**T**h₂-specific DNase I-hypersensitive sites in the murine IL-13 and IL-4 intergenic region

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**Abstract**

IL-4 and IL-13 are cytokines preferentially produced by T⁰₂ cells, and their genes are located in close proximity on human chromosome 5 and mouse chromosome 11. To identify potential regulatory elements that confer T⁰₂-specific expression of IL-4 and IL-13 genes, we constructed a physical map of the IL-13/IL-4 locus and conducted DNase I-hypersensitive (DH) site analysis using T⁰ clones and in vitro-differentiated effector T⁰ cells obtained from TCR transgenic mice. Three DH sites, HSS1, HSS2 and HSS3, were identified within the intergenic region between IL-13 and IL-4 genes. HSS3 was observed both in T⁰₁ and T⁰₂ cells as well as CD₄⁺ naive T cells, while HSS1 and HSS2 were detected exclusively in T⁰₂ cells. The correlation between differentiation into T⁰₂ subtype and the appearance of HSS1 and HSS2 suggests that these regions may play a role in subtype-specific expression of the IL-13/IL-4 locus.

The molecular basis for the difference between T⁰₁ and T⁰₂ cells had been a focus of research since the discovery of T⁰ cell subsets (1). T⁰₁ cells secrete IFN-γ and tumor necrosis factor-β, promoting cell-mediated immunity to intracellular pathogens; T⁰₂ cells secrete IL-4, IL-5, and IL-13, which function in allergy and humoral immunity to parasites (2). Priming of naive CD₄⁺ T cells in the presence of IL-4 leads to the development of T⁰₂ effector cells (3,4), while IL-12 priming of naive CD₄⁺ T cells leads to the generation of T⁰₁ effectors (5,6). Therefore, elucidation of mechanisms controlling T⁰₂-specific IL-4 gene expression should provide insights into the regulation of T⁰ cell subset development.

IL-4 shares many biological functions with IL-13. The genes for IL-4 and IL-13 are located in close proximity on human chromosome 5 and murine chromosome 11 within the cluster of other cytokine genes including IL-3, IL-5, IL-9 and granulocyte macrophage colony stimulating factor (7). To identify the regulatory region controlling the T⁰₂-specific expression of the IL-13/IL-4 genes, we initially made a physical map of a cosmid clone containing the 40 kb DNA segment of the murine IL-13/IL-4 locus (Fig. 1). The map was verified by genomic Southern blotting of DNA isolated from C57B/6 mice using the murine IL-13 and IL-4 cDNAs as probes (data not shown). These results show that murine IL-13 and IL-4 genes are arranged in a head-to-tail orientation, and are 12 kb apart on the genome.

Since both IL-13 and IL-4 genes are located closely on the chromosome, and are expressed in a T⁰₂ cell-specific manner, it is plausible that this genomic region in T⁰₂ cells takes on a chromatin configuration different from that of the T⁰₁ cells and ensures transcription of these genes. In many developmentally regulated and tissue-specific genes, DNase I-hypersensitive (DH) sites have been associated with regions active in transcriptional control and their appearance correlated with gene expression (8). Thus, we performed DNase I hypersensitivity assay to test this idea using HDK1 and D10...
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![Fig. 1. Physical map of the DNA segment containing both the murine IL-13 and IL-4 genes. Cosmid clone 129, containing mouse IL-13 and IL-4 genes, and derived from a 129 mice genomic library, was analyzed by restriction mapping. Gene names and the number of exons are indicated. Exons and 3' untranslated regions are denoted by filled and open boxes respectively. SacI–ScaI fragment (probe a) and BamHI–NspV fragment (probe b) used as probes in DNase I hypersensitivity assays are indicated by thick bars. Positions of HSS1, HSS2 and HSS3 are shown.](image)

...cells, which are T<sub>r</sub>1 and T<sub>r</sub>2 clones respectively (9,10). When ScaI-digested DNA of D10 cells was probed with fragment a (Fig. 1), we observed three DH sites, designated as HSS1, HSS2 and HSS3 (Fig. 2a). These sites were located 8.3, 8.5 and 9.5 kb upstream of the transcription initiation site of the IL-4 gene and were present both in unstimulated and phorbol myristate acetate (PMA)/A23187-treated cells. Intensity of the band corresponding to HSS1 increased after PMA/A23187 treatment. HSS3 was also observed in HDK1 cells, while HSS1 and HSS2 were observed exclusively in D10 cells. The band corresponding to HSS3 was already present in untreated nuclei of D10 cells, although its intensity increased significantly after DNase I treatment. It suggests that this genomic region has a labile nucleotide sequence, sensitive to mechanical stress as well as DNase I. To confirm hypersensitivity and subtype specificity of these DH sites, EcoR-digested DNA was hybridized with probe b (Fig. 1). As shown in Fig. 2(b), subfragments of 1.2, 1.4 and 2.4 kb which correspond to HSS1, HSS2 and HSS3 were detected in D10 cells, whereas only the HSS3-derived subfragment was detected in HDK1 cells. We also tested DNase I hypersensitivity of other fragments which cover the proximal promoter region of IL-13 or IL-4 genes, but no significant DH sites were detected in these regions (data not shown).

We further studied the subtype specificity of HSS1 and HSS2 using in vitro-differentiated CD4<sup>+</sup> effector T cells isolated from the ovalbumin (OVA)-specific TCR αβ (DO11.10) transgenic mice. CD4<sup>+</CD4<sub>44</sub><sup>low</sup> naive T cells were isolated by flow cytometry and were stimulated with OVA peptide in the presence of either IL-12 or IL-4. The stimulation was repeated weekly for two consecutive weeks. As shown in Fig. 3(a), in vitro-differentiated CD4<sup>+</sup> effector T cells exclusively produced IFN-γ or IL-4, as determined by intracellular cytokine staining (11), upon exposure to IL-12 or IL-4 respectively. DNA was prepared from DNase I-treated nuclei of these cells, restricted with ScaI, electrophoresed, blotted and hybridized to probe a. HSS1, HSS2 and HSS3 were observed in T<sub>r</sub>2 cells, whereas only HSS3 was observed in T<sub>r</sub>1 cells (Fig. 3b). We asked whether HSS1, HSS2 and HSS3 were formed in naive CD4<sup>+</sup> T cells using the CD4<sup>+</sup>-enriched splenic T cells of the same transgenic mice. Flow cytometric analysis showed that >70% of these cells were composed of CD4<sup>+</CD4<sub>44</sub><sup>low</sup> naive T cells (data not shown). One and a half times as much DNA as that of differentiated effector T<sub>r</sub>2 cells was digested with ScaI, hybridized with probe a. The result showed that HSS3, but not HSS1 and HSS2 were formed in these cells (Fig. 3b). Subtype specificity of these DH sites was further verified using EcoR I-digested DNA and probe b (Fig. 1 and 3c). HSS1 and HSS2 were observed exclusively in differentiated T<sub>r</sub>2 cells, whereas HSS3 was observed also in CD4<sup>+</sup> naive T cells and T<sub>r</sub>1 cells, as well as in T<sub>r</sub>2 cells. These observations suggest that HSS1 and HSS2 were...
induced during differentiation from CD4+ naive T cells into Th2 cells, whereas HSS3 existed constitutively during Th cell development. Taken together, we concluded that HSS1 and HSS2 represent differentiated Th2-specific DH sites.

The molecular mechanisms underlying Th cell subset-specific cytokine gene expression has been intensively investigated. Recently, it was reported that c-Maf and GATA-3 are preferentially expressed in Th2 cells, and up-regulate Th2 cytokine transcription (12,13). Kubo et al. identified the Th1-specific silencer element in the 3′ untranslated region of the IL-4 gene. Intriguingly, the negative effect of the silencer element is neutralized by STAT6, specifically in Th2 cells, leading to the specific transcription of IL-4 gene in Th2 cells (14). Detection and analysis of DH sites specific to Th subsets may allow us to identify such transcription factors or locus controlling factors that confer Th1/Th2 specificity. A recent study using transgenic mice containing three IL-4 proximal promoter region as well as those containing the IL-4 NFAT/AP-1 composite site revealed that these regions confer Th2-restricted expression, yet considerably less active than the intact IL-4 gene locus in Th2 cells (15). These results suggest that there may reside a distinct enhancer outside of the proximal promoter region to attain the optimal IL-4 gene transcription. It is tempting to speculate that HSS1 and HSS2, in combination with the proximal promoter region, confer high-level IL-4 gene expression specifically in Th2 cells.

IL-13 and IL-4 genes have been suggested to be coordinately expressed after T cell activation and possibly use a common mechanism for expression in Th2 cells (9,16,17). HSS1 and HSS2 exist in the intergenic region

Fig. 2. DNase I hypersensitivity assay of Th clones. HDK1 is a keyhole limpet hemocyanin-specific Th1 clone and D10 is a conalbumin-specific Th2 clone (9,10). Both clones were maintained by stimulating every 2 weeks with 100 μg/ml of antigen and 3000 rad-irradiated splenocytes from mice of the appropriate haplotype. Cells were grown in RPMI 1640 medium including 10% FCS, 200 U/ml IL-2, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under 5% CO2. Cells (2×10^6) were collected, and either unstimulated or stimulated (lanes 1–5) with 50 ng/ml PMA and 0.5 μM A23187 (lanes 6–10) for 2 h. After stimulation, cells were washed twice in ice-cold PBS and resuspended in 2 ml of ice-cold reticulocyte standard buffer (RSB; 10 mM Tris, pH 7.4, 10 mM NaCl and 5 mM MgCl2). An equal volume of RSB containing 0.5% NP-40 was added and cells were lysed by 15 strokes with dounce B homogenizer. Nuclei were washed in RSB, resuspended in RSB to be 1×10^8 nuclei/ml and divided into 100 μl aliquots. Each aliquot was treated with 0 (lanes 1 and 6), 5 (lanes 2 and 7), 10 (lanes 3 and 8), 15 (lanes 4 and 9) and 20 (lanes 5 and 10) μg/ml of DNase I (Worthington Biochemical, Freehold, NJ) at 37 °C for 12 min. An equal volume of 2×stop solution (1% SDS, 20 mM Tris, pH 7.4, 600 mM NaCl, 10 mM EDTA and 50 μg/ml proteinase K) was added, mixed vigorously and incubated at 37°C overnight. DNA was purified by successive ammonium acetate precipitation, phenol–chloroform extraction and ethanol precipitation. Genomic DNA was digested with Sca I (a) or EcoR I (b) and 4 μg of each digest was separated on a 0.8% (a) or 0.6% (b) agarose gel and blotted. Asterisks indicate positions of the parental fragment. The lower bands are 1.6, 1.8 and 2.8 kb (a) or 1.2, 1.4 and 2.4 kb (b) subfragments generated by DNase I digestion. Sizes of the DNA fragments in kb are indicated on the left.
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**Fig. 3.** (a) Intracellular synthesis of IFN-γ and IL-4 in *in vitro*-differentiated effector Th cells upon PMA/A23187 treatment (11). CD4⁺ splenic T cells isolated from OVA-specific TCR αβ (DO11.10) transgenic mice were enriched by negative selection using anti-mouse CD8α (53-6.7) and anti-mouse IgG antibodies. CD4⁺CD44⁺ T cells were further purified using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA). Primary stimulation of CD4⁺ T cells (2.5 × 10⁵/well) was carried out using 1 μM OVA peptide (OVA323–329) from chicken OVA and irradiated BALB/c spleen cells (2.5 × 10⁶/well) as antigen-presenting cells and incubated in either IL-4 (200 U/ml) or IL-12 (1.5 ng/ml). These cells were collected on day 7 and stimulated for an additional 1 week under the same conditions. For flow cytometric analysis of intracellular cytokine synthesis, cells were stimulated with PMA at 50 ng/ml plus 0.25 μM of A23187. Monensin (2 μM) was added 2 h before harvest. Cells were then washed and fixed in 4% paraformaldehyde for 5 min at 37°C. After washing twice, cells were permeabilized with a 0.5% Triton-100-containing lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA and 0.02% NaN₃, pH 7.5) for 10 min on ice, blocked with 3% BSA/PBS, 0.1% NaN₃ for 30 min on ice, and stained with anti-IFN-γ–FITC (XMG1.2) and anti-IL-4–phycoerythrin (11B11) for 60 min. Proportions of cells in the quadrants are shown. (b and c) DNase I hypersensitivity assay of *in vitro*-differentiated effector Th cells. DNA was digested with ScaI (b) or EcoRI (c) and 6 μg (naive CD4⁺ T cells) or 4 μg (in *in vitro*-differentiated effector Th cells) of each digest was separated on a 0.8% (b) or 0.6% (c) agarose gel and blotted. Asterisks indicate the positions of the parental fragment. The lower bands are 1.6, 1.8 and 2.8 kb (b) or 1.2, 1.4 and 2.4 kb (c) subfragments generated by DNase I digestion. Sizes of the DNA fragments in kb are indicated on the left.

between IL-13 and IL-4 genes, and thus are attractive candidates to mediate such coordinate expression of these cytokine genes. We are currently analyzing the role of HSS1 and HSS2 in transcriptional activation of IL-13 and IL-4 genes using constructs containing the IL-13/IL-4 locus with or without these DH sites linked to reporter genes in transgenic mice.

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Abbreviations

DH site  DNase I hypersensitive
OVA  ovalbumin
PE  phycoerythrin
PMA  phorbol myristate acetate
RBS  reticulocyte standard buffer

References


