco*RI and subcloned into the pBluescript (pBS) vector (Stratagene) for DNA sequence analysis. Dideoxy DNA sequencing was performed with universal and reverse sequencing primers and Sequenase (version 2.0; U.S. Biochemicals) to sequence nested deletions created by exonuclease III and mung bean nuclease digestion. 3′ RACE was performed to isolate the 3′ half of the cDNA, using the gene-specific primers GSP1 (CAGAAGTGTCCTCAGTAGAACACAC, which hybridizes to bp 785 to 810 of the cDNA) and GSP2 (CGAAGAGCTCTGTGCAGG GAAGCTGCAGA, which hybridizes to bp 815 to 840 of the cDNA) and a poly(dT) adapter primer, as described in the Clontech procedure manual. cDNA was prepared from three human T-cell lines with a poly(dT) primer, and following 3′ RACE, a *Sac*I-*Spe*I 900-bp amplified fragment was identified, purified from a 0.8% agarose gel, and subcloned into the pBS vector. Both strands of several different clones were sequenced, revealing identical DNA sequences. A full-length, in-frame 1.9-kb cDNA was generated by ligating the 5′ portion of the NF-IL3A cDNA (obtained by screening the λgt11 T-cell cDNA library) to the 3′ portion of the cDNA (obtained by 3′ RACE) at the unique *Sac*I site. The junction of the 5′ and 3′ portions of the cDNA was confirmed by dideoxy sequencing.

Cotransfection studies. To determine whether the DNA-binding protein encoded by the NF-IL3A cDNA was physiologically relevant, we subcloned the full-length cDNA into the BC12 expression vector (containing the cytomegalovirus promoter) for cotransfection studies with T cells. The generation of IL-3 promoter recombinant plasmids containing either 315, 173, or 61 bp of IL-3 5′-flanking sequences linked to the reporter gene for CAT has been described previously (35). The −315 mutant IL-3 promoter-CAT construct was generated by the PCR mutagenesis method of Chen and Przybyla (5). The wild-type −315 IL-3 promoter pCAT plasmid was used as the template, and four synthetic oligonucleotides (ordered from Integrated DNA Technologies, Inc.) were used as primers. Primer 1 is a primer complementary to noncoding strand sequences located upstream of the multiple cloning site of the −315 IL-3 pCAT plasmid; primer 2 and primer 3 are complementary and were used to introduce three nucleotide substitutions into the NF-IL3A consensus binding site in the A region. The sequence of the upper-strand mutant primer (primer 3) is 5′-CATGGAT GAATAATTACacgTGTGGTTTTCTATGG-3′ (the mutant nucleotides are indicated in lowercase letters; the wild-type sequence is GTC). Primer 4 is complementary to IL-3 promoter sequences from bp −24 to −8; the sequence of this primer is 5′-TGGCAACAACCTCGCGC-3′.

Separate PCRs were performed with primers 1 and 2 and with primers 3 and 4. We used 50 ng of template DNA, 20 μmol of deoxynucleoside triphosphate (dNTP) mix, 50 pmol of each primer, 10 μl of 10× Vent enzyme buffer, and 2 U of Vent DNA polymerase (New England BioLabs) in each 100-μl PCR. The PCRs were initiated at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 44°C for 1.5 min, and 72°C for 1 min, and then an additional 2 min at 72°C. The 272-bp and 153-bp PCR products were electrophoretically separated from the free primers and the template and purified with GeneClean (Bio 101). Then, 300 ng of the 272-bp product and 150 ng of the 153-bp product were mixed with 20 μmol of dNTP mix, 10 μl of 10× Vent enzyme buffer, and 2 U of Vent DNA polymerase in a 100-μl volume to generate a mutant IL-3 promoter fragment. The mixture was heated to 94°C for 1 min, and then the two DNA sequences were extended to full length by three cycles of amplification (each cycle consisting of 94°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min). Then 50 pmol of primers 1 and 4 and 1 μl of 10× Vent buffer were added to this PCR mix, and 30 cycles of amplification were performed to amplify the full-length mutant fragment. The PCR-generated mutant IL-3 promoter fragment was digested with *Stu*I and *Sma*I and subcloned into the −315 IL-3 pCAT plasmid, replacing the wild-type *Stu*I-*Sma*I fragment with the mutant fragment. The sequence, orientation, and junction of the mutant fragment were confirmed by dideoxynucleotide DNA sequencing. This construct is referred to as −315mut IL-3 CAT.

Additional plasmids containing one or more copies of the A region oligonucleotide subcloned in either the forward or reverse orientation at a *Bgl*II site 5′ of an enhancerless herpes simplex virus (HSV) thymidine kinase (Tk) promoter-CAT gene construct were also used for these studies. This construct is referred to as A pTE2, whereas pTE2 refers to the enhancerless HSV Tk-CAT plasmid.

Expression vector plasmid (10 μg of either the BC12 vector alone or the BC12 NF-IL3A construct) was introduced into resting MLA144 T cells together with 10 μg of a reporter gene plasmid (either the −315, −315mut, −173, or −61 IL3 promoter-CAT plasmids or the pTE2 or A pTE2 plasmid) by electroporation by previously published techniques (4). The following day, the cells were harvested, and CAT activity was determined by standard techniques (31). A human growth hormone expression vector, pXGH5 (Nichols Institute), was used to control for potential differences in the transfection efficiency of different plasmid preparations. The percent acetylations were divided by the human growth hormone concentrations to get the relative CAT activity; these values were used to define the transcriptional activity of the constructs.

In vitro transcription and translation of NF-IL3A. To examine the in vitro binding characteristics of the NF-IL3A protein, the pBluescript plasmid containing the full-length NF-IL3A cDNA was linearized at its 3′ end with the restriction enzyme *Hin*dIII, and RNA was made by using T7 RNA polymerase (Promega). In vitro translation was performed in a rabbit reticulocyte lysate with [^{35}S]methionine plus all other unlabeled amino acids (Promega) in a 20-μl reaction volume. The accuracy of the in vitro transcription and translation processes was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nonradioactive translation was performed in a reaction volume of 30 μl, replacing the [^{35}S]methionine with unlabeled methionine.

EMSA. For EMSAs, 2 μl of in vitro-translated NF-IL3A or control rabbit reticulocyte lysate was incubated with 2×10^5 cpm of radiolabeled oligonucleotide probe for EMSA analyses in a 20-μl reaction volume containing 1 μg of poly(dI-dC), 2 mM EDTA, 5 mM dithiothreitol, 2 mM spermidine, 50 mM Tris (pH 7.5), 7% glycerol, and 50 mM NaCl for 30 min on ice. After incubation, the samples were loaded onto a 5% polyacrylamide gel containing 0.25× TBE (Tris-borate-EDTA) and run at 10 mA constant current. The IL-3 wild-type oligonucleotide A probe contains the bp −165 to −128 sequences listed above. A gamma interferon promoter double-stranded oligonucleotide probe containing the sequence 5′-GTGAA<u>AATACGTAAT</u>CCTCA-3′ (28) and an adenovirus E4 promoter double-stranded oligonucleotide probe containing the sequence 5′-AAGCTTCTAAAAA<u>ATGACGTAAC</u>GGAAGCTT-3′ (7) were also used for these experiments. Various wild-type and mutant IL-3 oligonucleotides were also synthesized and used for competition experiments; the sequences of these oligonucleotides are shown in the figure legends and/or in Fig. 3.

To define the relative affinity of NF-IL3A for the various wild-type and mutant oligonucleotides, we used densitometry to measure the intensity of the specific gel shift bands generated with the A region oligonucleotide as the radiolabeled probe and a 10- or 100-fold molar excess of various oligonucleotides as competitors, using a Hewlett Packard ScanJet IIc scanner and the Scan Analysis program. The ability of an unlabeled oligonucleotide to compete for binding to the A region probe was expressed as $(a - b)/a \times 100$, where a is the band intensity without competitor and b is the band intensity with competitor.

We also performed EMSAs with 4 μg of unstimulated or PHA- or tetradecanoyl phorbol acetate (TPA)-stimulated MLA144 T-cell nuclear extracts with the radiolabeled A region oligonucleotide probe under the conditions described above. Unlabeled wild-type and mutant A region oligonucleotides were also used as competitors in these experiments.

Northern blot analysis of expression pattern of NF-IL3A. To examine the expression of NF-IL3A in T-cell lines, polyadenylated [poly(A)$^+$] mRNA was prepared from MLA144 cells and S-LB-1 cells with the Invitrogen Fast Track kit. Poly(A)$^+$ RNA (5 μg) was electrophoretically separated on a formaldehyde-agarose gel and transferred to an Amersham Hybond nylon membrane. Total cellular RNA was prepared from unstimulated and PHA- or TPA-stimulated HUT78 cells by standard techniques (6) and used for Northern blot analyses. These membranes were hybridized with a ^{32}P-random-prime-labeled full-length NF-IL3A cDNA probe and washed at high stringency under standard conditions. The blot was exposed to Kodak XAR film overnight at −80°C with an intensifying screen. A multiple-tissue Northern blot, containing 2 μg of poly(A)$^+$ RNA from various normal human tissues, was purchased from Clontech and probed with a radiolabeled NF-IL3A full-length cDNA probe. To examine expression in non-T-cell lines, cDNA was prepared from 1 μg of total cellular RNA obtained from HL60, KG-1, CMK, CMK-6, and U937 cells and used for reverse transcriptase (RT)-PCR. An amplified fragment of 1.1 kb was expected with the GSP1 primer and the adapter primer from the 3′ RACE kit.

DNase I footprinting. A pUC18 plasmid containing 900 bp of 5′-flanking IL-3 sequences was digested with *Ppu*MI (which cuts at bp −273 relative to the IL-3 cap site), dephosphorylated with calf intestine phosphatase, and then digested with *Sma*I, which cuts at bp −61. The 213-bp *Ppu*MI-*Sma*I fragment, which contains the A region (−165 to −128), was agarose gel purified with the GeneClean kit. The DNA probe used for DNase I footprinting of the coding strand was radiolabeled with polynucleotide kinase and [γ-^{32}P]ATP. The probe used to footprint the noncoding strand was labeled with [α-^{32}P]dCTP, unlabeled dGTP and dATP, and the Klenow fragment of DNA polymerase.

DNase I footprinting of the coding strand was performed by incubating 50 μg of MLA144 cell nuclear extract and 18 μl of in vitro-translated NF-IL3A protein (or H_2O alone to generate the DNase I ladder) in a total volume of 50 μl of gel shift buffer without probe on ice for 10 min. Labeled probe (30,000 cpm) was added, the mixture was incubated on ice for 20 min, and then 2 μl of 100 mM $MgCl_2$ was added to each tube, followed by addition of 0.3 to 3 μg of DNase I (Worthington Biochemical). The DNase I digestion was allowed to proceed for 60 s at room temperature; it was terminated by adding 250 μl of stop buffer (0.1 M Tris [pH 8.0], 0.1 M NaCl, 1% SDS, 10 mM EDTA) to each tube. The digested DNA was prepared by phenol-chloroform and chloroform extractions and then by ethanol precipitation. DNase I footprinting of the noncoding strand was carried out in a similar fashion except that 15 μl of in vitro-translated NF-IL3A, 15 μl of reticulocyte lysate without mRNA addition, or 50 μg of MLA144 nuclear extract was used in each reaction tube with 0.3 to 2 μg of DNase I.

The DNase I-digested DNA was dissolved in 6 μl of formamide loading buffer, heated for 5 min at 90°C, and separated electrophoretically on an 8% sequencing (denaturing) gel. Maxam-Gilbert A+G sequencing reactions (23) were per-

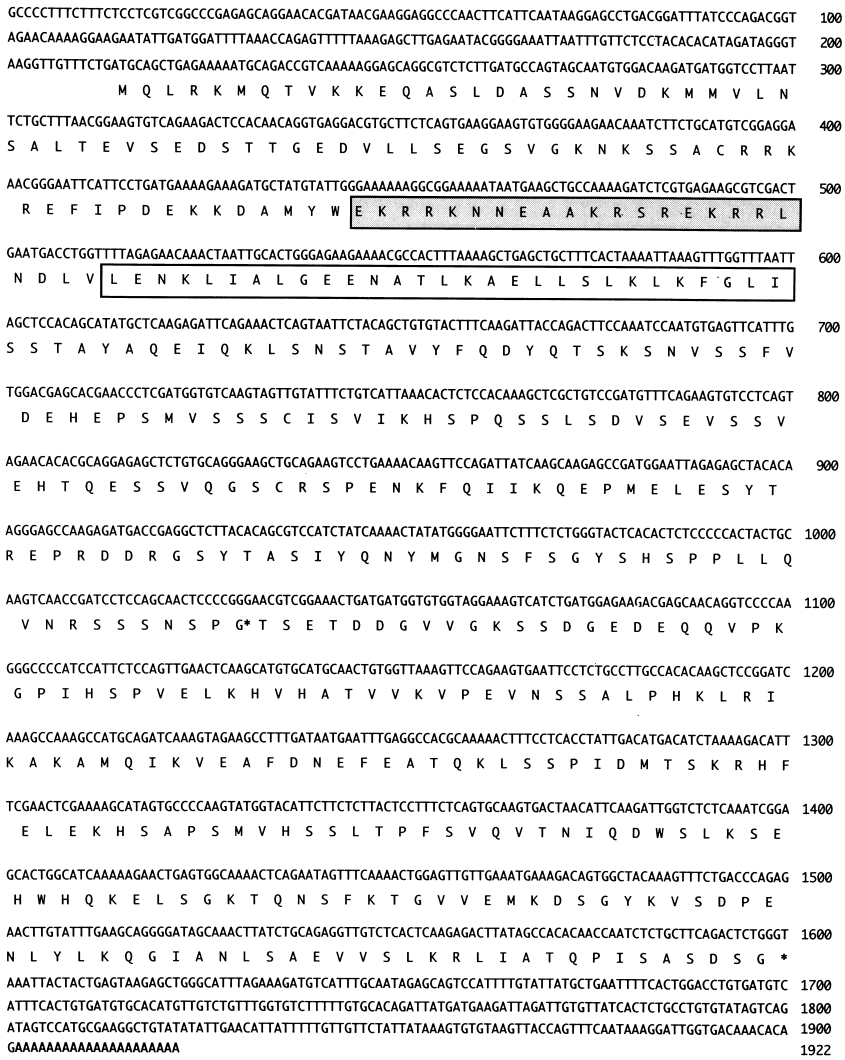

FIG. 1. Nucleotide and amino acid sequences of the full-length human NF-IL3A cDNA. The basic amino acid region (shaded rectangle) and the leucine repeat region (open rectangle) are indicated. The single-amino-acid difference between E4BP4 and NF-IL3A is indicated (*).

formed to locate specific IL-3 sequences on both the coding and noncoding strands.

RESULTS

Isolation of a full-length NF-IL3A cDNA clone. The screening of approximately 1.2×10^6 cDNA clones resulted in the isolation of a single cDNA clone that contained a long open reading frame encoding protein domains commonly found in transcription factors. This 960-bp cDNA clone was sequenced and found to contain a region of basic amino acids that could bind DNA, followed by a region containing leucine residues repeated every seven amino acids, forming a potential leucine zipper structure. These regions identified NF-IL3A as a member of the bZIP family of transcription factors (25), but the poly(A) tail and adjacent nucleotides were not contained in the cDNA.

The 3′ half of the full-length cDNA, approximately 1,000 nucleotides in length [extending from the GSP1 primer to the poly(A) tail], was obtained by the 3′ RACE technique. DNA sequencing of several subcloned RACE-generated cDNAs revealed the presence of sequences encoding two serine-rich regions and a glutamine-rich region, which could serve as transcriptional activation domains. The full-length sequence of NF-IL3A is shown in Fig. 1; the basic amino acid region and the leucine zipper region are indicated. The basic amino acid region of NF-IL3A appears to be more related to members of the proline- and acidic amino acid-rich (PAR) family of bZIP transcription factors, such as albumin promoter D-box-binding protein (DBP), thyrotroph embryonic factor (TEF), and hepatic leukemia factor (HLF), than to members of the AP-1 family (11, 15, 16, 26).

NF-IL3A transactivates the IL-3 promoter through the A region sequences. To define the transcriptional regulatory activity of NF-IL3A on the IL-3 promoter, we performed transient transfections into MLA144 cells. As shown in Fig. 2, introduction of the NF-IL3A BC12 expression plasmid into resting MLA144 T cells increased the CAT activity generated by the −315 and −173 plasmids 5.2- and 5.0-fold, respectively, compared with that generated by the BC12 expression vector alone. No significant change in the promoter activity of the −61 plasmid, which lacks the A region sequences, was seen. Introduction of 3-bp substitutions into the NF-IL3A consensus

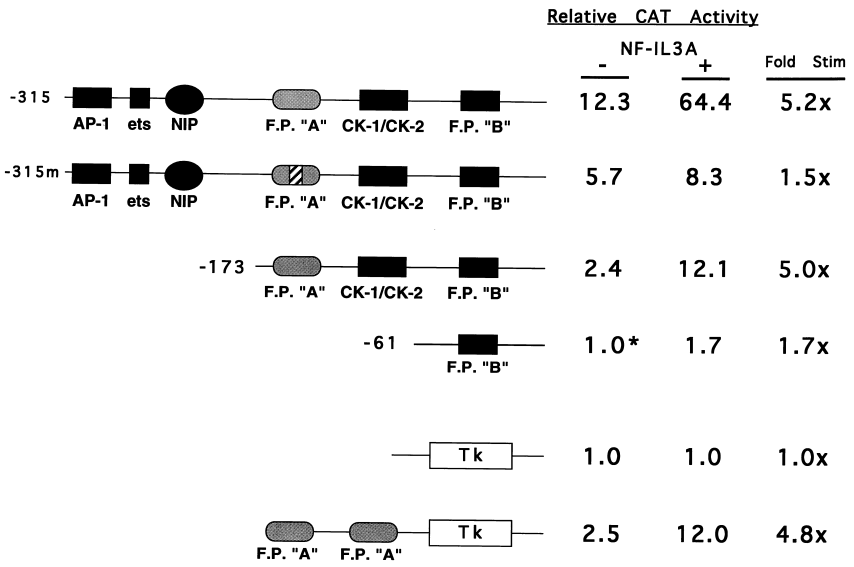

FIG. 2. Cotransfection experiments in MLA144 T cells. The CAT activity (calculated from percent acetylation divided by human growth hormone concentration) relative to that of the −61 plasmid in the absence of NF-IL3A (−) (which was assigned a value of 1.0) is shown for all constructs as fold induction. The location of the A region (bp −165 to −128) in the IL-3 promoter-CAT constructs is indicated; the presence of nucleotide substitutions in the −315mut IL-3 CAT construct is indicated by cross-hatching. Two copies of the A region oligonucleotide cloned upstream of the HSV Tk promoter are shown.

binding sequence (determined by the studies of DNA-protein interactions shown below and by the studies of Cowell et al. [7]) in the −315 construct (changing GTC to ACG) eliminated responsiveness to NF-IL3A. Similarly, cotransfection of the NF-IL3A BC12 plasmid had no effect on the enhancerless pTE2 plasmid, but it activated the pTE2 plasmid containing two copies of the A region sequences approximately fivefold (Fig. 2). These experiments demonstrate that the GTC nucleotides in the NF-IL3A consensus binding sequence (5′-AT TACGTCTG-3′, as shown in the experiments described below) in the A region are required for responsiveness to NF-IL3A and that the A region sequences are sufficient to confer NF-IL3A responsiveness on a heterologous promoter.

Binding of NF-IL3A to specific IL-3 promoter sequences. To demonstrate binding of NF-IL3A protein to the IL-3 A region sequences, we incubated 2 μl of in vitro-translated NF-IL3A with a ^{32}P-radiolabeled A region oligonucleotide in standard EMSAs and observed specific binding (i.e., binding was eliminated by a 50-fold molar excess of unlabeled A region oligonucleotide [see Fig. 7] but not by consensus AP-1 or NF-κB binding site oligonucleotides [data not shown]).

We operationally divided the A region into two halves, based on methylation interference experiments done by ourselves and others (22, 37), and synthesized oligonucleotides containing either the 5′ half of the A region sequences (extending from bp −160 to −141 [oligonucleotide AA]) or the 3′ half of these sequences (extending from bp −142 to −128 [oligonucleotide AA′]) (diagrammed in Fig. 3). NF-IL3A protein bound specifically to the AA oligonucleotide, as shown in Fig. 4, lanes 3 and 4. A 50-fold excess of the A region oligonucleotide also competed completely for binding (Fig. 4, lane 5), whereas an irrelevant 19-bp granulocyte-macrophage colony-stimulating factor (GM-CSF) oligonucleotide did not compete for binding (lane 9). On the basis of previously published DNase I footprinting and deletion mutant analyses (33), which proposed a binding sequence (5′-GATGAATAAT-3′) for an IL-3 promoter sequence regulatory protein, we mutated 5 bp in this sequence from 5′-GATGAATAATTAC-3′ to 5′-GAg cAAgggTTAC-3′. This mutant oligonucleotide (MA) also competed efficiently for binding (Fig. 4, lane 6), suggesting that the GATGAATAAT sequences were not the binding site for NF-IL3A. Several groups have reported the DNA sequence requirements for the specific binding of bZIP proteins similar to the NF-IL3A protein (E4BP4, HLF, E2A/HLF, and TEF/VBP) (7, 15, 17). Their data, obtained by using PCR-based oligonucleotide selection strategies, suggest that the five nucleotide substitutions introduced into the MA oligonucleotide would not disrupt the binding of NF-IL3A, which is consistent with our results. Rather, their studies suggest that a (G/A)T TAC sequence can serve as a half-site for the binding of this family of proteins; this sequence is intact in the MA oligonucleotide. An adenovirus E4 promoter sequence oligonucleotide (which contains the E4BP4 consensus binding site) efficiently competed for binding to the IL-3 sequences (Fig. 4, lane 7), and a gamma interferon promoter sequence oligonucleotide (described in reference 28) which is nearly identical to the E4BP4 consensus binding sequence and is identical to the

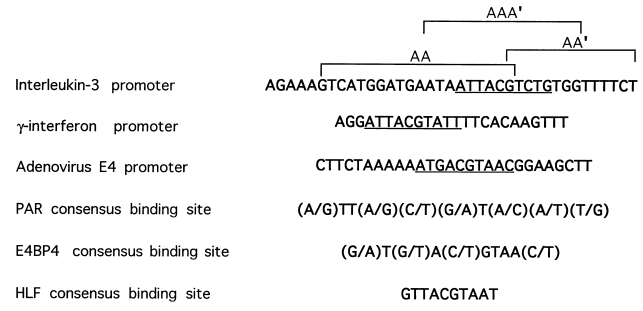

FIG. 3. Comparison of IL-3 promoter sequences (from bp −165 to −134), human gamma interferon promoter sequences (from bp −70 to −46), adenovirus E4 promoter sequences (from bp −61 to −34), and consensus binding sites for E4BP4, HLF, and VBP. Sequence homologies between these promoters are underlined. The sequences contained in the AA, AA′, and AAA′ oligonucleotides are indicated.

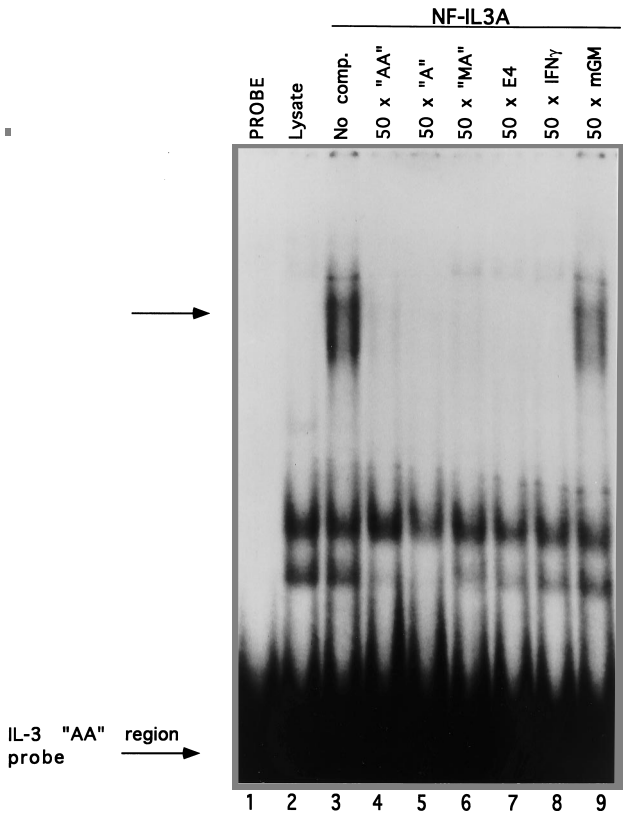

FIG. 4. EMSA with in vitro-translated NF-IL3A and the IL-3 AA region oligonucleotide (which contains the IL-3 sequences between bp −160 to −141, GTCATGGATGAATAATTACG, initially thought to be the NF-IL3A consensus binding sequence) as the probe. Lane 1, probe alone; lane 2, probe plus rabbit reticulocyte lysate; lanes 3 to 9, probe plus in vitro-translated NF-IL3A. Lane 3 contains no unlabeled competitor (comp.) oligonucleotide, whereas lanes 4 to 9 contain a 50-fold molar excess of unlabeled AA oligonucleotide (lane 4), A oligonucleotide (lane 5), MA oligonucleotide AGAAAGTCATGGAg cAAgggTTACGTCTGTGGTTTTCT (lane 6), adenovirus E4 promoter sequence oligonucleotide (lane 7), human gamma interferon promoter sequence oligonucleotide (lane 8), or a GM-CSF promoter mutant oligonucleotide (TCA CAAGGGATAGAAGCTT) (lane 9). The position of the free AA probe is shown at the bottom of the gel, and the gel shift complex containing NF-IL3A is shown by the upper arrow.

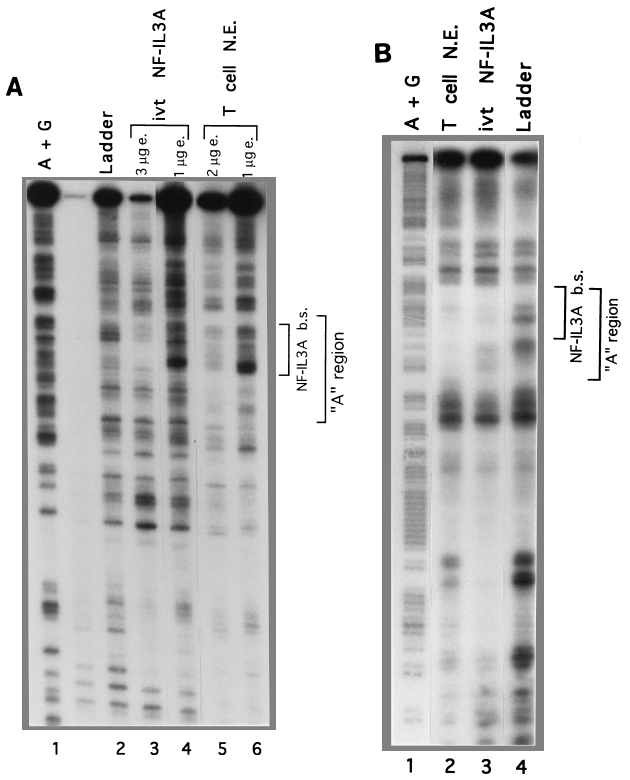

FIG. 5. DNase I footprinting assays with MLA144 nuclear extracts (N. E.) or in vitro-translated (ivt) NF-IL3A. A fragment of the IL-3 promoter from −273 to −61 was labeled on either the coding or noncoding strand. (A) DNase I footprinting of the coding strand. Lane 1, DNA subjected to Maxam-Gilbert A+G sequencing; lane 2, DNA ladder generated by digesting DNA in the absence of nuclear proteins; lanes 3 and 4, DNA incubated with in vitro-translated NF-IL3A prior to digestion with 3 or 1 μg of DNase I, respectively; lanes 5 and 6, DNA incubated with MLA144 T-cell nuclear extracts prior to digestion with 2 or 1 μg of DNase I, respectively. The NF-IL3A binding sequences (b.s.) protected by in vitro-translated NF-IL3A or MLA144 nuclear extract are bracketed; e, DNase I enzyme. (B) DNase I footprinting of the noncoding strand. Lane 1, DNA subjected to Maxam-Gilbert A+G sequencing; lane 4, DNA ladder generated by digesting the DNA probe in the absence of nuclear proteins; lane 2, DNA incubated with MLA144 T-cell nuclear proteins prior to DNase I digestion; lane 3, DNA incubated with in vitro-translated NF-IL3A prior to DNase I digestion. The protected NF-IL3A binding sequences (b.s.) are bracketed.

consensus binding site for the PAR family of bZIP proteins also competed for NF-IL3A binding (Fig. 4, lane 8).

DNase I footprinting assays. The results of DNase I footprinting experiments with either in vitro-translated NF-IL3A or MLA144 T-cell nuclear extracts are shown in Fig. 5. With the coding strand used as a probe, in vitro-translated NF-IL3A protected the IL-3 sequence TAATTACGTCTGTGG (Fig. 5A, lane 3), whereas with the noncoding strand as a probe, in vitro-translated NF-IL3A protected the IL-3 sequence AT GAATAATTACGTCTG (Fig. 5B, lane 3). Thus, the NF-IL3A binding sequences found on both the coding and noncoding strands are TAATTACGTCTG (the IL-3 promoter sequences similar to the consensus binding sequences for E4BP4 and members of the PAR family of bZIP proteins are underlined [7, 15, 18]). This region of the IL-3 promoter and approximately 28 additional nucleotides are protected from DNase I digestion when MLA144 T-cell nuclear extracts are used for these studies (Fig. 5A, lanes 5 and 6, and Fig. 5B, lane 2).

In vitro-translated NF-IL3A also binds to another region of the IL-3 promoter which is located upstream of the A region, extending from bp −210 to −197 (Fig. 5A, lanes 3 and 5, and Fig. 5B, lanes 2 and 3 [in the lower third of the gels]). This region contains the sequence tTTAaGTAAT, which is 80% homologous to the E4BP4 decanucleotide consensus binding sequence determined by Cowell et al. (7), (A/G)T(G/T)A(T/C)GTAA(T/C). Although we observed in vitro binding of NF-IL3A to this sequence, the CAT construct that contains this sequence (−315 CAT) is not more responsive to NF-IL3A transactivation than the −173 CAT construct (Fig. 2). Additional sequences downstream of the A region were slightly protected from DNase I digestion with the sense probe and in vitro-translated NF-IL3A (Fig. 5A, lane 3), but these sequences were not protected with the antisense probe (Fig. 5B, lane 3).

We also observed two DNase I-hypersensitive sites with in vitro-translated NF-IL3A or MLA144 T-cell nuclear extracts. One is located immediately downstream of the A region (from bp −127 to −122, ATGGGAG) (Fig. 5A, lane 3, and Fig. 5B, lanes 2 and 3), and the other is located from bp −187 to −178, TGTTTCACT (Fig. 5A, lane 3). The sequence immediately upstream of both hypersensitive sites is TTTTCT; the significance of this is not clear.

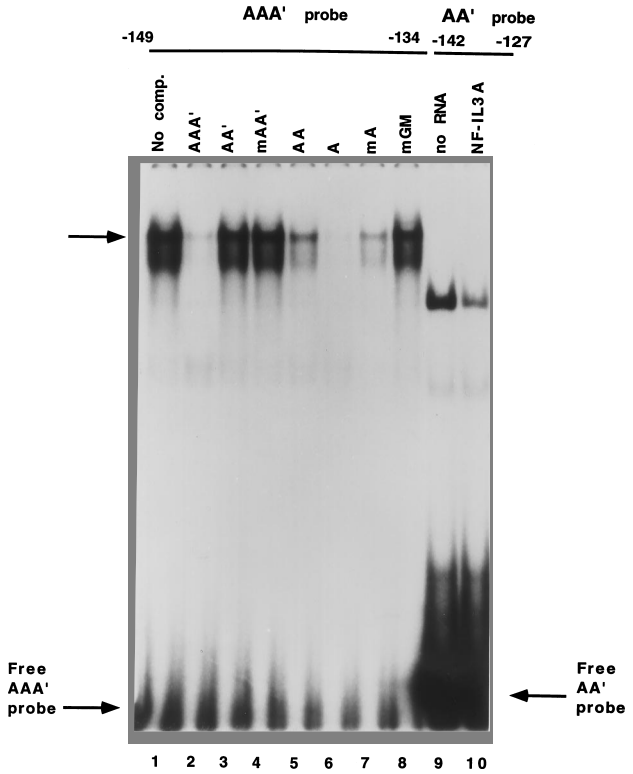

FIG. 6. EMSAs with in vitro-translated NF-IL3A protein and ^{32}P-radiolabeled AAA′ oligonucleotide probe, which contains the sequence ATAATACGTCTGTGG (first eight lanes), or the AA′ probe, which contains the sequence GTCTGTGGTTTTCT (lanes 9 and 10). The NF-IL3A consensus binding sequence is underlined. The competitor (comp.) oligonucleotides contain the following sequences. The A oligonucleotide contains sequences from bp −165 to −128; the mutant A oligonucleotide (MA) contains sequences similar to A except for a five-nucleotide substitution (CATGGAgcAAgggTTACGTCTGT); the AA oligonucleotide contains IL-3 wild-type sequences between bp −160 and −141; the AA′ oligonucleotide contains sequences from bp −142 to −128; and the mAA′ oligonucleotide contains sequences identical to the AA′ oligonucleotide except for a five-nucleotide substitution (GTCTcagacTTTCT). Lane 1, no competitor oligonucleotide; lanes 2 to 8, 100-fold molar excess of unlabeled AAA′ oligonucleotide (lane 2), unlabeled AA′ (lane 3), mutant AA′ (lane 4), AA (lane 5), A (lane 6), MA (lane 7), and the GM-CSF promoter mutant oligonucleotide (lane 8). ^{32}P-radiolabeled AA′ probe was incubated with rabbit reticulocyte lysate translated without NF-IL3A mRNA (lane 9) or with NF-IL3A mRNA (lane 10). The positions of the free AAA′ probe and the free AA′ probe are shown at the bottom of the gel, and the gel shift complex containing in vitro-translated NF-IL3A is indicated by the unlabeled arrow.

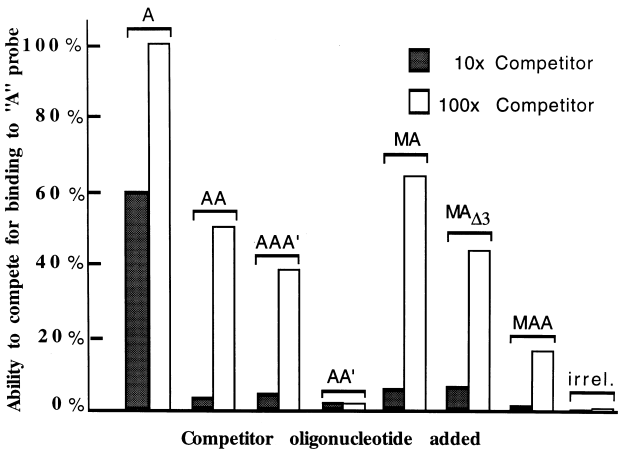

FIG. 7. Comparison of the binding affinities of various oligonucleotides for NF-IL3A protein. Radiolabeled oligonucleotide A was used as the probe in EMSAs. The sequences of several of the oligonucleotides used as competitors are shown in the legends to Fig. 4 and 6. The MAA competitor contains the same sequence as the wild-type AA except for three nucleotide substitutions, GTCAT GGATGAAgggTTACT, and the MA$_{\Delta 3}$ competitor contains the sequence CAT GGATGAATAATTACacgTGTGGTTTTCT. A 10-fold and a 100-fold molar excess of unlabeled competitors were used for these experiments, and the intensities of the specific gel-retarded bands were measured by densitometry and compared. The relative ability of an oligonucleotide to compete for binding to the A probe was calculated by the equation $(a - b)/a \times 100$, where a is the band intensity without competitor and b is the band intensity with competitor. Irrel., irrelevant oligonucleotide.

Binding of NF-IL3A to the IL-3 promoter consensus binding sequence. On the basis of the ability of the MA oligonucleotide to bind NF-IL3A and the DNase I footprinting data, we prepared the AAA′ oligonucleotide, which contains sequences from −149 to −134, including sequences 3′ of the AA oligonucleotide (see Fig. 3 and the legend to Fig. 6). Inclusion of these additional sequences generated a strong DNA-protein interaction involving the ATTACG sequences (Fig. 6 and data not shown). Addition of excess unlabeled AA oligonucleotide competed for binding of NF-IL3A to the AAA′ probe, supporting the notion that the ATTACG sequence constitutes the minimal NF-IL3A binding sequence (Fig. 6, compare lanes 2 and 5). The mutant A oligonucleotide (MA, described in the legend to Fig. 6) competed similarly to the wild-type AA oligonucleotide (compare lanes 5 and 7), providing additional evidence that mutations 5′ of the NF-IL3A consensus binding site (ATGAATAATTACGTCTG to AcgAAgggTTACGTCTG) do not disrupt the binding of NF-IL3A. The AA′ oligonucleotide, which contains sequences from the 3′ part of the A region, did not compete for binding to the AAA′ oligonucleotide (Fig. 6, lane 3), nor did a mutant AA′ oligonucleotide (lane 4). No specific binding of NF-IL3A to the radiolabeled AA′ oligonucleotide was seen (Fig. 6, compare lanes 9 and 10). The A region oligonucleotide competed completely for binding to the AAA′ oligonucleotide (Fig. 6, lane 6), as did the gamma interferon promoter regulatory element and the adenovirus E4 regulatory element (data not shown). The irrelevant GM-CSF promoter sequence did not compete for binding (Fig. 6, lane 8).

The A oligonucleotide binds NF-IL3A most efficiently; the AA and AAA′ wild-type oligonucleotides and the MA and MA$_{\Delta 3}$ mutant oligonucleotides bind NF-IL3A similarly but less well (Fig. 7). The data are most consistent with a minimal binding sequence of ATTACG, which we refer to as a half-consensus sequence because it contains 6 of the 10 nucleotides found in the E4BP4 decanucleotide consensus binding site. This half-consensus sequence is intact in both the AA and AAA′ oligonucleotides. Mutation of the first A residue to a G, in the context of the half-consensus sequence (as in the MAA oligonucleotide), completely eliminates the binding of NF-IL3A, whereas introduction of the same mutation into the decanucleotide consensus binding sequence in the MA oligonucleotide (which contains four additional nucleotide substitutions) impairs but does not abrogate NF-IL3A binding.

Mutations in the 3′ half of the E4BP4 decanucleotide consensus binding sequence (as in the MA$_{\Delta 3}$ oligonucleotide, which contains the sequence ATTACacgTG rather than AT TACGTCTG) reduces but does not eliminate NF-IL3A binding, which is also consistent with the ATTACG being the minimal NF-IL3A binding sequence. Although NF-IL3A can bind to the MA$_{\Delta 3}$ oligonucleotide in vitro, these three nucleotide substitutions completely abrogate the responsiveness of the IL-3 promoter to NF-IL3A transactivation in the co-

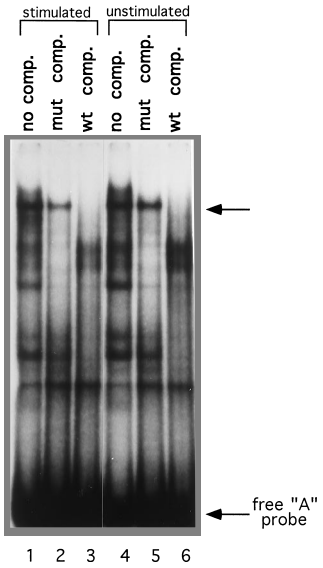

FIG. 8. EMSAs with PHA- or TPA-stimulated or unstimulated MLA144 T-cell nuclear extract and ^{32}P-radiolabeled A probe. Lanes 1 to 3, mitogen-stimulated nuclear extract; lanes 4 to 6, unstimulated nuclear extract. Lanes 1 and 4, no competitor (comp.) oligonucleotide; lanes 3 and 6, 500-fold excess of wild-type A oligonucleotide competitor; lanes 2 and 5, 500-fold excess of $MA_{\Delta 3}$ oligonucleotide competitor (5′-CATGGATGAATAATTACacgTGTGGTTT TCT-3′). The arrow indicates the position of the NF-IL3A gel shift complex. The position of the free probe is shown at the bottom of the gel.

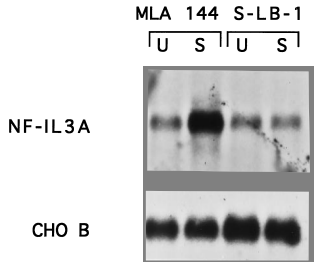

FIG. 9. Northern blot analysis with poly(A)$^+$ RNA from MLA144 and S-LB-1 T-cell lines. U, unstimulated cells; S, cells stimulated with PHA or TPA for 4 h. A single mRNA species is seen. The CHO B (ribosomal protein) control hybridization is shown below.

transfection experiments (Fig. 2). Additional evidence that the ATTACG sequence is the minimal NF-IL3A binding sequence is that the 3′ half of the E4BP4 consensus binding sequence (present in the AA′ oligonucleotide) neither binds NF-IL3A directly (Fig. 6, lanes 9 and 10) nor competes for binding of NF-IL3A to the A oligonucleotide (Fig. 7).

Several lines of evidence suggest that sequences 3′ and 5′ of the ATTACG consensus site stabilize NF-IL3A binding to the A oligonucleotide. The AAA′ oligonucleotide, which contains all 12 nucleotides protected by NF-IL3A in the DNase I footprinting experiments, is not as efficient a competitor as the A oligonucleotide. Rather, it binds NF-IL3A similarly to the AA oligonucleotide, which lacks the IL-3 promoter sequences 3′ of the half-consensus site. At the 5′ end of the A and AA oligonucleotides is the sequence GTCATGGATG, which is somewhat homologous to the E4BP4 consensus binding sequence (60%); the nucleotide substitutions in the MA oligonucleotide do not significantly affect this homology, which may account for some of the binding affinity of NF-IL3A for the A, AA, and MA oligonucleotides.

When MLA144 nuclear extracts were used in an EMSA with the A probe, several specific gel shift bands were observed (Fig. 8, lanes 1 and 4). The uppermost band was competed away by the wild-type A oligonucleotide (Fig. 8, lanes 2 and 5) but not by $MA_{\Delta 3}$ (lanes 3 and 6), demonstrating that it is the NF-IL3A-generated gel shift complex (indicated by an arrow). The position of this band is not precisely the same as that of the band generated by in vitro-translated NF-IL3A, but UV cross-linking of the DNA and protein(s) contained in the nuclear extract-generated gel shift band demonstrates proteins of 58 kDa, which is the molecular mass of NF-IL3A, and 105 kDa, which likely affects the position of the gel shift complex (37).

Expression pattern of NF-IL3A. NF-IL3A is expressed in resting MLA144 cells, but the amount of NF-IL3A mRNA increases following 4 h of PHA or TPA stimulation (see Fig. 9). NF-IL3A mRNA is also detectable in total cellular RNA from resting HUT78 T cells by Northern blot analysis, and the level increases following mitogen stimulation (data not shown). Although NF-IL3A mRNA is detectable in S-LB-1 T cells, a human T-cell lymphotropic virus-infected T-cell line that does not express IL-3, the amount of NF-IL3A mRNA in these cells does not change following PHA or TPA stimulation (see Fig. 9). A multiple-tissue Northern blot (purchased from Clontech) that contains poly(A)$^+$ RNA from colon, bladder, kidney, skeletal muscle, liver, cecum, basal cell, and stomach showed significant expression of NF-IL3A in the bladder and a very weak signal in muscle (data not shown), demonstrating some tissue specificity of NF-IL3A expression. Among hematopoietic cell lineages, NF-IL3A expression is not strictly restricted to T cells, as a sensitive RT-PCR assay demonstrated the presence of NF-IL3A mRNA in a variety of human hematopoietic cell lines (KG-1, HL60, U937, and CMK) (data not shown). The expression of E4BP4 by placenta cells has also been reported (7).

DISCUSSION

Like other lymphokines, the expression of IL-3 occurs following T-cell activation (8, 14, 27, 30). Two regions of the IL-3 promoter have been shown to play important positive regulatory roles in the mitogen responsiveness of this promoter. One region is located between bp −315 and −274 and contains an AP-1 site and several potential ets or elf-1 binding sites (1, 2, 13, 22). Another mitogen-responsive element, located between bp −168 and −125, binds T-cell nuclear proteins in gel shift and DNase I footprinting assays (10, 22, 33, 35, 37) and can confer mitogen responsiveness on a heterologous promoter (37). EMSA and UV cross-linking studies demonstrate the presence of protein(s) of 52 to 60 kDa (and other proteins) that can bind to this region in both resting and activated T cells (37).

Using an expression screening approach and 3′ RACE, we isolated a 1.9-kb full-length cDNA clone that encodes a leucine zipper-containing protein that can specifically bind to the IL-3 A region in vitro and can activate transcription of the IL-3 A region in cotransfection experiments. This transcription factor, which we call NF-IL3A, is approximately 58 kDa in size, similar to the size of the nuclear proteins that can be UV cross-linked to a radiolabeled A region oligonucleotide probe (37). Southern blot analysis has revealed that NF-IL3A is encoded by a single-copy gene (data not shown). A nearly identical cDNA was isolated from a human placenta cDNA library by virtue of its ability to bind to the E4 region of the adenovirus promoter (7). The nucleotide sequence of this protein, called E4BP4, differs from the NF-IL3A sequence by a single nucle-

otide in the coding region, which results in a single amino acid difference (a glycine instead of an arginine due to a G-to-A difference at nucleotide 1030). This likely reflects a polymorphism at this position. There are also nine nucleotide differences in the 3′ untranslated regions of the NF-IL3A cDNA and the E4BP4 cDNA. The 3′ untranslated region of NF-IL3A contains only one ATTTA sequence and no TTATTTA(T/A)(T/A) (20) or TTATTTATT (38) sequences, which appear to be the sequences responsible for increasing the rate of degradation of the mRNA.

Cotransfection studies performed by Cowell and colleagues, using an adenovirus E4 promoter element, demonstrated repression of transcription by the E4BP4 protein in HeLa cells (7). Our results demonstrate that NF-IL3A activates the IL-3 promoter in a sequence-specific fashion in T cells, and studies performed in collaboration with Tom Look and his colleagues have demonstrated that NF-IL3A has no effect on transcription from a GTTACGTAAT sequence (the HLF consensus binding site) in B cells (16). Our data and the data from Cowell et al. (7) suggest that NF-IL3A may stimulate the transcription of certain promoters, such as that for IL-3, and inhibit transcription from other promoters (e.g., exogenous DNA viral promoters), perhaps depending upon the nucleotide sequences surrounding its binding sequences, the presence or absence of other interacting nuclear proteins, and the cell type used for the transfection studies. YY1 transcription factor is another example of a factor with varying regulatory activity; it activates transcription in the presence of the adenovirus E1A protein but represses transcription in the absence of E1A (32).

We have demonstrated specific in vitro binding of NF-IL3A to the human IL-3 promoter sequences extending from bp −148 to −137 in both DNase I footprinting assays and EMSAs. Using a PCR-based oligonucleotide binding site selection strategy, Haas et al. (15) defined a consensus binding site for VBP, a member of the PAR family of bZIP proteins, which has a basic amino acid region very similar to that of NF-IL3A. Using full-length protein, they determined that this family of proteins can recognize asymmetric half-sites, with one half-site being most consistent with a PAR binding site [especially (A/G)TTAC] and the other half-site being similar to a CREB or C/EBP binding site. They determined that PAR proteins such as VBP can recognize a variety of DNA sequences, with a consensus binding site of (A/G)TT(A/G)(C/T)(G/A)T(C/A)(A/T)(T/G). This is also consistent with the E4BP4 consensus binding sequences (defined by Cowell et al. [7] using a similar PCR-based oligonucleotide selection technique) and with the consensus binding sequence for the related bZIP protein HLF, which binds preferentially to a GTTACGTAAT sequence (17).

Our data suggest that the recognition sequence for the binding of NF-IL3A may not be as strict as that published for E4BP4 by Cowell and colleagues (7), who used an oligonucleotide binding site selection strategy. Competition EMSAs suggest that the minimal IL-3 promoter sequence required for NF-IL3A binding is ATTACG, although the larger region, TAATTACGTCTG, is protected by in vitro-translated NF-IL3A in DNase I footprinting assays. IL-3 promoter sequences at the 5′ end of the A region can increase the binding affinity of NF-IL3A to this region but are not essential for NF-IL3A binding (37). Our limited mutagenesis studies suggest that the A residue at position −146, the first nucleotide of the NF-IL3A binding site, can be replaced by a G residue without disrupting recognition by NF-IL3A in the context of an intact A region (Fig. 4, lane 6).

The complete 10- or 12-nucleotide binding site for NF-IL3A includes the sequence TTACGTCT, which was shown to be critical for IL-3 expression and was thought to bind a member of the CREB/ATF family of transcription factors (22). Mutation of 3 bp in the middle of this consensus sequence (in the $MA_{\Delta 3}$ oligonucleotide) greatly reduced the binding of T-cell nuclear proteins and in vitro-translated NF-IL3A to the IL-3 promoter (Fig. 7) and eliminated its responsiveness to NF-IL3A (the −315mut construct, shown in Fig. 2). Previous studies have shown that mutation of these IL-3 sequences, from AATTAC (−147 to −142) to AtcgAC (33), or deletion of the ATTA sequences from this critical region (22) eliminates mitogen-inducible promoter function. The consensus CREB binding sequence from the somatostatin promoter does not compete for protein binding to the A probe (data not shown), demonstrating that the proteins binding to the A region are not CREB/ATF proteins. The adenovirus E4 promoter was also thought to bind a CREB/ATF-like factor, prior to the cloning of E4BP4 (7).

We examined the promoters of several other lymphokine genes to determine whether they contain sequences capable of binding or responding to NF-IL3A. The 5′-flanking regions of the human, murine, and rat gamma interferon genes contain the sequence ATTACGTATT, which nearly matches the consensus binding site for NF-IL3A and the PAR family of bZIP proteins. NF-IL3A can bind to these positive-acting regulatory sequences, but unlike the A region oligonucleotide, the gamma interferon sequences do not confer NF-IL3A responsiveness on the Tk promoter in T cells (36a). The sequences in the adenovirus E4 promoter region and the IL-3 and gamma interferon promoters are shown in Fig. 3. Although the sequence homology between these regions is not exact, all these sequences can bind NF-IL3A and can compete for its binding to the A region. Like the gamma interferon promoter sequence, the E4BP4 sequences cannot confer NF-IL3A responsiveness on the Tk promoter in T cells, suggesting that cooperativity between NF-IL3A and the other proteins that can bind to the A region is critical for NF-IL3A transactivation (36a). The sequence immediately 3′ of the NF-IL3A binding site, TGT GGT, is a consensus binding site for the CBFα/PEBP2α/AML1 family of proteins (3, 24); the possibility of interactions between these proteins is being explored. Additionally, binding of Oct-1 to IL-3 sequences 5′ of the NF-IL3A binding site in the A region has been demonstrated (22), although the interaction of Oct-1 and NF-IL3A has not been studied yet.

Although NF-IL3A mRNA levels increase after PHA or TPA stimulation of MLA144 cells, EMSAs with stimulated and unstimulated MLA144 nuclear extracts show no difference in NF-IL3A binding activity. There are several potential explanations for this discrepancy; the DNA-binding activity of NF-IL3A may be controlled via posttranslational events (e.g., phosphorylation). It is also possible that NF-IL3A interacts with other cellular proteins before or after stimulation, altering the amount of NF-IL3A available for DNA binding. Although the expression of NF-IL3A mRNA is not restricted to T cells, its expression pattern is limited. NF-IL3A mRNA can be detected in a variety of hematopoietic cell lines by RT-PCR, although it is not known whether NF-IL3A mRNA is expressed in normal hematopoietic cells (rather than cell lines). The ability of NF-IL3A to transactivate the IL-3 promoter in a sequence-specific fashion in T cells suggests that NF-IL3A is important in the activation-dependent expression of IL-3 (and possibly other lymphokines). Its presence in resting T cells suggests that post-transcriptional changes following T-cell activation may regulate its transcriptional activation activity.

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