The Human IL-3 Locus Is Regulated Cooperatively by Two NFAT-Dependent Enhancers That Have Distinct Tissue-Specific Activities

Abbas Hawwari, Joanna Burrows, Mathew A. Vadas and Peter N. Cockerill

*J Immunol* 2002; 169:1876-1886; doi: 10.4049/jimmunol.169.4.1876

http://www.jimmunol.org/content/169/4/1876

**References**

This article cites 43 articles, 24 of which you can access for free at: http://www.jimmunol.org/content/169/4/1876.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Human IL-3 Locus Is Regulated Cooperatively by Two NFAT-Dependent Enhancers That Have Distinct Tissue-Specific Activities

Abbas Hawwari, Joanna Burrows, Mathew A. Vadas, and Peter N. Cockerill

The human IL-3 gene is expressed by activated T cells, mast cells, and eosinophils. We previously identified an enhancer 14 kb upstream of the IL-3 gene, but this element only functioned in a subset of T cells and not in mast cells. To identify additional mechanisms governing IL-3 gene expression, we mapped DNase I hypersensitive (DH) sites and evolutionarily conserved DNA sequences in the IL-3 locus. The most conserved sequence lies 4.5 kb upstream of the IL-3 gene and it encompassed an inducible cyclosporin A-sensitive DH site. A 245-bp fragment spanning this DH site functioned as a cyclosporin A-sensitive enhancer, and was induced by calcium and kinase signaling pathways in both T cells and mast cells via an array of three NFAT sites. The enhancer also encompassed AML1, AP-1, and Sp1 binding sites that potentially mediate function in both T and myeloid lineage cells, but these sites were not required for in vitro enhancer function in T cells. In stably transfected T cells, the −4.5 kb enhancer cooperated with the −14 kb enhancer to activate the IL-3 promoter. Hence, the IL-3 gene is regulated by two enhancers that have distinct but overlapping tissue specificities. We also identified a prominent constitutive DH site at −4.1 kb in T cells, mast cells, and CD34+ myeloid cells. This element lacked in vitro enhancer function, but may have a developmental role because it appears to be the first DH site to exist upstream of the IL-3 gene during hemopoietic development before IL-3 expression. The Journal of Immunology, 2002, 169: 1876–1886.

Interleukin-3 is a cytokine that regulates the proliferation, differentiation, activation, and survival of myeloid progenitor cells and the mast cells, eosinophils, basophils, neutrophils, monocytes, megakaryocytes, and erythroid cells that they give rise to (1–3). Although IL-3 was first defined as a CSF (multi-CSF), it is not essential to hemopoiesis, and it may function in vivo predominantly as a proinflammatory cytokine activating mature myeloid cells. The IL-3 gene exists within a one megabase conserved cytokine gene cluster that also contains the genes for IL-4, IL-5, IL-13, and GM-CSF (4, 5). The IL-3, IL-5, and GM-CSF genes have arisen by gene duplication, and their regulation shares many features. They can all be activated in T cells via TCR signaling pathways, and are also expressed by activated NK cells, mast cells, eosinophils, and basophils (1–3, 6). However, there also exist significant differences in their patterns of expression, which suggest that they are independently regulated. Whereas IL-3 and GM-CSF are expressed in all classes of T and NK cells, IL-5 is expressed by Th2, but not Th1 T cells (7, 8), and by an analogous NK2 subset of NK cells (9). GM-CSF has a much broader expression pattern than IL-3 or IL-5 because it is also expressed by monocytes, endothelial cells, epithelial cells, and fibroblasts (1–3, 10). The IL-3 and GM-CSF genes exist just 10 kb apart (4, 5), and could potentially share regulatory elements that function in cell types where they are coexpressed. Alternatively, they may have evolved as independently regulated loci to accommodate their different expression patterns.

Many previous studies have been devoted to studying the transcriptional regulation of the IL-3 and GM-CSF genes. These closely linked genes, together with their known regulatory elements, are located within a 30-kb segment of DNA (Refs. 10–14 and Fig. 1A). We have used the approach of mapping DNase I hypersensitive (DH) sites across the entire IL-3/GM-CSF locus to identify distal DNA elements that govern the correct developmentally regulated and differential expression of the IL-3 and GM-CSF genes (10–14). These studies identified inducible DH sites 14 kb upstream of the IL-3 gene, and 3 kb upstream of the GM-CSF gene that function as inducible enhancers. These enhancers are dependent upon a combination of kinase and calcium signaling pathways, which in T cells are linked to the TCR. Both enhancers are activated via arrays of NFAT sites, and are repressed by the immunosuppressant cyclosporin A (CsA) that blocks the calcium-dependent induction of NFAT (10–14). Despite these similarities, the two enhancers have distinct tissue-specific functions that largely mirror the expression patterns of the IL-3 and GM-CSF genes. The −14 kb IL-3 enhancer functions exclusively in T cells, and this specificity is mediated by a composite NFAT/Oct element that recruits NFAT and Oct factors together with the NFAT cofactor NIP45 and the lymphoid-specific Oct cofactor OCA-B (10). In contrast, the −3 kb GM-CSF enhancer functions in a much wider range of NFAT-expressing cells such as T cells, myeloid cells, and endothelial cells (12), and is activated via composite...
The DNA samples analyzed in this study have been selected on the basis that the alleles are different and to replace the copy of the same region from a different allele. DNA samples were digested with either EcoRI-BamHI fragment, which indicates that the activation of the IL-3 locus, whereas the downstream sites are ubiquitous (12) and may serve to segregate the IL-3 and GM-CSF genes. The array of upstream DH sites include the highly inducible tissue-specific IL-3 promoter that functions in T cell lines (15–20) and in megakaryocytic cell lines that express IL-3 (21). The promoter binds an array of inducible and tissue-specific transcription factors that include AP-1 and NFIL3/E4BP4 hZIP family proteins; GATA, Sp1, and Egr1 zinc finger proteins; and AML1, Oct, and NFAT family proteins. The −120 to −310 regions of the human IL-3 promoter both associate with NFAT (P. N. Cockerill and A. G. Bert, unpublished observations). Developmentally regulated factors such as NFAT, AML1, and GATA proteins potentially act in concert to establish a powerful mechanism of combinatorial regulation likely to drive tissue-specific IL-3 expression in both the T and myeloid lineages. The aim of this study was to further characterize the tissue-specific distribution of DH sites upstream of the IL-3 gene, and to determine their role in its regulation. This investigation led to the identification of a previously unidentified inducible DH site at −4.5 kb that is closely linked to a constitutive DH site at −4.1 kb. The −4.5 kb DH site functions as an inducible NFAT-dependent enhancer, and it cooperated with the −14-kb IL-3 enhancer to activate the IL-3 promoter. However, the −4.5-kb enhancer had a broader tissue-specific range than the −14-kb enhancer because it functioned in both T cells and mast cells.

Materials and Methods

DH site analysis

DH sites were assayed essentially as described previously (11–14, 22). In brief, cells were either unstimulated, or stimulated for 4 hr with various agents. DH sites upstream of an internal IL-3 gene were probed with a 0.7-kb EcoRI-BamHI fragment. DH sites upstream of an internal IL-3 gene were probed with a 0.7-kb EcoRI-BamHI fragment.

DNA sequence homology

The human and mouse IL-3/GM-CSF locus sequences were compared using the Blastp program (23) located on the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/). A region extending from 20 kb upstream of the IL-3 gene to the GM-CSF promoter (13 kb downstream of the human IL-3 promoter) was compared with the mouse GenBank file GI:7542829. The human sequence was compiled from GenBank files AC004511, AC009175, and L77036, and our own unpublished primary sequence data. This compiled sequence is almost identical with the newly released GenBank file AC034216 that corresponds to the completed sequence of the same region from a different allele. DNA sequences were highly conserved encompassed 200–360 bp of 80–83% homology. DNA sequences were analyzed using the ClustalW program (24).

Plasmids

The pXPI luciferase reporter gene plasmid was a gift from Dr. S. Nordeen (University of Colorado, Denver, CO; Ref. 24). pXPG is a modified version of pXPI containing the Luc gene from pG3 (Promega, Madison, WI) and a novel high copy plasmid origin of replication, and is designed to avoid serious read-through transcription artifacts that appear to occur with the pGL3 vector (25). pXPG-SS245 (25) contains the minimum GM-CSF promoter core region (−55 to +28) in pXPI. pXPG-Hermes simplex thymidine kinase (TK)229 was generated by cloning a 229-bp HindIII/NotI fragment spanning the TK promoter (obtained from the Promega pRL-TK vector) into pXPG. pXPG-TK229-SV40E5 was generated by cloning a 175-bp BamHI fragment containing SV40 enhancer sequences from SV40 positions 11 to 120 into pXPG-SS245. pXPG-TK229 (referred to as pIL3 in Ref. 14) contains the −559 to +50 fragment of the IL-3 promoter in pXPI. pXPG-IL3H was generated by subcloning a 611-bp HindIII-3′ Flank-3′ Flank promoter from pIL3 into pXPG. pIL3H-S1.3 and pS1.3 were generated by subcloning a 13-kb SacI fragment from 3.3–4.6 kb upstream of the IL-3 locus from x66 (11) into the SacI sites of pIL3H and pBclH19. pXPG-GM55-IL3E (referred to as pIL3 in Ref. 10) contains a 330-bp NruI/AccI fragment from the human −14-kb IL-3 enhancer cloned into pXPG-GM55, pXPG-GM55-GME (referred to as pGME in Ref. 10) contains a 425-bp BamHI/MscI fragment from the human GM-CSF enhancer cloned into pXPG-GM55, pXPG-GM55-SS245, pXPG-IL3H-SS245, and pXPG-TK229-SV40E5 were generated by cloning a 245-bp EcoRI/NotI 5′ fragment of pS1.3 into pXPG-GM55, pXPG-IL3H, and pXPG-TK229, respectively. pIL3H-S1.3-121.2, and pIL3H-S1.3-1.2 were generated by cloning a 1.2-kb EcoRI fragment spanning the human IL-3 enhancer into pXPG. pIL3H-S1.3 and pXPG-GM55-SES46 were generated by cloning a 546-bp StuI/EcoRV (SES46) fragment of pS1.3 encompassing the −41 DH site into pXPG-GM55.

Site-directed mutagenesis was performed on a plasmid carrying the SS245 enhancer using PCR and the following double-stranded oligonucleotides (altered bases are depicted in lowercase): ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The PCR-generated fragments were digested with SmaI and NotI, and cloned into pXPG-GM55 to give the SS285, SS146, and SS95 derivatives, respectively. The mouse IL-3 gene was inserted into a 197-bp MscI/StuI fragment of the SS245 enhancer isolated from pXPG-GM55.

Site-directed mutagenesis was performed on a plasmid carrying the SS245 enhancer using PCR and the following double-stranded oligonucleotides (altered bases are depicted in lowercase): ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The PCR-generated fragments were digested with SmaI and NotI, and cloned into pXPG-GM55 to give the SS285, SS146, and SS95 derivatives, respectively. The mouse IL-3 gene was inserted into a 197-bp MscI/StuI fragment of the SS245 enhancer isolated from pXPG-GM55.

Site-directed mutagenesis was performed on a plasmid carrying the SS245 enhancer using PCR and the following double-stranded oligonucleotides (altered bases are depicted in lowercase): ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The PCR-generated fragments were digested with SmaI and NotI, and cloned into pXPG-GM55 to give the SS285, SS146, and SS95 derivatives, respectively. The mouse IL-3 gene was inserted into a 197-bp MscI/StuI fragment of the SS245 enhancer isolated from pXPG-GM55.

Site-directed mutagenesis was performed on a plasmid carrying the SS245 enhancer using PCR and the following double-stranded oligonucleotides (altered bases are depicted in lowercase): ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The PCR-generated fragments were digested with SmaI and NotI, and cloned into pXPG-GM55 to give the SS285, SS146, and SS95 derivatives, respectively. The mouse IL-3 gene was inserted into a 197-bp MscI/StuI fragment of the SS245 enhancer isolated from pXPG-GM55.

Site-directed mutagenesis was performed on a plasmid carrying the SS245 enhancer using PCR and the following double-stranded oligonucleotides (altered bases are depicted in lowercase): ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. ΔAML1: CTGAGCT CCAGTTTGAGGAGG TGTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The 3′ primer was TGAGCAGGATTTAAGGAGG GTTTGACcAACAGGCGGTCGATCATC. The PCR-generated fragments were digested with SmaI and NotI, and cloned into pXPG-GM55 to give the SS285, SS146, and SS95 derivatives, respectively. The mouse IL-3 gene was inserted into a 197-bp MscI/StuI fragment of the SS245 enhancer isolated from pXPG-GM55.
by activating PBMCs with 2 μg/ml PHA for 3 days followed by culture for 6 days in 5 ng/ml recombinant human IL-2.

With the exception of 5637 cells that were grown in 7% FCS, all cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mM HEPES (pH 7.3), 2 mM l-glutamine, 28 mM NaHCO3, 50 U/ml penicillin, and 50 μg/ml streptomycin. Human T cells were cultured in media supplemented with 50 μM 2-ME.

Transient transfection and luciferase assay

A total of 4.5 × 10^6 cells were transfected with 5–10 μg of pCMV-linearized reporter plasmid DNA by electroporation as described (11). Cells were cultured for 20–24 h, and then stimulated with a combination of 25 ng/ml PMA plus 1.2 μM calcium ionophore A23187 (PMA/I) in the presence or absence of 0.1 μM CsA and incubated for 9 h before harvesting, and assayed for luciferase activity as described (11).

Stable transfection assays

Jurkat cells were transfected with 10 μg of the Fsp-I-linearized reporter plasmid and 1 μg of the Pmol-linearized pEETKneo (26) as described above. Cells were allowed to recover for 2 days before commencing G418 selection. The cells were incubated with 600 μg/ml G418 for 20 or 21 days after which time cells in the unelectroporated control flask were all dead. Cells were removed from G418 selection for at least 24 h before stimulating 1 × 10^6 cells in 10 ml with PMA/I. Cells were stimulated for 9 h, and then harvested and assayed for luciferase activity as described (11).

EMSA

Nuclear extracts were prepared from stimulated and nonstimulated Jurkat cells by a modification of the method of Lavery and Schibler (30). Jurkat cells were stimulated with PMA/I for 2 h. Cells were washed with PBS and resuspended in five times the pellet volume of a hypotonic buffer (solution 1: 10 mM HEPES (pH 8.0), 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) so that the nuclei concentration was at 4 × 10^6 nuclei/ml. Nine times the nuclei suspension volume of NaCl-urea-Nonidet P-40 buffer (1.1 M NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glyceraldehyde 3-phosphate dehydrogenase [cetrimide] for 20 min and dried before autoradiography.

To determine whether enhancer function mirrored chromatin structure in these cell types, we assayed the −14-kb IL-3 enhancer in a subset of IL-3-expressing cell lines, but its role in normal T cells and other IL-3-expressing cells has not previously been investigated. To further define the tissue-specific functions of this enhancer, we mapped DH sites in a variety of cell types (Fig. 1). As models of T cells that efficiently express IL-3, we analyzed freshly isolated peripheral blood T cells and the Jurkat and CEM T cell lines (10). We also assayed Hut78 T cells and HMC-1 mast cells that produce low levels of IL-3, and the T cell line HS2B that does not express IL-3 (expression data not shown). As a model of primitive undifferentiated myeloid cells, we assayed the CD34+ cell line KG1a that has not yet acquired the capacity to express IL-3 (10). Cells were stimulated with PMA/I to activate signaling pathways in which T cells are linked to the TCR.

DNase I analyses of these cells confirmed that the −14-kb region exists as an inducible DH site in Jurkat and CEM T cells. In contrast, the −14-kb DH site could not be induced in peripheral blood T cells, HS2B or Hut78 T cells, HMC-1 mast cells (please ignore the blemish), or in KG1a cells (Fig. 1B). The previously identified −24-kb DH site (14) was seen in this study to be highly induced in HS2B cells, and a novel constitutive DH site existed at −11 kb in Hut78 cells that was not present in any other cell types.

To determine whether enhancer function mirrored chromatin structure in these cell types, we assayed the −14-kb IL-3 enhancer in the context of a minimal promoter upstream of the luciferase gene in plasmids transfected into CEM, HS2B, and HMC-1 cells (Fig. 1C). As observed previously, the IL-3 enhancer was efficiently induced in CEM cells to a level similar to that supported by the GM-CSF enhancer. However, in contrast to the GM-CSF enhancer, the IL-3 enhancer functioned poorly in HS2B cells, and not at all in HMC-1 mast cells. Because the −14-kb enhancer only functioned in a subset of IL-3-expressing cells, the need arose to seek regulatory elements in other regions of the IL-3 locus.

An array of tissue-specific DH sites exists upstream of the IL-3 gene

The human IL-3 gene is embedded within an extensive array of constitutive DH sites (11). To define the tissue-specific distribution and properties of these elements, we mapped DH sites upstream of the IL-3 gene in a wide range of cell types (Fig. 2). The ability of each cell type to express IL-3 is indicated below the DNase I assay.
FIGURE 1. Chromatin structure and function of the −14-kb IL-3 enhancer. A, Map of the human IL-3/GM-CSF locus with the locations of the IL-3 and GM-CSF enhancers relative to the IL-3 gene indicated by boxes. B, DH site analysis of cells that were either unstimulated (0) or stimulated for 4–6 h with PMA/I (+). Nuclei were digested with DNase I and DH sites were mapped within a 26-kb region upstream of a BamHI site at −10.1 kb. A size marker is shown on the left, intact genomic DNA is shown in lane 1, and a partial HindIII digest of genomic DNA in lane 2. Arrows indicate the positions of DH sites. Note that the blemish seen just below the −14-kb region in stimulated HMC-1 cells is not a DH site band. Each lane represents just the midpoint of a DNase I titration. C, Transient transfection assay showing the activity of luciferase reporter gene constructs in CEM and HSB2 T cells and HMC-1 mast cells in the presence (+) or absence (0) of PMA/I. Plasmids contained the minimal −55 to +28-bp GM-CSF promoter alone (pXPG-GM55, Prom), or the promoter linked to either the -14-kb IL-3 enhancer (pXPG-GM55-IL3E) or the GM-CSF enhancer (pXPG-GM55-GME). For the stimulated cells, activities are expressed as an average of three to six independent transfections on a scale where the promoter alone has an activity of one. Error bars indicate SEM.

(FIG. 2A). Upstream DH sites were mapped from an EcoRI site located 2.0 kb downstream of the transcription start site (Fig. 2A). Note that each lane in Fig. 2A represents just the midpoint in a DNase I titration that has been previously optimized for the detection of ubiquitous DH sites downstream of the IL-3 gene, as in a previous study (12). We detected a very prominent DH site at −4.1 kb in all T lineage cells, the mast cell line HMC-1, and the primitive CD34+ myeloid cell lines KG1 and KG1a, but this site was absent in other more mature myeloid cells, B cell lines, and all nonhemopoietic cell types. Five additional DH sites were detected at −0.1, −1.0, −1.5, −3.1, and −5.5 kb. Whereas the −0.1-kb DH site within the IL-3 promoter was present in both T cells and KG1a cells, the four DH sites at −1.0, −1.5, −3.3, and −5.5 kb were confined exclusively to T lineage cells. The −4.1-kb and −1.5-kb sites were present in all T lineage cells, but the DH sites in the promoter and at −1.0, −3.1, and −5.5 kb were only detected in some of the T cell lines, and they varied considerably in their intensities. In a more extensive series of analyses of primary T cells and cultured T lymphoblasts, the promoter was occasion-

ally detected as a weak DH site (data not shown). Seeking inducible DH sites, the same cell lines were also assayed in parallel after stimulation with PMA/I, but no change in the patterns of sites was detected by the EcoRI probe (data not shown).

To examine the −4.1-kb region in greater detail, a subset of this series of T and myeloid lineage cells was assayed for DH sites by probing from the SpeI site located 6.6 kb upstream of the gene. This analysis revealed a previously undetected inducible DH site located at −4.5 kb, immediately upstream of the constitutive DH site at −4.1 kb (Fig. 2B) that pre-exists in T cells as a weak DH site and is further induced by PMA/I. The −4.5-kb DH site was most strongly induced in T lymphoblasts cultured from human blood, and was also present in freshly isolated peripheral blood T cells that had been stimulated with PMA/I. This site was also induced to variable degrees in the Jurkat, CEM, HSB2, and HuT78 T cell lines. At least in the case of Jurkat and HSB2 cells, its induction was suppressed to the pre-existing level in the presence of CsA, which typically functions in the repression of cytokine genes by suppressing induction of NFAT. In those lanes where the upper part of the gel is clearly resolved, it is possible to see a parallel induction of the DH site in the NFAT-dependent GM-CSF enhancer, and its suppression by CsA. In KG1a and HMC-1 cells, DH sites are clearly visible extending from the −4.1-kb site to the +10-kb GM-CSF enhancer, but little or no induction of the −4.5-kb DH site is evident in these cells. However, it is possible to detect weak induction of the −4.5-kb DH site in HMC-1 cells by examining a darker reproduction of this image (Fig. 2B, right inset), and examination of the fluorescently stained DNA suggested that the HMC-1 sample was underrepresented in this analysis.

DH sites upstream of the IL-3 gene encompass conserved DNA elements

As a further aid in the identification of regulatory elements in the IL-3 locus, we searched for DNA sequences that are conserved between the mouse and human IL-3 genes (Fig. 2C). This analysis extended from 20 kb upstream of the human and mouse IL-3 genes to the GM-CSF promoter downstream of the IL-3 gene. This region encompasses all of the known genes and regulatory elements, and most of the known DH sites in the IL-3/GM-CSF locus (Fig. 2C, top row). Conserved regions are depicted on the two lower rows of Fig. 2C as either strongly (■) or moderately (□) conserved. Significantly, the most extensive highly conserved elements in this locus were not the coding regions, but the IL-3 −4.5-kb DH site (221 bp 79% or 376 bp 70%), the GM-CSF enhancer at +10 kb (227 bp 80% or 417 bp 76%), and the GM-CSF promoter at +13 kb (361 bp 83%). Only relatively short regions of the IL-3 promoter (93 bp 84%) and the IL-3 gene (176 bp 62%) were conserved, and the −14-kb IL-3 enhancer was not conserved at all. As a whole, the blocks of conserved sequences upstream of the IL-3 gene exhibit a striking association with the locations of DH sites. In addition to the above mentioned highly conserved elements, the −1.0 and −1.5-kb DH sites span a stretch of 484 bp of 63% conserved DNA, the −3.1-kb DH site is flanked by two blocks of DNA that contain 152 bp 66% and 301 bp of 61% conserved sequence, the −4.1-kb DH site includes 49 bp of 81% conserved sequence, and the −5.5-kb DH site is centered just upstream of an overlapping block of 290 bp of 65% conserved sequence.

To further investigate inducible mechanisms of IL-3 gene regulation, we focused on the inducible DH site at −4.5 kb. Shown in Fig. 3 is the 221-bp conserved sequence encompassing the
−4.5-kb site that is 79% homologous with a 218-bp sequence located 4.7–4.9 kb upstream of the mouse IL-3 gene. The 3′ boundary of the DH site is defined by a StuI site (Fig. 2B, lane 2) that also effectively defines the 3′ border of the conserved DNA sequence (Fig. 3). Given that the DH site itself spans 200 bp, there is a striking relationship between the location of the conserved domain and the DH site. Within the 221-bp conserved sequence lie three potential NFAT sites that could account for much of the inducible CsA-sensitive activity of the DH site. Based on their similarity to known NFAT sites, at least two of the NFAT-like elements are likely to be conserved in the mouse as NFAT binding sites (Fig. 3, NFAT and NFAT). Although there were no other perfect matches to transcription factor consensus sequences, we identified several sites that are likely to function as moderate to weak transcription factor binding sites. The best of these matches included a conserved AP-1 site that overlapped an AML-1 site, and an Sp1 site adjacent to the central NFAT motif. Although the AML1 and Sp1 sites were not conserved in the mouse, an ideal consensus AML1 site was present in the mouse sequence just 21 bp further downstream than the AML1 site in the human sequence. Four additional nonideal, partially conserved consensus sequences are underlined in Fig. 3. These include a second AP-1-like element (CgaTGTcaTCA) that coresides with the mouse AML1 site, a third AP-1-like element (cGAGGTCA) that exists as the more ideal tGAGGTCA in the mouse sequence, a fourth AP-1-like element (TGTCACA) downstream of the Sp1 site, and a GATA-like...
element (AGATtA) that most closely matches the GATA3 consensus (Ref. 32; the nonconserved bases in these elements are depicted in lowercase). In the human sequence, the GATA-like element (AGATtA) that most closely matches the GATA3 consensus (Ref. 32; the nonconserved bases in these elements are depicted in lowercase) was linked to a fragment (SS245; Fig. 4) centered 4.5 kb upstream of the human IL-3 gene, and 4.8 kb upstream of the mouse IL-3 gene. Close matches to known consensus sequences are highlighted in bold, and other weaker potential consensus sequences are underlined. Arrows indicate the end points of deletion constructs used in Fig. 7.

The SS245 enhancer functions as an inducible tissue-specific enhancer in T cells and mast cells

To assess the tissue-specific inducibility of the −4.5-kb IL-3 enhancer, the SS245 fragment was cloned upstream of the constitutively active TK229 promoter. These plasmids were transiently transfected into Jurkat T cells, HMC-1 mast cells, Raji B cells, KGA1 myeloid cells, and K562 proerythroid cells and stimulated with PMA/I (Fig. 5). The −4.5-kb SS245 enhancer increased inducible promoter activity by 2- to 3-fold in both Jurkat T cells and HMC-1 mast cells, but was inactive in cell types that do not express IL-3 (KGA1, K562, and Raji cells). The SS245 enhancer did not significantly influence the constitutive activity of the TK229 promoter, in contrast to the SV40 enhancer that was active in all of the cell lines and functioned to increase both inducible and constitutive promoter activity.

Transcription factor binding sites in the SS245 enhancer

To investigate mechanisms of SS245 enhancer function, EMSAs were performed on probes encompassing the transcription factor consensus sequences highlighted in Fig. 3 using nuclear extracts of unstimulated and PMA/I-stimulated Jurkat cells (Fig. 6, A–D). The sequences of the four AML1/AP-1, NFAT/Sp1, NFAT, and NFAT/AP-1-like elements used as probes (Fig. 6, A, B, C, and D, respectively) are shown below these EMSAs, and their locations depicted as bars above the map of the enhancer in Fig. 6. Probe A was supershifted by an AML1 Ab. The probe A AML1 and AP-1 complexes were also specifically competed by consensus binding sites for these factors (data not shown). Probe B was a moderately strong binding site for constitutively expressed Sp1-like factors and for inducible NFAT-like factors. The probe B Sp1 and NFAT-like complexes were specifically competed by the Sp1 or the GM430 NFAT-binding oligonucleotides, respectively. Probe C functioned as a moderate affinity binding site for inducible NFAT-like factors, and the NFAT-like complex was supershifted by an NFATC2 Ab (Fig. 6) and specifically competed by the GM430 NFAT-binding oligonucleotide (data not shown). Probe D, that included loose matches to NFAT and AP-1/CRE-like elements, generated a diffuse ladder of weakly inducible complexes that were specifically inhibited in the presence of the GM170 oligonucleotide that contains strong binding sites for both NFAT and AP-1, but not by the Sp1 oligonucleotide competitor. A comparison of the relative affinities of the three NFAT-like elements suggested that probe C was the strongest NFAT site, binding NFAT ~40% as efficiently as the GM430 high-affinity NFAT site (data not shown).

The locations of the identified binding sites in probes A, B, and C were confirmed in EMSAs where mutations in essential core regions of these sequences in each case eliminated formation of the respective complex (bases depicted in bold in Fig. 6 were altered to the bases shown underneath each probe in lowercase and data not shown). However, note that the precise location of the Sp1 site
FIGURE 4. Definition of an enhancer 4.5 kb upstream of the human IL-3 gene. A, Schematic diagram of the 5′ region of the IL-3 locus with DH sites marked by arrows. The region within a 1.3-kb ScaI fragment is expanded to indicate the locations of the 0.245-kb ScaI/StuI (SS245) and 0.546-kb (SE546) fragments encompassing the −4.5 and −4.1-kb DH sites. B, Transient transfection assay showing the activity of luciferase reporter gene constructs in Jurkat and CEM T cells in the presence (+) or absence (0) of stimulation by PMA/I. Plasmids contain the IL-3 promoter alone (pXPG-IL3H), or the promoter linked to either the −4.5-kb SS245 fragment (pXPG-IL3H-SS245) or the −14-kb IL-3 enhancer (pXPG-IL3H-NA330). Activities are expressed as an average of 3–12 independent transfections on a scale where the promoter alone has a activity in stimulated cells. C, Jurkat cell transient transfection assay of pXPG luciferase plasmids containing the GM55 promoter alone or the promoter linked to either the SS245 enhancer fragment, the SE546 fragment, or the GM-CSF enhancer (pXPG-GM55-GME). Transfected cells were either left untreated (0), stimulated with PMA/I alone (+), or stimulated in the presence of 0.1 μM CsA. Activities were expressed as a mean of three independent transfections. Error bars indicate the SEM.

in probe B is difficult to predict because it encompasses two overlapping sequences, GTGGCCGGAG and GGTCGGGAG, which both resemble the Sp1 consensus sequence, and the introduced mutation would strongly suppress binding to both. Also, note that the first of these two potential Sp1 sites closely resembles another Sp1-like element located just upstream of the 3′ NFAT site C (GTGGCGCGT, underlined in probe C2). However, probe C2 did not form any Sp1-like complexes with nuclear extracts (data not shown), which also makes it more likely that Sp1 contacts GGTCGGGAG rather than GTGGCCGGAG within probe B. Probe C also resembles an NF-κB site, but it did not form any NF-κB-like complexes, and its NFAT complex was not competed efficiently by an NF-κB site (29) from the E-selectin gene (data not shown).

Purified recombinant proteins were also used to assay binding of NFAT and AP-1-like complexes to regions A, B, C, and D of the enhancer (Fig. 6). This very sensitive approach uses relatively high concentrations of factors and detects binding even at very low-activity sites that are not detected when nuclear extracts are used. Probes A, B2, C2, and D were assayed in parallel with the stome-lysin gene high-affinity AP-1 site for binding to dimers of the DNA-binding domains of cFos and cJun. Probe A functioned as a medium-affinity AP-1 site, and probes B2 and D each contained at least one and potentially two low-affinity AP-1 sites. Probe C2 supported a very low level of AP-1 binding that is unlikely to be significant. EMSAs with the purified DNA-binding domain NFATC2 confirmed that probes C2 and B2 encompassed at least one medium-affinity NFAT site, and mutation of the GG core of NFAT site C within probe C2 essentially abolished NFAT binding. Probe D contained a low-affinity NFAT site, whereas probe A did not bind significant amounts of NFAT.

Because NFAT and AP-1 are often found bound cooperatively to sites that have the consensus sequence GAGGAG rather than GTGGCGGAG within probe B, which also makes it more likely that Sp1 contacts GGTCGGGAG rather than GTGGCCGGAG within probe B. Probe C

The SS245 enhancer requires three NFAT sites for function

To study the contribution of the identified binding sites to enhancer function, we introduced each of the previously tested transcription factor site mutations (Fig. 6) into the SS245 enhancer. Plasmid constructs containing the mutated enhancer fragments upstream of the GM55 promoter in pXPG-GM55 were tested in Jurkat T cells in transient transfection assays. The effects of these mutations are summarized below each site in the map in Fig. 6. Loss of the NFAT sites in regions B and C reduced the activity of these plasmids to 27 and 18% of that supported by the intact enhancer. This residual activity was not substantially greater than the activity obtained with just the GM55 promoter, which was 10% of the GM55-SS245 activity. In contrast, deletion of the AML1, AP-1, or Sp1 sites in regions A and B had little significant impact on enhancer activity.

To further define DNA regions needed for enhancer function, we created enhancer fragments that have 5′ deletions, as shown in Fig. 7. To broaden the scope of this analysis, the 5′ deletion series commenced at a point ~40 bp upstream of the ScaI site (SS285), and proceeded to a point 95 bp upstream of the StuI site (SS95). The boundaries of the SS146 and SS95 segments are indicated in Fig. 3. These enhancer fragments were cloned upstream of the minimal promoter in pXPG-GM55 and transiently transfected into Jurkat T cells (Fig. 7). Cells were either stimulated with PMA/I or left unstimulated. Deletions of the 5′ flanking sequences and the AML1/AP-1 region did not significantly affect the SS245 enhancer function, as indicated by the activities of the SS285, MS197, and SS146 plasmids that did not vary substantially from the SS245
plasmid. However, the deletion of an additional 51 bp resulted in an almost complete loss of enhancer activity in the SS95 plasmid (12% of the SS245 activity). This 51-bp region encompassed NFAT site D and nonideal consensus AP-1 and GATA-like elements (Fig. 3). Based on the above observations, we conclude that the essential core of the enhancer is located within a 146-bp region upstream of the \( \text{Stu} \) I site, and that its activity relies on three distinct regions that each encompass NFAT sites.

The \(-4.5\) and \(-14\)-kb enhancers cooperate in IL-3 promoter activation

We also used a stable transfection assay to examine the cooperativity between the IL-3 promoter, the \(-4.5/-4.1\)-kb region, and the \(-14\)-kb enhancer. For this purpose, the IL-3 promoter (\(-559\) to \(+50\)) fragment was cloned in the pXP1 luciferase plasmid (11) to give pIL3H. The 1.2 kb \( \text{Bgl} \) II fragment (B1.2) encompassing the

The Journal of Immunology

1883

Downloaded from http://www.jimmunol.org by guest on April 16, 2017
enhanced the activity of the IL-3 promoter by luciferase activities increased stepwise with the addition of each of the activities from each pair were averaged to create Fig. 8. The corrected for average gene copy number from each transfection, and was determined after stimulation with PMA/I. Activities were corrected relative to the stimulated activity of T cells in two independent transfection assays. Luciferase activity cloned in the pIL3H-S1.3, respectively. In addition, the B1.2 fragment was corrected in the pIL3H plasmid to generate pIL3H-B1.2. These constructs were stably integrated into the genome of Jurkat T cells in two independent transfection assays. Luciferase activity was determined after stimulation with PMA/I. Activities were corrected for average gene copy number from each transfection, and the activities from each pair were averaged to create Fig. 8. The luciferase activities increased stepwise with the addition of each of the S1.3 and B1.2 elements to the IL-3 promoter. The S1.3 element enhanced the activity of the IL-3 promoter by ~3.4-fold over the promoter alone. The addition of the B1.2 enhancer in pIL3H-S1.3-B1.2 increased the activity by a further 2.2-fold. However, it should be noted that this assay system tended to give somewhat more variable results overall than the transient transfection assay system, and was less reliable as a source of quantitative information.

Discussion

DNA sequence conservation

The combined approaches of studying chromatin structure and DNA sequence conservation have proved to be invaluable in the search for regulatory elements in the IL-3 locus. Although the ~4.5-kb IL-3 enhancer (11) and the 1.3-kb SacI (S1.3) fragment encompassing both the ~4.5-kb and ~4.1-kb DH sites (see Fig. 4A) were cloned in the BglII and SacI sites upstream of the IL-3 promoter in the pIL3H plasmid to generate pIL3H-B1.2 and pIL3H-S1.3, respectively. In addition, the B1.2 fragment was cloned in the BglII site of pIL3H-B1.2 to give pIL3H-B1.3-B1.2. These constructs were stably integrated into the genome of Jurkat T cells in two independent transfection assays. Luciferase activity was determined after stimulation with PMA/I. Activities were corrected for average gene copy number from each transfection, and the activities from each pair were averaged to create Fig. 8. The luciferase activities increased stepwise with the addition of each of the S1.3 and B1.2 elements to the IL-3 promoter. The S1.3 element enhanced the activity of the IL-3 promoter by ~3.4-fold over the promoter alone. The addition of the B1.2 enhancer in pIL3H-S1.3-B1.2 increased the activity by a further 2.2-fold. However, it should be noted that this assay system tended to give somewhat more variable results overall than the transient transfection assay system, and was less reliable as a source of quantitative information.

The roles of NFAT and CsA in enhancer function

The ~4.5-kb IL-3 enhancer shared many properties with the ~14-kb IL-3 enhancer and the GM-CSF enhancer. The ~4.5-kb IL-3 enhancer was first identified as a tissue-specific inducible DH site that was induced in T cells by stimuli that mimic TCR signaling pathways and repressed by CsA. The basis for the CsA-sensitivity in each case appears to reside on the dependency of these enhancers upon NFAT. The ~4.5-kb IL-3 enhancer relies on an array of three NFAT sites for its activity, and deletion of NFAT sites B or C or of a 51-bp region encompassing NFAT site D almost abolished its activity. CsA functions to block the calcineurin-dependent activation of NFAT (36), and it is likely that NFAT is required for both the chromatin remodeling and enhancer function of all three enhancers in the IL-3/GM-CSF locus. NFAT may in fact be the initiator of chromatin remodeling in these enhancers, because we have previously found that NFAT sites from the GM-CSF enhancer are sufficient to create inducible DH sites in transfected Jurkat cells (P. N. Cockerill, unpublished observations). Like many other cytokine genes, the IL-3 gene is suppressed by CsA (36), and the NFAT sites in the promoter (7) and the two upstream enhancers may play a major role in this effect.

Differential regulation of enhancers

One of the most significant findings of this study was the discovery that the IL-3 gene is regulated by two distinct enhancers that direct different activities. The ~14-kb enhancer is not conserved in the observed for other key enhancers that function in T cells such as the GM-CSF enhancer (227 bp 80%), the IL-4/IL-13 CNS-1 enhancer (401 bp 84%; Ref. 33), the IL-2 proximal enhancer/promoter region (580 bp 86%; Ref. 34), and the human TCRα and TCRβ enhancers (240 bp 84% and 370 bp 70%; Ref. 35). A common feature of many such elements is that they can be identified both as DH sites and as isolated blocks of conserved sequence distal to the coding regions. In contrast, the ~14-kb IL-3 enhancer is not in the least conserved and could not have been identified in silico.

The combined approaches of studying chromatin structure and DNA sequence conservation have proved to be invaluable in the search for regulatory elements in the IL-3 locus. Although the ~4.5-kb enhancer was identified by studying chromatin structure, it could equally well have been identified by seeking conserved DNA elements had this information been available at the beginning of this study. The ~4.5-kb IL-3 enhancer represents the most highly conserved region of the IL-3 locus, and it could perhaps be compared with the CNS-1 enhancer between the IL-4 and IL-13 genes that was originally identified solely on the basis of sequence conservation (33). The degree of homology between the human and mouse ~4.5-kb enhancers (221 bp 79%) is similar to that

FIGURE 7. Deletion analysis of the ~4.5-kb IL-3 enhancer. A, Schematic diagram showing constructs with 5' deletions of the SS245 enhancer and cloned in normal orientation upstream of the GM55 promoter in pXPG-GM55 and transiently transfected into Jurkat T cells. B, Activity of the above constructs in Jurkat cells that were unstimulated (0) or stimulated with PMA/I. Activities were expressed relative to the stimulated activity of the SS245 plasmid having a value of one. Data are expressed as the mean, with error bars indicating the SEM.

FIGURE 8. Stable cell transfection analysis of IL-3 gene enhancers. The 1.3-kb SacI fragment (S1.3) and the 1.2-kb BglII fragment (B1.2) encompassing the ~4.5 and ~14-kb IL-3 enhancers were linked individually or together upstream of the IL-3 promoter in pIL3H. Plasmids were stably transfected into Jurkat cells, and luciferase activity was assayed after stimulation with PMA/I. The data are presented as the mean of two independent transfections after correction for plasmid copy number. Error bars represent SEM.
mouse genome and may be a recent acquisition in the human genome, serving a highly specialized function. However, it is not unprecedented to find that genes expressed in T cells are controlled by more than one enhancer. The TCRγ locus encompasses two enhancers that have overlapping, but also unique, functions (37). The CD8 locus similarly contains more than one enhancer, and these are distinguished by the developmental stage at which they become active in the thymus (38).

Although they are both NFAT-dependent, the −14 and −4.5-kb enhancers are governed by distinct patterns of combinatorial regulation that direct very specific patterns of activity. The −14-kb enhancer does not function outside of the lymphoid lineage because it is absolutely dependent upon the lymphoid-specific Oct cofactor OCA-B (14). Unlike the T and B cell lines that we have analyzed (10), we were unable to detect significant OCA-B expression in HMC-1 mast cells (data not shown), and this probably accounts for the inactivity of the −14-kb enhancer in this cell type. In contrast, the −4.5-kb enhancer has a wider range of activity, and it appears to rely primarily on an array of three NFAT sites for its activity. However, it remains to be determined if these NFAT sites are sufficient to account for its functions. NFAT typically functions in strict cooperation with other classes of transcription factor, and further studies will be required to determine whether this is also the case in the −4.5-kb enhancer.

Although we did not identify any function for the AML1 or GATA consensus elements in our in vitro studies, it is nevertheless significant that the −4.5-kb enhancer encompasses the combination NFAT and AML1 binding sites, and a potential GATA site. These three families of proteins are widely expressed in the T cells and myeloid lineage cell types that express IL-3, and potentially direct a specialized pattern of combinatorial regulation that is appropriate for the IL-3 locus. A similar combination of regulatory elements exists within the IL-3 promoter. The presence of a potential GATA3 site in the −4.5-kb enhancer is interesting because GATA3 directs Th2 T cell differentiation (39), and Th2-specific elements such as the IL-5 promoter (40, 41) and the 3′ IL-4 enhancer (42) encompass NFAT and GATA3 sites. Hence, the −4.5-kb enhancer may be up-regulated in parallel with IL-4 and IL-5 in Th2 T cells. Conversely, we found that the −14-kb enhancer was down-regulated in the cell line HSB2 that exhibits high level IL-5 expression (43). This presents the interesting possibility that the −4.5 and −14-kb enhancers may be differentially regulated in Th1 and Th2 T cells. If it could be established that the −14-kb enhancer was preferentially active in Th1 rather than Th2 T cells, then this might account for the need to have two distinct enhancers directing IL-3 gene transcription. However, it was not possible to resolve these issues in this in vitro study, and a more physiological system will be needed to identify the true in vivo functions of the −4.5 and −14-kb enhancers.

Other regulatory elements in the IL-3/GM-CSF locus

The IL-3/GM-CSF locus is now known to encompass three inducible enhancers, but there still exist many other elements in this locus for which the function is unknown. There exists an extensive array of constitutive DH sites spanning the IL-3 gene that potentially provide mechanisms controlling the developmental regulation of the IL-3 gene, or for insulating the GM-CSF gene from the IL-3 gene. The presence of the −4.1-kb DH site in KG1, KG1a, and HMC-1 cells and in all T lineage cells was particularly interesting. KG1 is a CD34+ cell line that does not make IL-3, but it may be representative of an early stage of myeloid development (28). Because the −4.1-kb site is the only DH site detected upstream of the IL-3 gene in KG1 cells, it may be the first of the IL-3 DH sites to appear during hemopoietic development. Hence, the constitutive −4.1-kb DH site may represent a developmental marker present in primitive myeloid or lymphoid precursor cells that develop into IL-3-producing cells. In support of this concept, the −4.1-kb DH site can also be detected as an isolated DH site upstream of the IL-3 gene in primitive CD34+ M1 category AML samples (P. N. Cockerill, unpublished observations). However, we were unable to adequately assess the function of the −4.1-kb DH site using in vitro approaches because it lacked classical enhancer activity, and it may have a role that can only be detected in vivo. The −4.1-kb DH site may function by increasing accessibility within the IL-3 locus, thereby promoting subsequent activation of the enhancer and promoter regions by inducible factors. The −1.5-kb DH site may similarly represent a developmental marker of a primed IL-3 locus because this exclusively T cell-specific site is invariably present as a constitutive DH site in all T lineage cells.

In conclusion, the IL-3 locus is likely to be activated in a stepwise process by a combination of developmentally regulated elements that may prime the locus at the level of chromatin structure, and inducible tissue-specific elements that respond to agents that activate the immune system. The role of the downstream elements in the regulation of the IL-3 gene remains to be determined. Our future studies will attempt to determine whether the IL-3 gene is regulated independently of the GM-CSF gene and enhancer, and whether the downstream cluster of ubiquitous DH sites defines one of the boundaries of the functional IL-3 domain within the genome.

Acknowledgments

We thank J. Gamble for providing cultured endothelial cells; A. Lopez for assistance in the preparation of monocytes; M. F. Shannon, A. Boyd, L. Ashman, J. Butterfield, L. Northcote, and P. Simmons for the gifts of cell lines; A. Rao, S. Clark, D. Cohen, and N. Speck for the gifts of DNA clones and Abs; A. Kelso for helpful advice; A. Bert for assistance and preparations of oligonucleotides; and Kelly Frazer for access to the draft mouse genome DNA sequence. We thank Constanze Bonifer for comments on the manuscript.

References


