Identification of Transcription Factor Binding Sites Important in the Regulation of the Human Interleukin-5 Gene*

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The current study identifies three regions of the human interleukin (IL)-5 promoter involved in binding nuclear factors from activated T cells: DNAse I footprinting and mobility shift assays with nuclear proteins from the human T cell clone, SP-B21, demonstrated protein interactions with each of these response elements (REs), located between positions −79 and −45 (RE-I), −123 and −92 (RE-II), and −170 and −130 (RE-III). Two of these regions, RE-II and RE-III, have not previously been described to regulate IL-5 expression in T cells. The RE-II site was shown to be critical for inducible IL-5 promoter activity in transient transfection assays in D10.G4.1 T cells, while the RE-III site functions as a negative regulatory element. The activity of the RE-II site was specifically inhibited by cyclosporin A, and transfection assays with IL-5 constructs containing mutations in the RE-II site showed greatly reduced reporter gene activities with IL-5 constructs containing mutations in the RE-II site. Antibodies against at least two members of the nuclear factor of activated T cells (NFAT) family of transcription factors are capable of binding to the IL-5 RE-II complexes, although they can be distinguished from previously identified NFAT-specific complexes by several characteristics.

Interleukin-5 (IL-5) is an important cytokine involved in controlling the growth (1), differentiation (2), and activation (3) of eosinophils. Eosinophils play an important role in the chronic inflammation and pathology of the bronchial mucosa seen in asthma (4). IL-5 has been strongly implicated as a major factor controlling the local infiltration and activation of eosinophils in this disease (5–7).

T lymphocyte clones can be classified into two major subtypes, Th1 and Th2, based on the cytokines produced by the cells in response to T cell receptor (TCR) stimulation (8). Th1 cells produce IL-2, interferon γ, and lymphotxin and promote delayed type hypersensitivity responses. Th2 cells produce IL-4, IL-5, and IL-10 and participate in allergic or anti-inflammatory responses as well as provide help for some B cell responses. Both types of cells are capable of producing IL-3 and GM-CSF. Helper T cell subsets producing cytokines typical of both Th1 and Th2 clones have also been described for human T cells (9, 10). A third class of T cell clones that can express various combinations of these cytokines has been designated Th0 cells (11).

The expression of IL-5 is tightly regulated and highly tissue-specific, being expressed primarily in the Th2 subtype of T cells (4), mast cells (12), and eosinophils (13). The expression of IL-5 in Th2 cells is dependent on activation of the cells. Activation of T cells requires the interaction of the TCR complex with antigen in the context of major histocompatibility complex antigens (14). This activation can be mimicked in vitro by activating T cells with bound anti-CD3 monoclonal antibody (Ab) (15) and is sufficient for efficient induction of IL-5 synthesis in Th2 cells (16). The appearance of IL-5 message in T cells is inhibited when the cells are activated in the presence of protein translation inhibitors, such as cycloheximide (CHX), indicating that nascent protein(s) synthesized in response to the activation signals delivered through the TCR are required for expression of IL-5 (16, 17). The requirement for de novo protein synthesis for IL-5 gene expression is not shared by other cytokines produced by the same subset of Th2 cells in response to TCR activation. This distinction suggests that more than one induction pathway may be responsible for the apparently coordinate expression of multiple cytokine genes in Th2 cells (16, 17).

Like most cytokine genes, the regulation of IL-5 message is primarily at the level of transcription and is likely to be controlled to a large extent by regulatory elements in the promoter region that can influence the transcriptional activity of the gene (18). Numerous studies have examined the sequences and associated transcription factors that regulate the expression of a number of cytokine genes produced by T cells, including IL-2 (19), IL-3 (20), IL-4 (21), and GM-CSF (22). In contrast, although the IL-5 gene has been cloned for several years (23), only limited information has been reported regarding specific regulatory sequences that function in the transcriptional control of human IL-5 gene expression (24–27). None of these studies investigated the transcriptional control of human IL-5 gene expression in untransformed T cells in which the production of IL-5 was induced by TCR-mediated activation signals.

In the current study, sequences located in the 5′-flanking region of the human IL-5 gene were examined for the ability to respond to T cell activation signals mediated through the TCR and to regulate transcription of this gene. DNAse I footprinting and mobility shift assays with nuclear protein extracts from a human IL-5-producing T cell clone demonstrate that the hu-
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Materials and Methods

T Cells—The human CD3+, CD4+, CD8+ T cell clone, SP-B21, was obtained from Dr. Hans Yssel (DNAX Research Institute, Palo Alto, CA) and has been described previously (35). This cell line expressed significant levels of IL-5 mRNA and protein after TCR-mediated stimulation, but no IL-5 message or protein was detectable in the absence of stimulation (data not shown). The expression of IL-5 mRNA in these cells was inhibited when the cells were stimulated in the presence of the protein synthesis inhibitor, cycloheximide (data not shown), as has been previously reported for other T cell clones, such as the murine Th2 clone, D10.G4.1 (16, 17, 29). SP-B21 cells were maintained in culture by bimonthly stimulation with a feeder cell mixture consisting of irradiated (4000 rads) allogeneic peripheral blood mononuclear cells, an irradiated (5000 rads) Epstein-Barr virus-transformed B cell clone (JY), and phytohemagglutinin (0.1 mg/ml; Murex, Norcross, GA) in Yscel’s medium supplemented with heat-inactivated 1% human AB+ serum (Gemini Bioproducts, Calabasas, CA) as described (30). Three days after each restimulation, the cells were expanded in medium containing 20 units/ml recombinant human IL-2 (Biosource, Camarillo, CA). The medium was replaced with fresh medium containing IL-2 every 3 days until the cells were restimulated at the end of the 14-day period. The cells used for experiments 7–9 days after the last restimulation with feeder cells. For preparation of activated nuclear protein extracts, the cells were stimulated with anti-CD3 mAb (clone UCHT1, Pharmingen, San Diego, CA)-coated plates (coated at 1 mg/ml in 1 × phosphate-buffered saline, pH 7.4, overnight at 4 °C) and then washed with 1 × phosphate-buffered saline. Where indicated, cells were pretreated for 10 min with 10 μg/ml CHX (Sigma) or 2 μg/ml CsA (Sandoz Corp., East Hanover, NJ) prior to antibody stimulation. Antibody stimulation during this period of the feeder cell stimulation cycle is sufficient to induce significant levels of IL-5 message detectable by Northern blots and L-5 protein detectable by enzyme-linked immunosorbent assay (31).

The murine CD3+, CD4+, CD8+ Th2 T cell clone D10.G4.1 (ATCC, Rockville, MD) is specific for the conalbumin antigen, and it is H-2 I-A*-restricted. This T cell line is a model system widely studied for the regulation of cytokine gene expression (16, 17, 29). D10.G4.1 cells produced significant levels of IL-5 message and protein after TCR-mediated stimulation, while no IL-5 message or protein was detectable in the absence of stimulation, or when the activation of the cells occurred in the absence of protein translation (16, 17, 29). D10.G4.1 cells were maintained in Click’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES, 2 mM t-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. The cells were stimulated bimonthly with 100 μg/ml conalbumin antigen (Sigma) and irradiated (3000 rads) syngeneic AKRJ (Jackson Laboratories, Bar Harbor, ME) spleen cells. Concanavalin A-stimulated rat growth factor supernatant treated with α-methylmannoside (Collaborative Research Inc., Bedford, MA) was added at a concentration of 5% (w/v) during the expansion of the cells. The cells were used for electrophoresis experiments on day 8 of this 14-day antigen stimulation cycle. Where indicated, the cells were treated with 2 μg/ml CsA (Sandoz Corp.) 10 min prior to antibody stimulation following transfection.

Cloning the 5′-Flanking Region of the Human IL-5 Gene—A P1 clone containing an insert of approximately 100 kb, which included the human IL-5 gene, was identified in a human pdAd10SacBI library (32) constructed from the human lymphoblastoid cell line GM14165 (Human Genetic Cell Repository, Camden, NJ) by hybridization screening of the library by standard protocols (33). A 0.5-kb double-stranded DNA fragment containing the region between positions −505 and +42 of the human IL-5 gene was used as a probe for screening. A 3.26-kb PvUI fragment containing the human IL-5 gene was cloned from this P1 clone into the Pscl site of pGEM3Zf(+) (Promega Corp., Madison, WI). A 1.5-kb PstI-PspI1406I fragment was excised from the pGEM subclone and the overhanging ends were made blunt with the Klenow fragment of DNA polymerase. This fragment was inserted into the SmaI site of the luciferase expression vector, pGL2basic, in the 5′ to 3′ orientation to create the reporter construct HuIL5−(−1547)luc. The insert was sequenced by standard dye terminated sequencing methods (34) and was verified to be 5′-flanking to the human IL-5 gene to position −1547 upstream of the previously identified transcription start site (see Fig. 4). The 5′-flanking region sequence of the human IL-5 gene reported here is contained within the unpublished sequence deposited in GenBank™ (accession number L48477) from subclone 6-b10 from P1 H215.

IL-5 Reporter Gene Construct Cloning—A series of eight additional reporter gene constructs containing varying lengths of the 5′-flanking region of the human IL-5 gene (see Fig. 5A) were cloned in the luciferase reporter vector pGL2basic (Promega). DNA fragments extending from position −505, −384, −316, −236, −172, −127, −80, or −38, and position +42 relative to the start site of transcription (23) were generated by PCR with human PBL genomic DNA (33) as the template and a combination of one of the BglII-tailed oligonucleotides as the 5′-primer and the HindIII-tailed oligonucleotide as the 3′-primer. The sequences of these oligonucleotides are shown in Table I. The oligonucleotides were synthesized by standard β-cyanoethyl phosphoramidite chemistry (Life Technologies, Inc.). The resulting PCR fragments were digested with BglII and HindIII and cloned into the multiple cloning site of the pGL2basic reporter plasmid (Promega) up to 3′ orientation. The 5′-position at which each reporter construct was truncated is indicated by the number in the name of each construct (see Fig. 5A) and is relative to the transcription start site in the IL-5 gene. All constructions were verified by DNA sequencing (33). All constructs contain the IL-5 transcription initiation site (+1) but not the IL-5 translation initiation codon to ensure that translation of the luciferase reporter gene will initiate at the AUG of the luciferase mRNA.

Mutagenesis—Two synthetic oligonucleotides were designed to mutate the IL-5 RE-II region of the human IL-5 promoter. The SDM1 oligonucleotide (Table I) was synthesized by standard β-cyanoethyl phosphoramidite chemistry (Oligos, Etc., Wilsonville, OR) and contained altered sequence in the region corresponding to IL-5 RE-II as indicated. The altered sequence was randomly chosen and confirmed to contain no consensus sites for previously characterized DNA-binding proteins by searching against the Transcription Factor Database TFDSDITES;SUBSEQ.7.0.aa (MacVector, version 5.0, Oxford Molecular Group, Inc., Campbell, CA). This oligonucleotide was used to generate the mutant construct (designated mutant (−1547)luc) by site-directed mutagenesis with the Transformer site-directed mutagenesis kit (CLONTECH). For selection of mutant plasmids, the Scal/Stul selection primer (CLONTECH) was used to eliminate the unique Scal site in the pGL2basic vector portion of the wild-type HuIL5−(−1547)luc construct template. The AS1 oligonucleotide (Table I) was designed to generate a mutant plasmid (mutant (−127)luc) in the context of the HuIL5−(−127)luc construct. This oligonucleotide was used as the 5′-primer in a PCR reaction according to standard techniques (34) with the template HuIL5−(−127)luc and a 3′-primer (Table I) as the 3′-primer. The PCR product was subcloned into the BglII and HindIII sites of the pGL2basic vector. The mutated sequence in both mutant constructs was verified by DNA sequencing.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from unstimulated SP-B21 cells, cells stimulated by plate-bound anti-CD3 mAb in the absence or presence of 10 μg/ml CHX, or 2 μg/ml CsA by a modification of the procedure described by Dignam et al. (35). The modifications were as follows: phenylmethylsulfonyl fluoride was omitted from all of the buffers and was replaced by the protease inhibitor, Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) (Boehringer Mannheim) in the concentrations indicated below. The total volume of Buffer C used to resuspend the nuclear pellet did not exceed the volume of the nuclear pellet. The nuclear extracts were dialyzed for a maximum period of 1 h at 4 °C. The modified Dignam buffers had the following compositions: Buffer A, 10 mM HEPES (pH 7.9 at 4 °C), 1.5 mM MgCl2, 10 mM KCl, 0.2 mM Pefabloc, and 0.5 mM dithiothreitol; Buffer C, 20 mM HEPES (pH 7.9); 25% (w/v) glycerol, 1.2 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM Pefabloc, and 0.5 mM dithiothreitol; low salt Buffer C, the same as Buffer C except for a reduction in the KCl concentration to 0.02 M; Buffer D, 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 0.1 mM EDTA, 0.2 mM PEFabloc, and 0.5 mM dithiothreitol. The protein content of the nuclear extracts was determined by the method of Bradford (36) with bovine serum albumin as a standard.

DNase I Protection Assays—DNase I footprinting was performed as described by Galas and Schmitz (37). The double-stranded DNA tem-
plate was created by sequential digestion of the HuIL5(-384)lac construct with AccI and HindIII. The DNA was dephosphorylated between the two digestions. The resulting 347-bp template was therefore dephosphorylated on the 5’-end of either the coding or the noncoding strand, depending on which restriction enzyme was used first. The digestion of HuIL5 was separated by electrophoresis in 10% TBE-Tris acetate, 1 mM EDTA gel, and the template band was excised and purified using the Wizard DNA purification system (Promega). The DNA template was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Unincorporated nucleotide was removed from the sample by centrifugation through a Microcon 100 filter (Amicon, Inc., Keasbey, NJ).

The 50-μl digests for the footprinting experiments contained nuclear protein amounts as indicated in Figs. 1 and 3 in a reaction mixture that consisted of 8% (v/v) glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 2.5 μg of poly(dI-dC), and 50,000 cpm of 32P-labeled template. The reactions were incubated for 20 min at room temperature before digestion with 0.15 units of DNase I (Promega) for 60–120 s in the presence of Ca²⁺/Mg²⁺. The mixture was terminated by the addition of an equal volume of a stop solution containing 200 mM NaCl, 30 mM EDTA, 1% SDS, and 50 μg/ml tRNA. Following organic extraction and precipitation in the presence of ethanol, the reactions were resuspended in 4 μl of a loading dye solution, loaded onto a prewarmed 6% acrylamide-urea sequencing gel, and electrophoresed for 4 h at 75 watts. The gels were dried and exposed to x-ray film for 1–3 days at room temperature.

Cycle sequencing reactions for the coding and noncoding strands were used to orient the DNase I footprint regions within the template sequence. All cycle sequencing reactions were carried out with the dNTPase Cycle Sequencing System (Life Technologies). The template used was the same as that used for the DNase I footprinting reactions. Twenty-bp oligonucleotides complementary to the 5’-end of each template strand were synthesized with phosphoramidite chemistry according to standard protocols and used as primers in the cycle sequencing reactions.

**Transfection Assays—**D10.G4.1 T cell clones were transfected using electroporation-mediated DNA transfer with the Gene Pulser (Bio-Rad), and the luciferase activity in each sample (expressed in relative light units (RLU)) was assayed. Briefly, 100 μl of Click’s medium supplemented with 10 μM HEPES, 2 mM t-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 0.4-cm gap cuvette and electroporated at 320 V, 960 microfarads. Following transfection, the cells were transferred into 6 ml of supplemented Click’s medium and cultured at 37°C in 5% CO₂. After overnight culture, the cells were split equally between two 25-cm dishes. One dish of cells from each group was stimulated with 1 μg/ml plate-bound anti-CD3 mAb (clone 145–2c11) (Pharmingen, San Diego, CA), with or without a 10-min pretreatment with 2 μg/ml monoclonal IgG1 mouse anti-c-Jun (KM-1) (Santa Cruz Biotechnology, Inc.), monoclonal IgG mouse anti-NFATc1 (Affinity Bioreagents, Inc., Golden, CO), monoclonal IgG mouse anti-c-Jun (KM-1) (Santa Cruz Biotechnology), and polyclonal IgG rabbit anti-Pu.1 (Spi-1) (T-21) (Santa Cruz Biotechnology). All Abs are commercially available and have been reported to cross species and specifically react with the appropriate human proteins.

**RESULTS**

The Proximal IL-5 Promoter Contains Three DNA-binding Regions—To study the sequence-specific interactions of nuclear proteins with the human IL-5 gene promoter, we used a 347-bp DNA fragment (containing the IL-5 gene sequence between positions −308 and +39) as a template in DNase I protection assays with nuclear proteins isolated from the human Th0 cell line, SP-B21, following TCR-mediated stimulation with bound anti-CD3 mAb. Both the coding (lanes 1–5) and noncoding (lanes 6–10) strands of the human IL-5 promoter were subjected to partial DNase I digestion in the absence or presence of nuclear extracts from activated human SP-B21 T cells (Fig. 1A). Three distinct footprints on each strand designated as IL-5 response elements (RE-I, -II, and -III) were present. Sites hypersensitive to digestion were evident at the boundaries of both IL-5 RE-I and IL-5 RE-II on the coding strand and at the boundary of IL-5 RE-III on the noncoding strand, and these sites are indicated by asterisks. The appearance of at least two of these protected regions on the DNA template was dependent on the concentration of nuclear protein in each DNase I digestion and became more visible in the presence of increasing amounts of protein (compare lane 2 with lane 4 and lane 7 with lane 9). The specific bases protected on each strand were identified by alignment with cycle sequencing reactions run in parallel (data not shown) and are indicated by the sequence changes within the brackets in Fig. 1B. The alignment of protected bases on opposite strands varied slightly for each footprint region and probably reflects the three-dimensional conformation of the double-stranded DNA helix bound by the protein complex. In addition to the optimal protein concentrations for each reaction shown in Fig. 1A, footprinting reactions were performed with as little as 2 μg and as much as 100 μg of SP-B21 nuclear filters (Amicon, Inc.). Following centrifugation, the filters were rinsed with 10 mM Tris-Cl, pH 7.6, 1 mM EDTA, and the retentate was collected.

The 10-μl EMSA binding reactions contained 5 μg of total nuclear protein in 6% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, and 3 μg of poly(dI-dC) reaction (Boehringer Mannheim). Reactions were incubated with 50,000–100,000 cpm of 32P-labeled duplex oligonucleotide for 20 min at room temperature. Protein-DNA binding specificity was tested by competition assays in which the binding reactions were preincubated for 10 min at room temperature with excess unlabeled specific or nonspecific competitor duplex oligonucleotide prior to the addition of the labeled probe. In addition to the oligonucleotides defined in Table II, the following oligonucleotides were used as competitors in binding reactions: human IL-2 NFAT-1, AAGAGAGGAGAAAAACGTGGTATACAGAGGCCGT (38) (synthesized as described above); AP-1 consensus, CGCTTGATGATCACCCGGA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Following binding, the protein-DNA complexes were resolved by electrophoresis in non-denaturing 6% acrylamide gels at 200 V for 1.5 h at room temperature in 0.5× TBE buffer. Gels were dried prior to autoradiography.

For supershift experiments, 5 μg of nuclear extracts were preincubated with 1–3 h at 4°C with 0.5–3.0 μl of commercial antibody preparations that were specific for eukaryotic transcription factors. The volumes of each Ab preparation used in the reactions was based on the literature supplied by each Ab manufacturer. Following this incubation period, radiolabeled probe was added, the binding reactions were allowed to continue for 20 min at 4°C, and the reactions were separated electrophoretically as described above. The antibodies used were as follows: monoclonal IgG rabbit anti-NF-Y(A) (Rackland, Gilbertsville, PA), monoclonal IgG rabbit anti-NF-Y(B) (Rackland), ascs fluid containing monoclonal IgG mouse anti-NFAT-1 (Affinity Bioreagents, Inc., Golden, CO), monoclonal IgG mouse anti-c-Jun (KM-1) (Santa Cruz Biotechnology), and polyclonal IgG rabbit anti-Pu.1 (Spi-1) (T-21) (Santa Cruz Biotechnology). All Abs are commercially available and have been reported to cross species and specifically react with the appropriate human proteins.
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Fig. 1. Three regions in the human IL-5 proximal promoter are protected in DNase I footprinting assays. A, three 5' regions of the IL-5 promoter (IL-5 RE-I, positions -79 to -45; IL-5 RE-II, positions -123 to -92; and IL-5 RE-III, positions -170 to -130) are protected from digestion by DNase I in the presence of nuclear extracts from anti-CD3-stimulated human SP-B21 T cells prepared as described under "Materials and Methods." The template had been end-labeled either at the -308 terminus (coding strand, lanes 1-5) or the +39 terminus (noncoding strand, lanes 6-10). The positions of the protected regions are indicated at the sides of the gels. Two of the protected regions, IL-5 RE-II and IL-5 RE-III, contained sites at their boundaries that were hypersensitive to DNase I digestion. These sites are indicated by the asterisks. Lanes 1 and 5, coding strand template digested in the absence of nuclear proteins; lanes 2-4, coding strand template digested in the presence of 10, 30, and 60 μg of nuclear protein, respectively; lanes 6 and 10, noncoding strand template digested in the absence of nuclear protein; lanes 7-9, noncoding strand template digested in the presence of 2, 10, and 20 μg of nuclear protein, respectively. The dG, dA, dT, and dC cycle sequencing reactions used to align the protected regions with the template sequence are not shown. B, the DNA sequence of the three footprinted regions (IL-5 RE-I, IL-5 RE-II, and IL-5 RE-III) in the IL-5 promoter. The double-stranded DNA sequence contained within each of the three footprints is shown in the 5' to 3' orientation. The locations of each region within the promoter are indicated by the numbers and are relative to the cap site at position +1. The actual bases on each strand that are protected from digestion are contained within the brackets.

extract to optimize the reaction conditions required to achieve 50% digestion of the template (data not shown). When less than 50% digestion of the template was observed (e.g. lane 9 in Fig. 1A), the boundaries of the region considered to be footprinted were adjusted appropriately.

The first of these protected sites, designated IL-5 RE-I and located between positions -79 and -45 bp upstream of the IL-5 cap site, includes the conserved lymphokine element 0 (CLE0) sequence (Fig. 2) previously shown to be conserved among the IL-5, IL-3, and GM-CSF genes, and shown to be involved in the regulation of transcription of these genes (39). In addition, this region contains homology to binding sites, namely AP-1 (40), NF-IL6 (41), NF-GMb (42), GM-CSF CATT (43), and GATA-1 (44) (Fig. 2), previously found within the promoter regions of several cytokine genes.

The second protected footprint in the IL-5 promoter, IL-5 RE-II, located between positions -123 and -92, occurs in a region of the human IL-5 promoter that has not previously been shown to play a role in transcriptional regulation of this gene in T cells. The sequence of this protected region shows some homology to four previously characterized protein binding motifs, a 5-bp GM-CSF CATT motif (43), a partial NF-AT-like motif (45), a partial NF-Y consensus sequence (46), and a partial AP-1-like motif (19) (Fig. 2). The NFAT family of proteins have been reported to function in the IL-2 promoter through cooperative interactions with proteins bound to downstream AP-1 sites (45).

The third footprinted region in the IL-5 promoter template, IL-5 RE-III, is located between positions -170 and -130 and has homology to the 6-bp GATA-1 sequence (44) (Fig. 2).

To investigate whether the nuclear proteins that bind to these three promoter regions in SP-B21 cells are specifically induced and/or newly synthesized in response to T cell activation signals, nuclear extracts were prepared from unstimulated cells and from cells stimulated with anti-CD3 in the presence of CHX to inhibit nascent protein synthesis. The results of DNase I protection assays with the coding strand template and nuclear extracts from three different T cell treatments are shown in Fig. 3. The three protected regions, IL-5 RE-I, -II, and -III, were evident with extracts from all three treatment groups compared with template digested in the absence of nuclear protein, although the relative affinities of these protected regions varied between the treatment groups. These differences
**Fig. 3.** Protein binding to footprinted IL-5 promoter regions is altered in the absence of TCR stimulation and nascent protein synthesis. Nuclear extracts were prepared from SP-B21 T cells that were unstimulated, stimulated by anti-CD3 Ab, or stimulated by anti-CD3 Ab in the presence of CHX as detailed under “Materials and Methods.” The regions that are protected from digestion in the presence of the nuclear extracts, IL-5 RE-I, -II, and -III, are indicated by the brackets. The asterisks indicate the sites that are hypersensitive to DNase I digestion. Lanes 1 and 11, coding strand digested in the absence of nuclear proteins; lanes 2–4, coding strand digested in the presence of 10, 30, and 60 μg of nuclear protein, respectively, from unstimulated SP-B21 cells; lanes 5–7, coding strand digested in the presence of 10, 30, and 60 μg of nuclear protein, respectively, from anti-CD3-stimulated SP-B21 cells; lanes 8–10, coding strand digested in the presence of 10, 30, and 60 μg of nuclear protein, respectively, from SP-B21 cells stimulated with anti-CD3 Ab in the presence of 10 μg/ml CHX. The ddG, ddA, ddT, and ddC cycle sequencing reactions used to align the protected regions with the template sequence are not shown.

were consistently noted in multiple experiments. Specifically, the IL-5 RE-I footprint was readily detected with 60 μg of anti-CD3-stimulated SP-B21 cell nuclear extracts, but the protection of this region by equal concentrations of nuclear extracts from either unstimulated cells or cells stimulated in the presence of CHX was significantly reduced (compare lane 7 with lanes 4 and 10). Likewise, the IL-5 RE-II footprint was more pronounced with 60 μg of nuclear extracts from stimulated cells (lane 7) than it was with equal concentrations of protein extracts from the other two treatment groups (lanes 4 and 10). In contrast, the IL-5 RE-III region was footprinted only weakly by nuclear extracts from all three treatment groups and did not change significantly with additional protein. The weaker protection in this region made it more difficult to determine the boundaries of the footprint here, and it may actually include as many as three distinct regions, as indicated by the breaks in the bracket surrounding region III. Despite the slightly weaker footprint, this region was consistently protected from DNase I digestion in multiple experiments using both strands as templates and was therefore designated as a protein binding region. Thus, all three of these regions within the IL-5 promoter were able to bind nuclear proteins from a human T cell clone, and the components of least two of these protein-DNA complexes were affected by changes in protein composition or concentration in response to T cell activation signals.

**Functional Analysis of the Human IL-5 Promoter**—To examine the role 5′ regulatory sequences may play in controlling inducible IL-5 gene transcription, the activity of a series of IL-5 promoter-luciferase reporter gene constructs was assessed in transient transfection assays in D10.G4.1 cells. This nontransformed T cell clone has previously been shown to support the expression of a number of cytokine promoter reporter gene constructs, including IL-5 promoter constructs (16, 17, 29, 47, 48). The expression of IL-5 message and protein in this mouse T cell line is dependent on stimulation of the cells and de novo protein synthesis (16, 17, 29, 48) and parallels the pattern of expression of IL-5 in human SP-B21 cells.

Varying lengths of the 5′-flanking region between positions −1547 and −38 were inserted upstream of the luciferase gene in the pGL2basic plasmid vector. The full sequence of the human IL-5 promoter is shown in Fig. 4 and includes the 5′-position of each of the deletion constructs. Four of the constructs, (truncated at positions −172, −127, −80, and −38) were specifically designed to assess the relative contribution of each of the elements identified as protein-binding regions in the DNase I footprinting experiments by sequentially deleting the IL-5 RE-III, IL-5 RE-II, and IL-5 RE-I elements, respectively (Fig. 5A). All of the reporter gene constructs were transfected into D10.G4.1 cells, and the luciferase activity that resulted from each construct was determined with or without anti-CD3 stimulation of the transfected cells. The promoter activity of these constructs relative to the HuIL5(−1547)luc construct and the relative inducibility of each construct following anti-CD3 stimulation of the transfected cells compared with unstimulated transfected cells with the same construct are shown in Fig. 5, B and C, respectively.

Significant luciferase activity was detected in cells transfected with the HuIL5(−1547)luc construct and stimulated with anti-CD3 antibody. The level of induction of this construct in anti-CD3-stimulated cells compared with the basal level of expression in unstimulated transfected cells was 4.5-fold for these experiments. The removal of 5′-flanking sequences from the upstream position at −1547 to position −505 decreased the luciferase activity to approximately one-half of that seen with the longest construct. However, the ratio of induction of stim-
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**Fig. 5.** Transient transfection assay to measure IL-5 promoter activity in Th2 cells. A, the human IL-5 gene corresponding to positions -1547 to +34 relative to the transcription initiation site (+1) was cloned from a P1 library upstream of the luciferase reporter gene in plasmid pGL2basic in the 5' to 3' orientation. A series of reporter constructs in which the 5'-end of the IL-5 sequence was deleted as shown between positions -1547 and +34 while maintaining the same 3'-end position at +34 were cloned as described under "Materials and Methods." The relative positions of the DNA-binding regions, IL-5 RE-I, RE-II, and RE-III, are indicated by the shaded rectangles. B, each IL-5 luciferase reporter gene construct was transiently transfected into D10.G4.1 cells by electroporation and cultured with or without anti-CD3 Ab stimulation as described under "Materials and Methods." The luciferase reporter gene activity was measured in the cell lysates as described. The promoterless vector, pGL2basic, was used as a negative control (data not shown). The results for each construct are presented as the mean ± S.D. value < 0.6 is below the illustrative scale. Typical activity levels produced in stimulated cells transfected with the full-length HuIL5(-1547)luc construct were 20,000–50,000 RLU. C, the inducibility of each transfected construct is indicated as the -fold induction of each construct in anti-CD3-stimulated cells compared with unstimulated cells transfected with the same construct in the same experiment as described under "Materials and Methods." The results for each construct represent the mean ± S.D. -fold induction of 3–8 independent transfection experiments with each construct. S.D. value < 0.9 is below the illustrative scale.

ululated cells compared with unstimulated cells with the shorter construct remained the same. The decreased luciferase activity suggests that positive regulatory elements necessary for full activity may be present in the distal region of the IL-5 promoter. Further removal of the sequences between positions -505 and -172 did not significantly change the relative level of activity of the constructs or their inducibility. However, truncation of the sequences between positions -172 and -127 significantly increased the induced levels of expression. This increase in activity with the removal of 5’ sequences is attributed to a putative transcriptional silencer element between positions -172 and -127.

The IL-5 promoter construct deleted to position -127 expressed the highest absolute levels of luciferase activity (106% relative to the -1547 construct) and the most inducible activity following anti-CD3 activation of the transfected cells (15-fold). This level of activity and inducibility suggests that a regulatory element critical for IL-5 promoter activity is located within this region. Truncation of the promoter to -80 bp removed this regulatory region and decreased the overall expression of the luciferase reporter gene to a level similar to that seen with the intermediate length constructs. However, removal of the sequences between -127 and -80 bp completely abolished the inducibility of the promoter despite the presence of the entire CLE0 element in the -80 construct. This loss of inducibility resulted from an increase in basal activity in the unstimulated transfected cells (data not shown) rather than an increase in the levels of activity of the stimulated cells transfected with this construct (Fig. 5B). Thus, the CLE0 element alone can drive a moderate level of IL-5 promoter activity, but this activity is constitutive rather than inducible in response to T cell activation. Further truncation of the promoter between positions -80 and -38 resulted in the complete loss of activity of the promoter, indicating that the presence of a TATA box alone is not sufficient for IL-5 promoter expression. Taken together, these results suggest that the additional regulatory element, IL-5 RE-II, located between positions -127 and -80 is not only necessary for full expression of the IL-5 promoter, but it is also involved in the induction of activity in response to TCR-mediated signals.

**Mutation of the IL-5 RE-II Element Significantly Reduces IL-5 Promoter Activity**—To assess the function of the IL-5 RE-II region in the regulation of inducible IL-5 promoter activity, the activity of two mutant reporter constructs was measured in transient transfection experiments in D10.G4.1 cells. The IL-5 RE-II sequence was partially or completely altered to a randomly mutated sequence in the HuIL5(-1547)luc and HuIL5(-1547)luc constructs and compared with the corresponding parental constructs that exhibited high levels of promoter activity in transfected cells (Fig. 5). When the entire IL-5 RE-II region was mutated (SDM1) (Table I) in a construct that included the full-length IL-5 promoter to position -1547 (mutant (-1547)luc), the activity of the promoter was reduced to 44% of the activity of the parental construct (Fig. 6). When only a central portion of the IL-5 RE-II element was mutated (AS1) (Table I) in the context of the shorter HuIL5(-127)luc construct (mutant (-127)luc), the reduction in activity was significantly greater. In this mutated construct, the activity of the
transfected construct in stimulated cells was reduced to only 12% of the parental HuIL5\(^{-127}\)luc construct (Fig. 6). The mutated sequences used to replace the IL-5 RE-II region in these constructs were not able to bind specific protein complexes when used as probes in gel shift experiments with nuclear extracts from unstimulated or anti-CD3-stimulated SP-B21 cells (data not shown). In addition, neither of the mutant sequences were able to compete for protein binding to the IL-5 RE-II region defined by the H-117(20) probe, as well as several additional complexes that migrated more quickly (lane 3, 11), while cells stimulated in the presence of CsA produced only the faster, constitutive complex (I) (lane 15). The specificity of complexes I and II was demonstrated by the inhibition of these complexes in competition experiments using the labeled H-117(20) probe and excess unlabeled H-117(20) oligonucleotide in binding reactions with nuclear extracts from each of the four cell treatment groups (lanes 4, 8, 12, and 16). In addition to these specific complexes, the H-117(20) probe retarded a nonspecific complex (marked by the asterisk) that was not competed by unlabeled H-117(20) oligonucleotide in any of the competitive binding reactions.

The overlapping H-128(30) probe included 19 of the 20 nucleotides in the H-117(20) oligonucleotide, as well as 11 additional nucleotides at the 5'-end of the IL-5 RE-II region (Table II). The gel shift reactions with this probe and each of the four cell treatments produced specific gel shift bands identical in relative mobility to either complex I or II seen with the smaller H-117(20) probe, as well as several additional complexes that migrated more quickly (lanes 1, 5, 9, and 13). These additional complexes were determined to be nonspecific based on their inability to be competed in the presence of excess unlabeled H-128(30) or H-117(20) oligonucleotides (data not shown).

The inclusion of additional nucleotides 5' to the 20-bp region defined by the H-117(20) probe does not result in additional specific protein complexes, although an increase in non-

from unstimulated SP-B21 cells and cells that were stimulated with anti-CD3 mAb to look for changes in the pattern of RE-II binding complexes that result following activation of the T cells through the TCR. In addition, extracts were prepared from SP-B21 cells that were stimulated with anti-CD3 mAb after pretreatment with either CHX or CsA to look for changes in the bound complexes that result when the cells were activated in the absence of de novo protein translation (16, 17, 29) or calcineurin activity (49), respectively.

An EMSA with three of these oligonucleotides labeled as probes, H-128(30), H-127(20), and H-117(20), and one unlaabeled oligonucleotide as competitor, H-117(20), is shown in Fig. 7. The sequences of the oligonucleotides used as probes or competitor in the EMSAs and their relative positions within the IL-5 RE-II region of the promoter are shown in Table II. The H-117(20) oligonucleotide probe formed at least one prominent retarded band with each of the different nuclear extracts (lanes 3, 7, 11, and 15), although the relative mobility of the retarded complexes differed between treatment groups. In unstimulated SP-B21 cells, a constitutive complex (labeled as I in Fig. 7) was observed (lane 3), while a slightly slower migrating complex (labeled as II in Fig. 7) was evident in the nuclear extracts from T cells that had been stimulated by anti-CD3 mAb (lane 7). In cells that were stimulated in the presence of the protein synthesis inhibitor, CHX, both the constitutive and the activation-dependent complexes (I and II) were seen (lane 11), while cells stimulated in the presence of CsA produced only the faster, constitutive complex (I) (lane 15). The specificity of complexes I and II was demonstrated by the inhibition of these complexes in competition experiments using the labeled H-117(20) probe and excess unlabeled H-117(20) oligonucleotide in binding reactions with nuclear extracts from each of the four cell treatment groups (lanes 4, 8, 12, and 16). In addition to these specific complexes, the H-117(20) probe retarded a nonspecific complex (marked by the asterisk) that was not competed by unlabeled H-117(20) oligonucleotide in any of the competitive binding reactions.

**Nuclear Factors from Human SP-B21 T Cells Form Specific DNA-Protein Complexes with the IL-5 RE-II Region of the IL-5 Promoter**—To examine the transcription factors that associate with the IL-5 RE-II region of the human IL-5 gene promoter, EMSAs were performed with \(^{32}P\)-labeled oligonucleotides and nuclear extracts prepared from SP-B21 human T cells following one of four different cell treatments. Extracts were prepared
specific protein complex formation does occur with the longer probe.

To assess whether the 5'-end of the 20-bp sequence of the H-117(20) oligonucleotide without the 3'-end sequence was sufficient for specific protein binding, binding reactions were done with another related oligonucleotide probe, H-127(20). This oligonucleotide overlaps the 5'-end of the H-117(20) probe sequence by 10 bp but is lacking the 3'-end of the IL-5 RE-II region. Unlike the H-117(20) probe, this overlapping probe does not form a specific protein complex with any of the four different nuclear extracts (lanes 2, 6, 10, and 14), indicating that the 10 bp at the 3'-end of the H-117(20) sequence are required for specific complex binding. However, the same nonspecific band (indicated with the asterisk) seen previously was evident with the overlapping probe.

In an attempt to further define the sequences within the 20-bp core of the IL-5 RE-II element that are critical for complex II formation in anti-CD3-stimulated cell extracts, multiple overlapping oligonucleotides were used as competitors in the binding reactions with labeled H-117(20) probe (Fig. 8). The sequences of these oligonucleotides and their relative positions within the IL-5 RE-II element are shown in Table II. The protein-DNA complexes were resolved by PAGE on a nondenaturing 6% acrylamide gel. The specific retarded complexes, I and II, are indicated by the arrowheads. A nonspecific band that was not competed by any unlabeled oligonucleotide included in excess in the binding reactions (lanes 4, 8, 12, and 16 and data not shown) is indicated by the asterisk. The free probe is shown at the bottom of each lane.

**TABLE II**
Sequences of oligonucleotides synthesized for gel shift probes and competitors

The human IL-5 gene sequence between positions –128 and –88 relative to the transcription start site and its alignment with each probe sequence is shown for comparison. The minimal IL-5 RE-II sequence within the protein-binding region identified in the footprinting experiments is in boldface type, and the NFAT-like motif is underlined. The oligonucleotide sequences are shown in the 5' to 3' orientation. The complementary oligonucleotide for each sequence is not shown. Not included in each sequence is the 5'-GGG overhanging end included for radiolabeling the duplex oligonucleotides used as probes in EMSAs.

| Probe name | IL-5 gene: | Probe name | IL-5 gene: |
|------------|------------|------------|------------|
| H-128(30)  | AGATATAAGGCATTGGAAACATTTAGTTTCA  | H-128(30)  | AGATATAAGGCATTGGAAACATTTAGTTTCA  |
| H-127(20)  | GATATAAGGCATTGGAAACA  | H-127(20)  | GATATAAGGCATTGGAAACA  |
| H-117(20)  | ATTGGAAACATTTAGTTTCA  | H-117(20)  | ATTGGAAACATTTAGTTTCA  |
| H-107(20)  | CGATATGCCAT  | H-107(20)  | CGATATGCCAT  |
| H-122(12)  | AAGGCATTGGAA  | H-122(12)  | AAGGCATTGGAA  |
| H-120(12)  | GGCATTGGAAAC  | H-120(12)  | GGCATTGGAAAC  |
| H-118(12)  | CATTGGAAACAT  | H-118(12)  | CATTGGAAACAT  |
| H-116(12)  | TTGGAAACATTT  | H-116(12)  | TTGGAAACATTT  |
| H-114(12)  | GGAAACATTTAG  | H-114(12)  | GGAAACATTTAG  |
| H-112(12)  | AACATTTAG  | H-112(12)  | AACATTTAG  |

In an attempt to further define the sequences within the 20-bp core of the IL-5 RE-II element that are critical for complex II formation in anti-CD3-stimulated cell extracts, multiple overlapping oligonucleotides were used as competitors in the binding reactions with labeled H-117(20) probe (Fig. 8). The sequences of these competitors and their relative positions within the IL-5 RE-II region are shown in Table II. The protein-DNA complexes were resolved by PAGE on a nondenaturing 6% acrylamide gel. The specific retarded complexes, I and II, are indicated by the arrowheads. A nonspecific band that was not competed by any unlabeled oligonucleotide included in excess in the binding reactions (lanes 4, 8, 12, and 16 and data not shown) is indicated by the asterisk. The free probe is shown at the bottom of each lane.
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Proteins That React with NF-AT Abs Associate with the IL-5 RE-II Binding Site—The IL-5 RE-II region shares partial homology with binding sites that have been previously determined to be specific for the transcription factors, NFAT-1, NF-Y, and AP-1, as well as a CATTT motif described in the GM-CSF promoter (Fig. 2). Based on the gel shift experiments with overlapping probes and competitors described above, the sequences that are similar to these previously described binding sites are contained within the 20-bp core region critical for protein association (IL-5 RE-II) and the reporter construct responsible for the highest level of inducible promoter function (construct HuIL5(−127/lu)).

To examine the potential involvement of NFAT-1, NF-Y, and AP-1-like proteins in the IL-5 RE-II gel shift complex, Abs specific for the NFATc1, NF-Y(A), NF-Y(B), and c-Jun transcription factors were tested for the ability to recognize specific proteins from anti-CD3-stimulated SP-B21 cells bound to IL-5 RE-II in EMSAs. An Ab specific for the PU.1 transcription factor, a member of the ets gene family (50), was also used in the supershift experiment as an antibody control. As shown previously, the H-117(20) probe retarded the protein complex defined as complex II in nuclear extracts from stimulated SP-B21 cells (Fig. 9, lane 1), and the formation of this complex was specifically inhibited in the presence of excess unlabeled H-117(20) oligonucleotide (lane 2). In slight contrast to the gel shift pattern with this probe and anti-CD3-stimulated SP-B21 nuclear extracts shown in lane 7 of Fig. 7, a lower complex similar in mobility to complex I in unstimulated cell extracts (Fig. 7, lane 3) is also evident in this gel shift reaction. The appearance of complex II in these cell extracts has been shown...
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(Fig. 7) to depend on a stimulation-related change in the cells. Unstimulated cell extracts produce only the lower complex I with the same gel shift probe. However, differences in the efficiency of this type of cell stimulation regimen due to subtle differences in the viability or cell cycle characteristics of the cell preparations used may lead to incomplete stimulation of the cells. For this reason, the presence of the lower complex I in stimulated cell extracts is inconsistent and can be attributed to incomplete or inefficient stimulation of the cell culture used in this nuclear protein preparation.

The addition of anti-NFATc1 (lane 3) or anti-c-Jun (lane 7) antiserum to the binding reactions with nuclear extracts from stimulated SP-B21 cells and the H-117(20) probe resulted in a supershifted complex with either Ab (marked by an arrow). Additional antibodies reactive against several members of the NFAT and AP-1 families of transcription factors, such as NFATc (K-18), NFAT4 (C-20), JunB, JunD, c-Fos, and cFosB (all supplied by Santa Cruz Biotechnology) did not react in any way with proteins bound to IL-5 RE-II in supershift binding reactions (data not shown). This limited reactivity of IL-5 RE-II proteins with a large number of related Abs indicates that the IL-5 RE-II is not indiscriminately binding NFAT and AP-1 proteins in a nonspecific manner. In addition, neither NF-Y subunit antiserum reacted with the IL-5 RE-II complex in the stimulated cell extracts (lanes 4 and 5). The Ab against the c-Jun family member, PU.1, also did not react with this complex (lane 6). Although some sequence homology exists in the IL-5 RE-II for binding sites for NFAT, AP-1, and NF-Y, the specific proteins that form the IL-5 RE-II complex appear to include at least one NFAT- and AP-1-related protein but not proteins recognized by antibodies specific for NF-Y or PU.1 transcription factors.

The IL-5 RE-II Is a Specific Target of CsA Inhibition—To study the effect of CsA on the activity of the IL-5 promoter regions functioning as protein-binding elements, reporter constructs including one, two, or all three IL-5 REs, as well as the full-length IL-5 promoter construct, were transfected by electroporation into D10.G4.1 cells that were subsequently treated with 2 μg/ml CsA before anti-CD3 stimulation. The activity of the full-length HuIL5(-1547)luc construct was reduced to 55% in the CsA-treated cells (Fig. 10). The HuIL5(-172)luc construct, which contains all three protein binding regions identified in the footprinting experiments, was similarly affected by CsA treatment in that the activity of this construct was reduced to 43% compared with the untreated HuIL5(-172)luc construct. The effect of CsA on the activity of the HuIL5(-127)luc construct was more striking. In the CsA-treated cells, the activity of this construct in which IL-5 RE-III has been deleted was reduced to 14% compared with the untreated HuIL5(-127)luc construct. In contrast, removal of the IL-5 RE-II element to form HuIL5(-80)luc did not result in a significant loss of activity in the CsA-treated cells; the activity of this construct was only reduced to 76% compared with the untreated HuIL5(-80)luc construct.

The IL-5 RE-II Protein Complex Is Distinct from the Functional NFAT Site in the Distal Human IL-2 Promoter—Taken together, the experiments described above suggest that the specific protein-DNA complexes I and II bound to IL-5 RE-II include NFAT-1- and AP-1-like proteins. We compared the IL-5 RE II protein complexes to the protein complex that binds to the functional NFAT-1 site located at position −280 in the distal human IL-2 promoter (38). The results of this comparison are shown in Fig. 11. The NFAT-1 sequence probe retarded a protein complex in the stimulated cell extracts (lane 3) that was specifically competed by excess unlabeled NFAT-1 oligonucleotide (lane 4) but was not present in unstimulated cell extracts (lane 1). The relative mobility of this inducible complex is in agreement with previously published data from human T cells showing a specific protein complex bound to this IL-2 NFAT-1 sequence (38). The presence of an inducible complex binding to this IL-2 sequence reflects the ability of these Th0 cells to produce IL-2 after activation (31). The appearance of this inducible NFAT-1-specific band only in the stimulated cell extracts distinguishes this complex from the IL-5 RE-II complex I, which is retarded by the H-117(20) probe in unstimulated cell extracts (lane 5). As shown previously, the specificity of both complexes I and II is shown by the competition reactions with excess unlabeled H-117(20) oligonucleotide (lanes 6 and 8). In addition, the NFAT-1 oligonucleotide was able to compete for complex II binding to the H-117(20) probe (lane 9), while an oligonucleotide containing an AP-1 consensus sequence (38) is not able to inhibit the formation of complex II (lane 10). Thus, the IL-5 RE-II specific protein complexes are distinct from the inducible NFAT-1 complex that binds to the distal IL-2 promoter site, although some NFAT-1-like binding specificity appears to be involved in the IL-5 RE-II site.

DISCUSSION

The regulation of IL-5 gene induction during T cell activation is a complex process that is controlled primarily at the level of transcription. To investigate the molecular mechanisms involved in regulating transcription of the human IL-5 gene in activated T cells, the 5′-flanking region of this gene was examined for the ability to respond to T cell activation signals and to regulate transcription of this gene. Most regulation studies examining the activity of the human IL-5 gene have been performed in tumor cell lines, such as human Jurkat or mouse

**Fig. 10.** Effect of CsA treatment on IL-5 promoter activity. Each of the four IL-5 promoter reporter constructs indicated was transiently transfected into D10.G4.1 cells by electroporation and stimulated 24 h later with anti-CD3 mAb. Ten minutes prior to stimulation, one-half of each group to be stimulated (representing 107 cells from the original transfection) was pretreated with 2 μg/ml CsA. The luciferase reporter gene activity was measured in the cell lysates as described under “Materials and Methods.” The results for each construct are presented as the mean ± S.D. luciferase activity in stimulated cells with CsA (stippled bars) or without CsA (shaded bars) relative to the full-length HuIL5(-1547)luc construct stimulated in the absence of CsA (defined as 100%) and represent three independent transfection experiments with each construct. S.D. value < 2.0 is below the illustrative scale.

![Graph](https://example.com/graph.png)
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EL-4 cells, in which the stimulation-dependent regulation of the gene is uncoupled from cell proliferation (24, 25). Several examples of alternative cytokine expression profiles induced in response to a variety of stimuli in primary lymphocytes, transformed T cell clones, or untransformed T cell clones have been reported and suggest that differences may exist in the mechanisms of cytokine gene induction in these different types of cells (16, 17, 20, 51–53). For these reasons, the untransformed human SP-B21 T cell clone activated through the TCR to produce a complete set of cytokines, including IL-5 (16, 17, 29, 47, 48). In addition, IL-5 message induction in activated D10.G4.1 cells is inhibited in the presence of CHX and indicates that the requirement for de novo protein synthesis for IL-5 expression is maintained in this untransformed cell line (29). The IL-5 promoter-driven activity was 4.5-fold higher in the anti-CD3-stimulated D10.G4.1 cells transfected with the HuIL5(−1547)luc construct compared with transfected cells that were not stimulated, which suggests that the reporter gene activity in the transfected cells is up-regulated in a manner similar to the physiological induction of IL-5 transcription in these cells in response to TCR stimulation.

The pattern of inducible expression with truncated IL-5 promoter constructs transfected into D10.G4.1 cells suggests that both positive and negative regulatory regions as well as a constitutively active region contribute to the regulation of the human IL-5 promoter. The transfection results indicate that an important regulatory element, IL-5 RE-II, critical to inducible IL-5 promoter activity in T cells is located within 127 bp of the transcription initiation site. The deletion of this element resulted in a significant loss of IL-5 promoter activity as well as a complete loss of inducibility of the promoter in response to activation of the transfected cells through the TCR. The functional importance of the IL-5 RE-II element in regulating activation-dependent IL-5 promoter activity was confirmed by the transfection experiments with the IL-5 RE-II mutant reporter constructs in which more than 50% of the activity was lost compared with the parental constructs. This region of the human IL-5 gene has also been shown to function as an inducible regulatory element in an allergically triggered mouse mast cell line and to bind an NFAT-like factor from these cells (27). A common mechanism for induction of the IL-5 gene in these two cell types would explain why both T cells and mast cells are able to express a similar lymphokine pattern after stimulation.

This region of the promoter is similar in relative location and sequence to the IL-5P and CLE1 (54, 55) elements defined in the mouse IL-5 gene and reported to bind NFAT-like proteins from EL-4 thymoma cells.

The decrease in activity with the HuIL5(−172)luc construct compared with the HuIL5(−127)luc construct can be attributed to the additional regulatory element in this region, IL-5 RE-III, that appears to function as a negative regulator of IL-5 promoter activity. This region of the human IL-5 promoter has not been previously described as a regulatory element for IL-5 expression.

The reporter activity detected in cells transfected with the HuIL5(−80)luc construct is similar to that expressed by the full-length construct and indicates that the IL-5 RE-I element
alone is capable of driving an intermediate level of IL-5 gene transcription. However, the complete loss of inducibility with this construct in stimulated transfected D10.G4.1 cells compared with unstimulated transfected cells indicates the IL-5 RE-I region does not function as an inducible regulatory element. Instead, this region appears to act constitutively, as evidenced by the increase in basal levels of reporter activity in unstimulated cells transfected with this construct. This region includes the conserved CLE0 element that has been shown to be critical for inducible GM-CSF promoter activity in Jurkat and EL-4 thymoma cells stimulated with phorbol ester and calcium ionophore (39). Similar to its role in regulating the mouse IL-5 gene (29), the CLE0 element in the human IL-5 promoter functions in a manner that is distinct from its role in controlling GM-CSF promoter expression. Specifically, the IL-5 CLE0 element does not appear to be critical for inducible expression of the human IL-5 promoter and functions constitutively in both stimulated and unstimulated T cells. In addition to the three promoter regions defined here, additional undefined regulatory elements located upstream of position −505 appear to contribute to the expression of the IL-5 gene, based on the 50% drop in reporter gene activity when the promoter sequences between positions −1547 and −505 are deleted.

Using overlapping oligonucleotides from the footprinted IL-5 RE-II region as probes and competitors in EMSAs, the 20-bp sequence between positions −117 and −98 was identified as the minimal region within this regulatory element capable of stable protein complex binding. Although the 14-bp sequence located between positions −118 and −105 was shown to be critical for stable complex formation in competition reactions with overlapping 12-bp oligonucleotides from this region, the competition with these oligonucleotides was incomplete. Additional overlapping 12-bp oligonucleotides positioned either 5′ or 3′ to this critical binding region were unable to compete for any IL-5 RE-II protein binding. The lack of complete competition indicates that nucleotides both 5′ and 3′ to the 14-bp core-binding region defined by the two competing 12-bp oligonucleotides are necessary for complex formation. Thus, the minimal IL-5 RE-II-binding site required for stable protein binding is defined by these experiments to be the 20-bp sequence located between positions −117 and −98 of the IL-5 promoter.

The relative mobility of the complex that bound to the 20-bp oligonucleotide containing this minimal IL-5 RE-II site changed in response to activation of the T cells. The sequence-specific binding proteins that form the faster migrating complex (I) appeared to be constitutively expressed in these T cells, since their appearance did not depend on activation of the cells. Nuclear extracts prepared from stimulated T cells, however, produced a complex (II) that migrated more slowly. This alteration in the mobility of the protein complexes bound to the same binding site in extracts prepared following different cell treatments suggests that the compositions of the protein complexes in these two types of extracts are not identical. At least one member of the DNA-binding complex specific for the IL-5 RE-II sequence is altered, by a modification of preexisting proteins and/or by synthesis of new protein(s), in response to activation of the T cells. In EMSAs with extracts prepared from cells that were stimulated in the presence of the protein synthesis inhibitor, CHX, two complexes (I and II) were detected with mobilities identical to those in both unstimulated and stimulated cell extracts. Thus, the change in the nature of the DNA-binding complex in activated T cell nuclei is likely to involve a newly synthesized protein, although the appearance of the activated complex was not completely inhibitable by CHX.

In binding reactions with nuclear extracts prepared from activated cells that had been pretreated with CsA, only complex I is formed. Thus, the activation-dependent alteration in the IL-5 RE-II protein complex is completely inhibitable by CsA, indicating that at least one component in complex II is CsA-sensitive, while complex I is not. Based on previous reports that NFAT is a target for the inhibition of cytokine gene expression by CsA (49), this CsA sensitivity suggests a functional role for NFAT-like transcription factors in the activity of the IL-5 RE-II regulatory element. CsA is known to target the calcium-regulated NFAT family of transcription factors (52, 56–61) and block their translocation to the nucleus (49). Thus, the sensitivity of complex II formation in the gel shift experiments and sensitivity of the inducible activity of the IL-5 RE-II site to inhibition by CsA indicates NFAT-like factors are likely to be members of the IL-5 RE-II-specific complex. The relative insensitivity of the constitutively active IL-5 RE-I site to inhibition by CsA in the transfection assay indicates that NFAT family proteins are not required for this regulatory region to function. A comparative sequence analysis of the 20-bp IL-5 RE-II region involved in protein binding provided additional support for the involvement of NFAT-like proteins in the complexes that bound to this site. This sequence includes a 7-bp sequence that differs from a previously characterized NFAT-specific motif (45) by only 1 bp, and an AP-1-like motif that differs from a variant of the AP-1 consensus binding site by 1 bp (19).

The reactivity of both NFATc-1- and c-Jun-specific Abs with the proteins bound by the IL-5 RE-II in supershift EMSA experiments indicates that both NFAT- and AP-1-like proteins are present in the complex in stimulated SP-B21 cells. The Abs specific for the NF-Y(A) and NF-Y(B) subunits of NF-Y as well as the Ab specific for PU.1 did not react with the IL-5 RE-II specific complex, despite the presence of a NF-Y-like motif and a GGAA core in this sequence. The GGAA core has been reported to be included in the recognition site for several proteins in the ets family (62). Thus, NF-Y and PU.1 factors do not appear to be members of the protein complexes that bind to IL-5 RE-II. Taken together, these results provide strong evidence for the involvement of at least one member related to the NFAT-1 and AP-1 families of transcription factors, but not NF-Y or PU.1, in the regulatory complex bound to the IL-5 RE-II.

In an effort to characterize the degree of relatedness to a functional NFAT regulatory site, we compared the binding specificity of the IL-5 RE-II with that of the distal NFAT-1 site in the human IL-2 promoter. This element is a composite element that is bound by NFAT proteins as well as Fos and Jun family (AP-1) proteins and is antigen-inducible and CsA-sensitive (38, 45). Similarly to previously published reports using cultured T lymphocytes and phorbol myristate acetate and ionomycin stimulation (38), a probe corresponding to this NFAT-1 sequence was able to retard a specific inducible protein complex in anti-CD3-stimulated SP-B21 cell extracts. The presence of the induction-specific NFAT-1 binding activity parallels the induction of IL-2 expression following activation of the cells. In contrast to the results with the NFAT-1 probe, binding activity specific for IL-5 RE-II was repeatedly detectable in both unstimulated and stimulated SP-B21 nuclear extracts, although the relative mobility of the specific protein complex was altered in response to activation of the cells. In addition, the binding of the inducible complex to this IL-5 promoter element was not inhibitable by a consensus AP-1 sequence, unlike the blocking of IL-2 NFAT-1 binding by an AP-1 binding site reported by Boise et al. (38). The IL-2 NFAT-1 sequence does compete for inducible complex binding to IL-5 RE-II, however, indicating that there is some degree of relat-
edness between the IL-2 NFAT-1 and IL-5 RE-II binding sites and the individual proteins that form the regulatory complexes bound to these promoter regions.

In summary, these results demonstrate that multiple DNA-binding regions are involved in the activity of the human IL-5 gene promoter in activated T cells, and these regions include a highly inducible positive regulatory region (IL-5 RE-II) as well as a negative regulatory region (IL-5 RE-III) and a constitutively active region (IL-5 RE-I). The 20-bp minimal binding sequence within the IL-5 RE-II region described here appears to be the most critical region for inducible promoter activity in T cells, and it binds at least one specific protein complex related to a NFAT-1 transcription complex. The binding characteristics of this inducible complex are distinct from that of the distal NFAT-1 site in the human IL-2 promoter and suggest that the combination of proteins in each of these regulatory complexes is likely to differ. The identification of the proteins that bind to the IL-5 RE-II regulatory site in human T cell nuclei will be an important step in elucidating the mechanism by which the transcription of the IL-5 gene is specifically controlled in an activated T cell in response to immune signaling events.

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