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Molecular Regulation of the Human IL-3 Gene: 
Inducible T Cell-Restricted Expression Requires Intact 
AP-1 and Elf-1 Nuclear Protein Binding Sites

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Summary

Interleukin 3 (IL-3) is a hematopoietic stem-cell growth and differentiation factor that is expressed 
solely in activated T and NK cells. Studies to date have identified elements 5' to the IL-3 coding 
sequences that regulate its transcription, but the sequences that confer T cell–specific expression 
remain to be clearly defined. We have now identified DNA sequences that are required for T 
cell–restricted IL-3 gene transcription. A series of transient transfections performed with human 
IL-3-chloramphenicol acetyltransferase (CAT) reporter plasmids in T and non-T cells revealed 
that a plasmid containing 319 bp of 5' flanking sequences was active exclusively in T cells. Deletion 
analysis revealed that T cell specificity was conferred by a 49-bp fragment (bp -319 to -270) 
that included a potential binding site for AP-1 transcription factors 6 bp upstream of a binding 
site for Elf-1, a member of the Ets family of transcription factors. DNaseI footprint and 
electrophoretic mobility shift assay analyses performed with MLA-144 T cell nuclear extracts 
demonstrated that this 49-bp region contains a nuclear protein binding region that includes consensus 
AP-1 and Elf-1 binding sites. In addition, extracts prepared from purified human T cells contained 
proteins that bound to synthetic oligonucleotides corresponding to the AP-1 and Elf-1 binding 
sites. In vitro–transcribed and -translated Elf-1 protein bound specifically to the Elf-1 site, and 
Elf-1 antisera competed and super shifted nuclear protein complexes present in MLA-144 nuclear 
extracts. Moreover, addition of anti–Jun family antiserum in electrophoretic mobility shift assay 
reactions completely blocked formation of the AP-1–related complexes. Transient transfection 
studies in MLA-144 T cells revealed that constructs containing mutations in the AP-1 site almost 
completely abolished CAT activity while mutation of the Elf-1 site or the NF-IL-3 site, a previously 
described nuclear protein binding site (bp -155 to -148) in the IL-3 promoter, reduced CAT 
activity to <25% of the activity given by wild-type constructs. We conclude that expression 
of the human IL-3 gene requires the AP-1 and Elf-1 binding sites; however, unlike other previously 
characterized cytokine genes such as IL-2, the AP-1 and Elf-1 factors can bind independently 
in the IL-3 gene. Thus, the exact DNA composition of these sites, flanking DNA sequences, 
and the distance between the AP-1 and Ets family binding sites determine the fine specificity 
of nuclear factors that bind to these sites and the resulting inducible, cell-restricted expression 
of a group of lymphokine genes.

The growth factor interleukin 3 (IL-3) is a major regulator of hematopoiesis. The role of IL-3 is particularly 
interesting because IL-3 is secreted from T and NK cells and primarily acts on both early stem cells and hematopoietic 
cells committed to distinct lineages (1, 2). Alone, IL-3 stimulates the proliferation and differentiation of bone marrow precursor 
cells to produce macrophages, eosinophils, and mast cells, and in combination with other growth factors, IL-3 influences 
the development of erythrocytes, megakaryocytes, and neutrophils (3–5). In addition, IL-3 supports the growth of cell lines derived from bone marrow cultures and enhances retrovirus-mediated gene transfer into bone marrow stem cells 
(6, 7). Thus, this lymphokine forms a bridge between the immune system and homeostasis in non-immune tissues.

IL-3 is distinguished from other growth factors by its restricted cellular expression and mode of induction. Thus, only 
activated CD28+ T cells and NK cells (8–10) produce IL-3, whereas growth factors such as GM-CSF and G-CSF are 
expressed in a variety of cell types (11–13). In addition, the agent of activation is critical to optimal IL-3 gene induction in human 
T cells. In contrast to other cytokines such as IL-2, TNF-α, lymphotoxin (LT), IFN-γ, and GM-CSF, incubation of T
cells with the lectin lymphotixin (LT) PHA alone, or with the phorbol ester, PMA, with and without anti-CD28 fails to induce substantial IL-3 expression (8, 14). To induce high-level IL-3 gene expression, T cells must be activated via the TCR/CD3 pathway or with agents that mimic this pathway (PMA and ionomycin) (8).

Although much is known regarding the biological activities and requirements for induction of IL-3 in vitro, relatively little is understood about the regulation of expression of this gene. The genes for both the murine and the human homologue of IL-3 have been cloned (15, 16), and comparison between the two sequences reveals a low level of sequence identity except for some short regions of sequence homology in the 5' and 3' flanking regions. Two recent reports have demonstrated that IL-3 mRNA accumulation is controlled at the transcriptional and posttranscriptional levels (17, 18). The studies describing transcriptional regulation of the IL-3 gene have shown that the IL-3 promoter consists of two activating regions separated by an inhibitory region (19-21). The 3' activating region contains two motifs common to many lymphokine promoters, the CK-1 and CK-2 elements (22). In addition, the 3' activating region contains a sequence similar to the Oct-1 motif (23, 24), NF-IL-3, and an element immediately 3' that is similar to a cAMP-responsive element (CRE) (25). These latter two motifs, referred to as ACT-1 (21) may be important for IL-3 activation. The inhibitory region includes a sequence referred to as NIP that binds a nuclear protein in electrophoretic mobility shift assays (EMSA)1 (19) thought to restrict IL-3 expression to T cells. However, as the NIP site also appears to be functionally inhibitory in activated MLA-144 gibbon T cells, this site alone cannot be responsible for the restriction of IL-3 expression to T cells.

A number of recent studies have shown that the regulatory regions of inducible T-cell-related genes contain an AP-1 nuclear protein binding site in close proximity to an Ets family nuclear protooncogene binding site, and these sites are required for inducible gene expression (26, 27). Of note, the 5' activation region of IL-3 contains a consensus binding site for the AP-1 transcription factors. Notably, the AP-1 motif has been shown to confer phorbol ester inducibility upon heterologous reporter genes (28, 29) and is thought to regulate activation of many inducible genes (30). In addition, the human IL-3 promoter contains a consensus Ets family binding site 6 bp 3' of the AP-1 motif. Thus, given the distinct lineage restricted and TCR-inducible pattern of IL-3 gene expression, it was of interest to address the molecular mechanisms underlying the role of these sites in regulating IL-3 gene expression.

In this report, we show that the AP-1 and an Ets family binding site, Elf-1, are required for inducible IL-3 gene expression. Moreover, the 49-bp fragment containing the AP-1 and Elf-1 nuclear protein binding sites contributes to T cell-restricted expression of the IL-3 gene. In contrast to the requirement of AP-1 factor complex formation for NFAT binding activity seen in the IL-2 enhancer (27), the AP-1 and Elf-1 sites in the IL-3 gene can bind their respective proteins or protein complexes independently. We also confirm that the NF-IL-3 site is required for IL-3 gene expression. These data provide the basis for a better understanding of the molecular mechanisms of T cell IL-3 gene regulation and its role in the cellular link between the immune system and nonimmune cell proliferation.

Materials and Methods

Cells and Media. Gibbon lymphosarcoma-infected T cells, MLA-144 (ATCC TIB 201) (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories) and 1% penicillin-streptomycin (Gibco Laboratories).

RNA Preparation and Northern Blot Analysis. Total cellular RNA was isolated from MLA-144 cells using the guanidinium isothiocyanate–cesium chloride gradient method (31). RNA samples were prepared from cells after incubation with one or more of the following reagents: PMA (Sigma Chemical Co., St. Louis, MO) alone and with ionomycin (Calbiochem Corp., San Diego, CA) or with PHA (Sigma Chemical Co.), PMA alone and finally, with media only. After equalization by spectrophotometry and visualization in agarose gels containing ethidium bromide, 10 μg of RNA was fractionated on a 1% agarose/formaldehyde gel and transferred to Hybond-N nylon filters (Amersham Corp., Arlington Heights, IL). The filters were hybridized to a human IL-3 cDNA, which was radiolabeled with α[32P]-dCTP by random nucleotide priming (32).

Isolation of Human IL3 Genomic Clone and Plasmid Construction. A genomic clone containing the human IL-3 gene and 5' flanking sequences was isolated from a human peripheral blood genomic library (the generous gift of John Lowe, Howard Hughes Medical Institute, Ann Arbor, MI) by hybridization to a human IL-3 cDNA probe. Restriction mapping of the IL-3 clone was performed using standard techniques (31).

A set of overlapping DNA sequences from the 5' flanking region of the human IL-3 were cloned in 5' to 3' orientation directly 5' of the bacterial chloramphenicol acetyltransferase (CAT) gene in the promoterless/enhancerless vector, pCAT-Basic (Promega, Madison, WI).

Transfections. Various cell types were transfected with 10–20 μg purified supercoiled plasmid DNA prepared by alkaline lysis and cesium–chloride banding (31). The MLA-144, Clone 13, PEER, and Jurkat cells were transfected using the DEAE-dextran method as previously described (33). HeLa cells were transfected using 25 μg of Lipofectin (Gibco Laboratories). 2 μg of either pRSV/GAL or pRSVLuciferase DNA was included in each transfection to correct for differences in transfection efficiencies between samples as previously described (33).

CAT, β-Galactosidase, and Luciferase Assays. Cells were harvested 48 h after transfection by four cycles of freeze–thaw lysis, and protein levels were determined using a protein assay (Bio-Rad Laboratories Inc., Richmond, CA). The β-galactosidase and CAT activities were determined as previously described (33). Luciferase assays were performed essentially as described by De Wet (34) using luciferin substrate (Sigma Chemical Co.) and a Pharmacia luminometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

DNA Sequencing. DNA sequence analysis was performed on
double-stranded DNA templates using the dideoxynucleotide chain termination protocol (35).

DNAse I Footprint Analysis. A 193-bp subfragment (bp -319 to -126; sense strand) of the flanking IL-3 gene was end labeled with [α-32P]-dGTP and [α-32P]-dUTP with the large fragment of DNA polymerase 1 (Klenow) and isolated by PAGE. The labeled 193-bp fragment was subjected to partial DNAse I digestion as previously described (33), after incubation with 100–200 μg of MLA-144 nuclear protein extracts. Maxam and Gilbert purine sequencing reactions (33) were prepared and electrophoresed in parallel to the DNase I digested subfragments.

EMSA: Complementary oligonucleotides corresponding to the AP-1, Elf-1, NF-IL-3, and AP-1/Elf-1 nuclear protein binding sites were synthesized with overhanging BamHI/BglII ends and were end labeled using Klenow as described above before EMSA:

AP-1/Elf-1: GATCTGAGCTAGTCAGGGCTCTGGCCTCCCTTGACAGGG

MSV LTR: TCGGAGGACGGACGCACGGC

Elf-1: GATCTTCCCTTCTTTCGACAGGG

NF-IL-3: GATCCATGATGAATATTA

AP-1: GATCTGAGCTAGTCAGGGCTCTGGCCTCCCTTGACAGGG

EMSA: As performed as previously described (33) except that the binding buffer included 4% Ficoll, 10 mM Tris, 1 mM EDTA, 1 mM DTT, 75 mM KCl, and 500 ng poly (dI-dC), and the gels were run in 0.25 × Tris-boric acid-EDTA running buffer. Unlabeled competitor oligonucleotides (50–200 ng) were included in cold competition experiments. EMSA included either 3–5 μg of nuclear extracts prepared from MLA-144 cells (unstimulated and stimulated cells), or 3 μl of in vitro–transcribed/translated protein or 1 μg of nuclear extract prepared from unstimulated or stimulated (10 ng/ml PMA and 400 ng/ml ionomycin for 6–8 h) normal human T cells purified by elutriation (generous gift of Drs. Lawrence Boise and Craig Thompson, Howard Hughes Medical Institute, Chicago, IL). The sequences of oligonucleotide competitors used in these studies include the above probes unlabeled and:

mELF-1: GATCCCTGTGTCTCCTTCGGAA

MNIP: GATCCACCTTCTTACTCCTTCCA

Supershift experiments were performed in the presence of 1 μl of rabbit or mouse antisera. These antisera were incubated with the protein extracts for 15 min before the addition of DNA probe in EMSA reactions.

Preparation of Mutated Constructs. Mutations of the NF-IL-3 nuclear protein binding site were introduced by site-directed oligonucleotide-mediated gapped-heteroduplex mutagenesis as previously described (33) using the following synthetic oligonucleotide:

TGAGTACTGAGATGGACATCGAGTCTGCTGTTTCTATGG

The wild-type NF-IL-3 site (ATGATAAA) was changed to a mutant sequence (GACTCCGAG). Underlined sequences denote regions of mutation. Mutations were introduced into AP-1 and Elf-1 nuclear protein binding sites using the PCR as previously described (33) with the following oligonucleotides:

AP-1: GGCAGGAGGCTCAGTGAGTGAGCCGGCTCCCTCCTGACCAGGG

Elf-1: CAGTGAGCTAGTCAGGGCTCTGGCCTCCCTTGACAGGG

These oligonucleotides were ligated in reverse orientation to pSPCAT, an enhancerless plasmid under the control of the minimal SV40 promoter (36). In Vitro Transcription/Translation Reactions. Linearized cDNAs corresponding to full-length and truncated Elf-1 cDNA (Elf-1101–360; the generous gift of Jeffrey Leiden, University Chicago, IL) were transcribed using 10 U T7 RNA polymerase, 40 U RNasin ribonuclease inhibitor (Promega) and 0.4 mM rNTPs. Transcribed RNAs were then translated in the presence of 0.8 μCi/ml [35S]methionine (Amersham Corp.), 40 U RNasin, 20 mM amino acids (minus methionine) and 35 μl rabbit reticulocyte lysate (Promega). The integrity of translated proteins was assessed by SDS-PAGE.

Antibodies. Rabbit polyclonal Elf-1 antiserum and preimmune rabbit serum (generous gift of Jeffrey Leiden), and anti-Jun family sera that reacts with all three Jun proteins: JunB, JunE, and c-Jun (the generous gift of Rodrigo Bravo, Bristol-Meyers Squibb Pharmaceutical Research Institute, Princeton, NY) were used in some EMSA reactions. The Elf-1 antiserum does not react with Ets-1 or Ets-2. Anti–IL-2 receptor (IL-2R; Becton Dickinson, Mountain-view, CA) mouse mAb was used in control reactions.

Results

Isolation and Characterization of a Genomic Clone Containing the Human IL-3 Gene. We isolated a clone containing the full-length IL-3 gene from a human genomic library with a human IL-3 cDNA as a probe and standard techniques. This clone includes 3 kb of 5' flanking sequence and 5 kb of 3' flanking sequence. To identify regulatory elements in the 5' flanking region, we sequenced 1,400 bp of DNA 5' of the transcriptional start site (Fig. 1). A computer homology search of the human IL-3 DNA sequence revealed similarities to potential regulatory motifs not previously observed. These include an ets-1 family nuclear protooncogene binding site (EBS) with one mismatch (bp -288 to -278; antisense strand; GGCAGGAAAGG) (37–40). Of note, this EBS contains an
Identification of cis-Acting Transcriptional Regulatory Sequences that May Be Involved in T Cell-Restricted IL-3 Gene Expression

In an effort to define transcriptional regulatory elements that modulate IL-3 gene expression, we examined a series of heterologous reporter constructs containing the bacterial CAT gene and human genomic IL-3 subfragments in various cell lines. Initially, a set of contiguous DNA segments from the 5' flanking region of the IL-3 gene was cloned in 5' to 3' orientation directly 5' of the CAT gene in the promoterless/enhancerless vector, pCAT-basic. As shown in Fig. 2, constructs containing 150 bp (which includes the CK-1, CK-2, and CRE elements) and 175 bp (which includes the CK-1, CK-2, CRE, and NF-IL-3 elements) of 5' flanking sequence (with 30 bp of 5' untranslated sequences) increased CAT activity after activation in all cell lines tested. CAT activity was also observed in transfected HeLa cells and lower level activity was demonstrated in unstimulated T and B cells. In contrast, a construct containing 319 bp of 5' flanking sequence was active exclusively in T cells. Deletion analysis demonstrated that this activity was lost in T cells when 49 bp was removed from the 319-bp construct, and this construct was also inactive in non-T cell lines. The 49-bp DNA segment (bp -319 to -270) contains a consensus AP-1 nuclear protein binding site described above. Such an AP-1/Ets family member nuclear protein binding cassette has been implicated in the activation of other cytokine genes (26, 27).

Although -270CAT included the putative silencer NIP, mutation of the NIP site from GCTC~CATGC to TCTTCA~CATGC did not affect CAT activity in MLA-144 T cells or Clone 13 B cells. The 49-bp DNA segment was included in the constructs. The 319-bp construct was also inactive in non-T cell lines. The 49-bp DNA segment (bp -319 to -270) contains a consensus AP-1 nuclear protein binding site described above. Such an AP-1/Ets family member nuclear protein binding cassette has been implicated in the activation of other cytokine genes (26, 27).

Although -270CAT included the putative silencer NIP, mutation of the NIP site from GCTC~CATGC to TCTTCA~CATGC in the context of -270CAT failed to increase CAT activity in MLA-144 T cells or in Clone 13 B cells to the level demonstrated by -175CAT (data not shown). This suggests that additional DNA sequence is necessary for full activity in T cells regardless of the status of NIP site integrity, or that the low-level activity given by -270CAT is not en-
tirely due to the NIP site. Together, these data provide evidence that the 49-bp fragment is required for the T cell-restricted pattern of IL-3 gene expression and that it may function in concert with other negative regulatory elements within the IL-3 promoter.

**Activation of MLA-144 Cells Induces High-level IL-3 Gene Transcripts and Nuclear Proteins that Bind to DNA Sequences within the 49-bp Fragment in the 5′ Flanking Sequences of the IL-3 Gene.**

To study further the role of the 49-bp fragment in IL-3 gene transcription, it was necessary to identify cells that could be induced to express high levels of IL-3. Northern blot analysis of RNA obtained from Jurkat and PEER T cells revealed that these cells produced extremely low levels of IL-3 gene transcripts (data not shown). However, as shown in Fig. 3A, MLA-144 cells can be induced by incubation for 8 h with PMA (5 ng/ml) and PHA (2 μg/ml) to produce high levels of IL-3 gene transcripts and to a lesser degree after incubation with PMA (5 ng/ml) and ionomycin (400 ng/ml) or with PMA (100 ng/ml) alone. These levels were comparable with levels of IL-3 expression obtained in primary human T cells (L. Gottschalk, unpublished results). Notably, no IL-3 gene transcription was detected in RNA prepared from cells incubated with media alone.

These data confirm that, unlike other inducible cytokine genes, IL-3 gene expression is not induced with PHA alone (8) and suggest that relatively unique molecular mechanisms may regulate IL-3 gene transcription. To identify nuclear protein binding sites within the 49-bp subfragment of the human IL-3 gene promoter that conferred T cell specificity in CAT assay studies, we subjected a 193-bp DNA subfragment (bp -319 to -126; sense strand) containing the 49-bp segment (bp -319 to -270) to DNase I footprint analysis. Fig. 3B and C shows that nuclear extracts prepared from MLA-144 cells protected the AP-1 site, Elf-1 site, and intervening DNA from digestion by DNase I (bracketed and boxed) and that no other regions in the 49-bp segment within the 193-bp fragment bound nuclear proteins. Because these sites were protected by nuclear extracts prepared from both unstimulated and stimulated MLA-144 cells, further characterization of the proteins that interact with the IL-3 gene DNA was performed by EMSAs.

**MLA-144 Cells Contain Multiple Specific Nuclear Proteins that Bind to DNA Sequences in the IL-3 Promoter.**

To determine the number and the specificity of nuclear proteins that bind to the protected sequences present in the 49-bp IL-3 gene subfragment, we performed a series of EMSAs with MLA-144 nuclear extracts and double-stranded synthetic oligonucleotides corresponding to the AP-1 and Elf-1 nuclear protein binding sites. Fig. 4A shows that when nuclear extracts from resting MLA-144 cells were tested with the AP-1/Elf-1 DNA probe (including intervening DNA), four low-mobility bands (arrows) bound specifically, as demonstrated by competition with wild-type oligonucleotides (lane 2) but not by unrelated DNA competitor (MNIP, lane 6) or a mutant Elf-1 DNA competitor (lane 4). Specific bands were also demonstrated using stimulated MLA-144 extracts; however, with the exception of the lowest mobility band, the bands were difficult to distinguish. Of note, the highest mobility bands do not appear to be specific because they are present despite the addition of wild-type DNA competitors. Further cold competition experiments revealed that an oligonucleotide corresponding to a consensus AP-1 nuclear protein binding site competed the middle complexes in both unstimulated and stimulated nuclear extracts (lanes 3 and 11). In contrast,

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Northern blot analysis of MLA-144 RNA and DNase I footprint analysis of the 5′ flanking region of the human IL-3 gene. (A) Northern blot analysis of human IL-3 expression in MLA-144 cells during activation. Total cellular RNA was prepared from MLA-144 cells incubated for 8 h with either media alone, or with PMA (5 ng/ml) and ionomycin (400 ng/ml), or with PMA (5 ng/ml) and PHA (2 μg/ml), or with PMA (100 ng/ml) alone or with PHA (2 μg/ml) alone. Ten μg of RNA from each condition was equalized for 28S and 18S ribosomal RNA, fractionated by gel electrophoresis, and transferred to nylon. The nylon filter was hybridized to a radiolabeled human IL-3 DNA probe and later hybridized to a human β-actin DNA probe and the resulting autoradiograms are shown. (B) DNase I footprint analysis of the 5′ flanking sequences of the human IL-3 gene. A 193-bp fragment (bp -319 to -126; sense strand) was end labeled and incubated with unstimulated (lane) or with stimulated (stim) or without (Control) MLA-144 nuclear extract before partial digestion with DNase I. Standard Maxam and Gilbert purine (G + A) sequencing reactions of the same fragment were prepared and run in parallel. The protected DNA segment that includes AP-1 and Elf-1 nuclear protein binding sites is marked with brackets in B and boxed within the DNA sequence in C. The AP-1 and Elf-1 nuclear protein binding sites are underlined in C.
competition with an oligonucleotide that corresponds to the Elf-1 site alone showed that the lowest mobility specific band is competed when mixed with the AP-1/EIf-1 probe and both stimulated and unstimulated extracts. In addition, when an Elf-1 DNA competitor is added in EMSA at the same time with AP-1 DNA competitor, the lower predominant band evident in lanes 5 and 11 (AP-1 DNA competitor alone) is reduced suggesting that there are two bands that run with similar mobility, one binding to the AP-1 site and one binding to the Elf-1 site (data not shown). Together, the EMSAs demonstrate specific and distinct complex formation with the AP-1/EIf-1 oligonucleotide probe and proteins from both unstimulated and stimulated MLA-144 cells.

Fig. 4 B shows that an oligonucleotide corresponding to the AP-1 binding site in the IL-3 gene specifically bound nuclear proteins from stimulated MLA-144 cells in the absence of the Elf-1 site (lanes 1, 3, and 4). However, comparison of the complexes formed with the AP-1/EIf-1 probe described above to those formed with the AP-1 probe alone (Fig. 4 B) and the Elf-1 probe alone (see Fig. 6, lane 5) demonstrate that at least some of the binding activity observed with the AP-1/EIf-1 probe may require cooperative binding of AP-1 and Elf-1 or of yet undefined transcription factors. Of note, the addition of unlabeled NF-IL-3 competitor eliminated all complex formation observed with a labeled AP-1 consensus probe (Fig. 4 B, lane 2) and stimulated extracts, suggesting that the NF-IL-3 nuclear protein binding site may bind AP-1 transcription factors.

The AP-1/EIf-1 DNA Probe Binds In Vitro Transcribed/Translated Elf-1 Protein and Is Competed by Anti-EIf-1 Sera. To test whether Elf-1 protein binds to the EBS in the IL-3 promoter, truncated and full-length in vitro–transcribed/translated Elf-1 protein was used in EMSAs with the AP-1/EIf-1 DNA probe. Because truncated Elf-1 protein (Elf-1{109-304}) binds with equivalent specificity as full-length Elf-1 protein (44) and because of higher translation efficiency of smaller transcripts in vitro, the truncated version was used in competition experiments. As seen in Fig. 5, the AP-1/EIf-1 DNA probe bound truncated Elf-1 (t-Elf-1, lane 2). This binding activity was abolished by the addition of unlabeled wild-type Elf-1 competitor (lanes 3 and 4) but was still present when nonspecific DNA competitor was added (lane 5). As expected, no binding activity was observed when in vitro translation reactions without transcribed RNA was used in EMSAs (lanes 1 and 6). Finally, a control probe that contains an Elf-1 nuclear protein binding site from the MSV LTR (MSV) bound the in vitro–transcribed and translated tElf-1 protein (lane 7). These EMSA data demonstrate that Elf-1 protein can bind directly to DNA sequences in the IL-3 promoter.

Addition of Elf-1 and Jun Antisera Block Complex Formation in EMSA Reactions with MLA-144 Nuclear Extracts. To identify the proteins within MLA-144 nuclear extracts that bind to IL-3 promoter DNA sequences, we performed EMSAs as above with anti-Jun and anti-EIf-1 antisera. Fig. 6 shows that addition of anti-Jun family serum which recognizes JunB, JunD, and c-Jun blocked the formation of the middle complexes with both unstimulated and stimulated MLA-144 nuclear extracts (compare lanes 1 and 8 with 3 and 10). As described above, the same bands were blocked by addition of consensus AP-1 DNA competitor. When specific anti-EIf-1 serum was added into the EMSA reactions, it was difficult to demonstrate the disappearance of a band using the AP-1/EIf-1 DNA probe (lane 2); however, the higher mobility band (closed arrow) formation was blocked in EMSA reactions containing the shorter Elf-1 DNA probe (t-Elf-1; compare lanes 5 and 6). This lower band ran with the same mobility as

In Vitro Translated Protein: t-Elf-1 t-Elf-1 t-Elf-1 t-Elf-1 elf-1

Competitor (ng): 0 100 200 0 0

Figure 5. EMSA with either a radiolabeled AP-1/EIf-1 probe or MSV probe and in vitro–transcribed/translated truncated Elf-1 (t-Elf-1) or translation products prepared without RNA (–). Lanes in which unlabeled competitor oligonucleotide competitor was added and the amounts used are noted.
Intact AP-1 and Elf-1 Nuclear Protein Binding Sites Are Required for Full Functional Activity. We next asked whether the band observed in EMSA reactions containing in vitro-translated full-length Elf-1 protein and the AP-1/Elf-1 probe (data not shown). In addition, a faint supershifted complex (open arrow) was observed in reactions containing either DNA probe with unstimulated or stimulated MLA-144 nuclear protein extracts in the presence of various antisera. Various antisera, as noted, were incubated with the nuclear proteins for 15 min before the addition of the radiolabeled probe. Lanes 1–7 were run on one gel while lanes 8–11 were run on a separate gel. The complex corresponding to full-length Elf-1 (f-Elf-1) is denoted by a closed arrow as well as the supershifted complexes (open arrows) evident in lanes 2, 6, and 11. Preimmune sera and anti-IL-2 receptor (α-IL-2R) were used as controls.

Figure 6. EMSA using radiolabeled AP-1/Elf-1, Elf-1, or MSV oligonucleotide probes with unstimulated or stimulated MLA-144 nuclear protein extracts in the presence of various antisera. Various antisera, as noted, were incubated with the nuclear proteins for 15 min before the addition of the radiolabeled probe. Lanes 1–7 were run on one gel while lanes 8–11 were run on a separate gel. The complex corresponding to full-length Elf-1 (f-Elf-1) is denoted by a closed arrow as well as the supershifted complexes (open arrows) evident in lanes 2, 6, and 11. Preimmune sera and anti-IL-2 receptor (α-IL-2R) were used as controls.

the NF-IL-3, AP-1, and Elf-1 sites that bound nuclear proteins played important functional roles in IL-3 gene transcriptional activation in intact MLA-144 cells. A series of mutant IL-3 promoter constructs were produced and tested for transcriptional activity after transfection into MLA-144 cells. Mutations were introduced into the –319-bp promoter in the AP-1, the Elf-1, the NF-IL-3 nuclear protein binding sites or in a combination of sites (changes noted in Materials and Methods and in the figure legend). In addition, constructs containing multimerized oligonucleotides corresponding to the wild-type and mutant AP-1 and Elf-1 sites with interfering nucleotides were cloned into a vector under the direction of the minimal SV40 promoter and tested for transcriptional activity. Fig. 7, A and B shows that the AP-1 and the Elf-1 nuclear protein binding sites were necessary for full activity in stimulated MLA-144 cells both in the context of the endogenous IL-3 promoter (Fig. 7 A) and in constructs containing the multimerized DNA segments controlled by the minimal SV40 promoter (Fig. 7 B). Finally, mutation of the NF-IL-3 site in –319CAT reduced CAT activity by 80%, demonstrating a role for this site as well. Interestingly, the reduced CAT activity produced by –319CAT with the NF-IL-3 mutation in comparison to that produced by the wild-type construct is less dramatic when transfected into Jurkat T cells (data not shown). The studies detailed above demonstrate the requirement of intact AP-1, Elf-1, and NF-IL-3 nuclear protein binding sites for inducible IL-3 gene expression.

Human T Cells Contain Multiple Inducible Specific Nuclear Proteins That Bind to DNA Sequences in the IL-3 Promoter. To investigate the interaction of human T cell nuclear proteins with the 49-bp IL-3 promoter sequence, we performed EMSAs using various oligonucleotide probes and nuclear extracts prepared from uninduced and induced, purified human T cells. Before extract preparation, a portion of the human T cells was stimulated for 6–8 h with PMA and ionomycin, which induces high levels of IL-3 transcripts (data not shown). Fig. 8 A shows that multiple complexes were formed with induced T cell extracts and both the AP-1/Elf-1 (lane 2, arrows) and the AP-1 DNA probes (lane 4, arrows). In contrast to EMSA analysis performed using nuclear extracts from unstimulated MLA-144 cells, very little complex formation is evident using uninduced human T cell extracts (lanes 1 and 3). However, consistent with results obtained with nuclear extracts prepared from MLA-144 cells, the band pattern seen with human T cell extracts and the AP-1/Elf-1 probe (lane 2) compared with the pattern observed with these extracts and the AP-1 probe (lane 4) is distinct, with fewer and less intense complexes formed with the AP-1 probe.

The specificity of nuclear protein binding of induced T cell nuclear proteins to the AP-1/Elf-1 DNA subfragment was assessed using unlabeled DNA competitors, as shown in Fig. 8 B. The two higher mobility bands denoted by closed arrows were specific as demonstrated by competition with wild-type competitor (lane 2) but not by unrelated competitor (MNIP, lane 5) or mutated competitor (ME1fl, lane 6). Clearly, the lowest mobility band denoted by a closed arrow is nonspecific because it is competed by both specific and
Figure 7. Functional analysis of three nuclear protein binding sites in the human IL-3 gene. MLA-144 cells were transfected with a CAT construct under the control of 319 bp of the human IL-3 gene promoter (A) or with a CAT construct containing three copies of the AP-1/Elf-1 nuclear protein binding sites found in human IL-3 under the control of the minimal SV40 promoter (B). CAT constructs with mutations in up to three of the nuclear protein binding sites were also tested. In addition, 2 μg of pRSVβgal was included in each transfection to normalize efficiencies. After transfection, the cells were divided into two groups: one that received PMA (5 ng/ml) and PHA (2 μg/ml) 8 h before harvest and a second group that was incubated with media only. An activity of constructs containing mutated nuclear protein binding sites in the context of the endogenous IL-3 promoter. The mutations were (AP-1) TGAGTCAG to TCTCGAGC, (Elf-1) GGCAGGAAGGG to TGTCTCCTTCG, and (NF-Ib3) ATGAA-TAA to GACTCGAG. Activity shown is that of stimulated MLA-144 cells only. Relative CAT activity is that given each construct compared to the activity given by the pCAT vector alone. Each construct was transfected at least three times and the data shown are from a representative experiment. (B) Activity of intact and mutant multimerized AP-1/Elf-1 nuclear protein binding sites in constructs under the control of the heterologous minimal SV40 promoter. The mutations introduced into the AP-1 site were identical to A. The Elf-1 site in this construct was mutated to TCTCTCTGCC. Cultures stimulated with PHA/PMA are denoted by (+). Relative CAT activity here represents the activity given by the individual construct in extracts from induced cultures compared to the activity given by the construct in extracts from uninduced cultures. The uninduced and induced samples were assayed on the same day but run on two thin layer chromatographic plates. Each construct was transfected three times with different preparations of plasmid DNA and the data presented are representative of one experiment.

Discussion

IL-3 is a potent inducible hematopoietic growth factor that is produced solely by activated T and NK cells. In this report, we demonstrated that multiple nuclear protein binding sites are required for T cell-restricted expression of the human IL-3 gene, including sites that bind to AP-1 transcription factors and to Elf-1, an Ets family transcription factor. With oligonucleotides corresponding to the AP-1 and Elf-1 binding sites, we have shown by direct binding and competition that these sites, in fact, bind AP-1 and Elf-1 transcription factors. In the absence of a DNA subfragment containing the AP-1 and Elf-1 binding sites, CAT constructs including a segment of the IL-3 promoter/enhancer were active in both T and non-T cells. In addition, mutation of an element thought to be involved in restricting IL-3 expression, NIP, failed to
increase transcription to the level seen with a smaller subfragment when transfected into activated T and non-T cells. Together, these data support the conclusion that the AP-1/Elf-1 cassette is critical to cell-restricted and inducible IL-3 gene expression.

The AP-1 and Elf-1 binding sites were found to be absolutely required for IL-3 gene expression, because constructs with these sites deleted were inactive in induced T cells. DNaseI footprint and EMSA analyses revealed that the AP-1 and Elf-1 binding sites as well as intervening DNA bound multiple specific nuclear protein complexes, and there were differences in the mobilities but not the intensities of the complexes observed in EMSAs using extracts from resting or induced MLA-144 cells. These results suggest that there may be distinct nuclear proteins that bind to the IL-3 promoter in induced and uninduced extracts, or that one or more of these proteins might undergo posttranslational modifications after activation, or that different protein–protein interactions may exist in resting and induced cells. Further studies with antisera revealed that both Elf-1 and Jun family binding proteins individually formed complexes with the AP-1/Elf-1 DNA probe. The presence of proteins that were blocked by Jun family antiserum in extracts from resting MLA-144 cells may be attributed to constitutive expression of Jun and Fos family proteins in these cells. Evidence for high constitutive expression of JunD, and low but detectable expression of Jun-B, c-Jun, and Fra-1 has been demonstrated in other resting cells (45, 46). Alternatively, distinct combinations of Jun and Fos proteins may bind to the IL-3 gene during different stages of activation (47). Because it is evident that protein–protein interactions between the various Jun and Fos transcription factors mediate the differential effects observed in regulation of AP-1 proteins (30), examination of the individual constituents of the Fos/Jun or Jun/Jun complexes binding to the IL-3 gene AP-1 site in uninduced or induced MLA-144 T cells will be important to elucidate. Finally, EMSA studies using purified human T cells demonstrated that binding activities were largely limited to extracts prepared from induced cells. This result is not unexpected, and the demonstration of binding activities in unstimulated MLA-144 cell extracts possibly reflects the transformed state of the MLA-144 cells.

The exact DNA composition of the AP-1 and Ets family nuclear protein binding sites, flanking DNA sequences, and the distance between these sites may determine the fine specificity of nuclear factors that bind to these sites. Members of the Ets protooncogene family encode transcription factors that bind to purine-rich DNA sequences present in the promoters and enhancers of several cellular and viral genes (26, 33, 38, 40, 49, 50). The core GGA sequences are absolutely required for DNA–protein interaction, whereas the flanking sequences determine the specificity of DNA binding by the different Ets family members (39). With respect to the IL-3 gene, examination of the EBS sequence predicts that the IL-3 gene can bind Elf-1 because of a permissive A at nucleotide 8 (41). Whether an Ets family transcriptional protein actually forms a complex with AP-1 dimers or a third protein bridge may depend on the distance separating the two nuclear protein binding sites. For example, Boise et al. (27) have shown that NFAT complex formation requires cooperation between an Ets family factor and AP-1 dimers, and the EBS and AP-1 binding sites in the NFAT motif are 1 bp apart. Likewise, Wasylyk et al. (26) demonstrate that the ets-1 protein interacts with AP-1 dimers to activate transcription through the PEA3 motif in the polyoma enhancer. In the oncogene-responsive domain of the polyoma enhancer, the ets-1 and AP-1 sites overlap by several bases, and analyses using chimeric constructs revealed that maximal levels of activity were possible only when these sites were adjacent (48). In contrast, the Elf-1 and AP-1 nuclear protein binding sites in the IL-3 gene are separated by six nucleotides, and the resulting spatial orientation may account for the observation that these sites can bind to some proteins or protein complexes independently.

Although we have demonstrated the critical importance of the AP-1 and Elf-1 sequences to inducible, T cell–restricted IL-3 gene expression, both AP-1 and Elf-1 transcription factors themselves are found in non-T cells as well as in T cells. Therefore, the manner in which AP-1 and Elf-1 binding proteins might confer T cell IL-3 specificity is of extreme interest including several possibilities. First, T cell–specific proteins may interact with the AP-1 or Elf-1 binding factors but not directly with DNA. Second, there may be yet undefined regulatory protein binding sites and binding factors not detected by DNaseI and EMSA analyses. Finally, the AP-1 and Elf-1 transcription factors may undergo posttranslational modification in vivo leading to T cell–restricted gene expression.

Previous investigators have suggested that two different nuclear protein binding sites, the NF-IL-3 and the NIP sites, may play roles in T cell–specific IL-3 gene expression. For example, Davies et al. (21) implicated the NF-IL-3 site (within the ACT-1 motif) in IL-3 expression, yet the effect of a mutation in this site is only evident in the context of a construct truncated just 5' to the NF-IL-3 site. Within a larger construct (300 bp of 5' flanking DNA), the effect of an ACT-1 mutation is negligible (21). In contrast, our data show that mutation of the NF-IL-3 site within the context of 319 bp of 5' flanking sequence dramatically reduces CAT activity as compared to that of the native IL-3 promoter. There are several possibilities for this discrepancy. First, the designs of mutant constructs were different. We mutated specific bases within the NF-IL-3 site, whereas Davies replaced the entire NF-IL-3 site and the 3' CRE element with a short DNA spacer (15 to 6 bp). Thus, in the latter construct the spatial interactions were dramatically altered and the NF-IL-3 site mutations were not isolated. In fact, when we tested a construct truncated immediately 3' of the NF-IL-3 site, -150CAT (including the CRE or 3' section of ACT-1), this construct gave 50–100% of the activity given by the larger -175CAT construct. Thus, Davies' deletion of the CRE site would be expected to impact greatly on transcriptional activity. Second, the modes of IL-3 gene induction in the transfected MLA-144 cells were different. We induced IL-3 gene expression in MLA-144 cells with PHA and PMA, whereas Davies et al. activated these cells with PMA alone. Our Northern blot analysis shows that IL-3 transcripts in MLA-144 cells are significantly lower following incubation with PMA in con-
Contrast to the levels shown with PHA/PMA stimulation. Therefore, the effects of mutations in the larger construct described in the other report (21) may not be evident during low-level IL-3 gene transcription. Interestingly, our construct with NF-IL-3 site mutations which produced significantly less CAT activity compared to wild-type constructs after transfection into MLA-144 T cells, reduced CAT activity only moderately when tested in Jurkat T cells. However, the data derived from experiments using Jurkat cells must be carefully assessed because these cells produce extremely low levels of IL-3.

Two groups have suggested that this lineage-restricted expression is primarily regulated by the NIP element in the IL-3 promoter (19–21). However, mutation of the NIP sequence in the context of −270CAT failed to increase CAT activity to the level observed with the smaller −175CAT. This implies that another negative regulatory element in addition to or instead of NIP may be involved in repressing IL-3 gene expression. Alternatively, in the absence of upstream sequences, NIP mutations are unable to restore CAT activity to the levels observed with −150CAT and −175CAT. In any event, our data do not support a major role for the NIP binding site in the cell specificity of IL-3 gene expression in cells that produce significant quantities of IL-3 mRNA.

Our results are of particular interest in light of recent studies on both the molecular basis of T cell anergy and T cell cytokine deficiency after bone marrow transplantation. Schwartz and Kong et al. (51, 52) found that antigen-specific anergic T cell clones display profound deficiencies in the binding of AP-1 transcription factors to AP-1 nuclear protein binding sites. Similar AP-1 nuclear protein deficiencies could well explain the T cell anergy routinely observed following bone marrow transplantation in which T cells arising after transplant fail to secrete either IL-3 or GM-CSF in response to mitogens (53, 54). Thus, T cell activation via AP-1 and EBS nuclear binding proteins may be a major molecular checkpoint in the expression of the developmental program of T cells in diverse clinical settings.

In summary, these data indicate that 5′ flanking sequences containing nonadjacent AP-1 and Elf-1 nuclear protein binding sites play a major role in conferring T cell specificity to IL-3 gene expression. In contrast to the NF-IL-3 and NIP sites, the effects of the AP-1 and Elf-1 binding sites in reporter constructs with 5′ flanking IL-3 gene sequences distinguish T cells from non-T cells and are most evident under conditions of T cell activation that maximally stimulate IL-3 production per se. Future studies identifying the particular members of the Jun family, as well as additional proteins involved in activation at the AP-1 and Elf-1 sites will be extremely interestingly in illuminating the molecular basis of the specificity of T cell cytokine gene expression.

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