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The Inducible Tissue-Specific Expression of the Human IL-3/GM-CSF Locus Is Controlled by a Complex Array of Developmentally Regulated Enhancers

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The closely linked human IL-3 and GM-CSF genes are tightly regulated and are expressed in activated T cells and mast cells. In this study, we used transgenic mice to study the developmental regulation of this locus and to identify DNA elements required for its correct activity in vivo. Because these two genes are separated by a CTCF-dependent insulator, and the GM-CSF gene is regulated primarily by its own upstream enhancer, the main objective in this study was to identify regions of the locus required for correct IL-3 gene expression. We initially found that the previously identified proximal upstream IL-3 enhancers were insufficient to account for the in vivo activity of the IL-3 gene. However, an extended analysis of DNase I-hypersensitive sites (DHSs) spanning the entire upstream IL-3 intergenic region revealed the existence of a complex cluster of both constitutive and inducible DHSs spanning the −34- to −40-kb region. The tissue specificity of these DHSs mirrored the activity of the IL-3 gene, and included a highly inducible cyclosporin A-sensitive enhancer at −37 kb that increased IL-3 promoter activity 40-fold. Significantly, inclusion of this region enabled correct in vivo regulation of IL-3 gene expression in T cells, mast cells, and myeloid progenitor cells. The Journal of Immunology, 2012, 189: 000–000.

The closely linked IL-3 and GM-CSF genes are tightly regulated and are both expressed in a highly inducible and tissue-specific fashion (1). IL-3 and GM-CSF are closely related cytokines that activate the same signaling pathways and have similar functions (2, 3). However, they also have important unique roles in vivo. IL-3 has more wide-ranging functions, as it regulates the proliferation, differentiation, activation, and survival of hematopoietic progenitor cells, and it promotes the differentiation of mast cells, eosinophils, basophils, neutrophils, monocytes, megakaryocytes, and erythroid cells (2, 3). Although IL-3 is not essential for adult hematopoiesis, it can function as a proinflammatory cytokine in vivo, and as a CSF in vitro. During embryogenesis, IL-3 is known to play an important role in mobilizing and amplifying the earliest hematopoietic stem cells (HSCs) emerging from the endothelium of the developing aorta, and it has been shown that IL-3 mutant embryos are deficient in HSCs (4). Within the lumen of the aorta-gonad-mesonephros region of the embryo, IL-3 is expressed by cells adhering to the endothelial wall, but the identity of these cells remains unknown (4). IL-3 is also required for expansion of hemangioblasts in the aorta-gonad-mesonephros region at an even earlier stage of embryogenesis (5). GM-CSF functions primarily as a powerful proinflammatory cytokine (3, 6), acting more specifically on granulocyte-macrophage lineage cells and mediating some of the key actions of TNF-α (7). The IL-3, GM-CSF, and IL-5 genes all evolved from a common ancestor and remain linked within a 1-Mb cluster of cytokine genes (8). The human IL-3 and GM-CSF genes remain just 10.5 kb apart as a single compact locus, whereas the IL-5 gene lies 465 kb downstream. Although they are coinduced upon activation in some cell types, such as Th2 cells and mast cells, they are differentially expressed in other cell types, and are likely to have independent mechanisms of regulation. Furthermore, the IL-5 gene is functionally linked to the IL-4/IL-13 locus as part of a region known as the Th2 cluster, which is regulated by a shared locus control region (LCR) (9, 10).

Despite their close proximity within the same locus, the human IL-3 and GM-CSF genes appear to be regulated independently of each other as two distinct genes. Although they are coexpressed in
T cells and mast cells, just GM-CSF is expressed in many types of nonhematopoietic cells such as endothelial and epithelial cells (2, 3). This distinct expression pattern is made possible by a CTCF-dependent insulator located between the two genes (11). The human IL-3 gene is associated with at least two upstream enhancer elements. The most significant of these is a conserved inducible enhancer at −4.5 kb upstream that functions in both mast cells and T cells, and can therefore help to account for the inducible and tissue-specific regulation of IL-3 expression (1, 12). An additional nonconserved T cell-specific enhancer of unknown function exists 14 kb upstream of the IL-3 gene (1, 13, 14). However, there have never been any in vivo studies performed to determine just which regulatory elements in this locus are actually either necessary or sufficient for correct IL-3 gene expression in vivo.

The independent expression of the human GM-CSF gene is controlled by a conserved −3-kb enhancer that supports its efficient inducible expression in a transgenic mouse model containing just a 10-kb segment of the human GM-CSF locus (1, 15–17). The GM-CSF gene is also associated with additional highly conserved far downstream sequences, one of which is known to function as a BRG1/NF-κB-dependent enhancer in the mouse, where it was defined as conserved noncoding sequence a (CNSa) (18). This element is at +34 kb in the mouse and at +30 kb in the human genome relative to the start of the GM-CSF gene.

The IL-3 −4.5- and −14-kb enhancers, and the GM-CSF −3-kb enhancer all form inducible DNase I-hypersensitive sites (DHSs) (1, 12, 14, 16). These enhancers are each activated via cooperation between kinase and calcium signaling pathways, which in T cells are linked to the TCR, and they each encompass binding sites for the Ca2+-inducible transcription factor NFAT, which supports chromatin remodeling (1, 19). Induction of these DHSs is suppressed by cyclosporin A (CsA), which blocks the Ca2+-dependent induction of NFAT by inhibiting calcineurin (12, 14, 16).

IL-3 and GM-CSF can also be aberrantly expressed in myeloid leukemia, where they function as autocrine growth factors (20). For example, IL-3 functions as a mediator of autocrine growth in chronic myeloid leukemia (CML) (21), but the mechanisms responsible for its aberrant expression remain unknown. Additional studies are, therefore, needed to identify the significant in vivo sources of IL-3, and the DNA elements underlying the normal induction of activation of IL-3 expression in mature hematopoietic cells and in the developing hematopoietic system, as well as the aberrant induction of IL-3 expression by BCR-ABL signaling in early myeloid progenitor cells in CML patients (21–23).

In this study, we aimed to identify mechanisms controlling the correct inducible and developmental regulation of the human IL-3 gene. We initially found that the proximal −14- and −4.5-kb enhancer regions were not sufficient to direct efficient IL-3 promoter activation in transgenic mice. However, a more expansive analysis revealed that a transgene incorporating a powerful −37-kb inducible enhancer was able to direct the correct pattern of inducible and developmentally regulated IL-3 gene expression in vivo.

Materials and Methods

All experiments involving animals or humans were authorized by research ethics committees.

Transgenic mice

The previously described B38, C42, and D48 IL-3/GM-CSF transgenic mouse lines contain one to six copies of a 130-kb AgeI DNA fragment of bacterial artificial chromosome (BAC) clone CTD2004C12 (24). Line ES2 contains one copy of the same BAC fragment, but has a 15.5-kb deletion at the 3′ end, terminating 21 kb downstream of the GM-CSF gene, and lacking the +30 kb region homologous to mouse CNSa +34-kb enhancer element (18). Copy number was estimated made by Southern blot hybridization of mouse and human DNA, with human IL-3 and GM-CSF and mouse CD19 gene probes.

Cell preparation and stimulation

Unless indicated otherwise, all cell stimulation was for 4 h with 20 ng/ml PMA and 2 μM calcium ionophore A23187 (PMA/I). Human cell lines Jurkat T cells, HMC-1 mast cells, KG1a myeloblastic cells, and Raji B cells were all cultured, as previously described (25). HMC-1 mast cells were obtained from J. Butterfield (Mayo Clinic, Rochester, MN) (26).

Mouse cells used in this study include the following: 1) freshly isolated thymocytes obtained by passing a thymus through a cell strainer; 2) spleen-derived CD19+ve B cells purified on magnetic beads; 3) actively dividing T blast cells prepared from splenic T cells (24); 4) mast cells grown from bone marrow by long-term culture in IL-3 and stem cell factor (25); 5) myeloid progenitor (MP) cells and macrophages derived from fetal liver; and 6) mouse embryonic fibroblasts (MEFs) cultured from embryos. The MP cells were grown from day 13.5 transgenic mouse fetal liver by culture at starting density of 1 × 107 cells/ml for 5–8 d in IMDM supplemented with 10% FCS, 150 μM monothioglycerol, 500 U/ml penicillin and streptomycin, 10 ng/ml mouse rIL-3, and 10 ng/ml recombinant mouse stem cell factor. Nonadherent cells were moved into a new flask each time they were expanded so as to separate them from any emerging adherent macrophages from the culture. We confirmed that these cells grew as bunches of round nonadherent cells, stained for ERMP12 Abs, and were not granular, as is expected for MP cells. In parallel, the adherent macrophages were maintained in isolation from the MP cells in the presence of 10% L cell-conditioned media containing M-CSF until they reached confluence. The MEFs were prepared from decapitated, eviscerated day 13.5 transgenic mouse embryos dispersed by digestion for several hours with 0.25% collagenase, followed by culture for 14 d in DMEM plus 5% FCS, splitting the cells each time they reached confluence. The T lymphoblasts were prepared by culture with CD81-CD28 Ab beads for 2 d, followed by culture in IL-2 for an additional 2 d without beads. Liver nuclei were prepared by homogenizing liver in nuclei digestion buffer containing 0.3 M sucrose (25), passing it through a 70 μm strainer, and centrifugation in an angle rotor at 18,500 × g through a cushion of digestion buffer containing 1.8 M sucrose for 30 min at 4°C.

mRNA analyses

mRNA was extracted from cells using TRIzol (Invitrogen), according to the manufacturer’s instructions. cDNA was generated from purified mRNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen), according to the manufacturer’s recommendation. Unless indicated otherwise, all the gene expression analyses were performed by quantitative real-time PCR using SYBR Green I reagents (Applied Biosystems, Foster City, CA) on an ABI Prism 7500. The data from each set of primers were analyzed against a standard curve made with a serial dilution of human DNA from PMA/I-stimulated T blast cells or mast cells. Levels of expression of mRNA encoding both human and mouse GM-CSF and IL-3 were determined relative to mouse GAPDH expression. Semiquantitative analysis of mRNA was performed using 25 cycles of PCR.

Primers used for mRNA analyses were as follows. Human GM-CSF: forward, 5′-CAGCTCTGAGTAAGTGAATGAAG-3′; reverse, 5′-GTGCCAGGCAGGTGCGCTC-3′. Human IL-3: forward, 5′-GGACTTCAACACCTCAATGGG-3′; reverse, 5′-TTGATGCTCCTGAGTGGTTG-3′. Mouse GAPDH: forward, 5′-CAGATCGTTAAGGTGGACCATG-3′; reverse, 5′-GGACTTCAAACACCTCAATGGG-3′; reverse, 5′-TTGATGCTCCTGAGTGGTTG-3′. Mouse GM-CSF: forward, 5′-ATGCGTGTAGTGAAGTGAATGAAG-3′; reverse, 5′-CATCGTCTAAACACCTCAATGGG-3′. Mouse GAPDH: forward, 5′-TTGATGCTCCTGAGTGGTTG-3′; reverse, 5′-GTGCCAGGCAGGTGCGCTC-3′. Because the sequences of the mouse and human IL-3 and GM-CSF coding regions have diverged considerably, these primer sets do not cross-react between the human and mouse mRNAs (and the human proteins do not recognize the mouse receptors).

DHS analysis

DNase I digestions and analyses were performed on either isolated nuclei or permeabilized cells, as previously described (24, 25). Samples with optimal extents of DNase I digestion were selected for Southern blot hybridization.

Probes were based on previously published strategies for mapping DHSs were as follows: 1) DHSs upstream of the IL-3 gene −10.1 kb BamHI site were probed with a 0.7-kb EcoRI/BamHI fragment of DNA (−10.1 to −10.8 kb); 2) DHSs across the IL-3 promoter region were mapped from the −6.6-kb SpeI site with a 0.5-kb SpeI/BamHI fragment of DNA; 3) DHSs across a 9.4-kb region spanning the GM-CSF enhancer and promoter were mapped from an EcoRI/BamHI fragment of DNA (24). Line ES2 contains one copy of a 1.5-kb BamHI fragment of DNA as a probe (2); 4) DHSs spanning the insulator downstream of the IL-3 gene were mapped from a BamHI site using a 1-kb BglII/BamHI fragment of DNA (12, 15).
Priming assays used to amplify DNA segments as probes for mapping novel DHSs are described in Supplemental Table I.

Transfection assays

Transfection assays and luciferase assays were performed using the luciferase reporter gene plasmid pXPG (27) containing either the −559- to +50-bp segment of the human IL-3 promoter (pIL3H) or a 229-bp fragment of the thymidine kinase (TK) promoter, plus the indicated upstream DNA fragments, as described previously (25), or a DNA segment spanning the human IL-3 +4.5-kb DHSs. All transfection assays were performed by electroporation using 4.5 × 10⁶ cells and 5 μg test plasmid. With the exception of Fig. 5D, in each assay cells were cotransfected with 1 μg of an appropriate Renilla luciferase plasmid to control for transfection efficiency. Transfected cells were first cultured for 21 h to equilibrate and then stimulated for 8 h with 20 ng/ml PMA and 1 or 2 μM A23187.

Construction of plasmids used in transfection assays

DNA fragments spanning the human IL-3 −34-, −37-, and +4.5-kb DHSs were amplified from the BAC CTD2004C12 by PCR using primers with restriction enzyme sites added to allow cloning, as follows. IL-3 −37-kb DHS with XhoI linkers (defined in this study as the X73 element because of the XhoI sites): forward, 5'-CCGGGCTGGAC- TAAATGCTTCTCCACC-3'; reverse, 5'-CCCGCCTGAGGTCGA- TGGAGGATTACG-3'. IL-3 −34-kb DHS with KpnI linkers: forward, 5'-GGGGGTACCTGAAATCACAGAGTGCCCACC-3'; reverse, 5'-GGG- GGGGTCTAATCTACAGAGTCAGGTCACCGAGGCCACACC-3'. IL-3 +4.5-kb DHS with BamHI linkers: forward, 5'-CCGCGGATCCACCGCAGCTCCTAAAC- CAACATCTAGC-3'; reverse, 5'-CCGCGGATCCGGGTGGTATCCATACCTGC- TCAAGG-3'. IL-3 +4.5-kb DHS with BglII and KpnI linkers: forward, 5'-TTTCCTGATCTGACGTCGAGCAAGTGACTGTCG-3'; reverse, 5'-CTTGG- TCTCTGATCCAGCTGCTGTTACAGTGAGCGAG-3'. After amplification and restriction enzyme digestion, DNA fragments were cloned into either pIL3H (12) or pTK229 (12), containing a 229-bp fragment of the TK promoter in the sense orientation relative to the human IL-3 and GM-CSF genes, using the mouse CNSA enhancer. Lines B38, C42, and D48 contain the complete 130-kb transgene, and line E50 has a 15.5-kb deletion, as indicated by the bracket underneat. Underneath is the map of the minimal IL-3 Enhancer transgenic construct containing just the proximal human IL-3 gene elements plus the −14-kb enhancer linked to a human CD4 reporter gene. (B) Copy number determination of the 130-kb transgenes based on segments of the human IL-3 and GM-CSF genes, using the mouse CD19 locus as an internal reference, and human genomic DNA as a two-copy control. (C) Human IL-3 and GM-CSF mRNA expression in stimulated transgenic mouse T blast cells, expressed relative to expression of the mouse IL-3 and GM-CSF genes, corrected for copy number. (D) Hematopoietic differentiation pathway showing the predicted sources of IL-3 and GM-CSF.

EMSAs and chromatin immunoprecipitation

EMSAs of the IL-3 −37-bp enhancer EGR-1 site were performed, as previously described (28), and used the following primers: forward strand, 5'-TTTCCTGATCTGACGTCGAGCAAGTGACTGTCG-3'; reverse strand, 5'-CTTGG-TCTCTGATCCAGCTGCTGTTACAGTGAGCGAG-3'. EMSAs employed nuclear extracts from Jurkat T cells, cultured with and without stimulation for 4 h with 20 ng/ml PMA and 0.57 μM calcium ionophore A23187. The supershift EMSAs included Abs against Sp1 (Millipore; 17-601), Sp3 (Santa Cruz Biotechnology; sc644X), or EGR-1 (Santa Cruz Biotechnology; sc110X).

FIGURE 1. Developmental regulation of human IL-3/GM-CSF transgenes. (A) Map of the 130-kb BAC transgene with the intact genomic locus showing the predicted sources of IL-3 and GM-CSF. (B) Human IL-3 and GM-CSF mRNA expression in stimulated transgenic mouse T blast cells, expressed relative to expression of the mouse IL-3 and GM-CSF genes, corrected for copy number. (C) Hematopoietic differentiation pathway showing the predicted sources of IL-3 and GM-CSF. (E) Inducible expression of human GM-CSF and IL-3 mRNA in line C42. Columns depict mRNA expression in stimulated cells relative to mouse GAPDH and corrected for copy number. Fold induction relative to nonstimulated cells is shown underneath.
EGR-1 chromatin immunoprecipitation (ChIP) assays of the human IL-3 promoter in Jurkat cells were performed, as described (28), with enrichment levels normalized against a negative control within the human VEZF1 gene body, using the following primers: IL-3 promoter forward, 5'-GGTTGTGGGACCTTGGCT-3'; IL-3 promoter reverse, 5'-TCTGTCTTGGTGCTCCTTGCT-3'; VEZF forward, 5'-GACAGCCGCGAACTTGGT-3'; VEZF reverse, 5'-TGCTGCCGAGAGTAGG-3'.

Results

A 130-kb segment spanning the human IL-3/GM-CSF locus supports correct developmentally regulated and inducible IL-3 and GM-CSF gene expression

Our initial aim was to determine whether the previously defined regulatory regions of the human IL-3 locus are sufficient to support its correct inducible and tissue-specific expression in vivo. We created eight independent mouse lines from a transgene containing the IL-3 -14- and -4.5-kb enhancers and promoter linked to a human CD4 reporter gene (Fig. 1A). However, this combination of DNA elements was clearly insufficient for in vivo expression because three lines displayed low-level variegated expression, whereas the remaining five lines showed no expression upon stimulation in T blast cells (Supplemental Fig. 1).

To aid identification of additional essential IL-3 gene regulatory elements, we performed detailed analyses of a much larger transgene spanning the intact human IL-3/GM-CSF locus. We created several lines of transgenic mice from a 130-kb AgeI genomic DNA fragment (lines B38, C42, D48, and E50; Fig. 1A), which includes the entire upstream 49-kb intergenic region separating the IL-3 gene from the upstream ACSL6 gene. Lines B38, C42, and D48 also include sequences extending to 36 kb downstream of the start of the GM-CSF gene, whereas line E50 has a 15-kb 3' deletion and terminates 21 kb downstream. Transgene activity was assessed in actively proliferating T blast cells generated by stimulating spleen T lymphocytes with CD3 and CD28 Abs and then culturing them for several days with IL-2 in the absence of stimulus. These cells were then restimulated with PMA/I to activate TCR signaling pathways and induce cytokine expression.

FIGURE 2. Distribution of tissue-specific and inducible DHSs in the IL-3/GM-CSF locus. (A and B) Strategies employed for mapping DHSs. The indirect end-labeling probes used to map DHSs are marked as horizontal black arrows. Brackets define the regions between specific restriction enzyme sites covered by each probe. (A) Map of the previously defined inducible DHSs (vertical black arrows) and constitutive DHSs (vertical gray arrows) located within proximal regions of the human IL-3 and GM-CSF genes, as defined in T cells. The black boxes indicate the previously defined conserved -4.5-kb IL-3 and -3-kb GM-CSF enhancers. (B) UCSC genome browser view of conserved regions upstream of the human IL-3 gene (http://genome.cse.ucsc.edu), with the strategies for mapping DHSs shown underneath. The vertical arrows indicate the three conserved regions that were the principal targets for analyses of DHSs and enhancer function. (C) Summary of DHS mapping data. Inducible DHSs are black, stable DHSs are gray, and weak DHSs are shown as thin arrows. Levels of inducible IL-3 and GM-CSF expression relative to GAPDH, taken from Fig. 1E, are indicated at the left of each row.
gene expression. For all four lines, the IL-3 and GM-CSF genes were efficiently induced in a copy number-dependent fashion (Fig. 1C). The average level of expression was in each case slightly higher than the levels determined for the endogenous mouse IL-3 and GM-CSF genes (24). Line E50, which lacks the CNSa 3′ GM-CSF region, supported approximately the same level of IL-3 expression as the other three lines, and a slightly lower level of GM-CSF expression, but this difference was not considered significant.

To define the pattern and mechanisms of developmentally regulated inducible human IL-3 and GM-CSF gene expression during different stages of hematopoietic differentiation, we used transgenic mouse line C42 as a model and prepared the following cell types: 1) thymocytes as a model of immature T cells; 2) actively dividing spleen-derived T blast cells; 3) nonadherent MP cells cultured from fetal liver; 4) mast cells cultured from the bone marrow; 5) adherent macrophages derived from MP cells; and 6) fibroblasts cultured from MEFs. The differentiation pathways linking these cells are depicted in Fig. 1D. Within this scheme, HSCs and MP cells potentially give rise to both macrophages, expected to express just GM-CSF, and mast cells that efficiently express both IL-3 and GM-CSF.

Cells were stimulated with PMA/I, and human IL-3 and GM-CSF gene mRNA expression was measured relative to mouse GAPDH mRNA (Fig. 1E). The level of induction relative to unstimulated cells is shown underneath this graph depicting levels measured in stimulated cells. As anticipated, the IL-3 and GM-CSF genes were efficiently induced in activated T blast cells and mast cells. The level of expression was substantially higher in mast cells than in T cells, possibly because of the following: 1) mast cells express GATA-2 in addition to T cell factors such as Runx1, NFAT, and AP-1, and 2) the IL-3/GM-CSF locus includes many regulatory elements containing GATA motifs (1, 25). This is consistent with our findings that the GM-CSF enhancer is more active, and GM-CSF expression is higher, in cells expressing GATA factors (25).

MP cells also produced high levels of GM-CSF mRNA, equivalent to T blast cells, and moderate levels of IL-3 mRNA. In agreement with previous studies (3), IL-3 was not expressed in macrophages or MEFs. However, macrophages and MEFs similarly produced barely detectable levels of GM-CSF mRNA, in the order of 1000-fold lower than T blast cells. This was consistent with our previous studies of primary CD11b+ myeloid cells derived directly from the spleens of GM-CSF transgenic mice, where the mouse and human GM-CSF genes were both expressed at similar low levels (25). Hence, our findings are inconsistent with the prevailing dogma that macrophages and fibroblasts are assumed to be significant sources of GM-CSF.

Properties of DHSs located between the IL-3 and GM-CSF genes

To both identify novel potential regulatory elements, and define the pattern of developmental and inducible regulation of the IL-3/GM-CSF genes, we used the human IL-3/GM-CSF locus in transgenic line C42. (A–F) Southern blot hybridization analysis of DHSs, as summarized in Fig. 2C, using the probes defined in Fig. 2A and 2B. DHSs were mapped before and after stimulation for 4 h with PMA/I in line C42 mast cells, B cells, thymocytes (Thy), T blast cells (T BI), MP cells, macrophages (MΦ), fibroblasts (MEF), and liver. DHSs are marked by arrows plus their distances in kb relative to the IL-3 gene transcription start site.

![FIGURE 3](http://www.jimmunol.org/Download/5) DHS analysis of the human IL-3/GM-CSF locus in transgenic line C42. (A–F) Southern blot hybridization analysis of DHSs, as summarized in Fig. 2C, using the probes defined in Fig. 2A and 2B. DHSs were mapped before and after stimulation for 4 h with PMA/I in line C42 mast cells, B cells, thymocytes (Thy), T blast cells (T BI), MP cells, macrophages (MΦ), fibroblasts (MEF), and liver. DHSs are marked by arrows plus their distances in kb relative to the IL-3 gene transcription start site.
CSF locus, we performed an exhaustive analysis of DHSs in line C42 in the cell types employed above, both before and after stimulation (Figs. 2–4), and after stimulation in the presence of CsA (Supplemental Fig. 2). As additional nonexpressing controls, we included splenic B cells and liver nuclei. For each cell type, the optimal DNase I-digested samples were selected from a series of DNase I titrations, and the same set of samples was then used for each subsequent analysis. This selection was based on the efficiency of DNase I digestion and PMA/I induction, as confirmed by examining the intensities of the DHS bands detected across the ubiquitous CTCF sites within the insulator downstream of the IL-3 gene (Fig. 3A) and the inducible DHS within the GM-CSF enhancer (Fig. 3B). DHSs were mapped using the strategies depicted in Fig. 2A. These assays demonstrated strong induction of the −3-kb GM-CSF enhancer DHS in T blast cells and mast cells; somewhat weaker induction of this DHS in MP cells, macrophages, and MEFs; and no induction in thymocytes or B cells that do not express GM-CSF.

Within the insulator region, we also identified an inducible CsA-resistant DHS at IL-3 +4.5-kb in GM-CSF-expressing cells in addition to the ubiquitous DHSs (Supplemental Fig. 2A). Unlike the other PMA/I-inducible DHSs in the IL-3/GM-CSF locus, this DHS was not suppressed by CsA, which functions by blocking the Ca2+/calcineurin-dependent induction of NFAT. Furthermore, this DHS functioned as a noncoding RNA promoter that is transcribed toward the +4.9-kb DHS and the GM-CSF gene (Supplemental Fig. 2B). Interestingly, the +4.9-kb DHS, which encompasses a low-affinity CTCF site, was weakest in the cell types that have the weakest induction of the +4.5-kb DHS and show the lowest GM-CSF expression. This element may, therefore, function as a chromatin opening element for the intergenic region separating the GM-CSF gene from the high-affinity CTCF sites within the insulator.

IL-3 gene activation is associated with a cluster of far upstream DHSs

The above results suggested that the 130-kb BAC transgene is a reliable model to study the regulation of the IL-3/GM-CSF locus. To search for additional essential IL-3 gene regulatory elements within this transgene, we used the mapping strategies depicted in Figs. 2B and 4A to identify DHSs within the entire 39-kb region between the −14-kb enhancer and the upstream ACSL6 gene. These assays identified a tightly regulated complex cluster of novel far upstream DHSs present only in cells capable of IL-3 gene expression (summarized in Fig. 2C) and colocalizing with conserved noncoding sequences (Fig. 2B). This region included a highly inducible DHS at −37.5 kb, which was strongest in mast cells and T blast cells, weak in MP cells, and absent in all other cell types (Fig. 3C). The −37.5-kb–inducible DHS was flanked by stable T blast-specific DHSs located at −40.3 and −33.7 kb, and additional inducible DHSs present in activated mast cells and T blast cells at −40.6 and −34.0 kb, which were weak in stimulated MP cells, and absent in other cell types (Fig. 3C). Parallel assays of the IL-3 proximal region identified tissue-specific DHSs at −4.1 and −4.5 kb in mast cells, T blast cells, and MP cells; a DHS at −1.5 kb specifically in T blast cells; and inducible DHSs at the −4.5-kb enhancer and promoter in T blasts and mast cells (Fig. 3F). The IL-3 −34.0, −37-, and −40.6-kb–inducible DHSs

FIGURE 4. DHSs associated with a L1 LINE repeat element upstream of the IL-3 gene. (A) UCSC browser view and map spanning the conserved and repeat regions upstream of the IL-3 gene (http://genome.ucsc.edu). Probes used for mapping DHSs in (B) and (C) are indicated by horizontal arrows, with locations of DHSs indicated as vertical arrows. The −16.4-kb DHS lies within the 5′ UTR of the L1PA13 LINE, and includes motifs for factors such as NFAT, AP-1, ETS, PU.1, and RUNX1, similar to the enhancers in this locus. The −23.3-kb DHS lies just outside the L1PA13 LINE, within a coding region segment of a separate incomplete L1 LINE repeat. The −23.3-kb DHS was also detected using the −34-kb BamHI probe used in Fig. 3E, which employed the same BamHI Southern blot filter as the one depicted here in (C). (B) Higher resolution mapping of the −23.3-kb IL-3 DHS in stimulated C42 blast cells probed from an upstream KpnI site, and using internal EcoRI and EcoRV sites as size markers. The sizes of these markers are indicated on the right-hand side, and the positions of each band relative to the IL-3 transcription start site are shown on the left. (C) Mapping DHSs in transgenic tissues across the repeat-region spanning the −14-kb enhancer from a BamHI site at −10.3 kb. Shown underneath is an additional DHS identified at −10.3 kb mapped in the same DNase I-digested samples, but assayed in a parallel analysis from a SpeI site at −8.8 kb.
Firefly luciferase activity is used to correct for transfection efficiency of nonstimulated and stimulated Jurkat cells. In containing the TK promoter alone, or with the IL-3 or with either the SV40 or the IL-3 infection assays of a luciferase plasmid containing the TK promoter alone, 

2 lines. ( Before and after stimulation for 4 h with PMA/I. ( Values represent the average of two independent constructs. ( Analyses relative to the IL-3 promoter is as in the genome. In each case, the DHSs. For −37/−40 and −34/−37/−40, the order of elements relative to the IL-3 promoter is as in the genome. In each case, values represent the average of two independent constructs. ( B) DHS mapping of the IL-3 −37-kb region as in Fig. 3C in human cell lines before and after stimulation for 4 h with PMA/I. ( C) Transient transfection assays of a luciferase plasmid containing the TK promoter alone, or with either the SV40 or the IL-3 −37-kb enhancer in stimulated cell lines. ( D) Transient transfection assays of a luciferase plasmid containing the TK promoter alone, or with the IL-3 −37-kb enhancer, in nonstimulated and stimulated Jurkat cells. In (A) and (C), cotransfected Firefly luciferase activity is used to correct for transfection efficiency of

were each substantially suppressed by CsA, suggesting a NFAT-dependent mechanism of activation (Supplemental Fig. 2C).

We also made the unexpected observation that a full-length LI-LINE repeat element, embedded within a 25-kb cluster of repeat elements, was associated with inducible DHSs located at −23.3 kb in mast cells and T blast cells, and −16.4 kb in mast cells, MP cells, and macrophages (Fig. 4). The −16.4-kb DHS was located within the 5' untranslated region (UTR), which represents the promoter of the ancestral retrotransposon. An additional inducible DHS was detected at −10.3 kb as a prominent DHS in mast cells, and a weaker DHS in MP cells and macrophages (Fig. 4C). The −14-kb region, which functions as an enhancer in Jurkat and CEM T cells (12), was not detected as a DHS in any of the cell types examined in this study.

The IL-3 −37-kb DHS functions as a powerful inducible enhancer

Overall, it was clear that the level of inducible IL-3 expression in different cell types correlated very closely with the presence of a highly complex cluster of tissue-specific DHSs occupying much of the upstream intergenic region. Because the pattern of induction of the −37-kb IL-3 DHS closely paralleled the induction of the gene itself, we tested the function of this element as an inducible enhancer of the IL-3 promoter in luciferase reporter gene assays in Jurkat T cells stimulated with PMA/I. Parallel assays were performed with the adjacent −34- and −40-kb DHSs, and the previously defined −14- and −4.5-kb–inducible enhancers. We found that the −37-kb element functioned as an exceptionally powerful enhancer, which increased IL-3 promoter activity by 40-fold (Fig. 5A). This was in the order of 20 times more powerful than the −14- and −4.5-kb IL-3 enhancers, and 10 times greater than the −3-kb GM-CSF enhancer (11). In contrast, the −34- and −40-kb DHSs contributed no enhancer activity, even when in combination with the −37-kb enhancer (−40 plus −37, and −40 plus −37 plus −34). The −37-kb enhancer was equally active in the reverse orientation, whereas the −40-kb DHS remained inactive when reversed (data not shown).

Because the IL-3 promoter is itself both inducible and tissue specific, the above assays are unable to assess either of these parameters for the −37-kb enhancer. To find a model system in which we could test these properties, we first screened a panel of human cell lines for the inducible −37-kb DHS. These included Jurkat T cells, HMC1 mast cells, and KG1a myeloid progenitor cells, where in each case the −37-kb DHS was inducible, and Raji B cells and 5637 epithelial cells, where this DHS could not be induced (Fig. 5B). To assess both tissue specificity and inducibility of the −37-kb enhancer, it was assayed in plasmids containing the constitutively active TK promoter in parallel with the well-defined SV40 enhancer, which was used as the reference point. The −37-kb enhancer showed the greatest activity in Jurkat T cells, where it was twice as active as the SV40 enhancer, and increased TK promoter activity by 25-fold (Fig. 5C). The −37-kb enhancer was also active, but to a lesser degree, in HMC1 and KG1a cells, where it increased promoter activity by 3– to 6-fold, whereas it was inactive in Raji B cells. The function of the −37-kb enhancer was also strictly inducible because it did not support any significant increase in TK promoter activity in the absence of stimulation (Fig. 5D).
The −37-kb enhancer encompasses conserved motifs for inducible and developmentally regulated factors

The 684-bp sequence used in Fig. 5A to define the −37-kb enhancer is displayed in Fig. 6A, in which the conserved transcription factor-binding motifs are highlighted in gray and the most highly conserved DNA sequences are double underlined. These include a 55-bp core region containing GATA, ETS-1, and NFAT consensus elements, plus downstream EGR-1, NFAT, and AP-1 elements. These elements are widely conserved across a broad range of mammalian species (http://genome.cse.ucsc.edu). Supplemental Fig. 3 depicts the alignment between nine representative mammalian genomes. For this group of mammals, it can be seen that the core region of the enhancer is well conserved across primates, dogs, cats, and horses, but less well conserved among rodents.

To further dissect tissue-specific mechanisms of enhancer function, we followed the parallel approaches of the following: 1) assaying subfragments of the enhancer, starting from the 684-bp fragment (termed the X37 element because it has XhoI adapters at the ends of the −37-kb DHS) defined above (Fig. 6B, 6C), and 2) making point mutations in motifs for the developmentally regulated factors GATA, ETS-1, and EGR-1 (Fig. 6D).

The most significant motif identified by point mutations within the 274-bp core region was the EGR-1 site, where enhancer activity was reduced to ∼17% (Fig. 6D). The two ETS-1 motifs also each contributed to enhancer function, whereby mutation of site 1 reduced enhancer activity to 62% and mutation of site 2 reduced enhancer activity to 26% (Fig. 6D). No effect on enhancer activity was observed after either specific mutation of the conserved GATA motif or deletion of the nonconserved weak consensus PU.1 motif, most likely because Jurkat cells do not express GATA factors (25), and T cells do not normally express PU.1 (29).

Because EGR-1 motifs frequently overlap with Sp1 motifs (30), we further investigated the nature of factors binding to the crucial EGR-1 motif within the −37-kb enhancer. The −37-kb EGR-1 element resembles an EGR-1 binding site in the IL-2 gene (31), whereby both elements encompass the sequence TCCCCCAC and overlap with Sp1-like motifs. It has been shown previously that Sp1 and EGR-1 compete for binding to overlapping sites, and it is
proposed that EGR-1 displaces Sp1 to mediate activation of some PMA-inducible genes (32). To determine which of these factors bind to the enhancer, we performed EMSA and ChIP analyses in PMA/I-stimulated Jurkat cells (Fig. 7A, 7B). In these studies, we also confirmed induction of both IL-3 and GM-CSF mRNA expression (Fig. 7C). EMSAs revealed that the $-37$-kb EGR-1 motif bound in vitro to both of the constitutively expressed factors Sp1 and Sp3, and to the inducible factor EGR-1 (Fig. 7B). Note, however, that although Sp1 and EGR-1 can both bind in EMSAs, where the probe is in excess, these factors cannot bind simultaneously to the same site in vivo. We used ChIP assays in Jurkat cells to confirm inducible binding of EGR-1 to the $-37$-kb enhancer, but not the Sp1/EGR-1–like element in the IL-3 promoter (Fig. 7A). However, neither Sp1 nor Sp3 was found by ChIP to bind to the $-37$-kb enhancer in Jurkat cells (data not shown). We suggest that the lack of in vivo Sp1 binding prior to activation may be due to lack of chromatin accessibility within the enhancer in nonstimulated cells, whereas EGR-1 may outcompete Sp1 binding after activation once the DHS has formed.

**Discussion**

**Independent regulation of the IL-3/GM-CSF locus**

This study establishes that a 130-kb region of the IL-3/GM-CSF locus includes all the DNA elements that are required for its correct developmental and inducible regulation. It also establishes that the IL-3/GM-CSF locus is controlled independently of the downstream Th2 cytokine gene cluster encompassing the IL-4, IL-5, and IL-13 genes, which are coregulated as a separate unit by the Th2 LCR (9). This conclusion is further supported by chromatin cross-linking (3C) assays that detected direct interactions within the nucleus between the Th2 LCR and the IL-4, IL-5, and IL-13 genes, but not between the Th2 locus and the IL-3/GM-CSF locus (10). Furthermore, our data provide further evidence suggesting that the closely linked IL-3 and GM-CSF genes are regulated independently of each other. This concept was first proposed on the basis of the following: 1) the GM-CSF gene is expressed efficiently in vivo as an isolated 10-kb transgene (15); 2) the IL-3 enhancer effectively blocks activation of the IL-3 promoter by the GM-CSF enhancer (11); and 3) IL-3 and GM-CSF have overlapping, but also unique functions in hematopoietic development. There is also the suggestion that coexpressed genes may actually require independent enhancers, which is based on evidence that the β-globin LCR can only activate one gene in this locus at any one moment in time (33).

In this study, we also obtained a fortuitous deletion in line E50 of a conserved sequence 30 kb downstream of the GM-CSF gene, which is homologous to the mouse GM-CSF +34-kb CNSa enhancer (18). However, the activity of this truncated GM-CSF transgene was not substantially different from the intact transgenes, suggesting that CNSa is not playing a major role in the inducible activation of the GM-CSF gene in T blast cells.

**Identification of elements controlling IL-3 gene expression**

Our analyses revealed the existence of a highly complex 40-kb region extending upstream from the IL-3 gene that encompasses at least three enhancers and a total of nine additional DHSs that each had inducible and/or tissue-specific properties that mirrored the expression pattern of the IL-3 gene. Whereas it remains likely that many of these upstream elements are required for the correct fine tuning of IL-3 gene expression, the cluster of DHSs that colocalized with conserved sequences in the $-33$- to 40-kb region was particularly prominent as a region closely linked to IL-3 gene expression. This region is reminiscent of LCRs, which typically include both enhancer and nonenhancer elements, such as the β-globin LCR, which exists as a complex series of five DHSs spread over at least 22 kb upstream of the β-globin gene cluster (34). However, the IL-3 upstream regulatory region also includes inducible tissue-specific DHSs associated with LI-LINE retrotransposons. These DHSs may also make a significant contribution to the normal regulation of the IL-3 gene as there is already some precedent for L1 LINE 5′UTRs directing transcripts within the human genome, and even contributing to tissue specificity (35). This finding is also reminiscent of the observation that a repeat element has been adopted as a normal component of an enhancer in the Tal1 locus (36), and our previous studies identified aberrant activation of repeat elements as a factor contributing to aberrant gene expression in Hodgkin’s lymphoma (37).

**Regulation of the $-37$-kb IL-3 enhancer**

The $-37$-kb enhancer stands out as the dominant element controlling the IL-3 gene, considering the following: 1) the $-37$-kb enhancer is 10–to 20 times more powerful than either the $-14$- or $-4.5$-kb enhancers; 2) the combination of the $-14$- and $-4.5$-kb enhancers was insufficient to support IL-3 gene promoter activity in vivo; 3) the $-14$-kb DHS has never been detected in nonleukemic cells; and 4) the IL-3 $-40$, $-34$, and $-4.1$-kb DHSs lack enhancer activity (12). Like the other previously defined enhancers in the IL-3/GM-CSF locus, the $-37$-kb enhancer contains ideal consensus binding sites for the Ca$^{2+}$-inducible factor NFAT and the MAPK-inducible factor AP-1. The $-37$-kb NFAT and AP-1 sites are, therefore, likely to represent significant targets in which the Ca$^{2+}$ and MAPK TCR signaling pathways converge to activate the IL-3 locus. However, unlike the GM-CSF enhancer and IL-2 promoter, these NFAT and AP-1 motifs do not exist as classical composite NFAT/AP-1 binding sites in which these factors bind cooperatively (1, 38).

The exquisite tissue specificity of the $-37$-kb enhancer may be dictated by a highly conserved 55-bp core segment encompassing GATA, ETS-1, and NFAT motifs. In addition, a highly conserved EGR-1 site exists just downstream of this region. The two ETS-1 motifs and the EGR-1 binding site all contributed significantly to

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**FIGURE 7.** Transcription factor binding within the $-37$-kb IL-3 enhancer. (A–C) Analyses of Jurkat T cells cultured with and without stimulation for 4 h with 20 ng/ml PMA and 0.57 mM calcium ionophore A23187. (A) EGR-1 ChIP assay of the $-37$-kb IL-3 enhancer performed as described (28), with enrichment levels normalized against a negative control within the VEUZ1 gene body. (B) EMSA performed using a probe encompassing the IL-3 $-37$-kb EGR-1 site and Abs against Sp1, Sp3, and EGR-1. (C) Semiquantitative real-time PCR analysis of mRNA expression.
enhancer function in Jurkat T cells. The PU.1 and GATA motifs were not required for function in Jurkat T cells, which lack these factors. However, it is highly significant that essentially all the major regulatory elements in the IL-3/GM-CSF locus encompass GATA motifs (1, 25). These GATA motifs are more likely to be important for promoting inducible expression in progenitor cells and mast cells than in T cells, as is established for GATA motifs in the GM-CSF enhancer and promoter (25). These motifs may also be critical for IL-3 and GM-CSF expression in the emerging hematopoietic system in the embryo (4). Furthermore, because GATA-2 expression is typically maintained in leukemic cells arising from MP cells, these GATA motifs are also likely to contribute to IL-3 and GM-CSF expression in myeloid leukemia. Future studies of this locus will now be better equipped to identify the elements that account for the activation of IL-3 expression in CML in response to constitutive tyrosine kinase signaling from the BCR-ABL fusion protein (21). It is also possible that some DHSs in the locus will be disease specific, as the IL-3 −14-kb enhancer DHS has only ever been detected in two cell lines derived from T cell leukemia (12).

The IL-3/GM-CSF locus undergoes a program of developmental regulation

The specific combination of developmentally regulated transcription factor motifs found in the −37-kb enhancer, together with the many RUNX motifs found elsewhere in the locus (1), represents an ideal combination adapted to support gene expression in progenitor cells, mast cells, and T cells (25, 39), but not in other cell types. GATA, RUNX, and ETS family factors belong to a tight cluster of factors directing combinatorial control in hematopoietic progenitor cells (39). However, because the −37-kb DHS is strictly inducible, it is likely that the binding of the constitutively expressed factors to the enhancer is dependent on the inducible factors NFAT, AP-1, and EGR-1. This mechanism resembles the GM-CSF enhancer in which binding of the tissue-specific factor RUNX-1 is dependent on accessibility to chromatin created by NFAT-dependent chromatin remodeling (25).

Cells with the capacity to express IL-3 and/or GM-CSF arise from two different types of progenitor cell, as follows: 1) lymphoid progenitors that can produce either T cells that express both genes or B cells that express neither gene, and 2) myeloid progenitors that start out with some capacity to express both genes, and can produce mast cells that upregulate IL-3 and GM-CSF expression, and monocytes/macrophages that downregulate both genes. Within the lymphoid lineage, the −4.1- and −1.5-kb DHSs were constitutively present in T blast cells and memory T cells, but absent in both thymocytes, naive T cells, and B cells (24). We also observed some specific differences between the lymphoid and myeloid pathways, with the IL-3 −34- and −1.5-kb DHSs being T cell specific, and the IL-3 −16.4- and −10-kb DHSs being myeloid cell specific. To a very large extent, the inducible expression levels seen in the different lineages reflect whether these cells had acquired these various DHSs.

We observed that some specific DHSs in the IL-3 locus, such as the IL-3 −4.5- and −4.1-kb DHSs, begin to be acquired early during the differentiation process in MP cells, and become stronger in IL-3-expressing mast cells, but are extinguished in an alternate differentiation pathway leading to macrophages. We previously proposed that the IL-3 −4.5-, −4.1-, and −1.5-kb DHSs, which are stably maintained in T blast cells, also play a role in maintaining the locus in a partially active state in memory T cells, priming the locus for activation by inducible enhancers (24). These stably maintained DHSs, and the IL-3 promoter (40), each encompass motifs for RUNX1, a factor known to be required for IL-3 gene expression and expansion of HSCs in the early embryo (4). Hence, it is significant that the constitutive IL-3 −40-, −34-, and −10-kb DHSs also each encompass RUNX1 motifs. Once the open chromatin regions have formed at each of these DHSs, the RUNX elements may be able to maintain stable RUNX1 binding and thereby maintain the locus in a primed state in both memory T cells and myeloid cells that have gained the capacity to express RUNX1. Most of these DHSs also encompass motifs for other inducible factors that may be responsible for the initial creation of these DHSs.

The finding that GM-CSF expression was also strongly downregulated during monococyte/macrophage differentiation was somewhat unexpected. This does not appear to be a cell culture artifact, as we obtained similar findings in our studies of freshly isolated spleen CD11b+ monocytic cells stimulated with either PMA/I or LPS (25). Hence, the concept that macrophage-lineage cells produce significant amounts of GM-CSF may be more representative of macrophage progenitor cells that express GATA-2, and primitive cell lines (25), rather than mature monocytes and macrophages that no longer express GATA-2.

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Disclosures

The authors have no financial conflicts of interest.

References


30. Liu, C., A. Calogero, G. Ragona, E. Adamsson, and D. Mersola. 1996. EGR-1, the reluctant suppression factor: EGR-1 is known to function in the regulation of growth, differentiation, and also has significant tumor suppressor activity and a mechanism involving the induction of TGF-beta1 is postulated to account for this suppressor activity. Crit. Rev. Oncog. 7: 101–125.


SUPPLEMENTARY SECTION

Supplementary table 1:

Primers used to create probes for DHS analyses

<table>
<thead>
<tr>
<th>Region probed</th>
<th>Enzyme and location</th>
<th>Enzyme sites and PCR primers used to create probes</th>
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<tr>
<td>Distal 5' IL-3</td>
<td>BamHI -37.7 kb from the IL-3 start, probing upstream</td>
<td>PCR fragment using primers 5' CTGGAATGGAAGGTATGGCTC and 5' CTGCTGCTGAGATGGCTCCCTTC</td>
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<td>Distal 5' IL-3</td>
<td>EcoRI -40.7 kb from the IL-3 start, probing downstream</td>
<td>PCR fragment using primers 5' GAGGTGACTTGGCCACAAACA and 5' TGGAAATAGGTCCTGGGTAG</td>
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<td>Distal 5' IL-3</td>
<td>Kpnl -29.0 kb from the IL-3 start, probing downstream</td>
<td>Restriction enzyme fragment 720 bp Kpnl (-29.0 kb) - SfI (-28.3 kb)</td>
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<tr>
<td>Distal 5' IL-3</td>
<td>SpeI -8.8 kb from the IL-3 start, probing upstream</td>
<td>PCR fragment using primers 5' CCTGGAGGGAAGATGGTGTTG and 5' ACTGCCCTGGAAGAGCACAG</td>
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Analysis of the proximal human IL-3 enhancers and promoter in transgenic mice

The human IL-3 -14 kb enhancer was linked to a proximal 5.2 kb segment spanning the human IL-3 -4.5 kb enhancer and the promoter, coupled to a human CD4 reporter gene, and used to generate 8 independent lines of transgenic mice. The proximal 5.2 kb region of the IL-3 locus also includes constitutive tissue-specific DHSs located at -4.1 and -1.5 kb. To establish a model system to measure transgene activities we first generated cultures of T blast cells by stimulating splenic cells with concanavalin A for two days, followed by at least 2 days culture with IL-2. These cells were then re-stimulated for 4 h with 20 ng/ml PMA and 2 mM calcium ionophore (PMA/I), and stained with either IgG control (grey line) or CD4 antibodies (black line). (A) Representative example of 3 transgenic lines showing low level variegated human CD4 transgene expression. (B) Representative example of 5 transgenic lines showing no CD4 expression. The map underneath depicts the DNA segments used to construct the transgene, depicting the enhancers (E), the IL-3 promoter (P), and the DHSs (vertical arrows).
Supplemental Figure 2

Cyclosporin A sensitivity of DHSs in the IL-3/GM-CSF locus

(A) IL-3 Insulator  GM-CSF Enhancer  B  IL-3 +4.5 kb ncRNA Promoter activity

BamHI probe  EcoRI probe

Fold increase in Luciferase activity

<table>
<thead>
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<th></th>
<th>pXPG</th>
<th>+4.5 kb DHS</th>
<th>IL-3 promoter</th>
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<td></td>
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<tr>
<td>PMA/I</td>
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<tr>
<td>PMA/I/CsA</td>
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(B) Luciferase reporter gene transfection assay of a non-coding RNA promoter within the inducible CsA-resistant IL-3 +4.5 kb DHS. This DHS is the potential source of non-coding transcripts emanating from this region, which are transcribed in the direction of the GM-CSF gene(4). DNA spanning the DHS was inserted in pXPG in the same orientation as in the gene and assayed in parallel with the IL-3 promoter plasmid pIL3H and the empty vector. Transfected cells were assayed after stimulation with PMA/I, and normalized values were expressed relative to values for pXPG. Assays were performed in triplicate on each of two independent clones of each construct. Error bars indicate S.D.

Cyclosporin A (CsA) sensitivity and function of inducible DHSs in the IL-3/GM-CSF locus.

(A and C) Mapping of DHSs in line C42 transgenic T blast cells, before and after stimulation for 4 h with PMA/I, and in the presence or absence of 0.1 \( \mu \)M CsA, using the probes defined in supplementary figure 2. In panel C, the positions of HindIII-lambda DNA size markers are shown on the right hand side, and the positions of DHSs relative to the IL-3 transcription start site are shown on the left.

[Diagram showing mapping of DHSs and the positions of HindIII-lambda DNA size markers]
Supplemental figure 3

Conservation of transcription factor motifs within the -37 kb IL-3 enhancer

A Vertebrate conversation

B Enhancer Core = PU.1 + GATA + Ets + Egr + central NFAT + AP-1 motifs

C 3' region of Enhancer = 3' AP-1 + NFAT motifs

(A) UCSC genome browser view of conserved regions within the core of the human IL-3 -37 kb enhancer (Vertebrate Multiz Alignment & PhastCons Conservation, http://genome.cse.ucsc.edu).

A map of predicted transcription factor binding sites is aligned underneath. (B and C) Alignment of representative mammalian genome sequences of the central core (B) and 3' region (C) of the enhancer. Motifs are highlighted in yellow if the consensus sequence predicted to be required for DNA-binding is conserved. The DNA sequences are from the human (Hu), chimp (Ch), rhesus (Rh), mouse (Mo), guinea pig (Gu), rabbit (Ra), dog (Do), cat (Ca) and horse (Ho) reference genomes. Gaps in the alignment are depicted by dashes.