Localization of the Inducible Enhancer in the Mouse Interleukin-5 Gene That Is Responsive to T-Cell Receptor Stimulation

By Peter F. Bourke, Barbara H. van Leeuwen, Hugh D. Campbell, and Ian G. Young

Transcriptional regulation of the interleukin-5 (IL-5) gene in T lymphocytes appears to be of central importance in the control of the eosinophilia characteristic of allergic responses and certain parasitic infections. Previous studies of IL-5 gene regulation have been hampered by the lack of a transfection assay, which detects the antigen-responsive enhancer in the IL-5 promoter. Here we show that stable transfection of the Th2 clone D10.G4.1 and the T lymphoma EL4.23 with chloramphenicol acetyltransferase reporter gene constructs carrying the region to −3859 gives inducible expression with the known regulatory characteristics of the endogenous IL-5 gene. To facilitate detailed analysis of the promoter region, 3.9 kb of DNA sequence immediately upstream of the start of transcription was determined and the minimum upstream region required for inducible expression was further localized, by stable transfection studies in EL4.23 cells, to the region up to −1016. A CTF/NF1 site in the upstream enhancer at −940 to −928 was shown to be required for regulated inducible expression. Mutation of this sequence motif abolished inducibility and also prevented binding of the sequence to a nuclear protein(s). A TCATTT-containing element in the proximal promoter region was also demonstrated to be essential for inducible expression of the IL-5 gene, similar to the role of this conserved element in the transcriptional regulation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 genes.

INTERLEUKIN-5 (IL-5) is one of a group of cytokines produced by activated T lymphocytes that are involved in the regulation of immune and inflammatory responses. IL-5 appears to play a central role in the regulation of the eosinophilia, which is characteristic of allergic diseases, such as asthma and certain parasitic infections. IL-5 is also active in regulating the growth and differentiation of murine B cells. 2,3

IL-5 is produced by the Th2 subgroup of murine CD4+ T cells when stimulated by antigen or mitogens such as Concanavalin A (ConA), but relatively little is known about the mechanisms governing its regulation. Unlike some other T-cell–produced cytokines, such as IL-3, IL-4, and granulocyte-macrophage colony stimulating factor (GM-CSF), the expression of IL-5 is resistant to the immunosuppressive drug cyclosporin A 4 suggesting that the antigen-responsive enhancer in the IL-5 gene may be linked to the T-cell receptor via a different signalling pathway. Recent work in our laboratory has shown that IL-5 gene expression can be selectively induced in the Th2 cell clone D10.G4.1 by treatment with phorbol 12-myristate 13-acetate (PMA) or by stimulation of the IL-1 receptor or CD45, without induction of the IL-3, IL-4, or GM-CSF genes. 5 Thus, although the IL-5 gene is part of a cytokine gene cluster on mouse chromosome 11 with IL-3, IL-4, and GM-CSF, 6 the regulation of expression of the IL-5 gene must involve gene-specific mechanisms. The use of nuclear run-on transcription assays has established that the IL-5 gene in D10.G4.1 cells or the T-cell lymphoma line EL4.23 is regulated primarily at the level of transcription. 7,8

The lack of a transfection assay, which measures inducible expression of the IL-5 promoter in response to T-cell activation signals, has greatly hampered studies of IL-5 gene regulation. In the present work, we show that stable transfection assays with the T-cell clone D10.G4.1 and the T-cell lymphoma line, EL4.23 can be used to study the transcriptional regulation of the IL-5 promoter. We have used this assay to localize the inducible promoter/enhancer in the IL-5 gene and have demonstrated the involvement of a CTF/NF1 site and a TCATTT-containing element in the regulatory mechanism.

MATERIALS AND METHODS

Cell lines. The T-cell clone, D10.G4.1 was obtained from Dr T. Mosmann, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA. EL4.23 is a subline of the murine lymphosarcoma EL4 and was obtained from Dr C. Sanderson, National Institute for Medical Research, London, UK.

Sequence. A 10-kb HindIII-HindIII mouse (BALB/c) DNA fragment containing the IL-5 gene was cloned into pSV2neo (pSV2neo-10kbIL-5G). The 3884-bp HindIII-Eco47III fragment (Fig 1A) was sonicated and subcloned into M13. 9 Sequence of M13 subclones and alkali-denatured double-stranded plasmid DNA 10 was obtained by the chain termination method. 11 Both strands were completely sequenced. DNA sequences were assembled and analyzed using the programs described by Staden. 12,13 The IL-5 gene 5′ flanking sequence was compared with the GenBank nucleotide sequence database and databases of known motifs to identify conserved sequence elements. All IL-5CAT mutants were sequenced by cycle sequencing using polymerase chain reaction (PCR).

Constructs for expression studies. The 10-kb HindIII-HindIII IL-5 gene fragment (−3859 to +6181) was cloned into the promoterless vector pSP73 (Promega, Madison, WI) after insertion of an EcoRV linker into the BstEII site in the 3′ untranslated region (creating pSP73.mIL-SG(EcoRV)) to distinguish expression from that of the endogenous gene. The 6.5-kb Xba I-HindIII fragment (−312 to +6181) from pSP73.mIL-SG(EcoRV) was cloned into pCExV3 14 to create pCExV3.mIL-SG(EcoRV). The IL-5 gene fragment encompassing −547 to +74 was obtained by PCR using primers corresponding to the known sequence of the gene 11 plus additional sequence to generate a HindIII site at its 5′ end and an EcoRI site at its 3′ end to facilitate cloning. This fragment was cloned into M13mp19 and its sequence confirmed. The blunt-ended 572-bp HindIII-EcoRVIII fragment was then cloned into the blunt-ended HindIII site of PSVOCAT 15 yielding a construct, which has 547-bp of IL-5 genomic sequence 5′ of the transcriptional start site.

From the Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, The Australian National University, Canberra, Australia.

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Address reprint requests to Ian G. Young, PhD, Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, The Australian National University, GPO Box 334, Canberra, ACT 2601, Australia.

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enzyme sites are shown in Fig 1A. Exonuclease digestion and religation of these two constructs. The largest IL-SCAT construct contained -3859 to +25 of IL-5 genomic sequence relative to the CAP site (-3859IL-5CAT) and was similar using the F'romega Erase-a-Base system. -3859IL-5CAT was digested using the restriction enzymes unique to the -547-bp of IL-5 sequence. The CTFVNF1 site at -940 to -928 and the TCAm site at -49 to -25 were used to detect expression from pSP73.mIL-5G(EcoRV) and pCEXV-Sacchi and cDNA prepared by conventional methods. The primers for mutagenesis were PB19 and PB26. The 5' primer PB19 (ATT-GAGTGT AAATGTGGAT CAAAGTTGTA GAAACATTCT TTTATGTTAT AGAAAATGCT TTTTAAGCAG GGGTGSGGGT) and the 3' primer PB26 (GTTCAGGGTCACAGATATCGTG) correspond to the sequence of the EcoRV linker. These primers amplify a fragment of 2546-bp in length and yielded -312IL-5CAT, Nsi I creating -140IL-5CAT, and EcoNI creating -312IL-5CAT, Nsi I creating -140IL-5CAT, and EcoNI creating -312IL-5CAT. The enzyme map of the 5' flanking sequence of the IL-5 gene. Restriction enzyme sites are AccI (A), BglII (B), EcoNI (C), Eco47III (E), Nhel (F), AgeI (G), HindIII (H), Xsa (N), Spin (P), Sad (S), Tth1111 (T), XbaI (X). (B) Nucleotide sequence of the IL-5 gene from -1299 to +29. Underlined are the TATA box, the TCATT motif (IL-5/GM-CSF conserved region), the CK1 and CK2 sequences, possible glucocorticoid response elements (GRE), and the potential binding sites for the CTF/NF1 and OCT transcription factors. The * marks the start of transcription.

(CAP) site and 25-bp 3' of the CAP site (-547IL-5CAT). The largest IL-5CAT construct contained -3859 to +25 of IL-5 genomic sequence relative to the CAP site (-3859IL-5CAT) and was similar using the 3884-bp HindIII-Eco47III fragment from pSV2neo-10kbIL-5G.

A number of 5' truncation mutants were obtained by restriction endonuclease digestion and religation of these two constructs. The 5' end of the IL-5 sequence in -547IL-5CAT has an Nhe I site, which was generated on ligation of the two blunt-ended HindIII plasmids. The enzyme digestion of -3859IL-5CAT with Nhe I removed a fragment of 2546-bp in length and yielded -1299IL-5CAT. The enzymes employed were AccI creating -458IL-5CAT, Xbal creating -312IL-5CAT, NsiI creating -140IL-5CAT, and EcoN1 creating -88IL-5CAT. Digestion of -3859IL-5CAT with Nhe I removed a fragment of 2546-bp in length and yielded -131IL-5CAT. Nhe I (SpH I and Nhe I) digestion of -170IL-5CAT and -1089IL-5CAT, respectively. These restriction enzyme sites are shown in Fig 1A. Enonuclease III was used to generate the remaining truncation constructs from -3859IL-5CAT using the Promega Erase-a-Base system.17 -3859IL-5CAT was digested with SacI I to prevent digestion of the vector with exonuclease III, and Age II (-3656) or Bgl II (-1483) before treatment with exonuclease III to generate the deletion mutants. Each construct was sequenced to determine the 5' IL-5 terminus using 17-mer corresponding to sequence 5' of the HindIII cloning site of pSVOCAT. The CTF/NF1 site at -940 to -928 and the TCATT site at -49 to -44 were mutated by oligonucleotide-directed in vitro mutagenesis in the -1170IL-5CAT construct. Briefly, a 1199-bp SpH I- Eco47III fragment of the IL-5 gene 5' sequence was directionally subcloned into SpH I-Sma I cut phagemid pTZ18U (Bio-Rad, Sydney, Australia). Mutagenesis was performed in Escherichia (E coli) strain RZ1032 (mut", ung") as described previously.18 The resulting double stranded heteroduplex phagemids were transformed into E coli strain MC1061.1 (mut", ung").19 The resulting double stranded heteroduplex phagemids were transformed into E coli strain MC1061.1 (mut", ung").20 Mutagenized fragments were then isolated by primed HindIII digestion, blunt-ended and cloned back into the blunt-ended HindIII cloning site of pSVOCAT. These plasmids were confirmed by sequencing. Supercoiled CsCl-purified plasmid DNA was used in transient transfection experiments. For stable transfection, IL-5CAT construct DNA was linearized with Bgl I, while pMC1neo PolyA DNA21 was linearized with SacI I. DNA was then extracted with phenol-chloroform and ethanol precipitated before electroporation.

Reverse transcriptase-polymerase chain reaction. RNA was prepared from cells according to the method of Chomczynski and Sacchi22 and cDNA prepared by conventional methods. The primers used to detect expression from pSF73.mIL-5G(EcoRV) and pCEXV-3.mIL-5G(EcoRV) were PB19 and PB26. The 5' primer PB19 (ATT-GACCCGCAAAAAGAGAGACTG) spans exons 3 and 4 in the IL-5 gene sequence and only detects mRNA. The first 15 bases of the 3' primer PB26 (GTTCAGGGTCACAGATATCGTG) correspond to the sequence of the EcoRV linker. These primers amplify a fragment of 587-bp and do not amplify endogenously expressed IL-5 mRNA. PCR reactions were analyzed on agarose gels.

Cell culture. EL4.23 and DIO.G4.1 cells were maintained in RPMI 1640 (GIBCO, Ontario, Canada) medium, 10% fetal calf serum, with the following additives: 10 mmol/L NaCl; 1 mmol/L sodium pyruvate; 62 mL/L monothioglycerol; 100 U/L benzylpeni-
cillin (Commonwealth Serum Laboratories [CSL], Melbourne, Australia); 100 µg/L streptomycin (Sigma, St Louis, MO); 2 mmol/L L-glutamine (CSL) at 37°C, 5% CO₂, and 95% relative humidity. The RPMI 1640 medium for the T-cell clone D10.G4.1 also contained 0.05 mmol/L β-mercaptopethanol (Eastman Kodak, Rochester, NY) and IL-2. The IL-2 was either 10 units/mL of human IL-2 (Amersham, Buckinghamshire, UK) or conditioned medium from a cell line transfected with a mouse IL-2 construct. 23 D10.G4.1 cell growth was induced by alloimmune stimulation every 10 to 14 days with irradiated spleen cells from 6 to 8 weeks old C57BL/6 female mice. 23 D10.G4.1 cells were treated with one or more of the following as indicated: 5 µg/mL ConA, 10 ng/mL PMA, 3D3 antibody 24 at 1 in 1,000 dilution, 0.1 µg/mL desamethasone or 1 µg/mL cyclosporin A. EL4.23 cells were treated with 10 ng/mL PMA where indicated.

**Transient transfection.** Cells were resuspended (at 1 × 10⁶ to 2 × 10⁷/mL) in fresh medium the day before electroporation. Transfection was performed only if greater than 90% cells were alive. A total of 2 × 10⁷ cells in complete medium were mixed with 30 µg of supercoiled IL-5CAT construct DNA in a total volume of 400 µL and electroporated using a Bio-Rad Gene Pulser and Bio-Rad Capacitance Extender. Optimum parameters were 250 V at 500 microfarad (µF) for EL4.23 and 300 V at 500 µF for D10.G4.1. The cells were immediately transferred to 20 mL of fresh medium and allowed to recover for 16 hours. The transfected cells were divided in half and PMA was added to one half for EL4.23, or ConA for D10.G4.1. The other half of the cells was retained as the untreated control. Cells were harvested after 12 to 16 hours for chloramphenicol acetyltransferase (CAT) analysis. The number of viable cells was routinely checked at the time of harvest.

**Stable transfection.** D10.G4.1 cells were electroporated 9 to 11 days after alloimmune stimulation. The day before transfection, D10.G4.1 cells were resuspended at 1 × 10⁶ cells/mL, and EL4.23 cells were resuspended at 5 × 10⁶ cells/mL in fresh medium. Transfection was performed only if greater than 90% cells were alive. A total of 2 × 10⁷ cells were mixed with 30 µg of linearized IL-5CAT construct and 3 µg of linearized pMC1neo PolyA in 400 µL complete medium. Electrical parameters for electroporation of each cell line were the same as those for transient transfection. Immediately after electroporation, cells were transferred to 30 mL of fresh medium. D10.G4.1 cells were alloimmune stimulated the day after electroporation. Selection with the neomycin analogue G418 (400 µg/mL Gibco) was commenced after a further 2-day incubation. EL4.23 cells were incubated for 2 days after electroporation before commencing G418 selection (800 µg/mL). Selection with G418 was continued for 10 days, at which time all mock (no DNA) electroporated control cells were dead. Selected polyclonal EL4.23 cells were then grown for 2 days without G418 before stimulation. The number of individual clones in the polyclonal populations was estimated from the growth rate of the transformed cell lines. The doubling time of transformed (3418- to 1,500-fold integration events after transfection) was estimated and found to vary from 350 to 1,500 for the different transfection experiments. This range is a minimum estimate of the base complexity of the polyclonal population because it assumes that the transfected cells grow immediately at their normal rate.

Selected polyclonal D10.G4.1 cells required 3 to 4 weeks growth before sufficient cells were available for the first stimulation and then a further 2 weeks growth for the duplicate stimulation. The estimation of the number of individual clones in the polyclonal populations was more difficult for these cells, as the doubling time of the cells decreased to 18 hours after alloimmune stimulation and then increased to 48 hours over the next 12 days. However, by basing the estimation on the overall growth between alloimmune stimulations (60- to 100-fold), it was possible to estimate that the minimum number of transfectants was at least as many as estimated for EL4.23 cells.

For CAT assay of the stably transfected polyclonal cells, 1 × 10⁷ cells were stimulated and harvested as for transiently transfected cells.

**CAT assay.** After decanting the cells, the tissue culture flask was rinsed once with 10 mL of phosphate-buffered saline (PBS) containing 5 mmol/L EDTA, which was pooled with the culture sample. Cells were washed with PBS and then resuspended in 150 µL of 0.25 mol/L Tris-HCl, pH 8.0. Samples were sonicated on ice, incubated at 65°C for 10 minutes and immediately centrifuged at 4°C for 10 minutes. The supernatant was assayed for CAT activity using the butyryl coenzyme A/liquid scintillation counting (LSC) CAT assay. 25

**Gel-retardation assay.** Nuclear extracts were prepared by a modification of the method of Miskimins and al 26 from unstimulated EL4.23 cells and cells stimulated with 10 ng/mL PMA for 6 hours. The cells were washed twice in PBS and resuspended at 3.5 × 10⁷ cells/mL in lysis buffer (10 mmol/L Heps, pH 8.0, 50 mmol/L NaCl, 5 mmol/L MgCl₂, 0.5 mol/L sucrose, 0.1 mmol/L EDTA, 0.5% Triton X-100, 1 mmol/L DTT, 0.1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). The nuclei were pelleted at 500g for 1 minute, and resuspended in the lysis buffer, on ice, at 7 × 10⁶ cells/mL. Spermidine was added to 5 mmol/L and NaCl to 0.5 mol/L. The suspension was incubated on ice for 30 to 60 minutes and then centrifuged at 200,000g for 15 minutes. The supernatant was carefully removed and dialyzed against 10 mmol/L HEPES, pH 8.0, 50 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L DTT, 50% glycerol at 4°C for 18 hours. The nuclear extract was then aliquotted, immediately frozen in a dry ice-ethanol bath, and stored at −70°C. The protein concentration of the extract was determined using the Bio-Rad Protein Assay kit.

Complementary oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) Model 380A DNA Synthesizer and purified by high performance liquid chromatography (HPLC). The sequence of the oligonucleotides included AATT at the 5’ end to generate single-stranded ends after annealing to enable labeling by the fill-in method. Oligonucleotide duplex CTF-1 is two complementary 37-mers containing the IL-5 gene sequence from −946 to −915 (Fig 1B); oligonucleotide duplex CTF-2 is the same sequence except for the mutations at the CTF/NFI site. Oligonucleotide duplex RS is two complementary 38-mers of random sequence (5’-AAT- TCCCTTGTTCGAGATAACACTACGTCTTATAGCGCCG-3’). Pairs of oligonucleotides were annealed by mixing 6 pmol of each in Klenow buffer (50 mmol/L Tris-HCl pH 7.2, 10 mmol/L MgSO₄, 0.1 mmol/L DTT) containing 50 µg/mL bovine serum albumin (BSA). The annealed oligonucleotides were labelled with [α-³²P]-dATP by filling in the single-stranded end regions.

The gel retardation assay was carried out according to the method of Singh et al. 27 Briefly, 10 µg of nuclear extract was added to the labelled DNA in binding buffer (10 mmol/L Tris-HCl pH 7.5, 50 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 0.05% Nonidet P-40, 5% glycerol) in a total volume of 25 µL and incubated at room temperature for 30 minutes at 15 fmoL of labelled annealed oligonucleotide duplex. Poly(dI-dC)·poly(dI-dC) (1.2 µg, Phar- macia) was included as nonspecific DNA. After binding, 5 µL of the reaction mixture was loaded onto a polyacrylamide gel (6%; acrylamide:bis, 55:1) in 1 X TAE buffer (6.7 mmol/L Tris-HCl pH 7.5, 3.3 mmol/L sodium acetate, 1 mmol/L EDTA). The gel was preelectrophoresed in 1 X TAE for 30 minutes at 11 V/cm, and after
sample loading was Electrophoresed at 15 V/cm for 60 minutes at room temperature. The gel was dried and then autoradiographed.

GenBank database submission. Nucleotide sequence of the 5' flanking region of the mouse IL-5 gene will be submitted to the GenBank database.

RESULTS

Determination of 5' flanking sequence of mouse IL-5 gene. A sequence of 6727 bp covering the mouse IL-5 gene was determined previously in this laboratory. This sequence included 547 bp of the 5' flanking sequence of the IL-5 gene. Because upstream enhancers involved in transcriptional regulation may be several kilobases away from their proximal promoter elements, we determined an additional 5' flanking sequence of the mouse IL-5 gene to -3859. The additional sequence information was used to facilitate the generation of reporter constructs carrying different lengths of 5' flanking region for attempted localization of the IL-5 enhancer responsive to T-cell receptor stimulation. Database searching of the new sequence showed the presence of murine B1 and B2 Alu-like elements at -2538 to -2403 (plus strand) and -1530 to -1374 (minus strand), respectively. Because the functional studies described below located the promoter/enhancer to the region to -1016, a more detailed analysis of this region of upstream sequence was performed for potential transcription factor binding sites. A few sequence elements were identified in this region of the gene, which closely matched consensus binding sites of known transcription factors. These include a CTF/NFI site at -940/-928 (TGGGTCAAGGCCA, consensus TGGCANNNTGCCA), an octamer binding site at -453/-446 (ATTTGAAT, consensus ATGCAAAT), two possible glucocorticoid response elements, which may be involved in the dexamethasone repression of IL-5 gene expression, and the conserved cytokine sequences CK1 and CK2 (Fig 1B). The TCATT motif, which is conserved in the IL-5 and GM-CSF proximal promoter regions, is also shown in Fig 1B.

Transient expression assays using reporter genes. Two cell lines, which show inducible expression of the IL-5 gene, were used to test expression of the reporter gene constructs. D10.G4.1 is a Th2 murine T-cell clone that requires T-cell receptor stimulation for growth and, of the cell lines used, is the most closely related to normal T lymphocytes. EL4.23 is a murine T-cell lymphoma line. Regulation of the IL-5 gene in each cell type is predominantly transcriptional. IL-5 mRNA expression is induced by ConA, PMA, or the antidiotype specific monoclonal antibody 3D3 in D10.G4.1 cells, and by PMA in the case of EL4.23 cells. A series of constructs carrying different lengths of the 5' flanking region of the IL-5 gene linked to the CAT reporter gene was prepared and electroporated into the two cell lines, and assays for transient expression of CAT were performed. Optimum conditions for electroporation were determined for each cell line using control plasmids such as pRSVCAT. Using these conditions, the positive control pRSVCAT gave good levels of expression in each cell line, but no inducible expression was seen with any of the reporter constructs (data not shown). This result was confirmed in three separate experiments. Doubling the amount of DNA used for the

-3859IL-5CAT construct to 60 µg/transfection did not affect the result.

There are a few possible explanations why the -3859IL-5CAT construct was not expressed after transient transfection into cell lines, which inducibly express IL-5. One possible explanation is that the required regulatory elements are not present in the construct. To test if the necessary sequences are present in an intron or the 3' untranslated region of the gene, whole gene constructs were prepared. The 10-kb HindIII-HindIII gene fragment (including 5' sequence to -3859) was modified by insertion of a linker into the BsrEII site in the 3' untranslated region of the IL-5 gene and cloned into the promoterless vector pSP73 (Promega), so that the expression of the gene construct could be measured independently of that of the endogenous gene by RT-PCR.

![Fig 2. CAT activity of the IL-5CAT constructs after stable transfection into D10.G4.1 cells or EL4.23 cells. DNA was stably transfected into D10.G4.1 cells (A) or EL4.23 cells (B). D10.G4.1 cells were stimulated with 5 µg/mL ConA and EL4.23 cells with 10 ng/mL PMA. The CAT activity of each construct is expressed relative to the activity of pRSVCAT in stimulated cells. The CAT activity is the average of two stimulations of the polyclonal stable cell lines, and the bars indicate the variation between these stimulations.](image-url)
A control plasmid was also constructed, which carried the modified IL-5 gene (including 5' sequence to –312) in pCEXV3, so that expression of the IL-5 gene was under control of the SV40 early promoter. No inducible expression was detected after transient transfection of pSP73.mILS(EcoRV) into EL4.23 cells. As expected, constitutive expression of the IL-5 gene was detected when the pCEXV3-mILS(EcoRV) control plasmid was transiently transfected into EL4.23 cells. The lack of inducible expression with pSP73.mILS(EcoRV) was confirmed in four separate experiments.

The failure of the 10-kb gene fragment to give inducible expression in transient assays suggested that either the necessary regulatory sequences were located further away from the gene, or that they do not function in the absence of the normal chromatin environment. The latter possibility was tested by stably incorporating the CAT reporter constructs into the host genome.

Demonstration of the inducible IL-5 promoter/enhancer using stable transfection assays in D10.G4.1 cells. Stable transfection assays were performed on the different reporter gene constructs using linearized plasmid DNA cotransfected with pMC1neo PolyA. The latter plasmid carries the neo gene under the control of the herpes simplex virus thymidine kinase promoter allowing stable transfectants to be selected using G418. Polyclonal lines consisting of a minimum of three hundred independent transfectants were generated to minimize any influence of the site of integration and copy number on expression. Copy number and expression levels in stable transfectants are often uncorrelated, so it is not possible to completely standardize experiments of this type. Our criterion for demonstrating the presence of the IL-5 enhancer was consistent high level inducible expression (minimum of 10-fold induction) in independent experiments.

When the IL-5CAT reporter constructs previously used in transient assays were stably transfected into D10.G4.1 cells, the constructs carrying up to –547 showed varying levels of constitutive expression but the –3859IL-5CAT construct was highly inducible (Fig 2A). Other detailed studies in this laboratory have shown that the IL-5 gene is expressed in D10.G4.1 cells in response to stimulation with ConA or the antiidiotype specific monoclonal antibody 3D3. In contrast to the IL-3, IL-4, and GM-CSF genes, the IL-5 gene is also stimulated in these cells by PMA, and its expression is resistant to inhibition by cyclosporin A. The expression of the –3859IL-5CAT construct in D10.G4.1 cells was also shown to have these characteristics (Fig 3).

IL-5 gene expression in D10.G4.1 cells is also strongly inhibited by the antiinflammatory drug dexamethasone, just as it is in human peripheral blood mononuclear cells. Expression of the –3859IL-5CAT construct induced by either ConA, 3D3 antibody, or PMA was strongly inhibited by dexamethasone (Fig 3). However, the constitutive expression shown by the shorter constructs, such as –547IL-5CAT, was not significantly affected by dexamethasone (data not shown).

Therefore, the upstream region of the mouse IL-5 gene to –3859 carries an inducible enhancer responsive to T-cell receptor stimulation and confers all of the known regulatory properties of the endogenous gene.

Localization of the inducible IL-5 promoter/enhancer using EL4.23 cells. Comparative studies of the mechanisms regulating the IL-5 gene and several other lymphokine genes in D10.G4.1 cells and EL4.23 cells have shown that transcriptional regulation is predominant in both cells and that the regulatory mechanisms have very similar characteristics. EL4.23 cells were used for more detailed localization of the enhancer because these cells are much easier to cultivate and to transfect than the T-cell clone D10.G4.1. Stable transfection assays showed that the –3859IL-5CAT construct also gave inducible expression in EL4.23 cells, whereas the expression of constructs carrying up to –547 showed no significant inducibility (Fig 2B). Thus, in this respect, the reporter constructs showed analogous expression characteristics in EL4.23 and D10.G4.1 cells, although the magnitude of expression levels relative to pRSVCAT was generally greater for the shorter constructs in EL4.23 than in D10.G4.1 cells.

A number of 5' deletion constructs were prepared using existing restriction endonuclease sites or exonuclease III deletion as described in Materials and Methods. Stable transfection assays were performed with the aim of establishing the minimum upstream region required for inducible expression. Representative data on the localization of the enhancer
are given in Fig 4. There was some variation in the absolute level of CAT expression obtained for each construct in different experiments, as would be expected for random polyclonal populations of stable transfectants. However, the -3859IL-5CAT construct consistently gave a high level of inducible expression with inducibility greater than 10-fold, indicating the presence of the IL-5 enhancer. The -3859IL-5CAT construct was used to generate four independent polyclonal lines, which showed inducibility levels of 41, 90, and 82-fold respectively (Fig 2B; Fig 4B, C, and D). In contrast, four independent polyclonal lines generated with the -547IL-5CAT construct all showed no inducibility (Fig 2B; Fig 4A, B, and D). Although the level of constitutive expression of the -547IL-5CAT construct was relatively high in the experiment described in Fig 2B, this was not a consistent finding. In general, the expression of this construct was much lower than the inducible expression of -3859IL-5CAT.

Initial localization experiments narrowed the functional enhancer to the region up to -1089 (Fig 4A). Further experiments reduced the 5' boundary of the enhancer to -1016 (Fig 4B and C). In a number of separate electroporations, the polyclonal lines containing the -1016IL-5CAT construct gave comparable levels of inducible expression to the -3859IL-5CAT construct. In general, reporter gene constructs whose 5' ends ranged from -1016 to -929 gave progressively lower levels of inducible expression (Fig 4). Polyclonal lines containing from -547 to -88 were not inducible (Figs 2B and 4). Overall, the 5' deletion experiments suggest that the region -1016 to -929 carries one or more elements important in the induction of IL-5 gene expression and that the IL-5 promoter/enhancer is contained within the region to -1016.

Potential regulatory elements in the distal IL-5 enhancer region. The region from -1016 to -929 and surrounding sequences were examined for the presence of sequences corresponding to the binding sites of known mammalian transcription factors. A consensus site for CTF/NFI (-940 to -928) was found within the region essential for high level inducible expression. DNA-binding experiments with nuclear extracts prepared from EL4.23 cells showed the presence of a nuclear protein(s) binding to this region (Fig 5). Mutations known to prevent CTF/NFI binding were introduced into the -1170IL-5CAT construct (Fig 6A) and Fig 5B also shows that mutation of this site prevents binding of nuclear protein(s) to this region of the mouse IL-5 gene.

Stable transfections of these mutated -1170IL-5CAT constructs were performed in EL4.23 cells and compared with the parent construct -1170IL-5CAT. Mutation of the CTF/NF1 binding site had the very interesting effect of converting the -1170IL-5CAT stable transfectants to constitutive expression (Fig 6B). It would appear, therefore, that the CTF/NF1 site is involved in the inducible expression of the IL-5 gene in EL4.23 cells. Although the 5' deletion experiments suggest the possibility that other binding sites may be present in the region -1016 to -929, we have not yet been able to demonstrate any further DNA binding in gel retardation experiments.

Role of a conserved TCATT-containing element in the proximal promoter region. A TCATT-containing element extending from -61 to -41 of the mouse IL-5 gene is highly conserved in the human IL-5 gene and the mouse...
TRANSCRIPTIONAL REGULATION OF THE IL-5 GENE

Fig 5. Binding of nuclear protein(s) to the CTF/NF1 site in the IL-5 gene. Gel retardation assay using labelled CTF-1 duplex DNA and nuclear extracts from unstimulated EL4.23 cells (lanes 2 to 5) or cells stimulated with 10 ng/mL PMA (lanes 6 to 8). Binding was performed in the absence of competitor DNA (lanes 2 and 6), and in the presence of 100-fold unlabelled CTF-1 (lanes 3 and 7), CTF-2 (lanes 4 and 8), or RS (lane 5). Lane 1 contains no nuclear extract. CTF-1 contains the sequence -946 to -915 of the IL-5 gene, CTF-2 contains this sequence mutated at the CTF/NF1 site, and RS is an oligonucleotide duplex of random sequence (see Materials and Methods and Fig 6A for nucleotide sequences). DNA-protein bound complexes and free unbound DNA are indicated.

and human GM-CSF genes.15 Evidence supporting the role of this element in transcriptional regulation of the GM-CSF gene has been previously reported.16 To test whether this element was involved in inducible expression of the IL-5 gene in EL4.23 cells, three nucleotide changes were made to the TCATTT motif present in the –1170IL-5CAT reporter gene construct. The base substitutions used (Fig 6A) have previously been shown to abolish inducible expression of human GM-CSF reporter gene constructs in transient expression assays.15

Comparison of the –1170IL-5CAT with the same construct containing the mutant TCATTT-containing element using stable transfections in EL4.23 cells showed that mutation of the TCATTT motif abolished inducible expression of the promoter (Fig 6B).

DISCUSSION

Other studies in this laboratory have shown that the control of IL-5 gene expression in the T-cell clone D10.G4.1 and the T-cell lymphoma EL4.23 is predominantly transcriptional.6,8 In the present work, we have established a stable transfection assay, using either of these two cell lines, which measures IL-5 promoter activity, and we have used this assay to localize the mouse IL-5 gene transcriptional enhancer, which is responsive to stimulation of the T-cell receptor. The proximal region of the promoter up to –547 was shown to be capable of directing constitutive expression in stable expression assays in the T-cell clone D10.G4.1. However, the effect of inclusion of additional upstream sequence to –3859 resulted in high levels of inducibility. Reporter genes carrying 3884 bases of 5′ sequence (–3859 to +25), when stably transfected into D10.G4.1 cells, showed all of the known regulatory characteristics of the endogenous IL-5 gene.5,6 Induction could be achieved with ConA, PMA, or the T-cell receptor idiotype-specific monoclonal antibody 3D3. Induction by these agents was inhibited strongly by

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Fig 6. Mutation of the CTF/NF1 site in –1170IL-5CAT. (A) Nucleotide sequence of –1170IL-5CAT and mutagenized –1170IL-5CAT constructs. (B) CAT activity of –1170IL-5CAT and mutagenized –1170IL-5CAT constructs transfected into EL4.23 cells and stimulated with 10 ng/mL PMA. The CAT activity of each construct is expressed relative to the activity of –1170IL-5CAT in stimulated cells. The CAT activity is the average of five separately transfected polyclonal stable cell lines before and after stimulation, and the bars indicate the variation between these transfected populations.
dexamethasone, but was relatively resistant to cyclosporin A.

Analogous results to those obtained with the T-cell clone D10.G4.1 were also obtained with the T-cell lymphoma EL4.23. The -3859IL-5CAT construct gave inducible expression in response to PMA stimulation, the same as for the endogenous gene, but only when stable transfection assays were used. Once again, the proximal region of the promoter up to -547 directed constitutive expression. In EL4.23 cells, maximal inducible expression was dependent on the presence of the region -1016 to -929. A consensus binding site for CTF/NF1 was located at -940 to -928 within the critical region for inducible expression. The lower levels of inducibility of constructs -965 to -929 compared with -1016IL-5CAT may be due to a requirement for flanking sequences for cooperative interaction between transcription factors. Gel retardation assays provided evidence for the presence of a nuclear protein(s), which was capable of binding to synthetic duplex DNA corresponding to the sequence of this region. Mutation of the binding site for CTF/NF1 in a reporter gene construct (-1170IL-5CAT) resulted in loss of inducible expression, suggesting involvement of CTF/NF1 in the regulatory mechanism. Although CTF/NF1 has more commonly been reported to function in the proximal regions of gene promoters, it also has been shown to be active at upstream enhancer sites. Our results clearly show that the CTF/NF1 element at -940 to -928 is part of a distal enhancer involved in inducible expression of the IL-5 gene and that induction of the gene may involve displacement or modification of CTF/NF1.

Mutation of a conserved TCATTCC-containing element in the proximal region of the promoter showed that this element was also involved in inducible expression of the IL-5 gene in EL4.23 cells. This supports analogous findings for the role of this conserved region in the inducible transcription of the human and mouse GM-CSF gene. Furthermore, a recent study has demonstrated the functional importance of the analogous element in the mouse IL-4 promoter. Responsiveness of both the GM-CSF and IL-4 promoters to T-cell activation signals appears to require other elements in addition to the intact TCATTCC-containing element, as is also the case with the IL-5 gene.

Our failure to detect the inducible mouse IL-5 gene enhancer in transient transfection assays was an unexpected finding. It does not appear to be due to low transfection efficiency because transient transfection of a whole gene construct driven by the SV40 early promoter gave detectable expression, and constitutive expression of deleted IL-5 promoter constructs was observed. One possible explanation is that the antigen-responsive enhancer is active only in the highly structured chromatin environment of the chromosome, as has been established for several elements of the globin locus control region. In a very recent study of IL-5 gene regulation using a different subline of EL4 and a reporter construct carrying 1200 bp of the promoter region, inducible promoter activity was detected in transient assays providing a combination of PMA and substances which elevate levels of intracellular cAMP was used for induction.

The present work has provided several significant advances in approaching the study of IL-5 gene regulation. First, stable transfection of target cells was found to be necessary to obtain high level, inducible expression, which mirrored the inducible expression of the endogenous gene in every respect we were able to test. Second, using stable transfection, we were able to obtain inducible reporter gene expression in the T-cell clone D10.G4.1. This is a valuable advance, because D10.G4.1 has the relevant surface molecules and signalling pathways for a more normal induction of cytokine genes than is possible in transformed T-cell lines. Nevertheless, the T lymphoma EL4.23 cells have been useful in the dissection of the regulatory pathway.

Future work will need to define any other elements necessary for regulated expression and address in detail the cooperation between distal and proximal elements in the induction of transcription. Extension of these studies to the human IL-5 gene may provide opportunities for the development of drugs that inhibit induction of IL-5 and are useful in treating allergic disease. Definition of the normal regulation mechanisms also may allow investigation of possible abnormal IL-5 regulation in disorders involving eosinophilia, such as the hypereosinophilic syndrome.

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The image contains a page from a scientific research paper discussing transcriptional regulation, specifically focusing on the IL-5 gene. The page includes several references to other studies and research papers, mentioning various scientists and their contributions to the field. The text is dense with scientific terminology and methodologies, typical of a research paper in a scientific journal. The page seems to be from a volume dedicated to the IL-5 gene, indicating a focused study on this particular gene and its regulation. The text is formatted in a standard academic style, with proper citation of sources, indicating a thorough review of the existing literature on the topic.
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