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T-cell subset-specific expression of the IL-4 gene is regulated by a silencer element and STAT6

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During development of CD4$^+$ T lymphocytes in the periphery, differential expression of cytokine genes, such as those of interleukin (IL)-2 and IL-4, occurs in distinct T-cell subsets. IL-4 is a cytokine produced by T-helper 2 (Th2) cells, and the IL-4 receptor (IL-4R)-mediated signaling pathway is thought to be required for commitment to the Th2 phenotype. However, the molecular basis for development of the Th subset-specific production of IL-4 remains unclear. We demonstrate here that the IL-4 promoter is functional in Th1 and B cells which do not normally form IL-4 transcripts as well as in IL-4-producing T cells. Based on studies of the effect of several different upstream and downstream regions of the IL-4 gene on IL-4 promoter activity, a Th1-specific IL-4 silencer element was identified in the 3′-untranslated region. The silencer region contained a consensus sequence for a transcriptional factor that is normally regulated by the IL-4 R signaling pathway, STAT6. Nuclear expression of STAT6 protein, which was shown to bind to the silencer region, was observed in Th2 cells but not in Th1 cells. Deletion of the STAT6-binding site from the silencer region and inhibition of STAT6 function resulted in the appearance of silencing function even in Th2 cells. These results provide evidence that the silencer element, and the binding of STAT6 to this element, play a permissive role in determining the commitment into Th2 phenotype.
Keywords: interleukin-4/promoter/silencer/STAT6

Introduction

T-helper (Th) lymphocytes secrete a panel of cytokines which determine their functional role in immune regulation. Th cells are known to be divided into at least four subsets based on their cytokine production profile (Mossmann and Sad, 1996). Most CD4$^+$ T cells in the periphery are naive T cells which produce mainly interleukin (IL)-2 and small amounts of IL-3 and granulocyte–macrophage colony-stimulating factor (GM-CSF). Th0 cells, which are at an intermediate stage of CD4$^+$ T-cell development between naive and Th1/Th2 cells, have the pattern of cytokine production of both Th1 and Th2 cells. Th1 cells are biased toward secretion of IL-2, interferon-γ (INF-γ) and lymphotoxin, and are responsible for delayed-type hypersensitivity responses. Th2 cells are biased toward secretion of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are responsible for T-cell-dependent antibody responses and allergic responses (Mosmann, 1992; Paul and Seder, 1994). Differentiation towards either the Th1 or Th2 phenotype is thought to be a consequence of several cellular influences directing T-cell differentiation. Several studies have clearly demonstrated that in addition to T-cell receptor (TCR) stimulation, a certain cytokine environment is required for the initiation of T-cell differentiation (Seder and William, 1994). IL-12 induces INF-γ production by T cells and biases development into Th1 cells (Hsieh *et al.*, 1993; Seder *et al.*, 1993; Guler *et al.*, 1996). In contrast, addition of IL-4 to primary cultures promotes Th2 development (Le Gros *et al.*, 1990; Swain *et al.*, 1990; Hsieh *et al.*, 1992; Seder *et al.*, 1992), and mice with germ line disruption of the IL-4 gene fail to generate Th2 cells *in vivo* (Kuhn *et al.*, 1991). Recently, signal transducer and activator of transcription 6 (STAT6) was isolated as a molecule regulating the IL-4 receptor (IL-4R)-mediated signaling pathway (Quelle *et al.*, 1995). Mice with germ line disruption of STAT6 revealed a failure to generate Th2 cells (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996). Therefore, the IL-4R signaling pathway appears to be necessary for Th2 development. However, the molecular events regulated by the IL-4R signaling pathway responsible for the differentiation of the Th2 phenotype remain unclear. Since IL-4 and IL-2 gene expression must be regulated at the transcriptional level, identification of differential gene regulation among Th1 and Th2 cells by the nuclear factors involved in IL-4 gene expression will be required.

Recent studies have demonstrated that transcription of the IL-4 gene is positively regulated by coordination of multiple promoter and enhancer elements. An enhancer element located between –79 and –60 bp of the flanking sequences of the IL-4 gene is responsible for cyclosporin A sensitivity of the IL-4 promoter (Tara *et al.*, 1993; Todd *et al.*, 1993; Kubo *et al.*, 1994a). This region contains the consensus sequence for the calcineurin-regulated transcriptional factor, NF(P) (Kubo *et al.*, 1994b). There are four additional sequences similar to the consensus sequence for NF(P) within 300 bp of the mouse IL-4 promoter region (Bruhn *et al.*, 1993; Szabo *et al.*, 1993). The NF(P) protein is thought to be structurally related and perhaps identical to the cytoplasmic component of IL-2 NF-AT, based on observations from supershift assays using polyclonal anti-NF-ATp antibodies and binding assays using recombinant NF-ATp (Rooney *et al.*, 1994, 1995; Tara *et al.*, 1995). Moreover, the mouse IL-4 promoter region also contains several consensus sequences for

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common transcriptional factors of the major histocompatibility complex (MHC) class II promoter (NF-Y), OCT-1-associating protein p40 (OAP40) and NF IL-6 (Chuvpilo *et al.*, 1993; Li-Weber *et al.*, 1994; Davydov *et al.*, 1995; Rooney *et al.*, 1995). There is a report indicating that the presence of a position-independent silencer element negatively regulates the IL-4 promoter in Jurkat human T-lymphoma cells (Li-Weber *et al.*, 1993). This cell line secretes limited amounts of IL-4 and the silencer element represses a positive regulatory element located between –239 and –224 bp, but the repression dose not seem to be generalized in mouse T-cell lines. A recent report indicated that expression of the proto-oncogene c-*maf* is responsible for the tissue-specific expression of IL-4 (Ho *et al.*, 1996). However, the question of how the IL-4R signaling pathway is involved in c-*maf* expression remains to be resolved.

In this study, we established a series of T-cell hybridomas between the BW5147 thymoma line, which secretes IL-2 but not IL-4, and Th2 clones to examine the transcriptional regulatory mechanisms of IL-4 gene expression. All T-cell hybridomas had the lymphokine production pattern typical of BW5147. However, since IL-4 promoter activity is observed in those T-cell hybridomas when the minimal promoter construct is used, we suspected that Th subset-specific negative regulatory mechanisms are responsible for the inhibition of IL-4 transcription. In transient transfection assays with the T-cell hybridomas, we have identified a silencer element which negatively regulates IL-4 promoter activity in Th1 cells. The silencer region contains a binding motif for the STAT6 molecule that is known to be a member of the IL-4R signaling pathway, and STAT6 binding to the silencer region was found to occur in Th2 cells but not Th1 cells. Here we discuss the physiological significance of the newly identified IL-4 silencer element and the role of the STAT6 molecule in the regulation of IL-4 expression in Th1 and Th2 cells.

Results

Establishment and pattern of cytokine production of T-cell hybridomas

We attempted to establish IL-4-secreting T-cell hybridomas by hybridization of the Th1 thymoma BW5147 (TCRα^- β^-) with a KLH-specific I-A^b-restricted Vβ8$^+$ Th2 clone, 24-2 (Nakayama *et al.*, 1988). Seventy four hybridomas were obtained as HAT-resistant cell lines, and eight lines had a Vβ8 TCR derived from the Th2 clone. The pattern of cytokine production in these T-cell hybridomas was determined using the IL-2- and IL-4-dependent cell line CTLL-2. Surprisingly, all sublines only produced IL-2 but not IL-4 (Table I). We further examined other T-cell hybridomas such as 2B4.11 (Saito *et al.*, 1989), AHK61 and AHK71-23 (Hoshi *et al.*, 1994) that were established elsewhere using BW5147 (Table I). These three hybridomas showed the same cytokine production pattern as our T-cell hybridomas. Expression of IL-4 mRNA in these T-cell hybridomas was not detected by RT–PCR even after extensive amplification (50 cycles) (Kubo *et al.*, 1994A). Southern hybridization analysis showed that the genomic configuration of the IL-4 gene was normal in H24-2-68-41 (68-41) cells as well as in BW5147 (data not shown).

Table I. Cytokine production pattern of T-cell hybridomas established between the Th2 cloned cells and BW5147

T-cell line	IL-2 (U/ml)	IL-4 (U/ml)
BW5147 TCR$\alpha^-\beta^-$	37.0	ND
24-2 (TCR Vβ8$^+$)	ND	640.0
T-cell hybridoma		
H24-2-4 (TCR Vβ8$^+$)	40.0	ND
H24-2-5 (TCR Vβ8$^+$)	113.0	ND
H24-2-66 (TCR Vβ8$^+$)	24.6	ND
H24-2-68-41 (TCR Vβ8$^+$)	1280.0	ND
H24-2-64 TCR$\alpha^-\beta^-$	40.0	ND
H24-2-70 TCR$\alpha^-\beta^-$	34.8	ND
H24-2-74 TCR$\alpha^-\beta^-$	49.5	ND
H24-2-75 TCR$\alpha^-\beta^-$	32.5	ND
2B4.11	689.5	ND
AHK61	7.0	ND
AHK71-23	298.5	ND

Cells expressing TCR were stimulated with the plate-bound anti-TCR mAb. Cells that did not express TCR were stimulated with PMA and ionomycin. ND indicates that lymphokine was not detectable or was below the detectable level.

Table II. Relationship between promoter activity and protein synthesis ability

Cell line	Cell type	IL-4 (U/ml)	IL-4 promoter activity	
			No stimulation	Stimulated
BW5147	thymoma (Th1)	ND	0.0	16.4
EL-4	thymoma (Th0)	24.2	12.4	23.5
68-41	T-cell hybridoma (Th1)	ND	5.2	20.7
MS-SB	cloned T cell (Th2)	294.0	1.4	3.4
P3U1	B cell	ND	0.0	9.1
L929	fibroblast	ND	1.0	1.0
PAM212	keratinocyte	ND	0.4	0.4
1308-1	thymic epithelial cell	ND	0.7	1.0

Cells expressing TCR were stimulated with the plate-bound anti-TCR mAb. Cells that did not express TCR were stimulated with PMA and ionomycin. The data are presented as the percentage activity divided by the luciferase light units obtained from the cells transfected with pSV232-Lu. ND indicates that lymphokine was not detectable or was below the detectable level.

Cell lineage specificity of IL-4 promoter function

We and others have shown that the IL-4 promoter is located within the 5′-flanking sequence of the IL-4 gene between –766 and +63 [pIL-4 (–766)] (Bruhn *et al.*, 1993; Szabo *et al.*, 1993; Tara *et al.*, 1993; Todd *et al.*, 1993; Kubo *et al.*, 1994a). The promoter region was cloned upstream of the luciferase gene in a reporter construct. This was then transfected into a panel of IL-4-producing and non-IL-4-producing cell lines, and IL-4 production and promoter activity were examined (Table II). The promoter activity observed in the non-IL-4-producing cells 68-41 and BW5147 was equivalent to that in the IL-4-producing EL-4 cells. IL-4 promoter activity was observed even in the B-cell line P3U1 when cells were stimulated with a combination of phorbol ester and ionomycin. These results indicated the intact ability of the

IL-4 promoter in the non-producing T and B cells, and suggest that there is a mechanism to regulate the IL-4 promoter activity negatively in Th1-type cells.

In contrast to Th1 and B cells, IL-4 promoter activity was not detected in the fibroblast cell line L929, the keratinocyte cell line PAM212 and the thymic epithelial cell line 1308-1 (Table II). Evidence that overexpression of the active form of calcineurin in L929 cells did not initiate the promoter activity (Kubo *et al.*, 1994a) suggested that the L929 cells might lack target molecules of calcineurin, the IL-4 transcriptional factor NF(P). Indeed, these non-lymphiod cell lines did not express cytosolic NF(P) protein (data not shown). Taken together these results show that the impairment of IL-4 production in non-lymphoid cells was due to a lack of the transcription factor responsible for IL-4 promoter activity.

Localization of cell subset-specific negative regulatory elements

To examine the localization of negative regulatory elements operative in the non-IL-4-producing T cell, four fragments (A, B, C and D) were inserted upstream of the pIL-4(–766) luciferase construct (Figure 1A). The E and F fragments spanned a region from the second intron to the 3′-untranslated region (3′-UTR) and were inserted downstream of the SV40 polyadenylation site of the luciferase gene (Figure 1A). These constructs were transfected into the IL-4-producing thymoma EL-4 and the non-IL-4-producing T-cell hybridoma 68-41, and the transfectants were stimulated with anti-TCR monoclonal antibody (mAb). In comparison with the luciferase activity of pIL-4(–766)Lu, neither the 5′-UTR (fragments A and B) nor the first and second intron (fragment D) inhibited the IL-4 promoter in 68-41 cells (Figure 1B). In contrast, fragments E and F clearly inhibited IL-4 promoter activity in 68-41 cells but not in EL-4 cells (Figure 1C). These results suggested that IL-4 transcription in Th1 cells might be controlled by the negative regulatory elements within a 4.5 kb sequence located between 0.5 kb upstream of exon 3 and 1.2 kb downstream of the 3′-UTR.

To identify the region responsible for negative regulation more precisely, the E fragment was divided further into four fragments by *Pst*I digestion (Figure 2A). Each fragment (E1–E4) was inserted downstream of the SV40 polyadenylation site of the luciferase gene and transfected into EL-4 and 68-41 cells. As shown in Figure 2B, the E4 fragment preferentially inhibited the IL-4 promoter activity in 68-41 cells, indicating that the 1.2 kb sequences of the 3′-UTR were responsible for the Th1-specific negative regulation of IL-4 transcription.

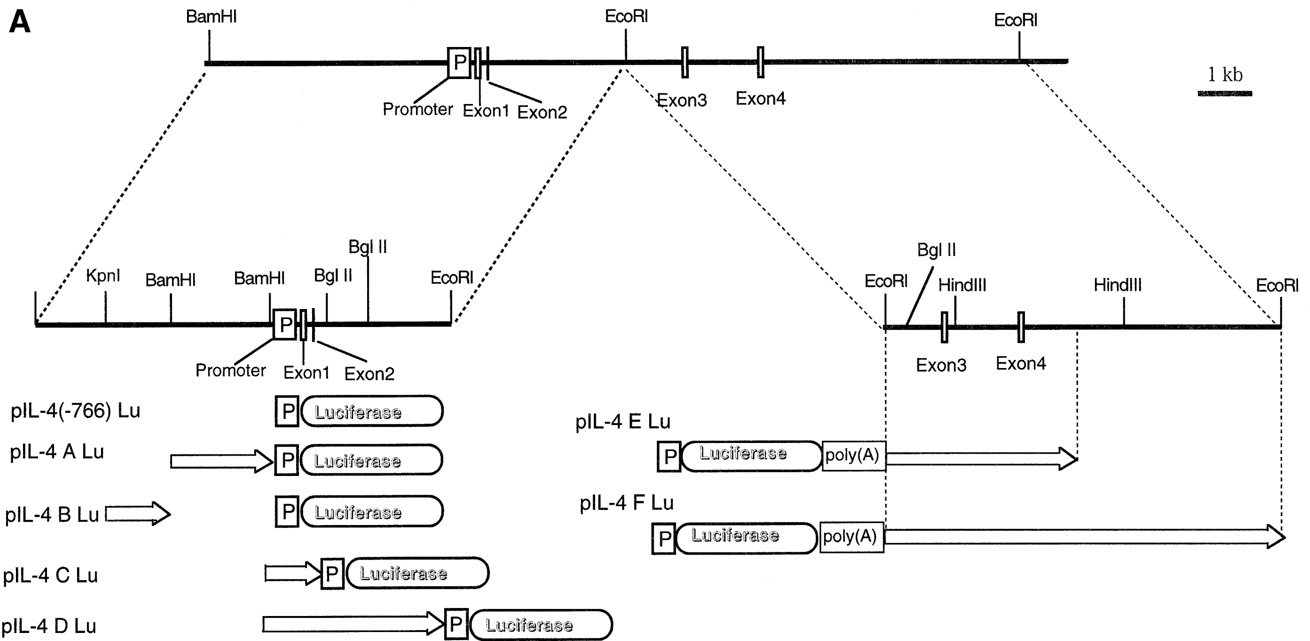
Characterization of the IL-4 silencer

The orientation and position dependence of the IL-4 negative regulatory element was examined by insertion of the 1.2 kb sequence (fragment E4) in either the sense or antisense direction upstream of pIL-4(–766) (Figure 3A). Insertion of E4 upstream of the promoter sequence resulted in inhibition comparable with that seen when the E4 fragment was inserted downstream of the polyadenylation site. Insertion of this fragment in the antisense direction was as inhibitory as insertion in the sense direction. Moreover, the negative regulatory element also acted on the minimal IL-4 promoter, pIL-4(–147). This result suggested that the target of negative regulation by the E4 fragment might be localized between –147 and +65 bp of IL-4 5′-flanking sequences.

Next, we further examined whether this negative regulatory element was able to act on heterologous promoters. The E4 fragment was inserted downstream of the polyadenylation site of the luciferase gene under the control of either the simian virus promoter (pSV) or the IL-2 promoter [pIL-2(–388)]. Figure 3B shows that the negative regulatory element for the IL-4 promoter was also functional against both of the heterologous promoters in 68-41 cells. Thus, the IL-4 negative regulatory element was functional in both an orientation- and position-independent manner and was also capable of acting on heterologous promoters. These results show that this element satisfies the criteria of a silencer element (Brand *et al.*, 1985).

To identify the IL-4 silencer precisely, the nucleotide sequences of the E4 and E3 fragments were determined. Possible binding sites of known transcription factors in the sequence of the 1.2 kb E4 fragment were searched for using the TFMATRIX transcription factor binding site profile database. We have focused mainly on lymphocyte-associated transcriptional factors. Three Ikaros sites (29–42, 1064–1074 and 1105–1148), three OCT sites (61–74, 523–545 and 940–954), two GATA sites (196–205 and 725–734), an Ets site (212–220), a Myc site (232–242), a IL-4 STAT (STAT6) site (338–349) and an IRF-2 site (752–764) were predicted from the sequences (Figure 4). To determine which transcriptional factors bind to the IL-4 silencer region, nuclear proteins were prepared from 68-41, EL-4 and Th2 clone MS-SB cells with or without stimulation by PMA plus ionomycin. Consensus-like sequences of IRF-2, Lyf-1, Ikaros, Oct, GATA, Ets, Myc, CF2-II and IL-4 STAT (STAT6) existing in the IL-4 silencer region were examined by electrophoretic mobility shift assay (EMSA).

There was no nuclear protein capable of binding to the Ikaros/Lyf-1 and IRF-2 site (Figure 5). The nuclear proteins binding to the Myc site, the CF2-II site and the combination site of GATA and Ets were expressed ubiquitously at equivalent levels in both Th1 and Th2 cells (Figure 5). However, there were no transcriptional factors in T cells that bound the IL-4 silencer Ets site because the addition of antibody and competitor for Ets binding had no effect on the EMSA patterns (data not shown). Expression of OCT proteins differed slightly between 68-41 and EL-4 cells (Figure 5). Competition analysis indicated that the upper two bands were OCT specific, but the third band was non-specific (data not shown). EL-4 cells showed higher expression of the second band than did 68-41 cells. Thus, we further studied the expression of these two OCT proteins within other Th1 and Th2 clones. Expression of the second band was also observed in the nuclear preparation from the Th1 clone, 28-4, indicating that there was no preferential difference in OCT protein expression between IL-4-producing and non-producing T cells (Figure 5). Taken together, the expression pattern of these eight nuclear factors appears not to be associated with the silencer function observed in Th1 cells.



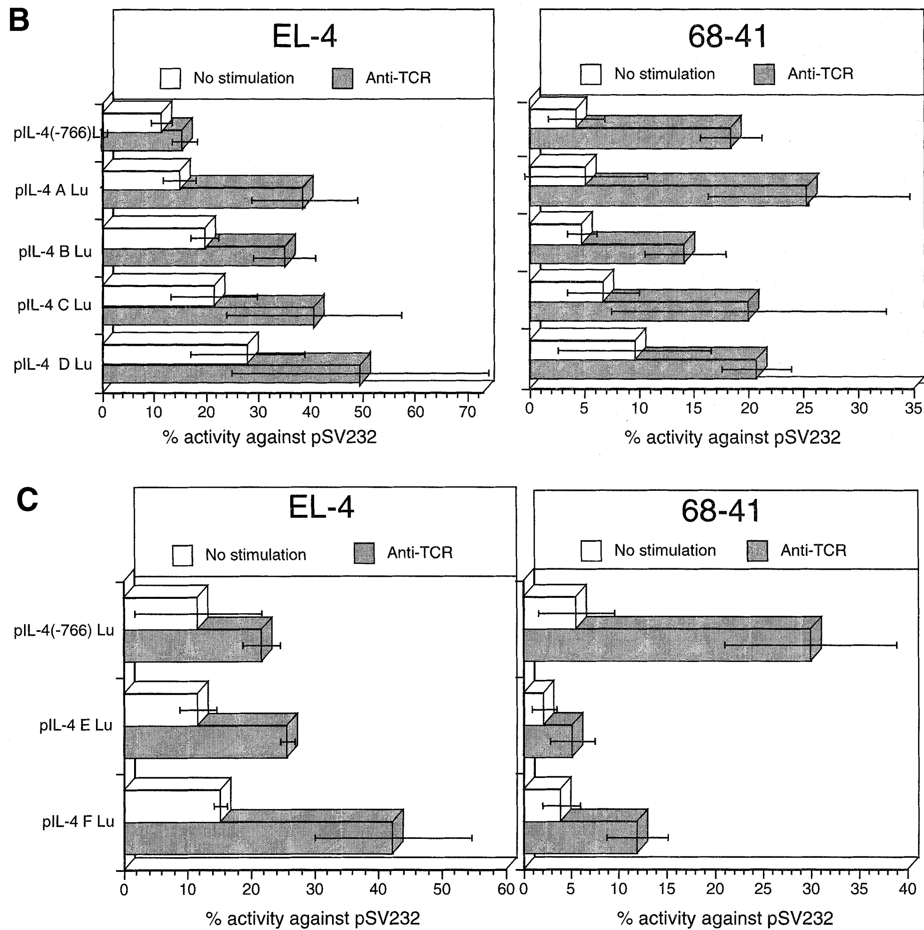


Fig. 1. Identification of a Th1-specific negative regulatory element in the IL-4 gene. (**A**) Genomic map of the IL-4 gene and scheme of the constructions. The left side represents four DNA fragments (A–D) that were prepared by digestion with the restriction enzymes *Bam*HI, *Bgl*II and *Kpn*I from the 5′-flanking sequences of the IL-4 gene. These fragments were inserted upstream of the pIL-4(–766) luciferase construct. The right side represents two fragments (E and F) that were obtained by digestion of the DNA fragment containing introns 1 and 2 with *Sal*I and *Eco*RI. They were inserted downstream of the polyadenylation signal for the luciferase gene controlled by the IL-4 promoter. (**B**) Effect of 5′-flanking and intronic sequences on the IL-4 promoter. (**C**) Effect of intron 1 and the first 3 kb of intron 2 on the IL-4 promoter activity. The pIL-4 reporter constructs containing fragments A–F were transfected into EL-4 and 68-41 cells, and the luciferase activity was measured with or without TCR stimulation. The data are presented as the percentage activity against relative luciferase light units obtained from pSV232-PAP as described in Table II.

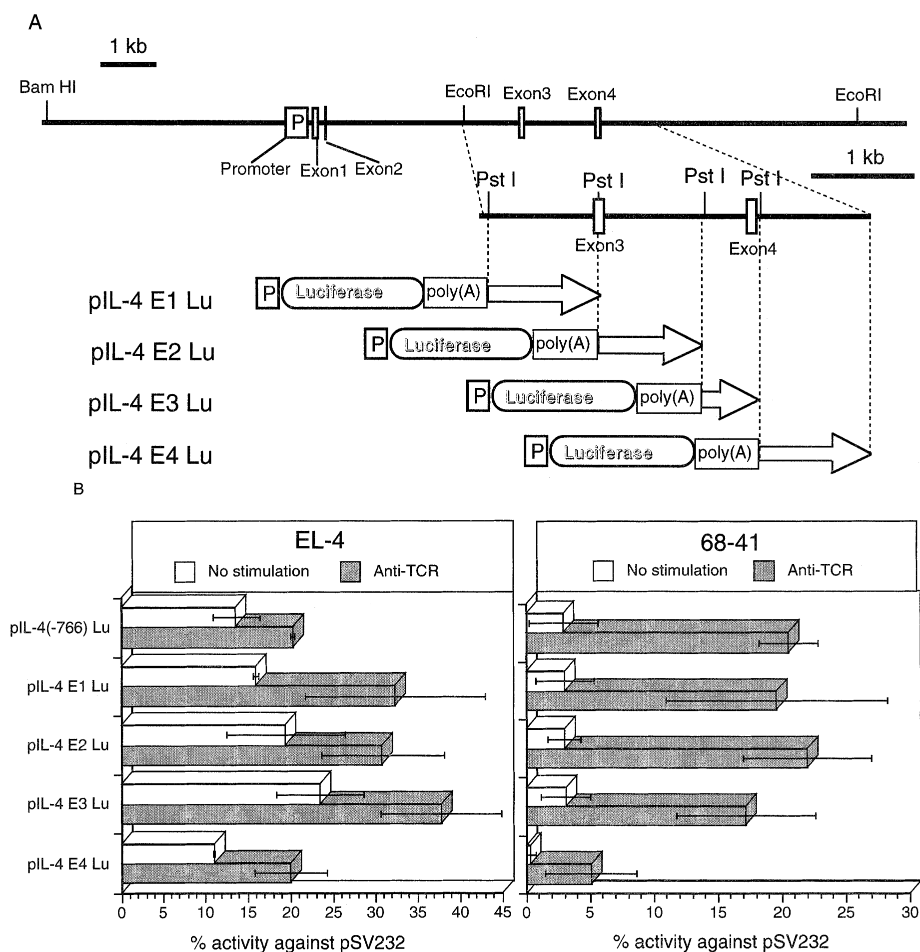


Fig. 2. The Th1-specific negative regulatory element is controlled by the 3′-UTR. (**A**) Scheme of the constructions. The E fragment was divided into four DNA fragments (E1–E4) by digestion with the *Pst*I, and inserted downstream of the polyadenylation signal for the luciferase gene controlled by the IL-4 promoter. (**B**) Effect of the E1–E4 fragments on the IL-4 promoter activity. The pIL-4 reporter constructs containing fragment E1–E4 were transfected into EL-4 and 68-41 cells, and the luciferase activity was measured with or without TCR stimulation. The data are presented as the percentage activity against relative luciferase light units obtained from pSV232-PAP as described in Table II.

Differential nuclear expression of STAT6 between Th1 and Th2 cells

In contrast to these sites, nuclear protein capable of binding to the IL-4 STAT site was expressed preferentially in the IL-4-producing cell lines EL-4, MS-SB and 24-2, but not in the non-producing T-cell hybridoma 68-41 or the Th1 clone 28-4 (Figure 6A). To determine whether this nuclear protein was STAT6, a transcriptional factor involved in IL-4R signaling, a supershift EMSA was performed with anti-STAT6 antibody and competition EMSAs were performed using mutant STAT6 oligonucleotides. The antibody and mutant oligonucleotides were added to mixtures of the IL-4 STAT site oligonucleotides and nuclear protein was obtained from the stimulated Th2 clone, MS-SB, or stimulated EL-4 cells. The addition of anti-STAT6 antibody clearly supershifted the binding of nuclear protein to the STAT6 site but had no effect on the binding to the OCT-1 site (Figure 6B). To examine the binding specificity further, three different mutations were introduced into the silencer STAT6 site (M1–M3 in Figure 6C). These mutant oligonucleotides were added to the mixture of the nuclear preparation from MS-SB and the silencer STAT6 oligo-probe. M1 and M3, containing mutations at the crucial sites for STAT6 binding, were not able to compete with binding of the nuclear protein, while M2 containing a mutation at the irrelevant site did compete (Figure 6C). Moreover, expression of this nuclear protein was induced more efficiently by IL-4 in the presence of concanavalin A, but not by IL-2, IL-3 or INF-γ (Figure 6D). These results indicate that STAT6 located in the nucleus is capable of binding to the IL-4 silencer element, and that the localization of STAT6 in the nucleus was only observed in the IL-4-producing T-cell lines.

Impairment of IL-4R-mediated tyrosine phosphorylation of STAT6 in Th1 cells

The lack of nuclear expression of STAT6 in the Th1 cells may be due either to loss of surface expression of IL-4R or to dysfunction of IL-4R-mediated signals. First, expression of IL-4R was examined by flow cytometric analysis of a panel of Th1 and Th2 cloned cells. All Th1 clones tested here expressed levels of IL-4R equivalent to that of Th2 cells (Figure 7A). However, the ability of IL-4R to function was clearly different between Th1 and Th2 cells (Figure 7B). The IL-4R expressed on the Th2 cells was able to transduce cell growth signals, and the

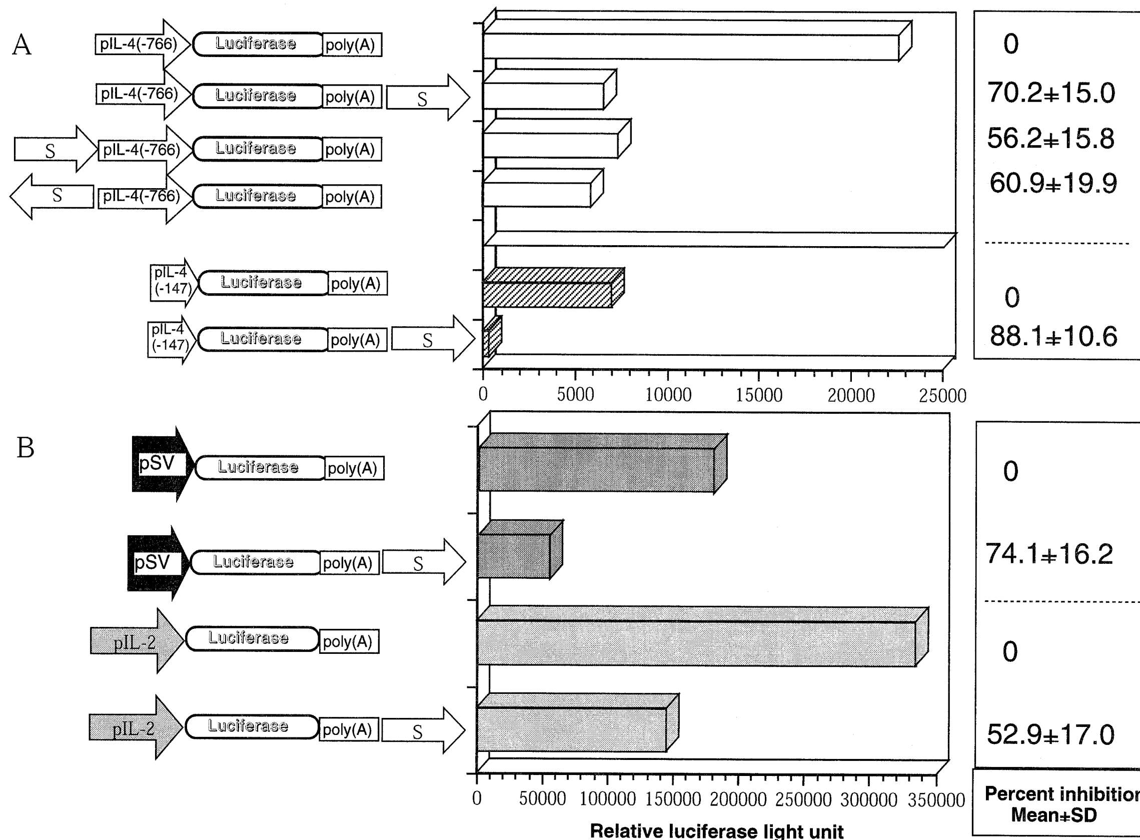


Fig. 3. Function of the Th1-specific negative regulatory element in different orientations and positions, and its function against the heterologous promoter. The fragment containing the negative regulatory element is indicated as S. The fragment was inserted upstream of the IL-4 promoter in sense and antisense orientations. The fragment was also inserted downstream of the polyadenylation signal of the luciferase gene controlled by the minimum IL-4 promoter region [pIL-4(–147)], the simian virus promoter and the IL-2 promoter. These constructs were transfected into 68-41 cells, and luciferase activity was measured with TCR stimulation. The data were collected as the relative luciferase light units, and the percentage inhibition was calculated from the luciferase activity of the construct without the negative regulatory element. Mean and standard deviation of the percentage inhibition were obtained from three independent experiments.

addition of PMA augmented these proliferative responses. In contrast, the Th1 cells were totally unresponsive to IL-4 stimulation even in the presence of PMA, indicating that the IL-4R expressed on Th1 cells had a defect in the ability to initiate a cell growth signal (Figure 7B).

To confirm further that the lack of the nuclear translocation of STAT6 in Th1 cells was due to the impairment of the IL-4R-mediated signaling pathway, we examined the amount of STAT6 protein and the tyrosine phosphorylation status of STAT6 in Th1 cells. The upper panel of Figure 7C shows the presence of STAT6 protein in Th1 cells. As shown in the lower panel, tyrosine phosphorylation occurred within 5 min of stimulation with IL-4 in the Th2 clone 24-2. In contrast, no phosphorylation of STAT6 was observed in the Th1 hybridoma (68-41) or the cloned cell line (28-4), even though the expression of STAT6 protein was equivalent. Since Th1 cells did not show IL-4-dependent cell growth or STAT6 phosphorylation, the IL-4R expressed on Th1 cells is dysfunctional.

Involvement of STAT6 expression in regulation of the IL-4 silencer

The tyrosine phosphorylation and nuclear translocation of STAT6 were only observed in the IL-4-producing T-cell lines. This raised the possibility that STAT6 might play a central role in regulating the silencer function in Th2 cells. Consequently, a point mutation was introduced into the IL-4 E luciferase reporter construct at the position described for the M1 mutant in Figure 6C. This mutant was transfected into three IL-4-producing T-cell lines, EL-4, D10.G4.1 and 24-2, to examine whether or not this mutation modified the IL-4 promoter activity in those cells as observed in Figure 2B. As shown in Table III, the addition of a silencer did not affect the IL-4 promoter activity in the IL-4-producing cells, but the M1 STAT6 mutation in the silencer region overcame the silencer function in all three IL-4-producing cell lines.

Next we examined the effect on the IL-4 silencer function under conditions where nuclear translocation of STAT6 was limited. In Th2 cells, the silencer function might be neutralized by the binding of STAT6 to the silencer region under normal conditions. If this is true, the silencer activity might become apparent even in Th2 cells if the availability of IL-4 is limited. To examine this possibility, three Th2 clones, D10.G4.1, MS-SB and 24-2, were maintained in 100 U of recombinant IL-2 in the presence of anti-IL-4 antibody (11B11) for 1 month. Although the 24-2 cells were not able to proliferate without an IL-4 supplement, the other two lines, MS-SB and D10.G4.1, grew with IL-2 alone. These two lines were then stimulated with anti-TCR antibody to assess IL-4 production and promoter activity. As shown in Figure 8A,

the amount of IL-4 production was decreased to almost one-tenth in MS-SB cells and to half in D10.G4.1 cells as compared with cells cultured in the presence of IL-4.

When the D10.G4.1 cells cultured without IL-4 (D10, no IL-4) and the control D10.G4.1 cells (D10, with IL-4) were transfected with the IL-4 promoter construct [pIL-4(–766)Lu], both lines showed IL-4 promoter activity, although the D10, no IL-4 line had a higher transfection efficiency than D10, with IL-4. However, transfection of the IL-4 promoter construct containing the silencer region (pIL-4 E4 Lu) into the D10, no IL-4 cells indicated the silencing effect, but the same construct did not show any silencing function in the D10, with IL-4 (Figure 8B). We also developed a different mutant construct at the position described for the M3 mutant in Figure 6C in addition to the pIL-4 E4-STAT-M1 Lu. With or without IL-4 supplement, both M1 and M3 mutants consistently reduced the promoter activity in the D10.G4.1 cells, indicating that the loss of the STAT6-binding motif from the silencer region diminished the ability to neutralize the silencer function (Figure 8B).

Furthermore, we examined IL-4 production in Th2 cells transfected with a dominant-negative STAT6 (STAT6ΔC) mutant. This mutant retains DNA and receptor binding activity but does not have transcriptional activation activity because of deletion of the C-terminal amino acids. Therefore, this mutant has transdominant negative activity that directly inhibits the function of STAT6 (Mikita *et al.*, 1996). As shown in Figure 8A, overexpression of STAT6ΔC apparently reduced IL-4 production to one-third, indicating that the inhibition of STAT6 activity might also diminish the ability to neutralize the silencer function. Taken together, the data in Table III and Figure 8 are consistent with our hypothesis that the IL-4 silencer function is inhibited by the binding of STAT6 to the silencer in IL-4-producing cells.

Fig. 4. Nucleotide sequence of the IL-4 silencer and position of the STAT6-binding site in the IL-4 gene. Ex-4 represents exon 4. The TAG in a box represents the stop codon for the IL-4 gene.

Discussion

We have examined the function of the IL-4 promoter in cell lines which do not normally form IL-4 transcripts. The data illustrated that the promoter was functional in Th1 cells. Based on the study of the effect of several different regions upstream and downstream of the IL-4

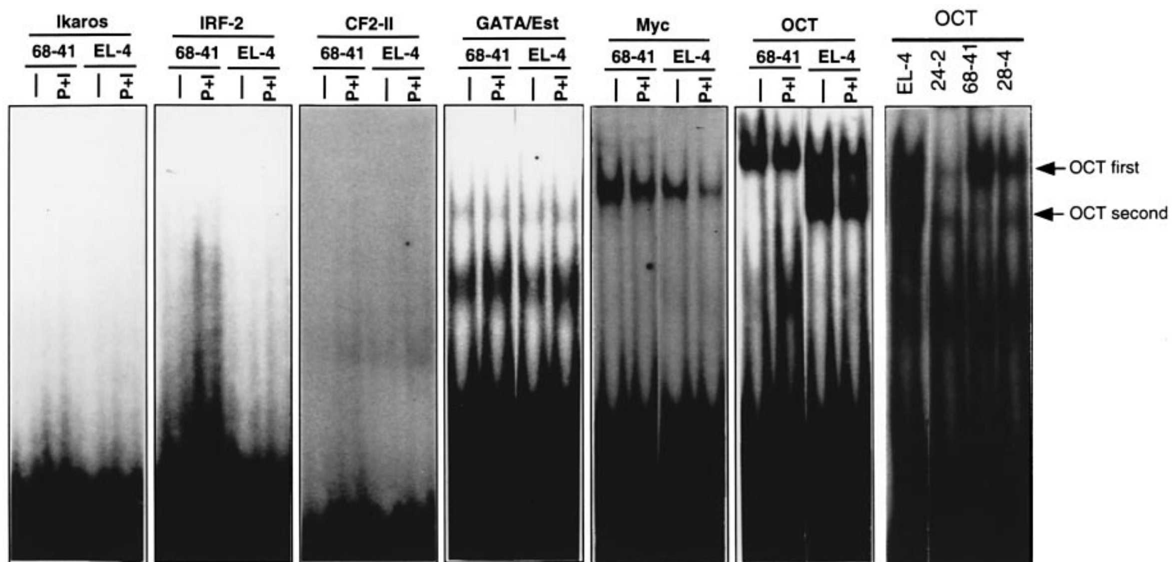


Fig. 5. Gel mobility shift pattern using possible transcriptional factor binding sites within the silencer region. Expression of nuclear proteins binding to Lyf-1/Ikaros, IRF-2, CF2-II, GATA/Ets, Myc and OCT sequences. Nuclear proteins were prepared from EL-4 and 68-41 cells with or without stimulation by PMA and ionomycin (P+I), and were incubated with a panel of end-labeled oligonucleotide probes. The sequences of the probes are described in Materials and methods.

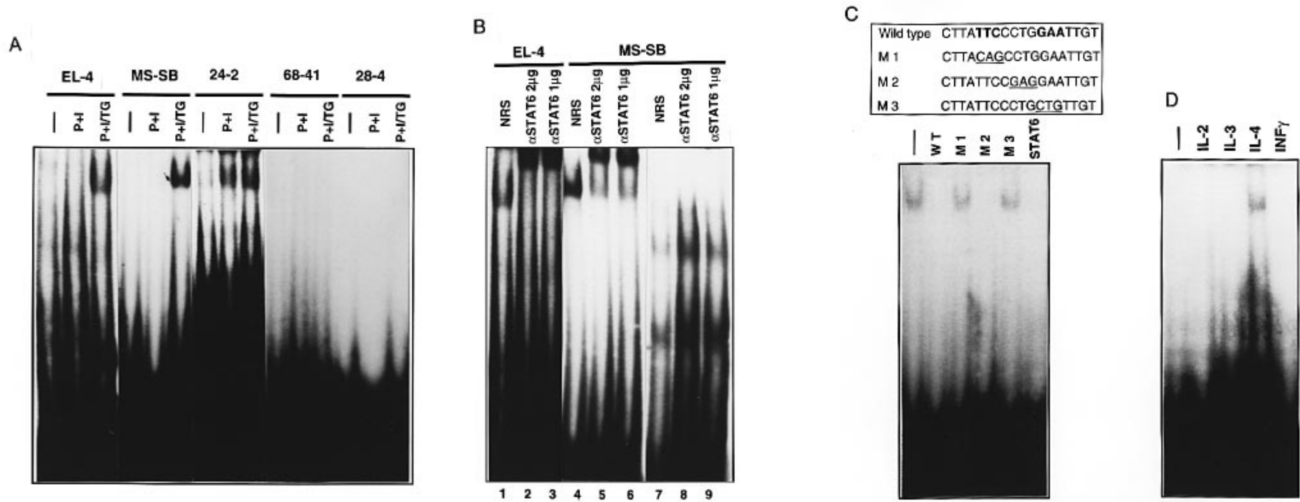


Fig. 6. Preferential binding of STAT6 protein to the silencer region in Th2 cells. (**A**) Nuclear protein binding to STAT6 consensus sequences in the IL-4 silencer. Nuclear proteins were obtained from IL-4-producing cell lines (EL-4, MS-SB, 24-2) and non-IL-4-producing cell lines (68-41, 28-4). Cells were stimulated with PMA plus ionomycin (P+I) in the presence or absence of TCGF (TG). (**B**) Supershift analysis by anti-STAT6 antibody. Nuclear proteins were prepared from the EL-4 and MS-SB cells stimulated with P+I in the presence of TCGF. The proteins were incubated with the probe containing STAT6 consensus sequences in the IL-4 silencer (lanes 1–6) in the presence of anti-STAT6 antibody (lanes 2, 3, 5 and 6). The nuclear proteins obtained from MS-SB were incubated with the OCT consensus (lanes 7–9) in the presence of the same antibody (lanes 8 and 9). (**C**) Competition analysis using mutant oligonucleotides. Mutations were introduced into the STAT6 consensus sequences in the IL-4 silencer (M1–M3), and these oligonucleotides were incubated with the mixture of the nuclear proteins from the stimulated MS-SB cells and the IL-4 silencer STAT6 probe for competition analysis. IgE STAT6 oligonucleotide was added as control competitor in the STAT6 lane. (**D**) Requirement of IL-4 for translocation of STAT6 protein to the nucleus. MS-SB cells were stimulated with concanavalin A in the presence of IL-2 (300 U), IL-3 (30 U), IL-4 (30 U) or INF-γ (200U), and nuclear proteins were extracted. These proteins were incubated with the probe containing STAT6 consensus sequences of the IL-4 silencer.

gene on IL-4 promoter activity, a T-cell subset-specific silencer region was identified in the 3′-UTR. This region contained the binding site for STAT6 which was regulated by the IL-4R signaling pathway, and STAT6 binding was observed preferentially in Th2 cells. This binding process might cancel the silencer function in Th2 cells. On the other hand, the silencer function was conserved in naive T cells and Th1 cells because STAT6 was not activated. In contrast, the IL-4 promoter was not functional in non-lymphoid cells, as observed in non-lymphoid cell lines such as fibroblasts, keratinocytes and epithelial cells. The simplest reason is a lack of the transcriptional factor which is responsible for activating the IL-4 promoter. There seem to be at least two distinct reasons for failure of the IL-4 gene to be expressed in different cell lineages.

The IL-4 promoter is active even in non-IL-4-producing T and B cells (Table II). This is consistent with observations that 6 kb of IL-4 5′-flanking sequences was capable of initiating IL-4 promoter activity in IL-4-producing Th2 cells as well as the INF-γ-producing Th1 cells in transgenic mice (Kamogawa *et al.*, 1993). Thus, IL-4 promoter activity does not reflect the profile of IL-4 mRNA expression among distinct T-cell subsets, and IL-4 transcription must be shut off by unknown mechanisms in Th1 and B cells. To understand the mechanism, hybridoma cell lines were established between the Th1 thymoma cell line, BW5147, and Th2 clones. Surprisingly, all T-cell hybridomas from these fusions had a cytokine production pattern typical of Th1 cells, even though TCR expression was inherited from the Th2 cells (Table I). These results suggested that negative regulation of IL-4 was dominantly inherited from the parental cell, BW5147. Therefore, the T-cell hybridomas were considered a useful tool to explore the molecular basis involved in T-cell subset-specific IL-4 expression (Scupoli *et al.*, 1994).

Based on the above observations, we predicted the presence of an active repressor located in a region distinct from the 5′ promoter region. Our findings strongly supported this hypothesis. A negative regulatory element capable of inhibiting IL-4 promoter activity in Th1 hybridomas was located 1.2 kb downstream of the polyadenylation site (Figure 3). This region inhibited IL-4 promoter activity in an orientation- and position-independent manner. Thus, the negative regulatory element fulfilled the criteria defined for a transcriptonal silencer element (Brand *et al.*, 1985), and knowledge of this silencer should provide new understanding of T-cell subset-specific expression of the IL-4 gene. A recent report demonstrated that repression was not involved in the negative regulation of the IL-4 gene and that expression of the proto-oncogene c-*maf* is responsible for the tissue-specific expression of IL-4 (Ho *et al.*, 1996). The discrepancy with our data may be due to differences in the experimental systems used. T-cell subset-specific IL-4 expression might be determined by the contribution of multiple regulatory elements just as the positive transcriptional regulation is controlled by the coordination of multiple enhancers. c-*maf* expression is one of the mechanisms determining cell specificity of IL-4 transcription. A silencer element identified here illustrates a novel mechanism for determining the cell specificity of IL-4 gene expression regulated by the IL-4R-mediated pathway.

In fact, stage-specific expression of other genes is known to be determined by negative regulatory elements other than the enhancer and promoter regions. Using transgenic mice and transient transfection assays, several

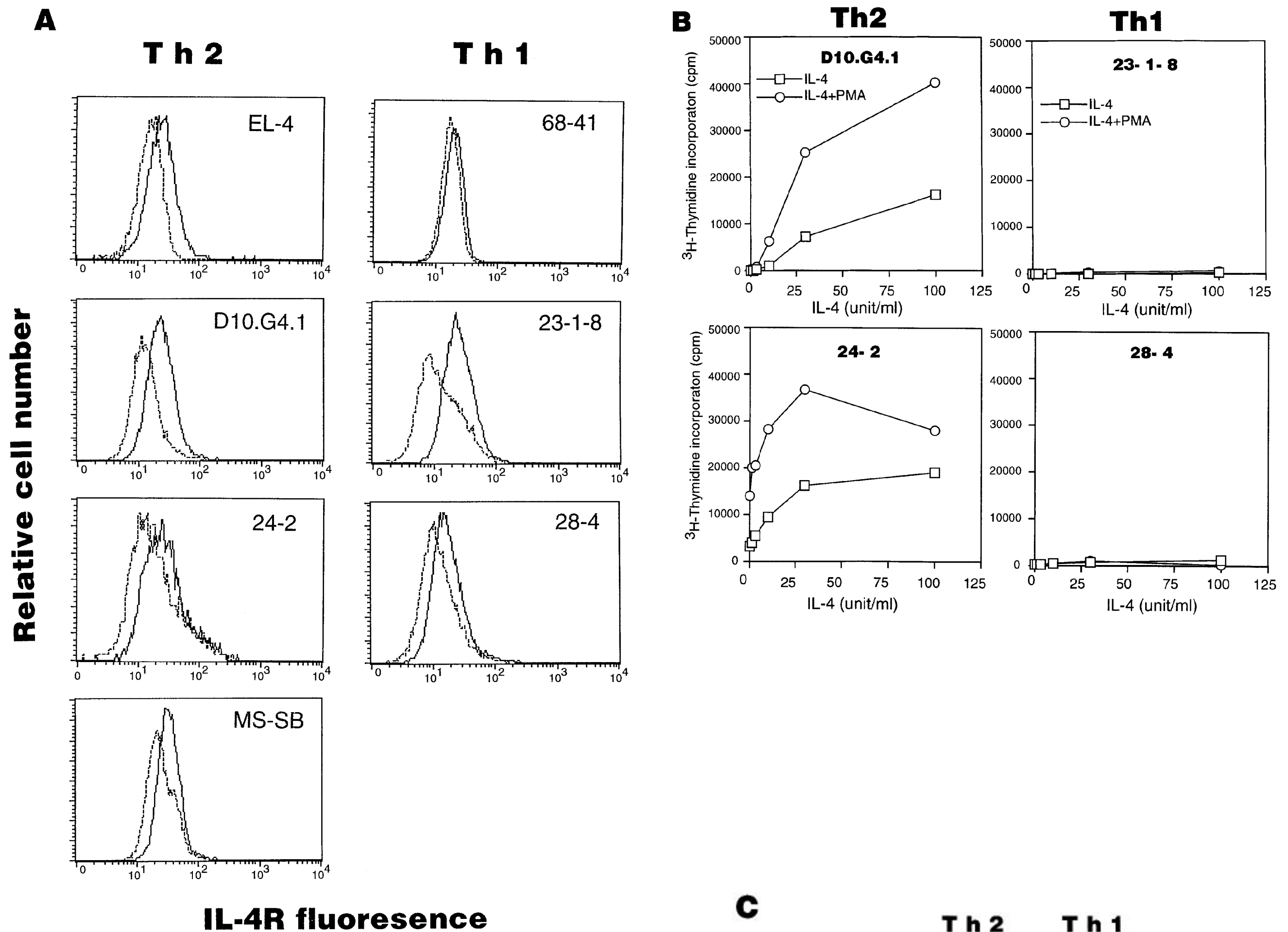


Fig. 7. Expression and function of IL-4R in Th1 cells. (**A**) Cell surface expression of IL-4R on Th1 and Th2 cells. IL-4R expression was analyzed by flow cytometry using rat anti-mouse IL-4R mAb, M1 antibody. The solid line indicates the anti-IL-4R antibody staining. The broken line indicates the FITC-conjugated anti-rat immunogloblin control staining. (**B**) IL-4-dependent proliferative responses. The cloned T cells (4×10^5 cells/ml) were stimulated with various concentrations of rIL-4 in the presence or the absence of PMA (100 ng/ml). After 48 h, proliferative responses were measured as [^{3}H]thymidine incorporation by scintillation counting. (**C**) Expression and tyrosine phosphorylation of STAT6. The top panel represents Western blotting of STAT6. Total cellular lysates of $3–5\times10^7$ cells were prepared from Th1 and Th2 cells, and 100 µg of protein lysates were separated by electrophoresis on 7.5% SDS–PAGE. After transfer to PVDF membrane, blots were probed with anti-STAT6 antibody. The lower panel represents phosphotyrosine blotting. The lysates of 10^7 cells were prepared from cells stimulated with IL-4, and 100 µg of protein lysates were separated by electrophoresis. Blots were probed with anti-phosphotyrosine antibody, PY20, and then stripped and reprobed with anti-STAT6 antibody to confirm the mobility of STAT6.

silencers of transcription were identified in a wide variety of tissue- and developmental stage-specific genes (Herschbach and Johnson, 1993). As observed in several systems, there does not appear to be a general rule regarding the position relationship between a promoter and a silencer. Some silencers such as those of the immunoglobulin κ light chain gene (Sakes and Baltimore, 1993), human β and ε globin genes (Peters *et al.*, 1993; Vitale *et al.*, 1994; Raich *et al.*, 1995) and angiotensin-converting enzyme gene (Goraya *et al.*, 1994) are located adjacent to their promoter. In contrast, the silencers of the TCR α-chain gene (Winoto and Baltimore, 1989), the CD4 gene (Sawada *et al.*, 1994), the tumor necrosis factor (TNF) gene (Kruys *et al.*, 1992), the sucrose isomaltose gene (Markowitz *et al.*, 1995) and the erythropoetin receptor gene (Liu *et al.*, 1994) are located at a distance from their promoter. The IL-4 silencer was found in the 3′-UTR region and was 9 kb away from the 5′ promoter region. Thus, the IL-4 silencer is of the second type. The positional relationship between silencer and enhancer

observed in the IL-4 gene was very similar to that of TNF and the embryonic ζ-globin gene. Those promoters are located in the 5′-flanking sequences, and the silencer elements controlling developmental expression of this gene are located immediately after the polyadenylation site (Kruys *et al.*, 1992; Liebhaber *et al.*, 1996).

Table III. Effect of a mutation at the silencer STAT6 site on IL-4 promoter activity in IL-4-producing cell lines

Reporter construct	IL-4-producing T-cell lines		
	EL-4	D10.G4.1	24-2
pIL-4(–766) Lu	73564	21751	4395
pIL-4 E4 Lu	67902 (7.6)	16816 (22.6)	5048 (0)
pIL-4 E4-STAT-M1 Lu	31905 (56.6)	9326 (57.1)	2637 (40)

The M1 mutation (Figure 6C) was introduced into the STAT6 site of the pIL-4 E4 luciferase construct. Cells were transfected with luciferase test construct and were stimulated with plate-bound anti-TCR mAb. The data are presented as relative luciferase light units. The numbers in parentheses represent the percentage inhibition with respect to the light units of pIL-4(–766) Lu.

Although silencers have been functionally demonstrated in numerous vertebrate genes, the question of how transcriptional silencers act against the target promoter is still unanswered. We found possible binding sites for previously characterized transcriptional factors in the sequences of the 1.2 kb silencer fragment. EMSAs indicated that the nuclear proteins that bind to Oct, GATA, Myc and CF2-II elements were expressed ubiquitously in Th1 and Th2 cells, and the amounts of binding protein were equivalent between the T-cell subsets. Initially, we expected that the transcriptional factors that regulate the IL-4 silencer would be restricted to non-IL-4-producing cells. However, no such factor was found. Thus, we proposed that STAT6 might cancel the silencer activity. In this case, Th1-restricted expression of transcriptional factors that activate the silencer is not required. As shown

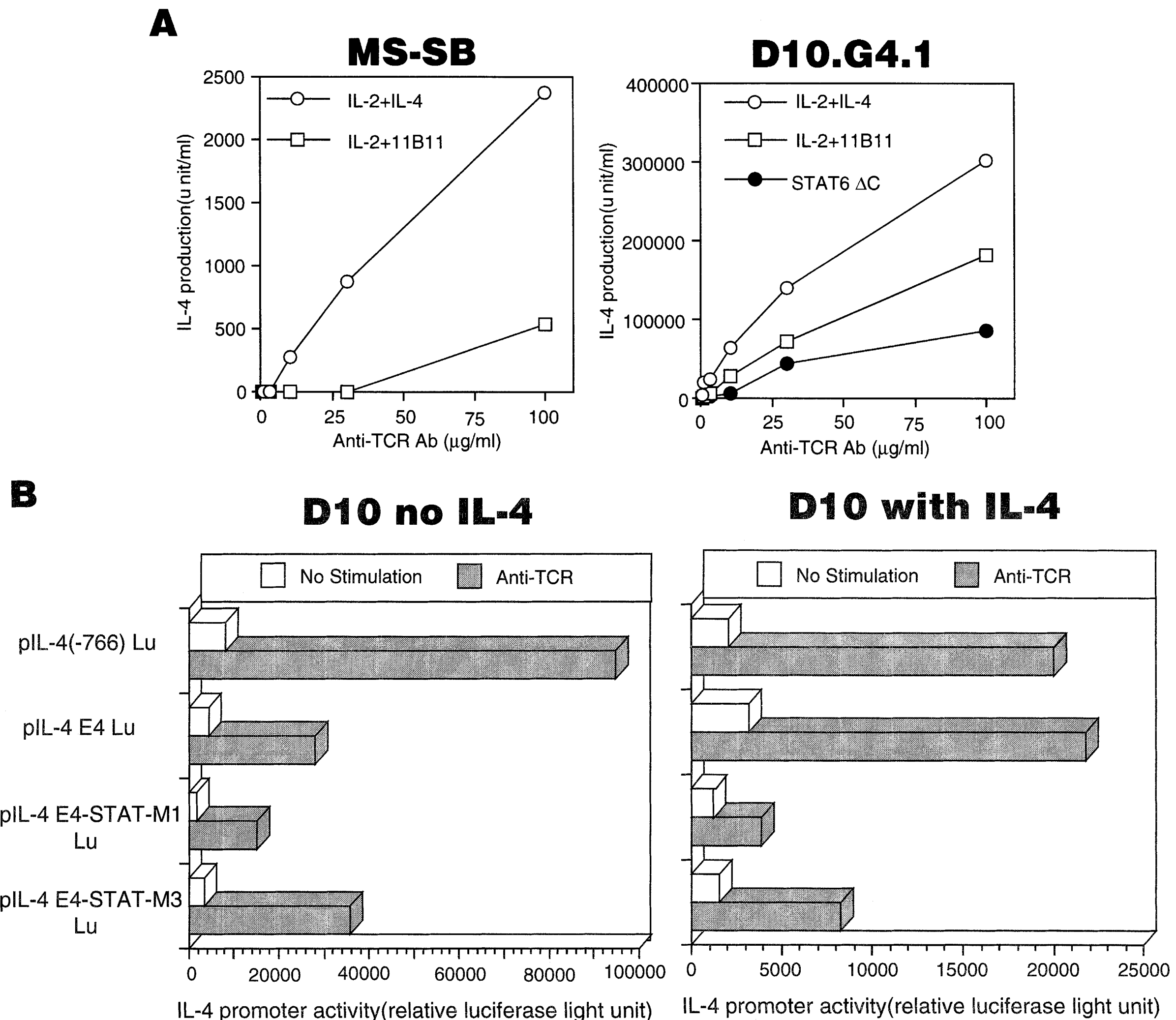


Fig. 8. (**A**) IL-4 production in Th2 cells cultured in the absence of IL-4. D10.G4.1 and MS-SB cells were divided into two groups. One group was cultured with rIL-2 (100 U/ml) and rIL-4 (100 U/ml). The other group was cultured with rIL-2 (100 U/ml) and anti-IL-4 mAb which could neutralize the IL-4 activity of 100 U/ml. These sublines were maintained for 1 month without antigen stimulation. The expression vector containing a transdominant negative form of STAT6 was transfected into D10.G4.1 cells (STAT6ΔC). After TCR expression was confirmed, these sublines and transfectants were stimulated with various concentrations of anti-TCR mAb. After 24 h, supernatants were harvested and the concentration of IL-4 was measured by ELISA. (**B**) Silencer activity in Th2 cells cultured under conditions in which IL-4 supplement is limited. The sublines of D10.G4.1 cells obtained as described above were transfected with the luciferase test construct, and were stimulated with anti-TCR mAb. The data are presented as relative luciferase light units.

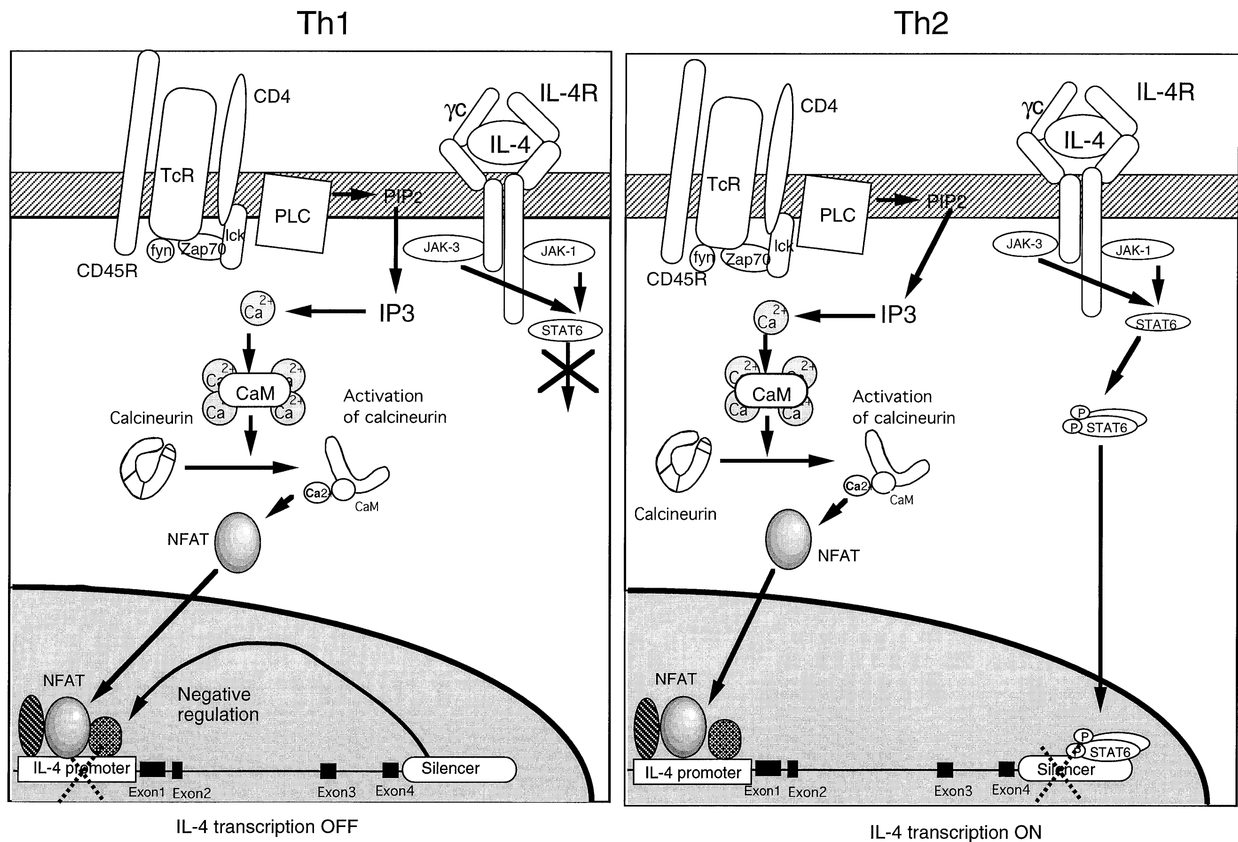


Fig. 9. Scheme of predicted mechanisms of $CD4^+$ T cell subsets.

in Figure 5, many of the transcriptional factors were capable of binding to the silencer region in both Th1 and Th2 cells. The E4 fragment containing the silencer function was divided further into two fragments by *Bst*1107I at the position described in Figure 5. These two fragments were again connected to the 3′ terminus of the IL-4 promoter luciferase construct, and the silencer function was examined in 68-41 cells. The silencer function was only observed in the 5′ end fragment which contained the OCT-, GATA-, Myc- and STAT6-binding motifs (data not shown). Recent reports demonstrated that GATA consensus sequences in the silencer region might be required for the developmental expression of the human ε-globin gene (Peters *et al.*, 1993; Raich *et al.*, 1995) and that the CD4 silencer region (Sawada *et al.*, 1994) also contained several GATA sites. This common occurrence raises the possibility that GATA is essential for the IL-4 silencer function. We did not show directly whether or not the nuclear proteins that bound the GATA/Ets site contained GATA protein.

The IL-4 silencer region contained a consensus sequence specific for STAT6 (IL-4 STAT), and the STAT6 protein was apparently capable of binding to this region. Nuclear localization of this transcriptional factor was observed only in the IL-4-producing but not in the non-IL-4-producing T cell lines. Previous reports indicated that IL-4 and a signal from the IL-4R were essential for commitment of IL-4 to producing the Th2 phenotype (Le Gros *et al.*, 1990; Swain *et al.*, 1990; Hsieh *et al.*, 1992; Seder *et al.*, 1992). However, regulatory elements controlled by the IL-4R have not been identified in the IL-4 gene. Our findings provide direct evidence that STAT6 is associated with transcriptional regulation of the IL-4 gene. Since the relationship between the IL-4 silencer function and the nuclear expression of STAT6 is reversible, we propose the model shown in Figure 9. Most peripheral T cells exhibit the naive T phenotype and do not secrete IL-4 (Hsieh *et al.*, 1992; Seder *et al.*, 1992). In naive T cells, IL-4 transcription is suppressed by the silencer element as shown on the left side of Figure 9. This silencer activity may be conserved even after the naive T cells differentiate into Th1 cells by the action of IL-12. During the course of IL-4-dependent differentiation from naive T cells to Th2 cells, occupancy of the IL-4R might result in tyrosine phosphorylation of JAK1, JAK3 and STAT6 (Kotanides and Reich, 1993; Hou *et al.*, 1994; Park *et al.*, 1995). Activated STAT6 then translocates into the nucleus and binds to the target sequences located at the IL-4 silencer region as we demonstrated in this study. We propose that the binding of STAT6 to the silencer region modifies the silencer function such that IL-4 transcription may commence after the appropriate activating signals are received at the IL-4 promoter. In fact, IL-4 gene expression in Th2 cells was significantly affected by inhibition of the function of STAT6 and of its binding (Table III, Figure 8). These results support the hypothesis that the modification of silencer function by STAT6 may be essential for determination of the Th2 phenotype (Figure 9, right). In contrast, Th1 cells have a defect in IL-4R-mediated signaling that leads to STAT6 activation, even though the expression of IL-4R and STAT6 in Th1 cells is equivalent to that in Th2 cells (Figure 7). This suggests that a defect in the activation of STAT6 might conserve the silencer function. A similar situation was reported previously in Th2 cells that have a defect in IL-12 receptor-mediated

signaling and receptor expression (Jacobson *et al.*, 1995; Szabo *et al.*, 1995). Moreover, our model is consistent with recent observations in STAT6 knock-out mice which show that STAT6 is essential for the expression of IL-4 mRNA as well as for the development of Th2 cells (Kaplan *et al.*, 1996; Shimoda *et al.*, 1996). Thus, the binding of STAT6 to the IL-4 silencer element may be a critical step in driving the commitment from naive T cells to Th2 cells.

Materials and methods

Plasmid construction

Mouse genomic IL-4 was isolated from a C57BL/6 liver genomic DNA library (Stratagene, La Jolla, CA) by plaque hybridization using ^{32}P-labeled mouse IL-4 cDNA. Two major IL-4 DNA fragments of 9.8 and 8.5 kb were isolated from the library. The 9.8 kb fragment contained 5 kb upstream from the IL-4 promoter, the first intron and part of the second intron. The 8.5 kb fragment covered from the second intron, 0.5 kb upstream of exon 3, to 4 kb of 3′-UTR. Four fragments (A–D) were obtained by digestion of the 9.8 kb DNA with the restriction enzymes *Bam*HI, *Bgl*II and *Kpn*I. Two fragments (E and F) were obtained by digestion of the 8.5 kb fragment with *Sal*I and *Eco*RI. Each fragment was subcloned into pBlusescript II SK(–) (Stratagene). The pIL-4(–766) and pIL-4(–147) promoter regions were amplified by PCR and inserted into the pGL-2 vector (Promega, Madison, WI). Fragments A–D were inserted upstream of the pIL-4(–766) promoter construct. Fragments E and F were inserted into the *Sal*I and *Bam*HI sites downstream of the SV40 polyadenylation signal for the luciferase gene controlled by the IL-4 promoter.

The mouse IL-2 luciferase construct was established by PCR amplification of the IL-2 promoter region from genomic DNA of the T-cell hybridoma 68-41. The PCR product encoding from –388 to +5 of the IL-2 5′-flanking region was inserted into a *Sac*I site of the pGL-2 vector (Kubo *et al.*, 1994a). A basic vector, pGL-2, and a simian virus enhancer/promoter luciferase plasmid pSV-Lu were purchased from Promega (Madison, WI). A simian virus enhancerless luciferase plasmid pSV232-Lu was provided by Roche Biosciences Inc. Placental alkaline phosphatase controlled by the simian virus promoter, pSV2A-PAP (Henthorn *et al.*, 1988), was obtained from Dr T.Kadesch (Department of Human Genetics and Howard Hughes Institute, University of Pennsylvania School of Medicine, Philadelphia, PA). An expression construct of the dominant-negative form of STAT6 was obtained from Dr U.Schindler (Tularik, Inc., South San Francisco, CA) (Mikita *et al.*, 1996).

Cell lines, cytokines and antibodies

Two thymoma cell lines, EL-4 and BW5147, and a plasmacytoma B-cell line, P3U1, were provided by Dr Y.Asano (Department of Immunology, University of Tokyo, Tokyo, Japan). Three immature B-cell lines, Ig6.3, 46-6 and 7OZ, a mature B-cell line, WEHI-231, a fibroblastoma cell line, L929, a keratinocyte cell line, PAM212, and a thymic epithelial cell line, 1308-1, were obtained from Y.Hashimoto (Syntex-Roche, Chiba, Japan).

Four clones, MS-SB, 24-2, 28-4 and 23-1-8 have been established from C3H or B6C3F1 mice as described elsewhere (Nakayama *et al.*, 1988). MS-SB is an I-A^k-restricted autoreactive Th2 clone. 24-2 is a TCR Vβ8$^+$, I-A^b-restricted, KLH-specific Th2 clone. 28-4 and 23-1-8 are H-2^k-restricted, KLH-specific Th1 clones. D10.G4.1 (a I-A^k-restricted *p*-azobenzene arsonate-ovalbumin-specific Th2 clone) was generously provided by R.Abe (RIBS, Science University of Tokyo). Most T-cell hybridoma lines were established by fusion between the TCR$\alpha^-\beta^-$ BW5147 and 24-2 cloned cells. The T-cell hybridomas AHK61 and AHK71-23 were established between BW5147 and splenic T cells obtained from B10.A(4R) mice immunized with B10.A(2R) (Hoshi *et al.*, 1994). T-cell hybridoma 2B4.11 was provided by T.Saito (Chiba University School of Medicine, Chiba, Japan).

Rat T-cell growth factor (TCGF) was prepared from 48 h concanavalin A-stimulated rat spleen cell supernatant and was used as a source of IL-2, IL-4 and INF-γ. Recombinant mouse IL-2, IL-3 and IL-4 were obtained from high titer culture supernatants from myeloma cell line X63-Ag8.653 transfected with the appropriate cDNA. Recombinant mouse INF-γ was purchased from Genzyme (Cambridge, MA). Anti-STAT6 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Transduction Laboratory (Lexington, KY).

Measurement of IL-2 and IL-4 activity

Cells (2.5×10^5 cells/ml) were stimulated with either plate-bound anti-TCR mAb (100 μg/ml) or PMA (50 ng/ml) and ionomycin (0.5 μM). The culture supernatant was harvested after 24 h and the biological activity of IL-2 and IL-4 was measured by the mouse indicator cell line, CTLL-2. IL-2 and IL-4 activities were distinguished by the addition of anti-IL-4 mAb, 11B11 (Ohara and Paul, 1985), and anti-IL-2 mAb, S4B6 (Mosmann *et al.*, 1986), respectively. IL-2 and IL-4 production were assessed as units, defined as the reciprocal dilution required to give 50% of the maximum response. In Figure 8A, IL-4 was measured by cytokine ELISAs (Pharmingen, San Diego, CA), and the ELISAs were performed as described previously in Kuchroo *et al.* (1995).

Transfection and luciferase assay

Cells were co-transfected with 5 μg of the test construct and 5 μg of pSV2A-PAP by electroporation. The PAP plasmid was used for normalizing the transfection efficiency. Each experimental group included pSV232-Lu and pGL-2 plasmid-transfected samples. pGL-2 was used for subtracting the background luciferase activity from all samples. The pSV232-Lu was used as an internal control to compare each different experiment. All data, except those of Figure 4, are presented as the percentage activity divided by the luciferase activity obtained from uninduced cells transfected with pSV232-Lu. The transfected cells were stimulated for 12–14 h with various stimuli at 24 h after transfection. Following stimulation, cells were harvested and divided into two groups. Then, 20% of the cells were used for measurement of alkaline phosphatase activity, and the rest of the cells were used for the measurement of luciferase light units.

After harvesting, cells were extracted with cell lysis reagent [25 mM Tris-phosphate, 2 mM dithiothreitol (DTT), 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol and 1% Triton X-100]. The extracts were mixed with luciferase substrate solution (Promega, Madison, WI), and the emitted light was measured with a luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Extraction of nuclear and cytoplasmic proteins

To prepare nuclear proteins, 5×10^7 EL-4 , 68-41 and MS-SB cells were cultured in the presence or absence of PMA (50 ng/ml), ionomycin (1 μM) and 10% TCGF for 2 h, and washed three times with phosphate-buffered saline (PBS). Then 1.5 ml of hypotonic buffer [10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 30 μg/ml leupeptin, and 1 mM benzamin] was added to the cell pellet and incubated on ice for 10 min. The cell membranes were disrupted with 0.15% NP-40, and disruption was judged by trypan blue staining. The nuclei were centrifuged at 2000 r.p.m. for 10 min. The pellet of nuclei was resuspended in extraction buffer (50 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, 30 μg/ml leupeptin and 1 mM benzamin] containing 0.3 M $(NH_4)_2SO_4$. After incubation at 4°C for 30 min with rocking to extract the nuclear proteins, the nuclear proteins were centrifuged at 100 000 r.p.m. for 10 min. The nuclear proteins in the supernatant were precipitated with 1.5 M $(NH_4)_2SO_4$ and centifuged at 50 000 r.p.m. for 5 min.

To prepare cytoplasmic protein, 5×10^7 cells were washed three times with PBS. Hypotonic buffer containing 0.15% NP-40 was added to the cell pellet and incubated on ice for 10 min. After centrifugation at 2000 r.p.m. for 10 min, the proteins were precipitated with 1.5 M $(NH_4)_2SO_4$ and centrifuged at 50 000 r.p.m. for 5 min. The pellet of nuclear or cytoplasmic proteins was aliquoted in the extraction buffer, and was dialyzed against the extraction buffer at 4°C. The protein concentration of nuclear and cytoplasmic extracts was estimated using the Bio-Rad Protein Assay kit.

EMSAs and synthetic oligonucleotides

The nuclear or cytoplasmic proteins were incubated with 1×10^4 c.p.m. of end-labeled oligonucleotide probe for 30 min at room temperature in 22 μl of binding buffer [20 mM Tris, pH 8.0, 60 mM KCl, 2 mM $MgCl_2$, 0.3 mM DTT, 12% glycerol and 2 μg of poly(dI–dC)–(dI–dC)]. The samples were loaded on a 4% polyacrylamide gel and run in low ionic strength buffer. The sequences of oligonucleotide probes were as follows: mouse IL-4 NF(P), TGTAATAAAATTTTCCAATGTAAACT-CATT; mouse IL-2 OCT, AAAATATGTGTAATATTGTAAAACATCG-TTGACA; IL-4 silencer STAT6, AGAAAACTTATTCCCTGGAATTGT-

CCTCTG; IL-4 silencer CF2-II, ATATATATATATATATATATATATATAT-ATATATATATAT; IL-4 silencer IRF-2, CTATAAAAGTGGAAAGAG-AAAATCAACTCT; IL-4 silencer Ikaros/Lyf-1, AAGCCCAGGCTATT-CCCAGTGTGTTCTCTC; IL-4 silencer GATA/Ets, GGAGCTGATGCT-CTCCTCTGGCCTCCTGTG; IL-4 silencer Myc, GAGTACACAGAC-CACATGCCTGTAGGCAAG; IgE STAT6, CCAAGCTAACTTCCCA-AGAACAGAATCAAA.

Flow cytometry analysis

Cells were suspended in PBS supplemented with 2% fetal calf serum (FCS) and 0.1% sodium azide. In general, 10^6 cells were incubated on ice for 30 min with anti-IL-4R mAb (Genzyme, Cambridge, MA), and stained using a standard method as described previously (Nakayama *et al.*, 1988).

SDS–PAGE and Western blot analysis

Total cellular lysates of 10^7 cells were prepared as described in Greenlund *et al.* (1994) and separated by electrophoresis on 7.5% SDS–PAGE. After electrotransfer to PVDF membrane, blots were probed with anti-phosphotyrosine antibody, PY20. Blots were stripped with the phosphate buffer containing 2% SDS and 0.1 M 2-mercaptoethanol and reprobed with anti-STAT6 antibody (Transduction Laboratory, Lexington, KY).

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