Regulation of IL-10 Gene Expression in Th2 Cells by Jun Proteins


J Immunol 2005; 174:2098-2105; doi: 10.4049/jimmunol.174.4.2098
http://www.jimmunol.org/content/174/4/2098

References
This article cites 29 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/174/4/2098.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Regulation of IL-10 Gene Expression in Th2 Cells by Jun Proteins

Zheng-Yu Wang, Hiroshi Sato, Saritha Kusam, Sarita Sehra, Lisa M. Toney, and Alexander L. Dent

IL-10 is a key regulatory cytokine produced by T lymphocytes. Although Th2 cells are a major source of IL-10, little is known about IL-10 gene regulation in Th2 cells. High levels of IL-10 mRNA transcription are induced in the Th2 clone D10 after PMA plus ionomycin (P/I) stimulation; however, we found that the IL-10 promoter was not inducible by P/I in D10 cells. We therefore sought regulatory regions in the IL-10 gene that could promote P/I-activated transcription in Th2 cells. Two strong DNase I-hypersensitive sites (DHSSs) were identified in the IL-10 gene in mouse T cells, and conserved noncoding sequences (CNSs) between the mouse and human IL-10 genes were also identified. One IL-10 DHSS maps within or next to a highly conserved CNS region, CNS-3. The CNS-3 region contains an AP-1 site that binds JunB and c-Jun proteins specifically in Th2 cells and not in Th1 cells. The CNS-3 element activates transcription from the IL-10 promoter after P/I stimulation and is responsive to c-Jun and JunB. Retroviral mediated-expression of either c-Jun or JunB in primary T cells led to a large increase in IL-10 expression, and inhibition of AP-1 activity by a dominant negative form of c-Jun in primary T cells strongly repressed IL-10 expression. IFN-γ was relatively unaffected by modulations in AP-1 activity. These data indicate that we have identified a novel regulatory element that can specifically activate transcription of the IL-10 gene in Th2 cells via the AP-1/Jun pathway. The Journal of Immunology, 2005, 174: 2098–2105.

Materials and Methods

Cell lines and primary T cells

The D10.G4.1 Th2 clone and RAW264.7 cells were obtained from American Type Culture Collection. The Th1 clone AE7 was obtained from Dr. G. Sui (Columbia University, New York, NY). The EL4 thymoma and NIH-3T3 cells were derived from the laboratory of Dr. L. Staudt, National Cancer Institute. Primary Th1 and Th2 cells were derived as previously described.

Abbreviations used in this paper: CNS, conserved noncoding sequence; CHIP, chromatin immunoprecipitation; DHSS, DNase I-hypersensitive site; P/I, PMA plus ionomycin; oligo, oligonucleotide; RSB, reticulocyte standard buffer.
described (13). Primary NKT cells were obtained by sorting NK1.1^+TCRb^+ cells from liver mononuclear cells as previously described (14). Freshly isolated NKT cells were stimulated with anti-CD3 Ab and expanded as previously described (15).

**Analysis of IL-10 expression and mRNA stability**

RNA and cDNA were prepared as previously described (16) from cells either left untreated or stimulated with PMA (20 ng/ml; Sigma-Aldrich) plus ionomycin (0.3 μM) for 4 h. RT-PCRs were run for 25 cycles to ensure linear amplification. All primers and oligonucleotides (oligos) were purchased from Invitrogen Life Technologies.

The following are primer sequences for RT-PCRs: IL-10, 5'-CTGGCTGCTCTTACTGAC-3' and 5'-CCGAGAGAGGCTCAAACAGA GG-3'; β-tubulin, 5'-CAGGGCGCAAGTGGTGCAAC-3' and 5'-GCTCTCATTATGTACACAGATTG-3'; and IFN-γ, 5'-TA GCCAAGACTGTGATTGCGG-3' and 5'-AGCAGATTTATGCTTAC TGCG-3'.

For mRNA stability, actinomycin D (10 μg/ml; Sigma-Aldrich) was added to cells either left untreated or stimulated with PMA (20 ng/ml) plus ionomycin (0.3 μM) for 4 h. RNA was prepared from the cells at various time points after actinomycin D treatment, and cDNA was synthesized and analyzed for IL-10 and GAPDH mRNA levels by real-time PCR. Quantitative real-time RT-PCRs were conducted on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using a SuperScript III Two-Step qRT-PCR Kit with SYBR Green (Invitrogen Life Technologies) and following the manufacturer's protocols.

The primers for IL-10 were 5'-ATGCTCTCAGAGTCTGGAGCTACT-3' and 5'-CTCGCATTAAAGGCGTGTTAGT-3'. The primers for GAPDH were 5'-CCAGGTGTGCTCTGAGACT-3' and 5'-ATACACGAGAAATCCTTGCAAG-3'.

IL-10 and GAPDH PCRs were performed in separate wells to avoid contaminate and the data were averaged. The levels of IL-10 mRNA were normalized to the levels of GAPDH mRNA.

**Luciferase assays and constructs**

Luciferase assays were performed on cell lysates from cells electroporated with plasmid constructs as previously described (17). The full-length IL-10 promoter (-1538 to +64) luciferase construct (10) was a gift from Dr. S. Smolen (University of California, Los Angeles, CA). The IL-4 promoter luciferase construct was obtained from Dr. M. Kaplow (Indiana University School of Medicine, Indianapolis, IN) and contains 250 bp of the proximal mouse IL-4 promoter. The CNS-3 IL-10 promoter construct was prepared by inserting the CNS-3 DNA fragment into the full-length IL-10 promoter luciferase construct via MluI and XhoI. The CNS-3 fragment was prepared by PCR from mouse genomic DNA and cloned into the pcR2.1-TOPO vector (Invitrogen Life Technololgies). MluI and XhoI sites were derived from the the pcR2.1-TOPO vector. The following primer sequences were used for generation of the mouse CNS-3 element (100 bp in length) by PCR: 5'-GTGGTCATTTTTTCAGTAAGACC-3' (Δ35 bp) probe, 5'-GGTGGTCATTTTTTCAGTAAGACC-3' and 5'-GCTCTCATTATGTACACAGATTG-3' (Δ50 bp) probe, 5'-GTGGTCATTTTTTCAGTAAGACC-3' and 5'-GATTGTACCAATTTCACTGAG-3' (Δ75 bp) probe, 5'-AATTCCTACGTAGGTC-3' and 5'-GCTCTCATTATGTACACAGATTG-3'.

**DNase I hypersensitivity assay**

The assays for DNase I hypersensitive sites (DHHs) on genomic DNA were performed essentially as previously reported (18). Briefly, cells were collected and either left unstimulated or stimulated with immobilized anti-CD3 Ab (10 μg/ml; BD Pharmingen; Th1, Th2, and NKT cells) for 3 h or with PMA (20 ng/ml) plus ionomycin (0.3 μM; D10, AE7, and NIH-3T3 cells) for 6 h. After stimulation, cells were washed twice with ice-cold PBS and resuspended in ice-cold radiolabeled standard buffer (RSB; 10 mM Tris-Cl (pH 7.4), 10 mM NaCl, and 5 mM MgCl2). An equal volume of RSB containing 0.5% Nonidet P-40 was added, cells were homogenized (DOUNCE; Kontes), then washed in ice-cold RSB and divided into 100-μl aliquots. Each aliquot was treated with 0, 2.5, and 5.0 μg/ml DNase I ( Worthington Biochemical) at 37°C for 12 min. Then, an equal volume of 2X stop solution (1% SDS, 20 mM Tris-Cl, 600 mM NaCl, 10 mM EDTA, and 0.5% sodium deoxycholate) was added, and cells were incubated at 37°C overnight. genomic DNA was extracted and purified by phenol/chloroform extraction and digested with Sau3A plus XhoI or with EcoRI (all from Roche). Each digest (10 μg/lane) was separated on a 0.8% agarose gel, blotted, and probed with 32P-labeled probes. The probes were designed from the mouse IL-10 gene genomic sequence (GenBank accession no. AL513351 and M84340), generated by PCR, and subcloned using pcR2.1-TOPO vector. Primers for the EcoRI probe (273 bp) were 5'-TAAGACTT TCCGCTTACAGACC-3' and 5'-TGCCATATGTCCTCCCCCTATTAC-3'. Primers for the 3'-probe (482 bp) were 5'-GCAAGCAAAACAGAAAG CAAAC-3' and 5'-CTGAGGGTTAGGAGGTCGAAATG-3'. DHSS assays were repeated several times, and representative experiments are shown in the figure.

**CNS analysis**

Comparison of CNSS between mouse and human IL-10 genes was performed using the VISTA program (19), located at www.gs.dlb.gov/vista/.

**Gel shifts and oligonucleotides**

EMSA was performed as previously described (16), using 32P-labeled blunt-ended DNA probes formed by annealing two complementary oligos or by PCR from mouse genomic DNA. Polyclonal Abs to c-Jun and JunB as well as control rabbit IgG were obtained from Santa Cruz Biotechnology.

The CNS-3 number 2 probe used in Fig, 5 is the same 100-bp fragment used for cloning into the pcR2.1-TOPO vector, as described above. The primer sequences used for the generation of the other probes in Fig, 5 (via PCR from the cloned CNS-3 probe) are: 4 (75 bp) probe, 5'-CCAGGTGTGCTCTGAGACT-3' and 5'-CCCTACTATCCTACAGAC-3'; Δ3 (75 bp) probe, 5'-GGTGGTCATT TTTTCAGTAAGACC-3' and 5'-GCTCTCATTATGTACACAGATTG-3'; Δ5 (50 bp) probe, 5'-GTGGTCATT TTTCAGTAAGACC-3' and 5'-GATTGTACCAATTTCACTGAG-3'; Δ75 (50 bp) probe, 5'-AATTCCTACGTAGGTC-3' and 5'-GCTCTCATTATGTACACAGATTG-3'. The oligo competitors used in Fig. 6 (sense strands alone shown) are: AP-1 consensus (12.5, 25, and 50 ng), 5'-GCGGTGTTAGCCTCCACGCG-3'; and AP-1 mutant (12.5, 25, and 50 ng), 5'-GCTCTCATTATGTACACAGATTG-3'. Each gel shift assay was repeated at least once; representative results are shown in the figure.

**Chromatin immunoprecipitation (CHIP)**

The CHIP assay was performed essentially as previously described (20), with the same Abs used for supershift analysis above. CNS-3 DNA was detected by PCR with the same primers as used to prepare the CNS-3 number 2 probe in Fig. 5. PCR products (100 bp) were analyzed on a 3% agarose gel. CNS-1 DNA (a 100-bp product) was detected by PCR with the following primers: 5'-AATTCCTACATTAGCTTCCAG-3' and 5'-AGGATTTGCAACCGGGATG-3'.

**Retrovirus infection**

The c-Jun- and JunB-expressing retroviruses were constructed by inserting c-Jun and JunB cDNAs (17) into the retroviral vector pMEGS (16) via NotI and XhoI sites. The Jun dominant negative vector was made by first preparing a version of c-Jun with the first 122 aa deleted. This cDNA was amplified by PCR from human c-Jun (17) with the following primers: sense, 5'-AAAAA-GAATTC-ATGACTAGCCGACACAGCCTGCCA GGTCC-3'; and antisense, 5'-AAAAA-GAATTC-ATGACTAGCCGACACAGCCTGCCA GGTCC-3'. The antisense primer adds an hemagglutinin tag to the C terminus of c-Jun. The PCR product was then cut with EcoRI and cloned into the retroviral vector pMEGS.

Mouse T cells were infected and sorted for GFP expression as previously described (21). Each individual retrovirus infection was performed with 20 million Con A-infected T cells, and the percent infection was 5-10%. At least one million retrovirus-infected cells of each type were sorted for analysis. The sorted T cells were activated with anti-CD3 Ab, and supernatants were measured for IL-10 and IFN-γ levels by ELISA as previously described (21).

**Results**

**Expression of IL-10 in T cells**

Previous studies of the regulation of IL-10 expression in T cells have used EL4 thymoma cells, which are considerably different from mature CD4 Th cells (10, 11). We therefore compared the expression of IL-10 mRNA in a cloned Th2 cell line, D10, with IL-10 mRNA produced by EL4 cells and the cloned Th1 cell line AE7. Before and after stimulation of the cells with P1, IL-10...
mRNA expression was assayed by RT-PCR (Fig. 1A). After stimulation, D10 Th2 cells produced abundant levels of IL-10 mRNA, whereas IL-10 mRNA was barely detected in EL4 cells. The AE7 Th1 cell line did not produce detectable IL-10 mRNA. Both EL4 and AE7 produced elevated IFN-γ mRNA after P/I stimulation. These data show that IL-10 is expressed in Th2 cells at very high levels compared with other types of T cells. To verify that the pattern of IL-10 expression seen in the cloned Th1 (AE7) and Th2 (D10) cell lines is representative of normal Th1 and Th2 cells, we assayed for IL-10 mRNA expression in primary T cells that were cultured under Th1 or Th2 skewing conditions. Because IL-10 is also produced by NKT cells, we examined IL-10 expression in primary NKT cells isolated from liver. We found that IL-10 was produced at very high levels by primary Th2 cells, at even higher levels by NKT cells, and not at all by primary Th1 cells (Fig. 1A).

Both primary Th1 and NKT cells produced elevated IFN-γ mRNA after CD3 stimulation. We concluded from these data that the D10 cell line, primary Th2 cells, and NKT cells all express similar high levels of IL-10 mRNA after stimulation.

We next addressed whether IL-10 expression in D10 cells is controlled by a post-transcriptional mRNA stability mechanism similar to what was observed in EL4 and RAW cells (12). We therefore treated unstimulated and P/I-stimulated D10 cells with actinomycin D, which blocks new transcription, at specific time points to examine the stability of IL-10 mRNA. Endogenous IL-10 mRNA expression was assayed by quantitative real-time PCR (Fig. 1B). In contrast to what was observed with EL4 and RAW cells (12), IL-10 mRNA was very stable in both unstimulated and P/I-stimulated D10 cells. Although IL-10 mRNA was slightly less stable in unstimulated than in stimulated D10 cells, this difference in stability cannot account for the strong increase in IL-10 mRNA after P/I stimulation. These results suggest that inducible IL-10 expression in D10 cells is not controlled to a significant extent by differential mRNA stability, but, rather, that the induction of IL-10 expression after P/I stimulation is mediated by increased transcription.

Because P/I stimulation increases IL-10 transcription in Th2 cells, we analyzed the ability of P/I stimulation to activate transcription from the IL-10 promoter. For this analysis, we used the full-length (1538-bp) IL-10 promoter previously found to have full activity in EL4 and RAW264.7 cells (10, 11). When a luciferase construct driven by this IL-10 promoter was electroporated into D10 cells, we found that P/I treatment did not stimulate transcription from the full-length IL-10 promoter; rather, P/I treatment repressed the promoter activity to a significant degree (Fig. 2). In contrast, the IL-4 promoter was activated over 10-fold after P/I stimulation of D10 cells. These data indicate that the IL-10 promoter is not activated by P/I stimulation, and that the induction of IL-10 mRNA after P/I stimulation requires regulatory elements outside the IL-10 promoter. We therefore decided to look for distal regulatory regions in the IL-10 gene that could drive the high level of IL-10 transcription in Th2 cells.

**Altered IL-10 chromatin in IL-10-producing cells**

To locate distal regulatory regions in the IL-10 gene, we analyzed the IL-10 gene locus for the presence of DHSSs, because these sites typically denote the altered chromatin found at regulatory regions of a gene. We initially characterized the presence of IL-10 DHSSs in D10 cells and found one strong DHSS, DHSS I, in unstimulated D10 cells and a second DHSS, DHSS II, that was induced in P/I-stimulated cells (Fig. 3). Both IL-10 DHSSs I and

---

**FIGURE 1.** Regulation of IL-10 expression in T cells. A, RT-PCR analysis of IL-10 and IFN-γ mRNA in different T cell populations before and after stimulation with P/I. N.D., not done. B, Analysis of IL-10 mRNA stability in D10 cells after actinomycin D (Act. D) treatment. IL-10 mRNA levels were quantitated by real-time PCR and normalized to GAPDH mRNA levels. The graphs show IL-10 levels at 0, 3, and 6 h of Act. D treatment in unstimulated or P/I-stimulated D10 cells. The level of IL-10 mRNA at 0 h was designated 1 for unstimulated and stimulated conditions; mRNA levels at 3 and 6 h are relative to the 0 h mRNA level. Error bars represent the SD. The data shown are representative of two separate experiments.

**FIGURE 2.** Analysis of IL-10 and IL-4 promoter activity in Th2 cells. D10 cells were transiently transfected via electroporation with luciferase reporter genes driven by either 1.5 kb of the IL-10 promoter or 250 bp of the IL-4 promoter. The electroporated cells were harvested after 24 h. P/I stimulation was initiated 6 h before harvest. Error bars represent the SE.
II were also observed in AE7 Th1 cells and primary NKT cells, but were not found in NIH-3T3 fibroblasts, indicating that the IL-10 DHSSs are cell type specific and possibly restricted to T cells. The IL-10 DHSS II showed a variable level of expression; it was induced in D10 cells after P/I stimulation, but was present in both unstimulated and stimulated AE7 and NKT cells. These data indicate that the IL-10 gene chromatin is modified in cells that transcribe IL-10, and that this modification can be induced by cellular activation in the case of DHSS II. These two DHSSs in the IL-10 gene mark potential regulatory regions for control of IL-10 gene transcription.

Conserved noncoding sequences in the IL-10 gene

With the availability of the complete sequences of the mouse and human genomes, novel regulatory regions can be found by the identification of CNSs. CNSs are regions of high sequence identity between mouse and human DNA found outside of gene-coding regions. CNS analysis has been successful in the identification of an important regulatory region, CNS-1, that controls expression of the Th2 cytokines IL-4, IL-5, and IL-13 (22, 23). We therefore wondered whether the identification of CNS regions in the mouse and human IL-10 genes would lead to the discovery of novel IL-10 regulatory elements. With the VISTA computer program (19), we aligned the mouse and human IL-10 genes to find highly conserved regions of DNA (Fig. 4). Using a cut-off of 75% sequence identity or higher, we identified four CNS regions in the IL-10 gene excluding the promoter region. CNS-3, located ~1 kb downstream of the IL-10-transcribed region, showed the greatest homology between mouse and human IL-10 genes, with 84% sequence identity (Fig. 4). Strikingly, CNS-3 localizes to approximately the same region of DNA (within 200 bp) as the DHSS I site we observed in the IL-10 gene.

Identification of Jun factors binding to CNS-3

From our previous data, we hypothesized that CNS-3 participated in the control of IL-10 expression in Th2 cells. We therefore tested whether CNS-3 DNA could bind to nuclear factors expressed in Th2 cells using a gel-shift assay (Fig. 5). We found three protein complexes (A, B, an C) in D10 Th2 cell extracts that bound strongly to the CNS-3 probe. We next characterized these CNS-3 binding factors A, B, and C by systematically deleting the CNS-3 probe to localize which regions of DNA bound the different factors. Using nuclear extracts from D10 cells, we localized factors A, B, and C to discrete regions of CNS-3 (Fig. 5). Factor B was of particular interest, because its expression increased after P/I stimulation. We then identified a canonical AP-1 site in the middle of the CNS-3B binding region (Fig. 6). Using a gel-shift competition assay, we assessed whether the AP-1 site is important for binding of factor CNS-3B. We were able to strongly compete away CNS-3B binding with an unlabeled consensus AP-1 binding site probe, but not an unlabeled probe containing a mutated AP-1 site (Fig. 6, lanes 1–7). These data strongly suggested that the CNS-3B binding factors were AP-1 or AP-1-like proteins. Next, we used Abs to c-Jun and JunB, AP-1 proteins expressed in activated T cells, to determine whether the CNS-3B DNA binding complex contained c-Jun or JunB (Fig. 6, lanes 8–11). We found that the CNS-3B complex contained both c-Jun and JunB. Although anti-c-Jun Ab strongly augmented the CNS-3B gel-shift band, this effect appeared to be nonspecific, because control IgG caused the same effect (Fig. 6, lanes 10 and 11). Because Abs to c-Jun and

FIGURE 3. DNase I hypersensitivity analysis of the mouse IL-10 gene. Nuclear DNA preparations were treated with two different titrations of DNase I indicated by the triangle above each Southern blot. A, Analysis of DHSS in D10 genomic DNA digested with SwaI plus XhoI using the IL-10 3’ probe. B, Analysis of DHSS in genomic DNA from the indicated cell types digested with EcoRI using the IL-10 Eco probe.

FIGURE 4. Genomic organization of the mouse IL-10 gene and identification of CNSs. The histogram plot shows the percent DNA sequence identity between mouse and human IL-10 genes. Major CNS regions were defined as 75% or greater identity between mouse and human genes over at least 100 bp. The positions of the DHSS from Fig. 3 are indicated.
JunB only shifted a fraction of the CNS-3B band, it is likely that the CNS-3B complex contains other AP-1 or AP-1-like proteins as well.

Next we tested whether c-Jun and JunB bind to the CNS-3 region in vivo. We therefore performed CHIP from primary Th2 and primary Th1 cells with control IgG, anti-c-Jun, and JunB Abs (Fig. 7). We found that both c-Jun and JunB bind to the CNS-3 region in Th2 cells, but not in Th1 cells. Although binding of c-Jun and JunB was weakly observed in unstimulated Th2 cells, the binding was greatly enhanced by P/I stimulation, consistent with the idea that the levels of these factors increase upon P/I stimulation. CHIP of c-Jun and JunB did not bring down the IL-10 CNS-1 region, which does not contain an AP-1 site, showing specificity to the binding of these proteins. Because Th1 cells express both c-Jun and JunB (data not shown), the fact that these proteins do not bind to the CNS-3 region in Th1 cells suggests there is a Th2 cell-specific mechanism that controls binding of Jun proteins to CNS-3 DNA in vivo.

**Functional activity of CNS-3 and activation of IL-10 by Jun proteins**

We next tested whether the CNS-3 element we characterized was capable of activating transcription. We constructed a luciferase construct with the CNS-3 element cloned upstream of the 1.5-kb

---

**FIGURE 6.** Gel-shift analysis of nuclear factors binding to the CNS-3 region. A. Schematic of the CNS-3 region, showing the probe design and mapping of CNS-3 binding factors. B. Gel shifts using nuclear extracts from D10 cells, with and without P/I stimulation, with the indicated probes. C. Sequence of CNS-3, showing the alignment between mouse and human sequences. Factor binding regions are indicated.

**FIGURE 7.** Binding of c-Jun and JunB to the CNS-3 region in vivo. CHIP assays were performed on primary Th1 and Th2 cells using control Abs, anti-c-Jun Abs, or anti-JunB Abs. Cells were assayed in the unstimulated condition or after P/I stimulation for 4 h. IL-10 CNS-1, and CNS-3 DNA were amplified from DNA obtained from the CHIP assays via PCR. INPUT shows amplification of CNS-1 and CNS-3 DNA from Th1 and Th2 cells before CHIP. Each sample was assayed by 3-fold serial dilutions (A) of the CHIP or INPUT DNA, followed by PCR. The experiment shown was repeated twice, with similar results each time.
IL-10 promoter. When electroporated into D10, cells we found that in contrast to the IL-10 promoter alone, the CNS-3-IL-10 promoter construct was able to stimulate transcription in response to P/I by >2-fold (Fig. 8A). These data indicate that the CNS-3 element can mediate activation of IL-10 expression in Th2 cells after P/I stimulation. Mutating the AP-1 site in the CNS-3 element destroyed the ability of this element to activate transcription, showing the key nature of the AP-1 site. We next tested the ability of Jun proteins to activate the IL-10 promoter, with and without the CNS-3 element (Fig. 8B). Luciferase expression plasmids were electroporated into D10 cells along with either c-Jun or JunB expression plasmids in the absence of P/I stimulation. Similar to what we observed with the IL-10 promoter alone after P/I stimulation, the IL-10 promoter alone was repressed significantly by both c-Jun and JunB proteins. In contrast, the CNS-3-IL-10 promoter construct was activated significantly by the expression of c-Jun and JunB. The expression of JunB led to greater activation than that of c-Jun, which is consistent with the greater binding of JunB to the CNS-3 element detected by CHIP. JunB therefore either may bind to the CNS-3 AP-1 site more efficiently than c-Jun or may cooperate with other factors more effectively in terms of transcriptional activation.

Next, we tested the ability of c-Jun and JunB to activate the expression of the endogenous IL-10 gene in T cells. For this assay, we used retroviruses expressing c-Jun and JunB to infect primary mouse T cells activated under neutral conditions, and then sorted infected cells on the basis of GFP expression. After isolation of the infected cells, we stimulated them with anti-CD3 and tested for secretion of IL-10 and IFN-γ by ELISA (Fig. 9A). We found that overexpression of either c-Jun or JunB in primary T cells increased IL-10 expression by several-fold, whereas IFN-γ expression was increased <2-fold. When the increase in IL-10 was calculated relative to IFN-γ, c-Jun and JunB proteins were able to activate IL-10 expression by 4-fold (Fig. 9B). We could not detect IL-4 expression from any of the retrovirus-infected T cells (data not shown), suggesting that the increase in IL-10 expression was not secondary to increased Th2 differentiation driven by the Jun proteins. Although JunB can promote Th2 differentiation in a transgenic system (24), JunB does not have this function in our retrovirus system. This may be due to the fact that IL-4 expression activated by JunB is particularly important during the earliest stages of T cell

![Figure 8](https://example.com/fig8.png)

**Figure 8.** Functional activity of the CNS-3 region and activation of IL-10 expression by Jun proteins. A. D10 cells were transiently transfected by electroporation with a luciferase construct driven by the full-length IL-10 promoter plus either the wild-type CNS-3 element or a CNS-3 element with the AP-1 site mutated. Luciferase activity was measured after 24 h, with and without P/I stimulation. P/I stimulation was performed for 6 h before cell harvest. B. D10 cells were transfected by electroporation with luciferase plasmids driven by either the full-length IL-10 promoter alone (top graph, □) or the full-length IL-10 promoter plus the CNS-3B element (lower graph, ■). Luciferase activity was measured after 24 h without P/I stimulation.

![Figure 9](https://example.com/fig9.png)

**Figure 9.** Jun proteins drive IL-10 expression in primary T cells. Primary mouse T cells activated with Con A were infected with retroviruses expressing GFP alone (GFP-cont), GFP-c-Jun, or GFP-JunB. Retrovirus-infected cells were sorted on the basis of GFP expression. Sorted T cells were cultured for 4 days in IL-2, then stimulated with plate-bound anti-CD3 Ab. IL-10 and IFN-γ levels in the supernatant were measured by ELISA (A). None of the T cells expressed significant levels of IL-4 (data not shown). The ratios of the levels of IL-10 and IFN-γ are shown for the respective T cell populations (B). The data shown are representative of four different experiments for c-Jun and two different experiments for JunB.
were measured by ELISA. The ratios of the levels of IL-10 and IFN-γ expression in T cells (26). We constructed a c-Jun dominant negative version of c-Jun, which interferes with AP-1 function by binding to other AP-1 proteins and creating a nonactivating DNA binding complex (25). Dominant negative-expressing retrovirus and infected primary T cells with the virus in a similar manner as in Fig. 9. We found that inhibiting AP-1 function with the c-Jun dominant negative protein resulted in a 6-fold suppression of IL-10 expression, on the average (Fig. 10). In contrast, IFN-γ expression was not significantly affected by inhibiting AP-1 activity.

Our data with retrovirus-infected primary T cells strongly support the idea that Jun proteins bind to the IL-10 CNS-3 region and that this leads to augmented IL-10 transcription.

**Discussion**

In this study we have analyzed IL-10 gene expression in Th2 cells and have identified a novel regulatory element in the IL-10 gene, CNS-3, that binds and is activated by Jun proteins. The CNS-3 nucleotide sequence is highly conserved between mouse and human IL-10 genes and localizes close to a strong T cell-specific constitutive DHSS in the IL-10 gene. The CNS-3 element is bound by Jun proteins in Th2 cells, but not in Th1 cells; therefore, binding of CNS-3 by Jun proteins correlates with the high levels of IL-10 produced by Th2 cells. Forced expression of either c-Jun or JunB strongly augments IL-10 expression in primary T cells, whereas inhibition of AP-1/Jun activity decreases IL-10 levels. The IL-10 promoter is not activated by AP-1/Jun proteins (Fig. 8) (10), whereas endogenous IL-10 expression is activated by retrovirus-mediated expression of c-Jun and JunB. These data support the idea that Jun proteins bind to the CNS-3 element and augment the transcription of the IL-10 gene in T cells after stimulation with P/L. Although Jun proteins may regulate IL-10 transcription via regulatory elements other than CNS-3, the fact that we have demonstrated binding of c-Jun and JunB to CNS-3 specifically in Th2 cells supports an important role for CNS-3 in controlling Th2-specific IL-10 gene expression.

Jun/AP-1 proteins have been implicated as important regulators of Th2 cytokine expression and differentiation (24, 27). AP-1 transcriptional activity is increased in Th2 cells compared with Th1 cells, and the AP-1 protein JunB is expressed at higher levels in Th2 cells than in Th1 cells (24, 27). Moreover, JunB has been shown to cooperate with c-Maf in promoting IL-4 transcription in T cells (24). Transgenic expression of JunB could promote IL-4 expression in T cells even in the presence of Th1-polarizing conditions (24). The same study also showed that transgenic JunB increased IL-10 expression in T cells in the presence of Th1-polarizing conditions (24). Thus, these data on JunB and IL-10 fit well with our findings that Jun/AP-1 proteins can promote the expression of IL-10. Moreover, because JunB is up-regulated in Th2 cells, this helps to explain why IL-10 is expressed at much higher levels in Th2 cells than in Th1 cells. Jun/AP-1 proteins can therefore be seen as critical positive regulators of two key Th2 cytokines: IL-4 and IL-10.

Our results also show that the identification of DHSSs and CNS elements has strong utility in finding regulatory regions of cytokine genes. Transcriptional regulation of the key cytokine, IL-4, has benefited greatly from analysis of DHSS (18, 28, 29). Studies of IL-4 gene regulation also led to the identification of a critical CNS between the IL-4 and IL-13 genes, CNS-1, which was identified as a coordinate regulator of Th2 cytokine expression and differentiation (24, 27). In this study we have identified two previously unknown DHSS in the mouse IL-10 gene that are likely to participate in the control of IL-10 transcription. We have also identified four CNS regions in the IL-10 gene. Similar to the CNS-1 region in the IL-4/IL-13 locus (22, 23), the IL-10 CNS-3 overlaps with the site of a strong DHSS. By analogy with the IL-4/IL-13 CNS-1, the IL-10 CNS-3 region is likely to participate in the control of IL-10 transcription. Although the IL-4/IL-13 CNS-1 element did not activate transcription in a transient transfection assay (22), the CNS-1 is extremely important in the context of genomic DNA, as revealed by both
transgenic and knockout approaches. CNS-1 probably plays an important role in controlling the structure of chromatin around the IL-4 and IL-13 genes. In contrast to the IL-4/IL-13 CNS-1, the IL-10 CNS-3 element is able to activate transcription in a transient transfection assay. We have found that the isolated CNS-3B element can activate transcription in a transient transfection assay, whereas the isolated CNS-3A and CNS-3C elements cannot (data not shown). Thus, the transcriptional activity of the CNS-3 element is most likely due to the canonical AP-1 binding site localized to the CNS-3B binding region. However, the CNS-3A and CNS-3C elements may still contribute to CNS-3 function, such as through modification of chromatin, similar to the IL-4/IL-13 CNS-1 element. A full analysis of CNS-3 function will therefore require identification of the CNS-3A and CNS-3C binding factors as well as transgenic and knockout experiments.

Previously we found that the transcriptional repressor BCL-6 regulates IL-10 in T cells (21). IL-10 is increased in BCL-6/− T cells, and BCL-6 can repress IL-10 expression when expressed by retrovirus in primary T cells. However, little was known about the regulation of IL-10 expression in T cells, and we did not understand the mechanism for repression of IL-10 by BCL-6. With the results of this study, however, we have a potential mechanism for the regulation of IL-10 expression by BCL-6 in T cells. In a separate study we found that BCL-6 inhibits transcriptional activation by AP-1 proteins (17). Thus, BCL-6 may inhibit IL-10 expression by dampening AP-1-mediated activation of IL-10 transcription. This idea is strengthened by the finding that IL-10 is up-regulated even in BCL-6/− Stat6−/− T cells (21), which implies that BCL-6 regulates IL-10 by a mechanism independent of the Th2 regulatory factor Stat6.

Although this study has revealed new mechanisms for the control of IL-10 by Th2 cells, much work remains for understanding how IL-10 transcription is regulated. Specifically, future work will focus on the in vivo function of the CNS-3 element as well as other CNS elements in the IL-10 gene.

Acknowledgments

We gratefully acknowledge the receipt of reagents and advice from Dr. Steven Smale, University of California-Los Angeles. We would acknowledge help from Shail Govani in constructing the JunB retrovirus construct.

References