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# A distal *cis*-regulatory element, CNS-9, controls NFAT1 and IRF4-mediated IL-10 gene activation in T helper cells

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ABSTRACT

IL-10 is a multifunctional cytokine that plays a critical role in maintaining the balance between immunity and tolerance. Previously, we identified proximal regulatory elements and alterations of chromatin structure in the IL-10 gene loci of Th1 and Th2 cells. We have now characterized a crucial *cis*-regulatory element, CNS-9, located 9 kb upstream of the transcription start site in IL-10 gene loci. The CNS-9 region is highly conserved in vertebrate genomes, and contains clustered NFAT and IRF binding motifs. *In vitro* binding of NFAT1 and IRF4 to the CNS-9 region was observed by EMSA. Furthermore, Th2-preferential *in vivo* binding of NFAT1 and IRF4 to the CNS-9 region was observed by ChIP. Cyclosporine A treatment on wild type Th2 cells or Th2 cells derived from NFAT1 knockout ($NFAT1^{-/-}$) mice showed significantly reduced *trans*-activity of CNS-9. The Th2 subset-specific enhancer activity of CNS-9 was upregulated synergistically by NFAT1 and its partner IRF4. Mutations in the binding sites for NFAT1 and IRF4 abrogated its enhancer activity of CNS-9. Collectively, our results establish crucial roles for enhancer element CNS-9, and NFAT1 and IRF4 that bind to it, for IL-10 expression in differential T helper subsets.


## 1. Introduction

IL-10 is an immuno-regulatory cytokine with potent effects on circulating and resident immune cells as well as epithelial cells (Moore et al., 2001). IL-10 is produced by many cell types, such as T helper cells, regulatory T cells, B cells, monocytes, macrophages and dendritic cells. It stimulates NK cell activity and B cell growth, but suppresses inflammation-associated immune responses (Pestka et al., 2004). IL-10-deficient mice develop inflammatory bowel disease, thus emphasizing the protective role of this cytokine in inflammatory and autoimmune conditions (Kuhn et al., 1993). Paradoxically, however, IL-10 stimulates polyclonal B cell activation and so has a pathogenic role in systemic lupus erythematosus (Beebe et al., 2002). IL-10 is produced in both differentiated primary Th1 and Th2 cells, though Th2 cells have much higher levels than Th1 cells (Im et al., 2004; Jones and Flavell, 2005; Saraiva et al., 2005). During differentiation into Th1 cells, naïve T cells merely develop their ability to express IL-10, while IL-10 production increases during the development of Th2 cells; this suggests that changes at the IL-10 locus are involved in T cell differentiation.

The genomes of humans and rodents (mouse and rat) have been sequenced (Blake, 2003; Venter et al., 2001). Since most of the proteins in human and rodents have similar functions and expression profiles, it seemed possible that the conserved non-coding regions in various genes might serve regulatory functions (Hubbard et al., 2002). Indeed, recent studies of the IFN-γ, IL-4 and IL-10 loci have revealed a correspondence between DNase I HS sites (HSS) and computer-predicted conserved non-coding sequences (CNSs) (Im et al., 2004; Lee et al., 2004; Loots, 2000). Vista (Mayor et al., 2000) and TRANSFAC (Wingender et al., 2000) analyses have identified possible regulatory elements involved in

*Abbreviations:* CNS, conserved non-coding sequences; IRF, interferon regulatory factor; HSS, hypersensitive sites; CsA, cyclosporin A; ChIP, chromatin immunoprecipitation.

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**Table 1**
Primers used for reporter constructs, ChIP, RT-PCR and conventional PCR.

| | Sense | Antisense |
|---|---|---|
| Luc[a] constructs | | |
| Minimal promoter | GCCTGAATAACAAAAACCTT | TAGACCTCCTGTTCTTGGTC |
| CNS-9 | CGCTCTTCCGAATAGATGTGGT | CCGCACTCCGATGTATTAGTTCC |
| CNS-4.5 | AGCTCAGAGATGCCACTGGT | CTAGCCTAGGGGCAAATCAA |
| CNS-0.12 | GGTTGAGTGGAGGAAACAAT | CTAAAGAACTGGTCGGAATG |
| CNS + 1.65 | CCACCCAACAAATACTGTCT | TCTGCATCCATGGTACTTGG |
| CNS + 2.98 | GGCGAGTGTAACAAGACCT | AAGGGAAGCATTCAGTCC |
| CNS + 6.45 | TTCTTCAAACTGAGGTCACA | CCCTAACCTTTCATCTCACA |
| ChIP | | |
| IL-4 promoter[b] | ACTCATTTTCCCTTGGTTTCAGC | GATTTTTGTCGCATCCGTGG |
| CNS-9 | CTTGAGGAAAAGCCAGCATC | TTTGCGTGTTCACCTGTGTT |
| RT-PCR and conventional PCR | | |
| HPRT | TTATGGACAGGACTGAAAGAC | GCTTTAATGTAATCCAGCAGGT |
| IFN-γ | GAGCCAGATTATCTCTTTCTACC | GTTGTTGACCTCAAACTTGG |
| IL-4 | CAACGAAGAACACCACAGAG | GGACTTGGACTCATTCATGG |
| IL-10 | ATAACTGCACCCACTTCCCA | TCATTTCCGATAAGGCTTGG |
| IRF4[c] | TCCGACAGTGGTTGATCGAC | CCTCACGATTGTAGTCCTGCTT |

[a] Luc, luciferase.
[b] Primer was reported previously (Fields et al., 2002).
[c] Primer was referred from the database of PrimerBank (Wang and Seed, 2003).

gene regulation. However, such *in silico* analysis is not able to provide information about lineage-specificity (Th1, Th2, B cells and antigen presenting cells), relationship to gene expression level and involvement of specific regulatory factors (Elnitski et al., 2006).

The NFAT family of transcription factors regulates the expression of a large number of cytokine genes that are induced by TCR signaling. NFAT1, NFAT2 are calcium-regulated, and expressed in T helper cells. When T cells are stimulated, NFAT proteins are dephosphorylated by calcineurin and translocate to the nucleus, where they activate several cytokine genes. The nuclear translocation of NFAT proteins is inhibited by cyclosporin A and FK506 (Rao et al., 1997), and NFAT1 and NFAT2 deficiency impairs expression of most of the cytokines (Luo et al., 1996; Peng et al., 2001; Yoshida et al., 1998). Differential access of NFATs to specific regulatory elements in cytokine loci results in differences in chromatin dynamics and the recruitment of lineage specific transcription factors, even though the NFAT proteins are themselves induced in a relatively non-specific manner (Agarwal et al., 2000; Hogan et al., 2003). Although the role of NFAT1 in the regulation of various cytokines is well known, its role in IL-10 gene regulation is poorly understood. NFAT proteins play an important integratory role in T cell differentiation by interacting with transcriptional binding partners (Macian, 2005). The IRF family consists of IRF1 to IRF10 (Taniguchi et al., 2001) and is involved in the differentiation of T helper cells (Lohoff and Mak, 2005). IRF4 expression is limited to immune cells and its expression in T cells can be increased by TCR stimulation (Marecki et al., 1999). The IRF4-NFAT1 interaction has a positive effect on IL-4 expression (Rengarajan et al., 2002) but its role in IL-10 expression during T cell differentiation is not well characterized.

In the present study, we identified a critical *cis*-acting regulatory element, CNS-9, crucial for IL-10 gene expression and analyzed the transcription factors involved in it. We showed that CNS-9 enhancer activity in Th2-cells is mediated by NFAT1 and IRF4.

## 2. Materials and methods

### 2.1. Animals

Mice were housed in specific pathogen-free barrier facilities and used in accordance with protocols approved by the animal care and use committees of the Gwangju Institute of Science and Technology. C57BL/6 mice were purchased from SLC (Hamamatsu, Japan). NFAT1$^{-/-}$ mice (kindly provided by Dr. A. Rao, Harvard Medical School) were maintained under specific pathogen-free conditions in the animal facility of the Gwangju Institute of Science and Technology.

### 2.2. Cell lines and primary T cells

The EL4 thymoma cells were obtained from the Korean Cell Line Bank (Seoul National University, Korea) and 293FT human fibroblast was purchased from Invitrogen. CD4$^+$ T cells were purified from the lymph nodes and spleen of 8–10-week-old mice with the use of magnetic beads (Miltenyi, L3T4). For Th differentiation, the cells ($5 \times 10^6$/ml) were stimulated with 1 μg/ml plate-bound anti-CD3ε and 2 μg/ml soluble anti-CD28 under Th1-skewing (10 ng/ml IL-12 plus 10 μg/ml anti-IL-4) or Th2-skewing (10 ng/ml IL-4, 10 μg/ml anti-IFN-γ plus 10 μg/ml anti-IL-12) conditions in RPMI 1640 medium (Welgene, Korea) supplemented with 10% fetal bovine serum, L-glutamine, penicillin–streptomycin, non-essential amino acids, sodium pyruvate, vitamins, HEPES and β-mercaptoethanol. 100 U/ml of recombinant human IL-2 (rhIL-2) was added after 24 h, and the cells were expanded in complete medium containing IL-2 for 7 days. On day 7, they were restimulated with 1 μM ionomycin plus 50 ng/ml PMA in the presence or absence of 1 μM CsA. Recombinant human IL-2 and anti-IL-4 (11B11) were provided by the National Cancer Institute, Preclinical Repository. IL-4 was added as supernatant of the I3L6 cell line and IL-12 was purchased from SIGMA. Anti-CD3 (145.2C11), anti-CD28 (37.51), anti-IFN-γ (XMG1.2) and anti-IL-12 (C17.8) were obtained from BD Biosciences.

### 2.3. RNA isolation, cDNA synthesis, quantitative RT-PCR and conventional PCR

Total RNA was extracted from the cells using easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Korea). For reverse transcription, cDNA was generated using 1 μg of total RNA, oligo(dT) primer (Promega) and Improm-II Reverse Transcriptase (Promega) in a total volume of 20 μl. One microliter of cDNA was amplified using the primers in Table 1. Mouse hypoxanthine-guanine phosphoribosyl transferase (HPRT) primer was used for quantitative