Transcription of the Interleukin 4 Gene Is Regulated by Multiple Promoter Elements

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Summary

Activation of T helper cell 1 (Th1) and Th2 results in transcription of the interleukin 2 (IL-2) and IL-4 cytokine genes, respectively. Whereas many of the regulatory elements and factors responsible for IL-2 transcription in T cells are well defined, little is known about parallel mechanisms that drive transcription of the IL-4 gene. Here we have analyzed the murine IL-4 promoter, both in vivo and in a Th2 clone. 3 kb of IL-4 upstream sequence is shown to be sufficient to achieve tissue-specific and inducible expression of a thymidine kinase reporter gene in vivo in a manner that mirrors the expression of endogenous IL-4. Tissue-specific and inducible expression is also demonstrated in a Th2 clone, but not in a B cell line. Deletional and mutational analysis of the IL-4 promoter demonstrated that sequences from −100 to −28 were necessary for a transcriptional response to Concanavalin A or anti-CD3 monoclonal antibody. An overlapping, yet smaller region, spanning the sequences from −60 to −28 bp was shown to be required for the response to ionomycin. Mutation of an 8-bp region from −43 to −35 of the IL-4 promoter completely abrogated IL-4 gene transcription in response to all stimuli tested. In addition, our results show that the effects of the immunosuppressive agent Cyclosporin A map to the same DNA sequences as the positive control elements. These results identify DNA sequences that are functionally important for the control of IL-4 gene transcription both in vivo and in vitro. Although these sequences are highly conserved in the human and murine IL-4 genes, they are largely not present in the IL-2 enhancer complex. Thus, cytokine-specific cis-acting elements may be one mechanism by which these two cytokine genes are differentially regulated.

The interaction of Th cells with peptide–MHC complexes on APCs, initiates a cascade of events culminating in an effective immune response. One consequence of this interaction is the synthesis and release of cytokines into the surrounding microenvironment. One such cytokine, IL-4, originally described as a B lymphocyte growth factor, is now known to regulate a broad spectrum of biologic activities including activation, growth and differentiation of T lymphocytes, B lymphocytes, macrophages, and cells of the inflammatory and hematopoietic systems (1, 2). The receptor for IL-4 is expressed on most cells of hematopoietic lineage, where it can be regulated in an autocrine manner (3, 4).

Subsets of murine CD4+ Th cells have been defined on the basis of their ability to provide help to B cells and on the patterns of their cytokine secretion (5–7). The Th1-type clones classically produce IL-2, TNF, lymphotoxin, and IFN-γ and participate in delayed-type hypersensitivity responses. Th2 cells, on the other hand, secrete IL-4, -5, -6 and -10, and

provide help to B cells. Both Th1 and Th2 cells secrete GM-CSF and IL-3. Thus, whereas there is some overlapping cytokine secretion between these subsets of cells, it appears that among differentiated Th populations, Th1 cells produce IL-2 and Th2 cells produce IL-4. One mechanism by which differential expression of IL-2 and IL-4 in vivo is accomplished may involve differences in DNA sequence elements in the two promoters. Dissection of the IL-2 promoter had identified several functional elements including those that bind nuclear factor of activated T cells (NFAT)[1], NFIL-2, and NF-κB (8–10). These elements, with the exception of the NFAT sequence, are not present in the IL-4 promoter sequence. Another mechanism may involve alternative T cell activation pathways that can differentially regulate transcription factors that bind the IL-2 and IL-4 promoters in Th1 versus Th2 cells (11, 12).

In this report, we describe the analysis of the murine IL-4 promoter. We demonstrate that ~3 kb of the IL-4 promoter

is sufficient for tissue-specific and inducible expression of IL-4 in vivo. In a Th2 clone, we demonstrate that the first 97 bp of the IL-4 promoter is sufficient to mediate induction from two distinct pathways initiated at the cell surface. Linker-scanning mutagenesis and multimerization experiments further localize response elements to several discrete regions within this proximal promoter. This control region is highly conserved between murine and human IL-4 promoters. Very little homology, however, is noted between this region of the IL-4 promoter and the well-characterized IL-2 enhancer–promoter complex with the exception of an NFAT-like sequence. Our data also suggest that Cyclosporin A (CsA) downregulates IL-4 gene transcription by inhibiting the function of the positive elements.

Materials and Methods

Cell Lines. D10 cells were grown in RPMI 1640 medium supplemented with 10% rat Con A supernatant, 10% FCS, 2 mM glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 100 μg/ml penicillin and streptomycin, and 10^{-4} M 2-ME. All other cell lines were grown in RPMI 1640 medium supplemented with 8% FCS, 2 mM glutamine, 100 μg/ml penicillin and streptomycin, and 10^{-4} M 2-ME. D10 is a murine Th2 clone specific for conalbumin in the context of I-A^k (13). EL4 cells were derived from a murine thymoma and M12.4.1 cells were derived from a mature B cell lymphoma.

Transgenic Mice. A BglII fragment containing $\sim$3 kb (-3 kb to $+58$ bp) of IL-4 upstream sequence, derived from a murine IL-4 genomic clone isolated from a C57BL/6 spleen cell library, was fused to the coding region of the HSV thymidine kinase (tk) gene. Using standard techniques, this fusion construct was injected into (CBA/J $\times$ C57BL/6)F$_1$ and transgenic animals from one founder line containing 5–10 copies of the transgene established (14). The mice were then bred to achieve homozygous expression of the transgene.

PCR Analysis of IL-4 mRNA. Total tissue RNA was isolated from one mouse by methods previously described (15). cDNA synthesis was performed with 5 μg of total DNase-treated RNA in 1$\times$ Superscript buffer (GIBCO BRL, Gaithersburg, MD), 10 mM DTT, and 300 μM of each dNTP, 1.5 μM oligo(dT), and 200 U Superscript-reverse transcriptase (GIBCO BRL). The reaction volume was 20 μl. After incubation at 42°C for 1 h, the oligo(dT) was removed using a G-50 column (Worthington Biochemical Corp., Freehold, NJ); the total volume was 50 μl. A mock reaction lacking reverse transcriptase was prepared as a negative control. 15 μl of the cDNA reaction mixture was serially diluted from 1:10 to 1:320. The negative control was diluted 1:10. 10 μl of each cDNA dilution was used for a PCR in 1$\times$ Taq buffer (Boehringer Mannheim Biochemica, Indianapolis, IN), 300 μM each dNTP, 25 pmol of each primer, and 2.5 U Taq DNA polymerase (Boehringer Mannheim Biochemica). The reaction volume was 50 μl. 25 cycles for hypoxanthine phosphoribosyl transferase (HPRT) primers, 30 cycles for IL-4 primers, and 35 cycles for IL-4/tk primers were performed for 1 min at 95°C, 2 min at 59°C, and 3 min at 72°C (10 min

during the last cycle). Primers for the fusion gene consisted of a 5′ primer in the IL-4 gene 5′ untranslated region and a 3′ primer in the tk gene. Sequences of PCR primers specific for the IL-4, tk, and HPRT cDNA are available upon request. 8 μl of each PCR reaction was electrophoresed on a 4% agarose gel denatured in 0.5 M NaOH, 0.8 M NaCl, neutralized in 0.5 M Tris (pH 7.6), 1.5 M NaCl, and Southern blotted onto GeneScreen membrane (New England Nuclear, Boston, MA). Southern hybridization was performed as described (15). The probe for IL-4 was a 373-bp EcoRI/HindIII fragment from the IL-4 cDNA (gift from W. Paul, National Institutes of Health, Bethesda, MD). The HSV-tk plasmid was a kind gift of R. Evans (Massachusetts General Hospital, Boston, MA). The probe for tk was an $\sim$450-bp XbaI/SphI fragment and the probe for HPRT was a PCR product made from cDNA with the same primers as those used in the experimental PCR reactions.

Stimulation of Primary T Cells. LN were collected from five transgenic mice, reduced to a single cell suspension and plated onto an anti-CD3 mAb (2C11) coated dish in RPMI media (GIBCO BRL) with 10% FCS, 100 mM glutamine, 100 μg/ml penicillin and streptomycin, and 10^{-4} M 2-ME. 200 U/ml rIL-2 and 500 U/ml IL-4 (J558L supernatant from cells transfected with the IL-4 cDNA, gift of Dr. R. Tepper, Massachusetts General Hospital, Boston, MA) were added to the cell cultures. The cells were stimulated for 24 h, lysed with guanidinium isothiocyanate, and prepared for PCR as above. As an unstimulated control, transgenic LN RNA was made directly from fresh tissue.

Plasmids and Mutagenesis. Standard recombinant technology was used (15). Numbering of IL-4 gene sequences designates $+1$ as the start of transcription as described by Otsuka et al. (16). A 3-kb fragment of the IL-4 5′ untranslated region (~ -3 kb to $+58$ bp) was derived from a BglII digestion of subcloned genomic sequences, blunted, and subcloned into the end-filled HindIII site of pSVOcatA (a gift of J. Strominger, Harvard University, Boston, MA). The 801-bp fragment (spanning 5′ noncoding sequences from -801 to $+58$ bp) was a BglII/HindIII fragment, blunted, and subcloned into pSVOcatA. pSVOcatA contains the chloramphenicol acetyl transferase (CAT) gene but no promoter or enhancer sequences.

Deletion mutations of the IL-4 promoter were generated by using PCR to amplify appropriate sequences. In each case, a SalI site was introduced at the 5′ end and a XbaI site was added at the invariant 3′ end. Substitution mutations of the IL-4 promoter were created using PCR to amplify two DNA fragments which, when cut with SalI and BglII or XbaI and BglII and ligated, reconstitute the IL-4 promoter sequences -157 to $+58$ with the appropriate base substitutions. The fragments were subcloned into pSVOcatApoly (the pSVOcatA plasmid containing a polylinker with SalI and XbaI sites). The nucleotide sequences of both strands were confirmed using the dideoxynucleotide method with Sequenase, as recommended by the manufacturer (U.S. Biochemical Corp., Cleveland, OH).

20-bp sequences of interest were multimerized with six random bases inserted between three repeats. These sequences were made into two complementary oligonucleotides with BamHI sites on the end. The oligonucleotides were kinased and then annealed. The multimer fragments were ligated into the BamHI site of pBLCAT2 which contains the tk promoter linked to the CAT reporter gene. All plasmids were sequenced as described.

Transfection. D10 and EL4 cells were transfected by electroporation using a Gene Pulsar (Bio-Rad Laboratories, Richmond, CA). 20 μg of plasmid DNA were added to 10^7 cells in 400 μl of RMPI 1640 medium in a 0.4-cm gap electroporation cuvette (Bio-Rad Laboratories) and incubated for 10 min at room temperature. Cells were electroporated at 270 V, 960 μF with an average time constant of 25. The cells were then placed on ice for 10 min and cul-

tured in D10 media or RPMI media described above. 24 h after transfection, D10 cells were stimulated with 10 μg/ml Con A (Sigma Chemical Co., St. Louis, MO), 1 μM ionomycin (Calbiochem Corp., La Jolla, CA), or on an anti-CD3 mAb coated plate at 100 μg/ml, or incubated with 1 μg/ml CsA (gift from A. Abbas, Harvard Medical School) for 16–18 h. Protein extracts were prepared and CAT activity was assayed and quantitated using thin layer chromatography and liquid scintillation counting as described (15). M12.4.1 cells were transfected using the DEAE-dextran method as described (15). 10 μg of plasmid DNA were transfected into M12.4.1. cells, and 24 h after transfection, the cells were stimulated with 1 μM ionomycin for 16–18 h. Cells were harvested and CAT activity assayed as described above.

Results

Approximately 3 kb of 5' IL-4 Upstream Sequence is Sufficient for Tissue-Specific and Inducible Expression In Vivo and In Vitro. IL-4 expression is both strictly tissue limited and highly inducible after T cell–activating stimuli (2). Specifically, IL-4 expression is limited to a subset of T helper cells (Th2), to mast cells, and to a non-T-non-B cell population in spleen and bone marrow (17). To determine whether sequences 5' to the IL-4 coding region were sufficient for in vivo tissue–specific and inducible expression, a murine IL-4 genomic clone containing ~7 kb of 5' sequence was isolated from a mouse spleen genomic library. A line of mice was generated that contains a transgene consisting of ~3 kb of IL-4 5' sequence fused to the herpes virus tk gene. Appropriate breeding led to the production of a transgenic line homozygous for the IL-4 tk transgene.

Immune (thymus, spleen, and LN) and nonimmune (kidney, liver, and heart) tissues were harvested from these mice and RNA prepared. Since levels of endogenous IL-4 are extremely low in freshly isolated, unstimulated T cells (18), organ RNA was used to generate first-strand cDNA and comparative PCR using primers specific for IL-4 or IL-4 tk sequences was per-

formed. Hybridization of amplified PCR products to an IL-4 or tk-specific probe revealed a single band of the correct size in the lanes containing thymus, LN, and spleen (Fig. 1 *A*). No hybridization was seen to liver, kidney, or heart (Fig. 1 *A*). All sample dilutions were approximately equal as demonstrated by the equivalent amount of PCR product obtained when control primers to HPRT were used (data not shown). We conclude that 3 kb of the IL-4 promoter is sufficient to drive tissue-specific expression in a manner which parallels endogenous IL-4 expression in vivo.

We next performed experiments to determine if 3 kb of the IL-4 promoter contained elements responsive to T cell activation stimuli in vivo. LN cells from IL-4 tk mice were plated in tissue culture dishes coated with anti-CD3 mAb, and RNA was prepared after culture for 24 h. This RNA was utilized as described above to produce cDNA which was then used in comparative PCR and probed with IL-4 or tk-specific probes. These samples were compared with samples that had not been stimulated with anti-CD3 mAb. In all cases, there was at least 10-fold increase in the amount of IL-4 tk message seen when the cells were exposed to activating anti-CD3 mAb (Fig. 1 *B*). The induction of IL-4 tk message paralleled induction of endogenous IL-4 message (Fig. 1 *B*). Therefore, 3 kb of IL-4 promoter contains sequences responsive to T cell activation stimuli in vivo.

To begin to dissect functionally important elements in the IL-4 promoter, we next examined the expression of IL-4 promoter sequences in vitro. IL-4 promoter sequences containing ~3 kb, 801 bp and 157 bp of upstream sequence were fused to the bacterial CAT reporter gene in the vector pSVOcatA. pSVOcatA contains the bacterial CAT gene and SV40 polyadenylation sequences, but no exogenous promoter or enhancer sequences. Promoter activity was measured by transfecting the IL4-3kb, IL4-801, and IL4-157 plasmid constructs (Fig. 2 *A*) into the well-characterized Th2 clone, D10, or into the thymic lymphomas cell line, EL4. Both of these cell
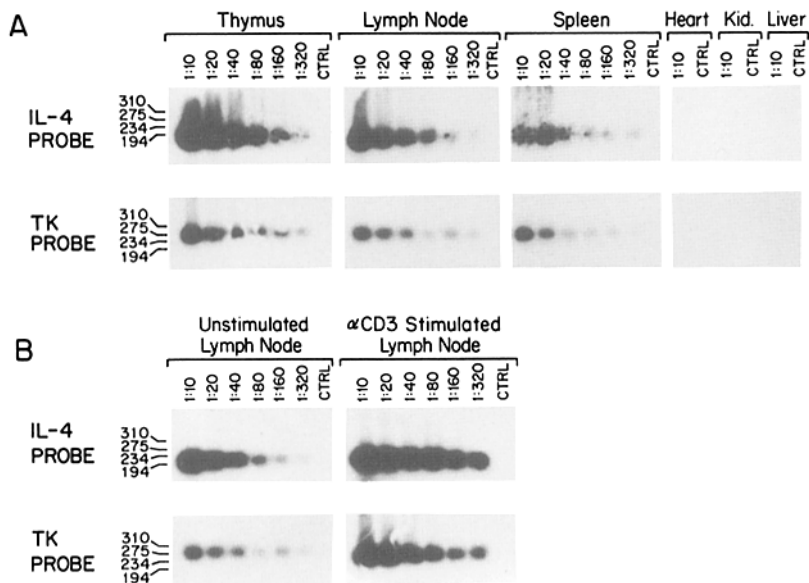

Figure 1. Tissue-specific and inducible regulation of IL-4 5' sequences in vivo. Immune and nonimmune tissue RNA was harvested from transgenic mice homozygous for a construct containing ~3 kb of the IL-4 promoter fused to the bacterial tk gene. First-strand cDNA was synthesized and serially diluted from 1:10 to 1:320 and used in comparative PCR. Only the 1:10 dilution was used for the nonimmune tissues. A negative control (*CTRL*) containing a 1:10 dilution of RNA was included for each tissue. Primers specific for the endogenous IL-4 mRNA or for the IL-4 tk fusion RNA were used to generate specific products of 180 and 250 bp, respectively. PCR products were electrophoresed on a 4% agarose gel and then Southern blotted. The blots were hybridized using either a probe specific for IL-4 or for tk DNA product. (*A*) Comparative PCR of unstimulated immune (*thymus, lymph node, spleen*) and nonimmune (*heart, kid., liver*) mRNAs using probes specific for endogenous IL-4 or tk messages. (*B*) Comparative PCR of unstimulated LN mRNA vs anti-CD3 stimulated LN mRNA using probes specific for endogenous IL-4 or tk messages.
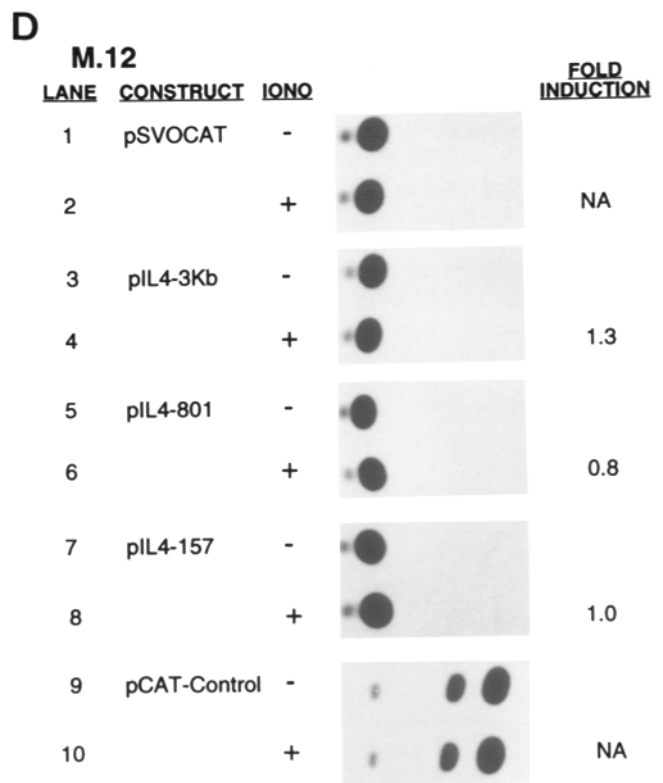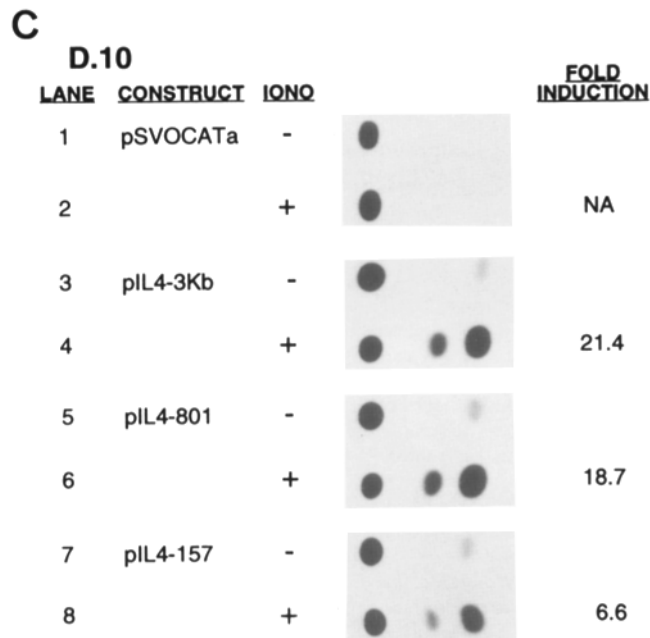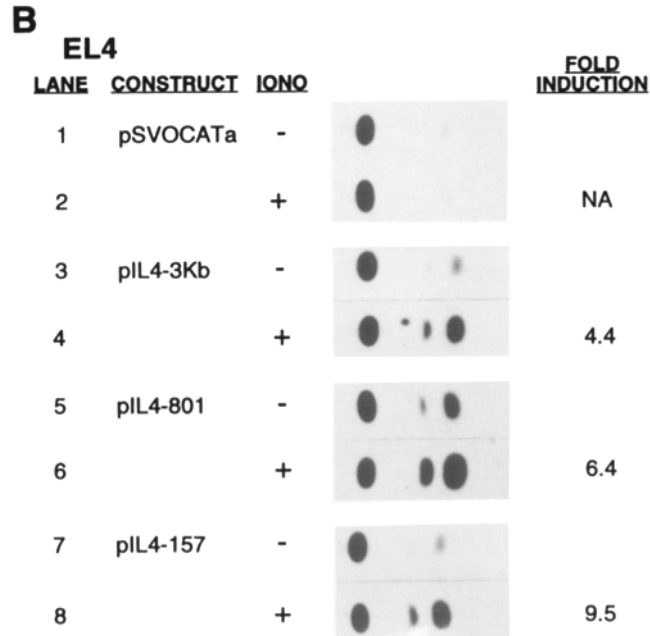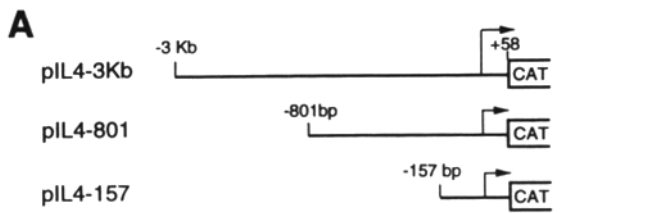
A

pIL4-3Kb -3 Kb ⌐ +58 CAT

pIL4-801 -801bp ⌐ CAT

pIL4-157 -157 bp ⌐ CAT

B

EL4

LANE	CONSTRUCT	IONO		FOLD INDUCTION
1	pSVOCATa	−		
2		+		NA
3	pIL4-3Kb	−		
4		+		4.4
5	pIL4-801	−		
6		+		6.4
7	pIL4-157	−		
8		+		9.5

C

D.10

LANE	CONSTRUCT	IONO		FOLD INDUCTION
1	pSVOCATa	−		
2		+		NA
3	pIL4-3Kb	−		
4		+		21.4
5	pIL4-801	−		
6		+		18.7
7	pIL4-157	−		
8		+		6.6

D

M.12

LANE	CONSTRUCT	IONO		FOLD INDUCTION
1	pSVOCAT	−		
2		+		NA
3	pIL4-3Kb	−		
4		+		1.3
5	pIL4-801	−		
6		+		0.8
7	pIL4-157	−		
8		+		1.0
9	pCAT-Control	−		
10		+		NA

Figure 2. Cell type specificity of the IL-4 promoter. (*A*) The structure of the pIL4-3kb, pIL4-801, and pIL4-157 reporter constructs containing IL-4 promoter sequences subcloned into the vector pSVOCATA. (*B*) EL4 thymoma cells were transfected with reporter constructs and appropriate samples were treated with 1 μM ionomycin beginning 24 h after transfection. Cells were harvested 48 h after transfection and CAT activity assayed by thin layer chromatography and liquid scintillation counting. (*C*) The Th2 clone D10 was transfected with the reporter constructs and stimulated with 1 μM ionomycin. (*D*) M12.4.1 cells were transfected with the reporter constructs and stimulated as above. A representative experiment is shown. At least three independent experiments were conducted for each cell line. (*NA*) No significant induction of CAT activity generated by pSVOCATA or pCAT-Control.

lines produce IL-4 after stimulation with T cell activation agents such as anti-CD3 mAb, Con A, or ionomycin, but produce minimal IL-4 at baseline. A transformed B cell line, M12, which expresses no endogenous IL-4 was used as a negative control. After transfection, the cells were cultured in the presence or absence of the calcium ionophore, ionomycin,

for 24 h. Ionomycin rather than Con A or anti-CD3 was chosen as an inducing stimulus since M12 cells do not express surface CD3 or TCR. Cell lysates were then prepared and the percent conversion of acetylated chloramphenicol was quantitated by thin layer chromatography.

In D10 cells, an ~15-fold induction was seen above background (Fig. 2 C) and in EL4 cells, an ~sevenfold induction was seen with the constructs after stimulation with ionomycin (Fig. 2 B). Transfection of these constructs into the B cell lymphoma M12 did not yield higher levels of CAT activity than that seen with the CAT vector alone (Fig. 2 D). Also, no expression was detected in the Th1 clone, D1.1 (Lederer, J., M. D. Todd, L. H. Glimcher, and A. H. Lichtman, manuscript in preparation). We conclude that 157 bp of the IL-4 promoter is sufficient to confer ionomycin-induced and tissue-specific expression in vitro.

Deletional Analysis Maps Inducible Promoter Activity to a Region Either 3' of −97 or 3' of −77 bp. To further identify DNA sequences in the IL-4 promoter involved in inducible activity, a series of 20-bp deletions was made starting at −157-bp upstream and ending at −77-bp upstream and the resulting fragments cloned into the pSVOcatA vector. Deletion constructs were transfected into D10 cells, and cells were stimulated for 24 h to induce IL-4 gene transcription. Several different stimuli were used to induce IL-4 transcription these including Con A, anti-CD3 mAb, and ionomycin.

D10 cells were stimulated with 10 μg/ml of Con A or plate-bound anti-CD3 mAb for 24 h after transfection of the deletion constructs. Con A and anti-CD3 mAb are both thought to act through the TCR–CD3 complex. In these experiments, Con A or anti-CD3 treatment led to an average fivefold induction of all deletion constructs 5' to and including pIL4-97 (Fig. 3, *A* and *B*). Activity decreased to ~1.5-fold or lower from pIL4-97 to pIL4-77 (Fig. 3, *A* and *B*). The CAT activity of the IL4-77 construct in Con A or anti-CD3 treated cells dropped off to uninduced levels (Fig. 3, *A* and *B*). From these results we conclude that deletion of the region from −97 to −77 of the IL-4 promoter perturbs Con A/anti-CD3 mAb–mediated stimulation of the IL-4 gene.

Another stimulus that can induce IL-4 gene transcription in D10 cells is ionomycin. Ionomycin is a calcium ionophore that allows influx of extracellular calcium into a cell. Classically, calcium ionophores are used in conjunction with protein kinase (PKC) activators such as PMA to activate T cells. In initial experiments, however, ionomycin alone worked as well as ionomycin plus PMA to activate IL-4 gene transcription in our subclone of D10 cells. D10 cells were therefore stimulated with 1 μM ionomycin alone after transfection with the deletion constructs. Ionomycin induced IL-4 gene transcription an average of 15-fold over basal activity in D10 cells using the deletion constructs from IL4-157 to IL4-77 (Fig. 3, *A* and *B*). When cells were treated with DMSO (the vehicle for ionomycin) alone, no activation of the IL-4 promoter was seen (data not shown). Therefore, the sequences 3' of −77 bp must contain an element(s) that is responsive to the ionomycin induction pathway.

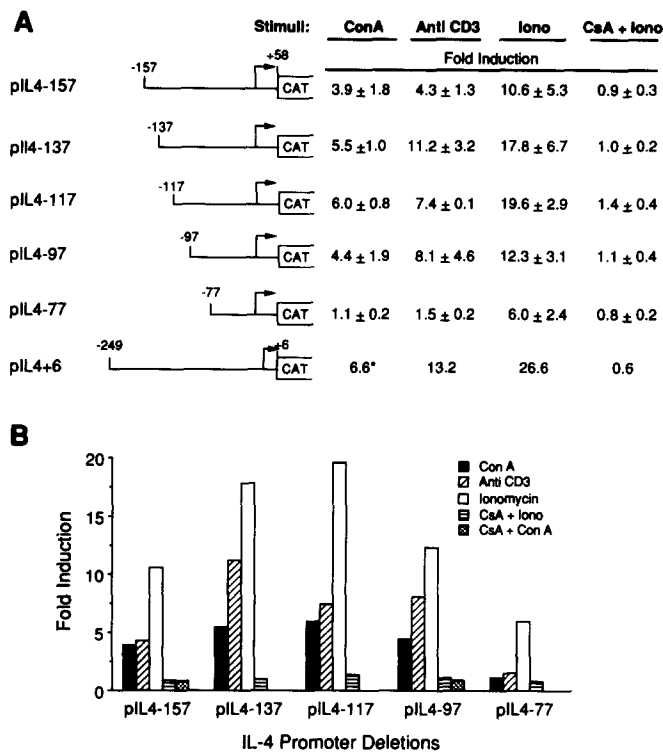The immunosuppressive drug, CsA, prevents IL-2 gene

transcription by interfering with T cell activation pathways (19). Recently, it has been shown that CsA interferes with IL-2 gene transcription by binding to calcineurin, a calmodulin-dependent protein phosphatase that is important in early T cell activation events (20, 21). Therefore, it could be expected that no single promoter element would be responsible for CsA activity. After transfection with IL-4 promoter deletion constructs, D10 cells were treated with 1 μM ionomycin and 1 μg/ml CsA. In controls using comparable levels of ethanol (vehicle for CsA) and ionomycin, no effect was seen on induction relative to ionomycin alone (data not shown). In these experiments it was found that CsA could fully suppress the inducible activity associated with any of the deletion constructs used (Fig. 3, *A* and *B*). Indeed, the effects of CsA were still apparent in the context of the first 77 bp of the IL-4 promoter (Fig. 3, *A* and *B*). CsA could also effectively suppress activation of the IL-4 promoter by Con A (Fig. 3 *B*).

A

	Stimuli:	ConA	Anti CD3	Iono	CsA + Iono
			Fold Induction		
pIL4-157		3.9 ± 1.8	4.3 ± 1.3	10.6 ± 5.3	0.9 ± 0.3
pIl4-137		5.5 ± 1.0	11.2 ± 3.2	17.8 ± 6.7	1.0 ± 0.2
pIL4-117		6.0 ± 0.8	7.4 ± 0.1	19.6 ± 2.9	1.4 ± 0.4
pIL4-97		4.4 ± 1.9	8.1 ± 4.6	12.3 ± 3.1	1.1 ± 0.4
pIL4-77		1.1 ± 0.2	1.5 ± 0.2	6.0 ± 2.4	0.8 ± 0.2
pIL4+6		6.6*	13.2	26.6	0.6

B

Figure 3. Effects of deletion on inducible expression of IL-4 promoter plasmids in D10 cells. (*A*) Structure of constructs containing progressive deletions of 5' flanking IL-4 sequence. D10 cells were transfected with promoter plasmids and 24 h later stimulated with 10 μg/ml Con A, anti-CD3 coated plates, 1 μM ionomycin or 1 μM ionomycin plus 1 μg/ml CsA. CAT activity was assayed 48 h after transfection. Each plasmid was assayed in at least three independent transfections. The data is shown as mean fold induction ± SE. Fold induction was calculated as induced percent conversion divided by uninduced percent conversion. The absolute percent conversion was in the range of 2–15%. (*) Mean fold inductions calculated from two experiments where SE not shown. (*B*) Fold induction generated by deletion mutations using Con A, anti-CD3, ionomycin, ionomycin plus CsA, or Con A plus CsA.

A construct was also designed which contains sequences from −249 to +6 bp of the IL-4 promoter deleting the 5′ untranslated region. When this construct was transfected into D10 cells and induced with Con A, anti-CD3 or ionomycin promoter activity was retained (Fig. 3 A). From these experiments we can conclude that the 5′ untranslated region is not necessary for activation by Con A, anti-CD3, or ionomycin. Also, CsA effectively blocks induction in the context of this construct.

Mutagenesis of the IL-4 Promoter and Analysis of the Effects of Substitution Mutations on Inducible Promoter Function. To define more precisely important cis-acting elements, linker-scanning mutations were introduced into the IL-4 promoter by PCR. Each linker-scanning mutation is 20 bp in length and lies within the context of the −157 region (Fig. 4). Each base in the 20-bp stretch was randomly mutated to one of the three remaining nucleotides. The mutation constructs were transfected into D10 cells and induced with the various agents as described above. In all experiments, the pIL4-157 wild-type construct was included as an internal control for fold induction levels.

Results obtained with the mutation constructs emphasize the importance of the region −97 to −77 bp in Con A and anti-CD3 inducible IL-4 gene transcription. Mut3 spans most of the region from −97 bp to −77 bp of the IL-4 promoter. The region encompassing mut3 is necessary for Con A and anti-CD3 induction based upon the deletion analysis (Fig. 3 A). The region mutated in mut3, however, is not solely responsible for induction through ConA or anti-CD3 because mut4, mut9, and mut10 in the context of −157 bp, can also block this induction (Fig. 5, A and B). Other linker-scanning mutations in the −157 region (mut1 and mut2) were inducible by Con A and anti-CD3 (Fig. 5 A). Therefore, induction of IL-4 gene transcription by stimulation with Con A or anti-CD3 involves several sites spanning the region extending from +1 to −97 bp.

The sequences that control the response to ionomycin, however, lie entirely within the first 77 bp of the IL-4 promoter. Linker-scanning mutation analysis allowed us to localize the responsive element(s) to mut9 and mut10 where fold induction in response to ionomycin dropped to background levels
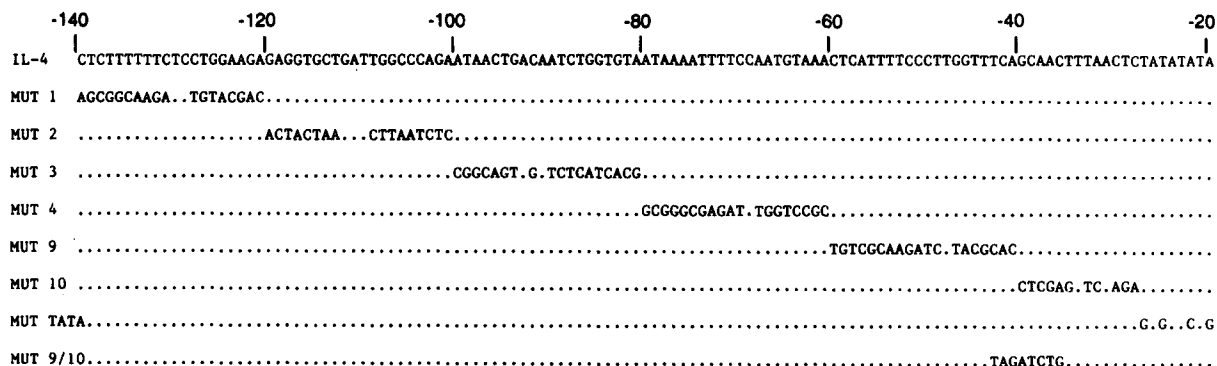

A

	Stimuli:	ConA	Anti CD3	Iono	CsA + Iono
		Fold Induction			
-157 WT		3.9 ± 1.8	4.3 ± 1.3	10.6 ± 5.3	0.9 ± 0.3
mut 1		5.4*	3.4	12.5	0.9
mut 2		3.8	5.0	8.7	1.3
mut 3		1.5 ± 0.3	1.6 ± 0.4	4.1 ± 0.6	1.1 ± 0.1
mut 4		1.6 ± 0.4	1.0 ± 0.3	12.2 ± 6.2	1.1 ± 0.1
mut 9		1.7 ± 0.2	1.4 ± 0.2	1.7 ± 0.4	1.0 ± 0.2
mut 10		1.2 ± 0.3	0.8 ± 0.3	1.4 ± 0.1	1.0 ± 0.5
mut 9/10		0.7 ± 0.1	1.0	1.0 ± 0.1	N.D.
mut TATA		0.9 ± 0.3	0.7	1.1 ± 0.6	N.D.

B

Figure 5. Effect of linker-scanning mutations on inducible expression of IL-4 promoter constructs in D10 cells. (*A*) Structure of constructs containing linker-scanning mutations of the IL-4 promoter. D10 cells were transfected with reporter plasmids and 24 h later stimulated with 10 μg/ml Con A, anti-CD3 coated plates, 1 μM ionomycin, or 1 μg/ml CsA plus 1 μM ionomycin. CAT activity was assayed 48 h after transfection. Each plasmid was assayed in at least three independent transfections. The data is shown as mean fold induction ± SE. Fold induction was calculated as described in Fig. 3. The absolute percent conversion was in the range of 2–15% (*) Mean fold induction calculated from two experiments, where SE not shown. (*B*) Fold induction generated by linker-scanning mutations using Con A, anti-CD3, ionomycin, or CsA plus ionomycin.

(Fig. 5, *A* and *B*). All other mutations in the −157 region were inducible by ionomycin, including mut3 and mut4 (Fig. 5, *A* and *B*).

Figure 4. Linker-scanning mutagenesis of the IL-4 promoter. IL-4 5′ flanking sequence from −140 to −20 and specific nucleotide substitutions are shown. Substitution mutations were made in the context of the pIL4-157 construct. All constructs were verified by sequencing of both strands.
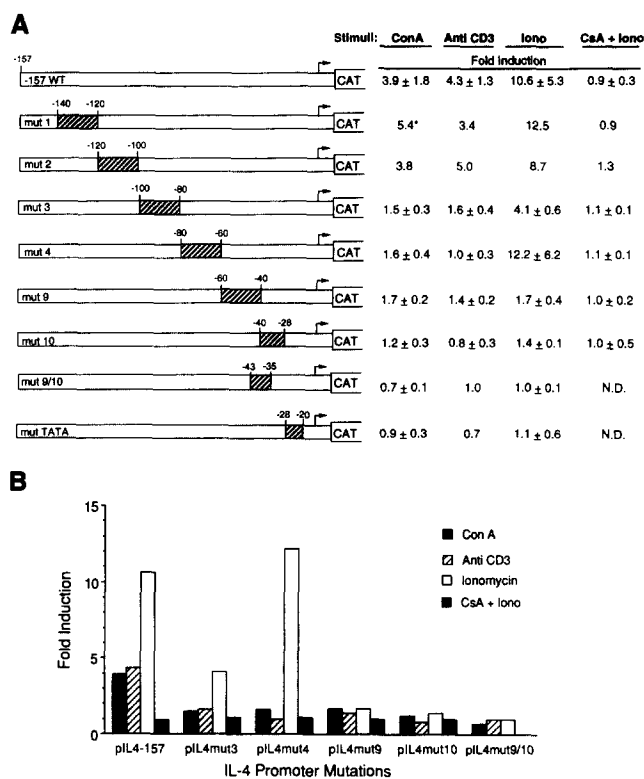
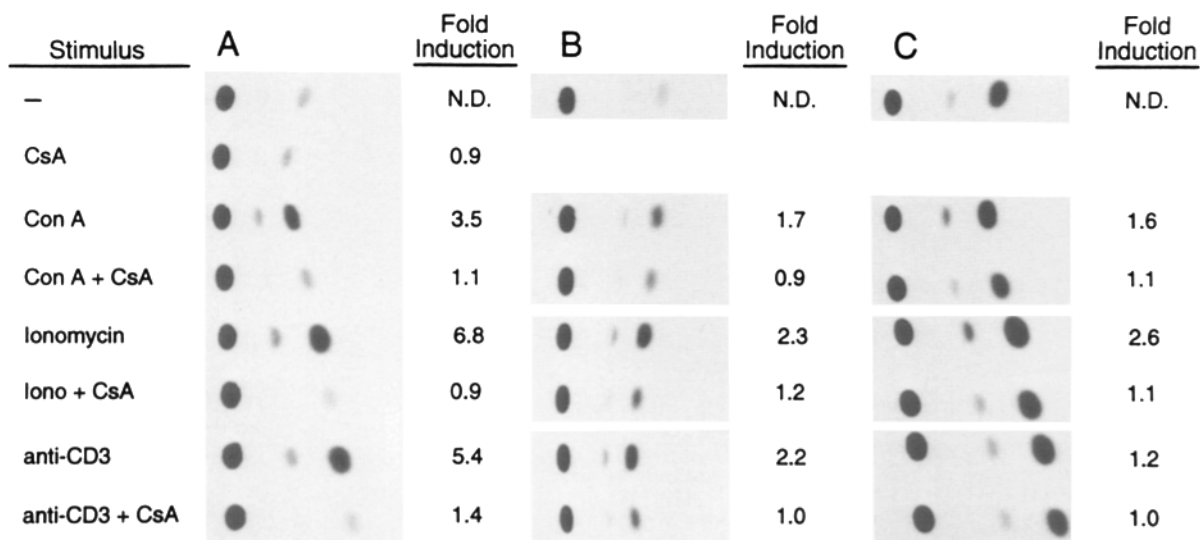| Stimulus	A	Fold Induction	B	Fold Induction	C	Fold Induction
—		N.D.		N.D.		N.D.
CsA		0.9				
Con A		3.5		1.7		1.6
Con A + CsA		1.1		0.9		1.1
Ionomycin		6.8		2.3		2.6
Iono + CsA		0.9		1.2		1.1
anti-CD3		5.4		2.2		1.2
anti-CD3 + CsA		1.4		1.0		1.0

Figure 6. Induction of a heterologous promoter with IL-4 5' sequences. D10 cells were transfected with multiple copies of IL-4 5' sequences in the pBLCAT2 vector which contains the bacterial tk promoter fused to the CAT gene. The cells were stimulated 24 h later with 10 μg/ml Con A, anti-CD3 coated plates, 1 μM ionomycin, 1 μg/ml CsA plus 10 μg/ml Con A, 1 μg/ml CsA plus plate-bound anti-CD3, or 1 μg/ml CsA plus 1 μM ionomycin. CAT activity was assayed in three independent experiments. A representative experiment is shown. Fold induction was calculated as described in Fig. 3. (A) D10 cells transfected with three copies of the wild-type mut4 region (20 bp) in the 5' to 3' orientation. (B) Three copies of the wild-type mut4 region (20 bp) were transfected into D10 cells in the 3' to 5' orientation. (C) D10 cells transfected with two copies of the wild-type mut9 plus 10 region (33 bp) in the 5' to 3' orientation.

Two additional constructs were made, one that mutates sequences overlapping mut9 and mut10, and another that mutated the putative TATA box. An 8-bp mutation, mut9/10, spanning bases −43 to −35 of the IL-4 promoter completely abrogated the responses to Con A, anti-CD3, and ionomycin in D10 cells (Fig. 5, A and B), thus defining more precisely a promoter element which plays a critical role in the inducible transcription of the IL-4 gene. Mutation of the TATA box also led to complete abolition of transcription (Fig. 5 A).

Linker-scanning mutation constructs transfected into D10 cells were induced with ionomycin or Con A in the presence of CsA. CsA was able to suppress to background levels induced transcription from any linker-scanning mutant construct (Fig. 5 A). Therefore, we conclude that CsA can block the pathways arising from the T cell surface that are modulated by Con A or ionomycin.

Induction of a Heterologous Promoter by 5' IL-4 Gene Sequences. To further confirm that specific IL-4 5' sequences contribute to IL-4 transcriptional regulation, heterologous promoter constructs were made. Sequences spanning regions that upon mutation showed a regulatory role in the IL-4 promoter were multimerized and placed upstream of the tk promoter in the pBLCAT2 vector. Specifically, three copies of IL-4 gene wild-type sequences corresponding to the mutated regions in mut3, mut4, mut9, and mut10 were placed in the 5' to 3' orientation upstream of tk promoter/CAT reporter gene sequences. An additional plasmid containing two copies of the region mutated by mut9 and mut10 (−60 to −28) was also constructed.

Results utilizing multimerized sequences showed that the wild-type sequences spanning −80 to −60 bp (mut4 region) and −60 to −28 bp (mut9 plus mut10 regions) were sufficient to confer significant inducible expression upon the heterologous tk promoter. When sequences −80 to −60 bp were multimerized, placed upstream of the tk promoter, and transfected into D10 cells, they allowed the heterologous promoter to be induced by Con A and anti-CD3, as well as ionomycin (Fig. 6 A). The induction was lower than that seen with the wild-type IL-4 promoter, but still significant. These sequences could also mediate a response to Con A, anti-CD3, and ionomycin when multimerized and placed in the 3' to 5' orientation in front of the tk promoter (Fig. 6 B). The inducible responses conferred on the tk promotor by the multimerized region −80 to −60 bp were completely suppressed by CsA (Fig. 6, A and B). When the wild-type sequences encompassing −60 to −28 bp (mut9 plus mut10 regions) were multimerized and transfected into D10 cells, inducible tk gene transcription by ionomycin was observed (Fig. 6 C). These sequences were also slightly responsive to Con A but not more than twofold above background transcription. No induction above basal transcription was seen using the stimulus anti-CD3 mAb. When multimerized, the region spanning the sequences −100 to −80 bp (mut3 region) could also increase the transcriptional response of the heterologous promoter tk to Con A and ionomycin (data not shown). Although measurable, no more than twofold induction was achieved. Sequences from −60 to −40 (mut9 region) and from −40 to −28 (mut10 region) had no effect on the tk promoter when transfected into D10 cells and stimulated with the activating agents Con A or ionomycin (data not shown).

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       -157
         |
Murine:  GGAA·AATTTACCTGTTTCT·····CTTTTTTCTCCTGGAAGAGAGGTGCTGATTGGCCC
         IIII I IIIIIIIIIII I      IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
Human:   GGAACATTTTACCTGTTTGTGAGGCATTTTTTCTCCTGGAAGAGAGGTGCTGATTGGCCC

Murine:  AGAATAACTGACAATCTGGTGTAA[TAAAATTTTCC]AATGTAAACTCATTTTCCCTTGGTT
         I I IIIIIIIIIIIIIIIIIII III IIIIIIIIIIIIIIIIIIIIIIIIIIIII IIII
Human:   CAAGTGACTGACAATCTGGTGTAA[CGAAAATTTCC]AATGTAAACTCATTTTCCCTCGGTT

Murine:  [TCAGCAAC]TTTAACTCTATATATAGAGAGACCTCTGCCAGCAT
         IIIIIIII IIIIIIIIIIIIIIIIIIIIIII I II II IIIIII
Human:   [TCAGCAAT]TTTAAATCTATATATAGAGATATCTTTGTCAGCAT
                                              |
                                             +1

       -125
         |
Murine:  GAAGAGAGGTGCTGATTGGCCCAGAATAACTGACAATCTGGTGTAA[TAAAATTTTCC]AAT
         IIIIIIIIIIIIIIIIIIIIIIII III IIIIIIIII IIIIIIIIII III IIIIIIII
Bovine:  GAAGAGAGGTGCTGATTGGCCCCAAATGACTGACAACCTGGTGTAA[TGAAAATTTCC]AAT

Murine:  GTAAACTCATTTTCCCTTGGTT[TCAGCAAC]TTTAATTTAACTCTATATATAGAGAGACCT
         IIIIIII IIIIIIIII IIIIIIIIII IIIII      IIIIIIIIIIIIIII I II
Bovine:  GTAAACTTATTTTCCCTCAGTT[TCAGCAAT]TTTAAAA····TCTATATATAGAGATATCT

Murine:  CTGCCAGCAT
         I III II
Bovine:  TTATCAGAAT
                 |
                +1

       -157
         |
Murine:  GGAAAATTTACCTGTTTCTCTTTTTTCTCCTGGAAGAGAGGTGCTGATTGGCCCAGAATA
         IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII IIIIIIIIIIIIIIII IIII
Rat:     GGAAAATTTACCTGTTTCTCTTTTTTCTCCTGGAAGAGAGATGCTGATTGGCCCGAAATA

Murine:  ACTGACAATCTGGTGTAA[TAAAATTTTCC]AATGTAAACTCATTTTCCCTTGGTT[TCAGCA]
         IIIIIIIIIIIIIIIIII IIIII IIIIIII II IIIIIIIIIIIIIIIIIIIIIII IIIIII
Rat:     ACTGACAATCTGGTGTAA[TAAAAATTTCC]AACGTAAACTCATTTTCCCTTGGTT[TCAGCA]

Murine:  [AC]TTTAACTCTATATATAGAGAGACCTCTGCCAGCAT
         II IIIIIIIIIIIIIIIIIIIIIII II II II III
Rat:     [AC]TTTAACTCTATATATAGAGATGTCTTTGTCACCAT
                                              |
                                             +1
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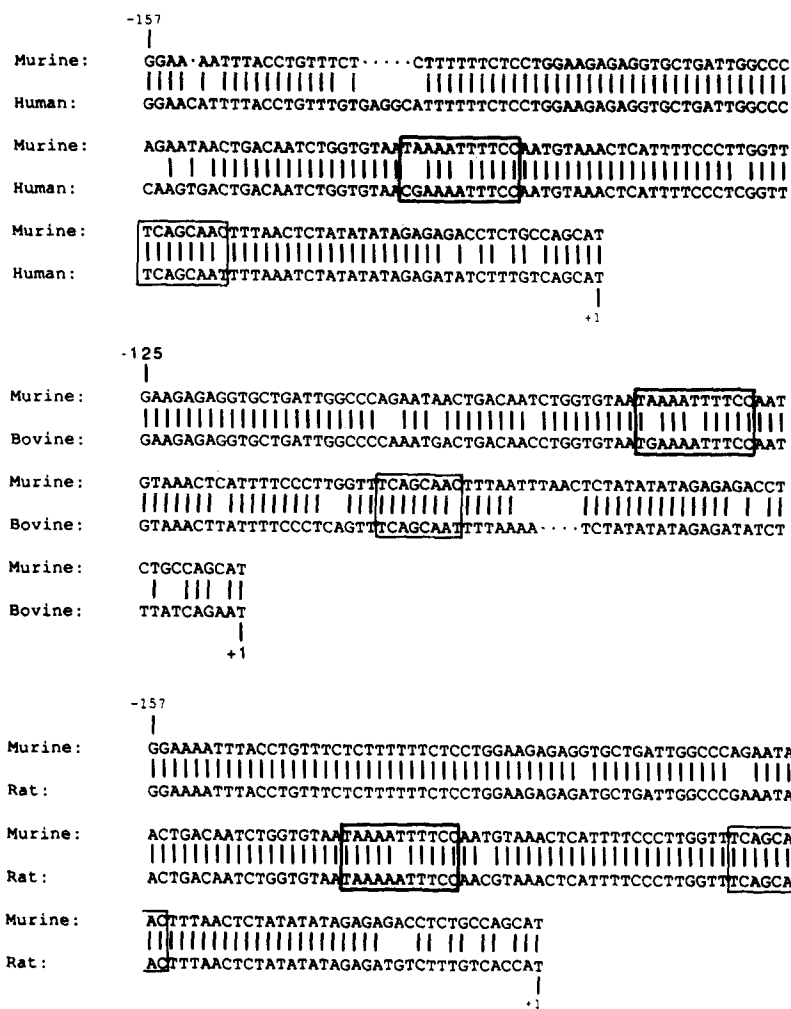
Figure 7. IL-4 5' region sequence homology. Comparisons of the murine IL-4 promoter with human, bovine, and rat IL-4 promoters are shown. The homologies of the murine IL-4 promoter to the bovine, human, and rat were, respectively, 82% (103/125), 86% (135/157), and 93% (146/157). (Dark box) The region representing mut4 or the P element (34); (light box) 8-bp region represented by mut9/10.

Discussion

In resting T cells, the IL-4 and IL-2 genes are quiescent. Upon occupancy of the TCR with its natural ligand (antigen plus MHC) or an artificial ligand, anti-CD3 mAb, or upon stimulation with calcium ionophores, T cells become activated and IL-2 and IL-4 gene transcription is rapidly initiated. In contrast to IL-2 gene transcription in Th1 cells, however, very little is known about how the signals transmitted through TCR occupancy lead to IL-4 gene transcription in Th2 cells. IL-4 regulatory sequences have not yet been defined, nor have the factors which bind these sequences been identified. In this report, we have begun an analysis of the murine IL-4 promoter in Th2 cells and have identified sequences in the proximal promoter which mediate the response of Th2 cells to both TCR crosslinking and calcium ionophore–dependent signals.

Initial experiments utilizing an IL-4 tk transgene demonstrated that 3 kb of upstream sequence is sufficient to achieve tissue-specific and inducible expression of the transgene in a manner similar to the endogenous IL-4 gene. Specifically, the tissue distribution of tk mRNA in spleen, thymus, and LN mirrors endogenous IL-4 expression. Moreover, the trans-gene promoter does not allow for expression in nonlymphoid tissues. TCR-mediated induction of IL-4 gene transcription also resides within this region, as evidenced by the induction of tk mRNA in anti-CD3 activated LN cells. In the transgenic mice, levels of tk message driven by 3 kb of the IL-4 promoter are lower than endogenous IL-4. There are several possible explanations for this observation. It may be that the specific PCR primers used bind with higher affinity to their target sequences in the IL-4 cDNA than in the tk cDNA. Alternatively, many reports exist showing that transgenes may be expressed at higher or lower levels than the endogenous gene (22, 23). This phenomenon may depend on factors such as copy number or integration site (24). It is also possible that an enhancer exists elsewhere in the IL-4 gene, either further upstream of 3 kb or in an intron. A recently published report, however, suggests that there are no T cell–specific enhancers located in IL-4 gene introns (25).

Deletional analysis established that sequences residing between −157 and +6 bp of the IL-4 gene confer tissue specificity and inducibility on a CAT reporter gene in two different T helper cells in vitro. The D10 T cell clone is a

prototypic Th2 cell which secretes IL-4, IL-5, and IL-6 upon stimulation through the TCR. D10 cells require antigen plus APC stimulation to survive in tissue culture and fulfill the criteria for a nontransformed, growth factor–dependent T cell clone. EL4, on the other hand, is a T cell thymoma that secretes both IL-2 and IL-4 and, therefore, cannot strictly be classified as either a Th1 or Th2 cell. The deletion data demonstrate that fold induction varies little as sequences are removed from 3 kb to 801 bp and from 801 bp to 157 bp in D10 cells. Additional deletional analysis through the region −157 to −77 bp further localized the active promoter to 97-bp upstream of the transcriptional start site. Sequences within this region are necessary for induction to occur after stimulation of the TCR–CD3 complex. However, there seems to be a divergence in the induction pathways for ionomycin and the TCR–CD3 complex. This is shown by the ability of ionomycin to stimulate transcription within the context of the −77-bp promoter region whereas both Con A and anti-CD3 mAb require the 20 bp between −97 and −77. These results indicate that the major elements necessary for a minimal inducible promoter responsive to all T cell activation signals tested lie within the first 97 bp of the IL-4 promoter.

Analysis using the linker-scanning mutants are in general agreement with the deletion mutants. Mut3, which spans most of the region between −97 and −77 bp, shows a sharp drop in its ability to be activated by Con A or anti-CD3. This mutation retains, however, the ability to be stimulated by ionomycin. This suggests that at least part of a cis element necessary for IL-4 transcriptional activation through the TCR–CD3 complex lies within mut3. Examination of this region revealed no known promoter motifs. Furthermore, all of the mutations that disrupt sequences within the −77 to +58 bp promoter region also lack the capacity to respond to Con A or anti-CD3, including mut4, mut9, and mut10. This data would suggest that more than one cis element is necessary to generate a response to Con A or anti-CD3 as the mutated sequences span 80 bp. This is in contrast to a pathway dependent upon ionomycin which requires a smaller yet overlapping region of the IL-4 promoter. Only the region spanning mut9 and mut10 (−60 to −27 bp) is necessary for an ionomycin response. This suggests that the response to ionomycin may be limited to a single element spanning mut9 and mut10. Indeed, when 8 bp of the sequence spanning mut9 and mut10 are mutated, the responses to Con A, anti-CD3, and ionomycin all decline to basal levels indicating that this 8-bp region plays a pivotal role in the induction pathways leading to IL-4 gene transcription. No known transcriptional elements were found to be homologous to the sequences in this region when a homology search was conducted. These data suggest that a transcription factor binding to the wild-type mut9/10 region may control the calcium-dependent response and is necessary along with other factors in controlling transcriptional responses originating from the TCR–CD3 complex which in D10 cells may be calcium independent (26).

Multimerization of the region spanning −80 to −60 bp (mut4) led to a response to Con A and anti-CD3. This region

in conjunction with the regions mutated in mut3 and mut9/10 mediates the response through the TCR, and is in fact a necessary part of this signal transduction pathway. When using the IL4-157 construct, the response to Con A was consistently severalfold lower than the response to ionomycin. Multimerization of several IL-4 promoter regions led to lower fold induction versus fold induction achieved when stimulating the wild-type construct. Therefore, it may not be possible in this system to measure the responses to Con A or anti-CD3 of weaker promoter regions (such as mut3 and mut9/10) when they are multimerized. The region spanning mut4, however, is not necessary for the pathway dependent upon ionomycin as demonstrated by the mutational analysis. Nevertheless, when the wild-type sequences corresponding to the mut4 region were multimerized they did respond to ionomycin. When multimerized, the region encompassing sequences from −60 to −28 bp (mut9 plus mut10) was also responsive to ionomycin and, to a lesser extent, to Con A. This is not surprising in light of the results with mut9/10 which demonstrated abolition of the transcriptional responses to both ionomycin and Con A. The region of the IL-4 promoter from −100 to −80 bp (mut3 region) also showed a small yet consistent response to Con A and ionomycin. One interpretation of all these data might be that the regions mutated in mut4 and mut3 can moderate the calcium-dependent responses to ionomycin, but that the element identified by the mut9/10 mutation is both necessary and sufficient. The region represented by mut3 likely works in concert with the regions mutated in mut4 and mut9/10 to accomplish the transcriptional response to TCR-mediated signals.

T cell activation pathways leading from the cell surface to events in the nucleus are complex. Stimulation through the TCR as accomplished by Con A or anti-CD3 forms one well-studied pathway (11). Other pathways that may intersect TCR-mediated pathways do exist. For example, treating T cells with calcium ionophores, such as ionomycin, leads to an influx of extracellular calcium into the cell (11). This, in turn, influences downstream activation events. It has been postulated that increased intracellular calcium may be a necessary event in PKC activation through the TCR (27). Others, however, postulate that calcium influx may actually trigger a distinct TCR activation pathway (11). Increased calcium may be necessary at several steps in T cell activation. However, there is no evidence that calcium is required for induction of IL-4 gene expression. In fact, certain Th2 clones, including D10, do not flux calcium in response to TCR stimulation (28, 29). These clones, however, can still produce significant amounts of IL-4 upon TCR engagement (30). Our data would support at least some divergence between calcium-dependent and -independent pathways leading to IL-4 gene transcription. Recently, an alternative pathway, involving the cell surface molecule CD28, has been implicated in T cell activation (31–33). Differential activation of these various signaling pathways could lead to distinctly different levels of IL-4 gene transcription. Therefore, IL-4 production would be tightly regulated in different situations depending upon the activation signals present.

From an analysis of a combination of the deletion and mu-

tation data, it becomes apparent that CsA is able to block transcription initiated through any positive promoter element. This inhibition also occurs with the combinations of CsA with Con A or anti-CD3. CsA can completely disrupt transcription even in the limited context of the first 77 bp of the IL-4 promoter. We have also found that CsA can inhibit inducible transcription in the limited context of the multimerized mut4 and mut9 plus 10 wild-type sequences. This indicates that CsA either acts directly upon *trans*-acting factors binding to sequences in these regions or that CsA affects events at an earlier point in the T cell activation cascade. Recent evidence suggests that CsA acts upon calcineurin, an early T cell activation protein (20, 21).

Using deletional and mutational analysis, two groups have identified elements important in the regulation of the human IL-4 promoter (34, 35). One element, the P element, lies within the region defined by our mut4 construct (34). This element was found to be responsive to PMA/calcium ionophore. In our studies, mutation of this region does affect responsiveness to Con A and anti-CD3 but not to ionomycin. However, when this region is fused to a heterologous promoter it can respond to ionomycin as well as to Con A and anti-CD3. The combination of these data indicate that this region, containing the P element, is important in inducible IL-4 gene transcription. Of interest, there is a perfect NFAT sequence within the mut4 region which we have recently found binds an inducible factor (Glimcher, L., unpublished data). Another group has identified two negative regulatory elements (NRE-I and NRE-II) in the human IL-4 promoter (35). No evidence has been found for any functional negative regulatory regions in the analysis of the murine IL-4 promoter. Perhaps there may be subtle differences between the transcriptional regulation of the human and mouse IL-4 promoters, although the striking sequence conservation (86%) in the region between −157 and +58 bp makes this rather unlikely (Fig. 7). Alternatively, the discrepancies observed may be attributable to the model system used. Both of the studies alluded to above have utilized the transformed T cell line Jurkat. Since this cell line produces very small quantities of IL-4, it was necessary in both reports to link IL-4 5' sequences to a heterologous promoter. This may complicate the analysis of additional regulatory signals on IL-4 gene transcription. Analysis of unmanipulated Th2 cell clones as performed here may give a more accurate representation of what occurs in vivo.

Another group has defined an element in the GM-CSF promoter that shares homology with human and murine IL-4 promoter sequences (36). This element, CLE0, binds a PMA/A23187 inducible factor. We have mutated this element by linker-scanning mutagenesis (mut9). When this region is mutated, the IL-4 promoter loses inducible activity with Con A, anti-CD3, or ionomycin as compared with the wild-type promoter. Upon multimerization of the mut9 wild-type region, however, a heterologous promoter cannot be activated above basal transcription with Con A or ionomycin. This may indicate that the element identified in the GM-CSF promoter is not functional in the IL-4 promoter, or that it cannot act alone but requires other factors in the region to form a functional complex.

IL-2 is regulated by an enhancer–promoter complex that spans ∼300 bp and that comprises several promoter elements (10). Some of these elements are necessary whereas others are contributory to transcriptional activation (8). Our data suggest that IL-4 is regulated in a similar manner. In the IL-4 promoter, the wild-type region spanning mut3 and mut4 (−100 to −60 bp) is clearly important for a calcium-independent response generated through the TCR–CD3 complex. This region contains one known functional element, the NFAT element, or the P element, and probably contains another element just upstream of the P element. This region, however, plays a minor role in the response to ionomycin. On the other hand, an element(s) that is mutated in mut9 and mut10 (−60 to −24 bp) is necessary for any induced activity of the IL-4 promoter. The IL-4 promoter also requires a TATA box for transcription to occur. One can envision several promoter elements acting together, as in the IL-2 gene, in regulating IL-4 gene transcription. Only one of the motifs identified in our analysis is homologous to elements within the IL-2 enhancer complex. Unlike the IL-2 gene, the transcription factors which bind IL-4 promoter elements may also be regulated by divergent TCR pathways. This would imply that the IL-4 and IL-2 genes are not regulated in a coordinate manner and that their actual promoter sequences, as well as alternative signaling pathways, play a pivotal role in establishing tissue-specific expression. It remains to be seen if transcription factors that bind to these sequences are also unique.

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