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Phylogenetic and Functional Analysis Identifies Ets-1 as a Novel Regulator of the Th2 Cytokine Gene Locus

Jannine M. Strempel,* Roland Grenningloh,†‡ I-Cheng Ho,†‡ and Donata Vercelli*§∥

The Th2 cytokine gene locus has emerged as a remarkable example of coordinated gene expression, the regulation of which seems to be rooted in an extensive array of cis-regulatory regions. Using a hypothesis-generating computational approach that integrated multispecies (∼11) sequence comparisons with algorithm-based transcription factor binding-site predictions, we sought to identify evolutionarily conserved noncoding regions (ECRs) and motifs shared among them, which may underlie coregulation. Twenty-two transcription factor families were predicted to have binding sites in at least two Th2 ECRs. The ranking of these shared motifs according to their distribution and relative frequency pointed to a regulatory hierarchy among the transcription factor families. GATA sites were the most prevalent and widely distributed, consistent with the known role of GATA3 as a Th2 master switch. Unexpectedly, sites for ETS-domain proteins were also predicted within several Th2 ECRs and the majority of these sites were found to support Ets-1 binding in vitro and in vivo. Of note, the expression of all three Th2 cytokines (IL-5, -13, and -4) was significantly and selectively decreased in Th2 cells generated from Ets-1–deficient mice. Collectively, these data suggest that Ets-1 contributes to Th2 cytokine gene regulation by interacting with multiple cis-regulatory regions throughout the Th2 locus. The Journal of Immunology, 2010, 184: 1309–1316.

The spatio-temporal control of eukaryotic gene expression is a complex process, orchestrated to a large extent by mechanisms that control transcription. Regulation of individual loci often relies on multiple cis-regulatory elements, located proximal (promoters) and distal (enhancers, silencers, and insulators) to the transcription start site. Clusters of functionally related genes may exhibit additional complexity and share multiple cis-regulatory elements that transduce developmental and/or environmental cues to specify sequential expression of individual genes, as in the β-globin gene cluster (1), or coordinated expression of several genes, as is the case for the Th2 cytokine gene locus (2), which includes IL5, IL13, and IL4.

The Th2 cytokine genes are grouped within a 150-kb region of human chromosome 5q31 and the syntenic region of mouse chromosome 11. IL13 and IL4 are adjacent to one another, whereas IL5 is separated from these two by a large (85 kb) DNA repair gene, RAD50, a configuration that has been preserved for ∼300 million years (3). The regulation of this locus has been studied most extensively in the Th2 lineage and was shown to provide a remarkable example of coordinated gene expression (4, 5). Such coordination seems to be highly adaptive, in that the distinct components of the Th2 cytokine trio cooperate, ensuring the deployment of complementary antiparasite defense pathways (6). In contrast, when dysregulated by genetic and/or environmental influences, coordinated Th2 cytokine expression becomes critical for the pathogenesis of allergic inflammation (7, 8).

An extensive array of highly conserved cis-regulatory elements essential for the coordinated expression of Th2 cytokine genes has been identified by a combination of DNase I hypersensitive site (HS) mapping and comparative sequence analysis (Fig. 2A). HSs 1 and 2 (9), located between Il13 and Il4, map to conserved noncoding sequence (CNS)-1, a potent enhancer of cytokine gene expression in T cells (10–12) (Fig. 2A). Two other regions, which map to the second intron of Il4 (HS II and III; Il4 intronic enhancer [IE]) and downstream of Il4 (HSV/Va; CNS-2), exhibit enhancer activity in Th2 cells and mast cells (13–15). Several HSs (RAD50 HS [RHS]-5–7) clustered within the 3′ end of the DNA repair gene RAD50 (16, 17) collectively function as a locus control region (LCR), conferring robust Th2-specific, position-independent and copy number-dependent expression to the Il4 and Il13 genes (16, 18). Finally, a single silencer located at the 3′ end of Il4 (HSIV) is required to suppress Il4 expression in naive CD4+ T and Th1 cells (14, 19). Of note, the major Th2 cis-regulatory elements typically map to extensive (300–600 bp) regions that are highly conserved between mice and humans.

Several lines of evidence point to a significant degree of interdependency among distinct regulatory regions within the Th2 locus. Indeed, analysis of transgenic models has shown that no single element is sufficient to fully recapitulate Th2 cytokine gene expression at the levels driven by an intact Th2 locus (16, 18). Conversely, deletion of any enhancer region is sufficient to markedly inhibit the expression of more than one Th2 cytokine gene (11, 15, 20). Moreover, characterization of long-range intrachromosomal interactions at the murine Th2 locus determined that in T cells, the Il5, Il13, and Il4 promoters are physically
clustered with one another and with the distant regulatory elements, forming a distinctive chromatin hub (21).

The advanced structural and functional characterization of the Th2 locus provides a unique opportunity to decipher molecular cues that might dictate the function of individual regulatory regions, as well as to specify coordinate gene expression. A simple model to orchestrate the coexpression of distinct genes predicts that their promoters will contain a common set of transcription factor binding sites critical for transducing relevant signals. Building on such a model, we took a comprehensive in silico approach, and complemented it with functional in vitro and in vivo studies, to decipher the regulatory logic underlying the coexpression of the Th2 cytokine gene cluster. Although computational predictions of transcription factor binding sites have been hindered by considerable false-positive rates, such predictions are substantially improved by integrating motif-finding algorithms with phylogenetic comparisons (22–24) and further strengthened by functional validation. In this study, we show that our hypothesis-generating, multipronged approach succeeded in identifying Ets-1 as a novel, important regulator of coordinated Th2 cytokine gene expression.

Materials and Methods

Multispecies comparative analysis

Genomic sequences corresponding to the human Th2 cytokine gene cluster (20 kb downstream of ILS through the 3‘ end of KIF3A) and the syntenic regions in Pan troglodytes (chimp pancreas), Papio anubis (baboon), Callithrix jacchus (marmoset), Otolemur garnetti (bush baby), Bos taurus (cow), Canis familiaris (dog), Rattus norvegicus (rat), Mus musculus (mouse), Monodelphis domestica (opossum), and Gallus gallus (chicken) were obtained from NCBI. Some of the sequence used was generated by the National Institutes of Health Intramural Sequencing Center (www.nisc.nih.gov).

Generation of a multispecies alignment and identification of evolutionarily conserved regions (ECRs) were performed with the MULAN program (http://mulan.dcode.org) (25). MULAN uses a local alignment strategy using the threaded blockset aligner program and uses the phylogenetic relationships of the sequences provided to build the multispecies alignment (25). Repeat masking was performed on all sequences with the species-appropriate filters prior to alignment. A large gap due to incomplete sequence data was detected at the 3‘ end of RAD50 in C jacchus. Therefore, our analysis of this region (which spans RH56, 1, 6, 2, and 7 within the Th2 LCR) did not include this species. Because of the very close relationship between human and marmoset, it is unlikely that exclusion of this species significantly altered the multispecies alignment or the transcription factor binding-site profiles generated for this region.

Identification of conserved transcription factor binding sites

Genomic regions (excluding exons) identified by the MULAN program as multispecies ECRs (≥70% identity over ≥100 bp in all species examined) were examined for putative transcription factor binding sites. Ungapped sequences from each species were analyzed individually with the MatInspector program, using the matrix library version 6.0, which contains 431 position weight matrices (PWMs) for vertebrate transcription factors, representing 1460 transcription factors from Genomatix, Munich, Germany, http://www.genomatix.de/). PWMs describe transcription factor binding sites in terms of the complete nucleotide distribution for each single position, allowing for quantification of similarity between the weight matrix and the putative binding motif (26). Detection criteria were set to only report matches that demonstrated a core similarity score ≥0.85 and a matrix similarity score that met or exceeded the optimization threshold defined for each PWM. The optimization threshold is a parameter designed to significantly reduce the rate of false positives by limiting detection to no more than three hits within 10 kb of nonregulatory sequence (26). We identified those transcription factor binding sites predicted independently in 100% of the species examined, including matches to different matrices within a family. Matrix families represent functionally related transcription factors that also have PWMs that are essentially indistinguishable (26). Of these sites, those that mapped to the same location within the multispecies alignment were included in the profile. For those cases in which multiple matrix families met the conservation criteria and mapped to the same location in the alignment, all families were included in the profile.

EMSA

Single-stranded complementary oligonucleotides were annealed and PAGE purified. Annealed oligonucleotides were end-labeled with γ-[32P] ATP using T4 polynucleotide kinase. Binding reactions consisted of 50 ng human recombinant Ets-1 (Axxon, San Diego, CA) in binding buffer (25 mM Tris-Cl, 1 mM EDTA, 60 mM KCl, 6 mM MgCl2, 10 mM DTT, 1 μg Poly (dIdC), 10% glycerol). Competition assays were performed by preincubation for 30 min at room temperature with unlabeled oligonucleotide competitors (30- and 90-fold molar excess) prior to addition of the probe. Samples were incubated with radiolabeled probes for 30 min at room temperature and run on a 5% (w/v) polyacrylamide gel (20 mAmp, 4 h, 4˚C), which was dried and subjected to autoradiography. Oligonucleotide sequences used as probes and/or competitors included a previously defined high-affinity Ets-1 motif (SC1_ETS1 CGGCAACCGGGAATGAGTGGCC; the core consensus is underlined) (27), a mutant of this motif (SC1_ETS1mut CGGGGCGAGAAGTGGGTTAG), and the conserved ETS sites predicted within the human Th2 cis-regulatory regions (ILSP: TGTTCTTGAGAAATGAAATAA; ILSP: GTTGCGGGGAAGAATGGGTTAG; ILAP: GATTTCAAGAGCAATATTAC, ILAP: TTCTTCCTGAGGAAGAGGTTG; ILAP: GATTTCAAGAGCAATATTAC, ILAP: GTGGGTTAG, RHSS: GGTCAACGGCAAATCTAGG, ILAP: GATTTCAAGAGCAATATTAC, ILAP: GTGGGTTAG, ILAP: GATTTCAAGAGCAATATTAC, ILAP: GTGGGTTAG, ILAP: GATTTCAAGAGCAATATTAC, ILAP: GTGGGTTAG).

Mice

Female 3-6-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Ets-1–deficient mice were described previously (28). The animals were housed under specific pathogen-free conditions and experiments were performed in accordance with the institutional guidelines for animal care at the University of Arizona and the Dana-Farber Cancer Institute (Boston, MA) under approved protocols.

T cell isolation and in vitro differentiation of Th2 cells

Murine Th2 cells were generated essentially as described (29). Naïve CD4+ T cells were isolated from spleens by negative selection, followed by enrichment using anti-CD62L–coated magnetic beads (Miltenyi Biotec, Auburn, CA). Cells (2–5×106) were cultured in the presence of anti-CD3e Ab (145.2C1, 1 μg/ml) and anti-CD28 (37.5.1, 1 μg/ml) (BD Pharmingen, San Diego, CA) in a flask coated with goat anti-hamster IgG (0.2 mg/ml), under Th2 skewing conditions (1000 U/ml IL-4, 3 μg/ml anti-IL-12, and 5 μg/ml anti–IFN-γ; complete medium) for 3 d. Then the cells were expanded in complete medium containing IL-2 (20 U/ml) for 7 d. Cytokine expression at the single-cell level was examined by intracellular staining using the following Abs: anti–IL-4 PE (11B11), anti–IL-13 Alexa eBio13A), and anti–IFN-γ FITC (XMG1.2). For restimulation, cells were incubated with increasing concentrations (0.3–3 μg/ml) of anti-CD3 mAb, in the presence of constant amounts of anti-CD28 Ab (2 μg/ml) for 24 h. Cytokine secretion was quantified by ELISA (Quantikine Immunoassays, R&D Biosystems, Minneapolis, MN).

Chromatin immunoprecipitation

Murine Th2 cells were treated with 1% formaldehyde for 10 min at room temperature followed by the addition of glycine (125 mM final concentration) to halt cross-linking. Cells were harvested, washed twice with 1× PBS, and resuspended in cell lysis buffer (5 mM PIPES [pH 8.5], 85 mM KCl, 0.5% Nonidet P-40) supplemented with protease inhibitors (10 μg/ml Proux, 1× EDTA-free complete protease inhibitor mixture [Roche, Basel Switzerland], and 1 mM PMSF). Nuclei were collected by centrifugation and lysed in nuclease buffer (10 mM Tris-HCl [pH 8.1], 50 mM NaCl, 0.5% SDS) supplemented with protease inhibitors as above. Chromatin was sheared by sonication to yield the majority of fragments in the 200–600-bp size range. An aliquot of chromatin (1× whole cell equivalents) was precleared with a 50% Protein A Sepharose slurry (150 μl) for 20 min at 4˚C. Precleared chromatin was then diluted 10-fold in IP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl) containing protease inhibitors. Immunoprecipitation reactions were performed with 5×106 whole-cell equivalents overnight at 4˚C with an anti–Ets-1 Ab (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG (10 μg/ml) for 20 min at 4˚C. Immunoprecipitation reactions were analyzed using anti-CD3e Ab (145.2C1, 1 μg/ml) and anti-CD28 (37.5.1, 1 μg/ml) (BD Pharmingen, San Diego, CA) in a flask coated with goat anti-hamster IgG (0.2 mg/ml), under Th2 skewing conditions (1000 U/ml IL-4, 3 μg/ml anti-IL-12, and 5 μg/ml anti–IFN-γ; complete medium) for 3 d. Then the cells were expanded in complete medium containing IL-2 (20 U/ml) for 7 d. Cytokine expression at the single-cell level was examined by intracellular staining using the following Abs: anti–IL-4 PE (11B11), anti–IL-13 Alexa eBio13A), and anti–IFN-γ FITC (XMG1.2). For restimulation, cells were incubated with increasing concentrations (0.3–3 μg/ml) of anti-CD3 mAb, in the presence of constant amounts of anti-CD28 Ab (2 μg/ml) for 24 h. Cytokine secretion was quantified by ELISA (Quantikine Immunoassays, R&D Biosystems, Minneapolis, MN).
7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The following primer pairs were used: IL5 promoter: 5’-ACCCTGAGTT-CAGGACTCG-3’; 5’-TCGAACACTCAAGTGCAGAAG-3’; Rad50S: 5’-GTTGGTCTGCCTCTGAAGAAGC-3’; 5’-GTCACATCTGCCATG-3’; 5’-ATGGGAG-ATGGAAATCTGCG-3’; Rad50H: 5’-CTGGCTCTTCTCTATACCTG-3’; 5’-CCTGCTACACTGCTGGAG-3’; 5’-GTCGATCTCTTGCACTG-3’; CNS-2: 5’-GAACACATGAGGAGGAGGAGAAGG-3’; 5’-GCCGCT-3’; 5’-GAGCCAGTGGAGAGAG-3’; and GAPDH (Quantitect Primer Assays, Qiagen). Products were verified by melting curve analysis. Th2 cytokine and Gata3 mRNA levels are expressed relative to Gapdh mRNA abundance within the same sample.

Results

Most Th2 cis-regulatory elements predate the divergence between placental and nonplacental mammals

To investigate the evolutionary history of the Th2 regulatory elements and establish an appropriate scope of species for subsequent binding-site analyses, we first assessed to what extent the extensive array of Th2 regulatory regions characterized in humans and mice is conserved in additional placental mammals and evolutionary more distant species. Beyond placental mammals, orthologs for IL5, IL13, and IL4 were recently identified for a marsupial, the laboratory opossum (Monodelphis domestica) (30), and the chicken (G. gallus) (3). Although a draft sequence of the platypus (Ornithorhynchus anatinus) genome was recently released (31), the genomic region expected to contain orthologs of the Th2 cytokine genes is incompletely covered and could not be included in our analysis. Furthermore, because comparative analysis of syntenic regions and mining of expressed sequence tag libraries failed to identify these genes in earlier vertebrate species (teleosts) (32), we did not extend our analysis beyond the avian order.

Fig. 1 shows the phylogenetic relationships and estimated divergence times (33–35) for the 11 species included in our study. A multiple DNA sequence alignment for the entire Th2 cytokine gene cluster (Fig. 2A) was generated with the MULAN program (25), which relies on a local alignment strategy and integrates the phylogenetic relationships among the sequences. Fig. 2B shows the MULAN-generated alignment for selected regions within the Th2 locus in a subset of species representative of the clades we analyzed. The ability to detect the exons for IL5, IL13, and IL4 by multiple species alignment decreased with increasing phylogenetic distance, consistent with previous reports that the opossum (30) and chicken (3, 32) Th2 cytokines exhibit relatively low homology to the human proteins. In contrast, all exons for the DNA repair gene RAD50 were highly conserved and readily identified in all species (Fig. 2B and data not shown). As expected, all of the Th2 regulatory regions previously identified in pairwise human–mouse alignments (Fig. 2A) and validated in functional studies (10, 11, 15, 17–20) were highlighted as ECRs in the seven placental mammalian species examined (Fig. 2B). In addition, this analysis identified a novel noncoding ECR, located ∼6 kb upstream of the IL5 gene (IL-5/-6). Of note, only a single noncoding element (110 bp within RH5S) was conserved in the syntenic region in chicken. In contrast, the majority of the noncoding ECRs were highly conserved in the orthologous opossum locus (Fig. 2B), with the exception of the functionally undefined ILS/-6, the distal region of the IL13 promoter (which encompasses a conserved GATA-3–responsive element) (36), and HSIIV, which is critical for IL4 silencing in murine Th1 cells (19).

Collectively, these data indicate that the majority of conserved regulatory regions within the Th2 cytokine locus predate the divergence of marsupials and placental mammals ∼180 million years ago (35, 37). The phylogenetic distance between chicken and mammals might preclude alignment-based detection of additional regulatory regions.

Phylogenetic profiling of Th2-responsive elements

The Th2 cytokine locus ECRs identified by comparative analysis were then examined to search for shared transcription factor binding sites that might contribute to coordinated regulation of the Th2 locus. Because some of the Th2 ECRs were undetectable in the opossum genome, we profiled transcription factor binding sites across nine placental mammalian species, representing four major clades (primates, carnivores, artiodactyls, and rodents). Multi-species comparisons of ECRs increase the overall sequence divergence, which serves to refine alignments and reveal invariant positions more likely to reflect true functional constraints associated with transcription factor binding sites (22–24, 38). Indeed, the sequence diversity within the noncoding Th2 ECRs captured by the multiple species alignment was ∼20% greater than that obtained by pairwise comparison between human and mouse sequences (data not shown). Th2 ECRs from placental mammals were examined for putative transcription factor binding sites using the MatInspector program (26). Searches were conducted on un-gapped sequence from each species using a vertebrate PWM library and stringent match criteria. Sites predicted independently in all species and residing in the same position within the multiple species alignment were included in the profile.

Within the Th2 ECRs, which collectively span ∼4.8 kb, we identified 103 conserved motifs representing targets for 52 distinct transcription factor families (data not shown). Fig. 3 shows that 22 transcription factor families were predicted to have binding sites within at least two Th2 regulatory regions. When multiple sites for a particular factor were found, they typically resided in different
regions, rather than clustering within one or few regulatory elements. In fact, the number of conserved sites and the number of distinct regulatory regions they are predicted to target were positively correlated ($R^2 = +0.81$). The number of motifs common to distinct regions did not correlate with GC content nor linear proximity (data not shown). Therefore, these data are unlikely to merely reflect genomic bias. Rather, they may highlight an underlying regulatory network within the Th2 locus.

**Identification and functional validation of Ets-1 binding sites in the Th2 locus**

The ranking of transcription factor binding sites shared across the Th2 locus, according to their distribution and relative frequency (Fig. 3, Supplemental Fig. 1), pointed to a regulatory hierarchy among transcription factor families. Interestingly, the most prevalent and widely distributed motifs were those for GATA proteins, consistent with the notion that GATA-3 acts as a master switch for Th2 cell differentiation and Th2 cytokine expression (36, 39–42). This finding validated our approach and by extension raised the possibility that members of the ETS and SORY/HMG transcription factor families, which like GATA proteins were predicted to target six or more Th2 cis-elements, might also play a role in the coordinated regulation of the Th2 cytokine locus.

Our subsequent analysis focused on the ETS family, which had not been previously implicated in orchestrating the coordinated expression of the Th2 cytokines, and particularly on Ets-1, the family member that is predominant in T cells (43). To functionally validate the ETS motifs predicted within the Th2 ECRs, we used EMSA and compared and contrasted the ability of oligonucleotides corresponding to individual putative ETS sites to bind recombinant human Ets-1 (rhEts-1). Fig. 4A shows the results of an experiment representative of this approach. Incubation of rhEts-1 with a high-affinity Ets-1 consensus probe (27) led to the formation of a complex (lane 1) that was competed almost completely upon incubation with a 30-fold molar excess of unlabeled Ets-1 consensus oligonucleotide (lane 2) and was specifically shifted by an anti–Ets-1 Ab (data not shown). Oligonucleotides spanning the putative ETS sites in HSIV (lanes 4 and 5) and the IL4 promoter (lanes 6 and 7) also competed Ets-1 binding, albeit less effectively. In contrast, an oligonucleotide encompassing one of the two putative ETS sites within the IL4 promoter (IL4P.1, lanes 12 and 13) failed to compete Ets-1 binding. Similar experiments were performed to test each predicted ETS motif, and the intensity of the relevant bands was measured by densitometry and compared with the intensity of the uncompeted Ets-1 complex. The Ets-1 consensus and an Ets-1 oligonucleotide containing two mutations in the ETS core were used as positive and negative controls throughout this study.

Fig. 4B shows the competition curves for the eight putative ETS sites identified within the Th2 locus. The motifs within RHS5 and the IL13 promoter competed Ets-1 binding almost as effectively as the Ets-1 consensus oligonucleotide. The sites within HSIV, CNS2,
and IL4IE exhibited intermediate binding ability. Competition by the IL5 promoter site was more modest but still appreciable. In contrast, individual ETS motifs within the IL4 promoter (IL4P.1 and IL4P.2) failed to compete Ets-1 binding.

Fig. 4C shows the core motif defined specifically for Ets-1 (27) and the sequences of the Th2 Ets-1 binding sites, ranked by relative Ets-1 binding ability. Relative to the Ets-1 core, all sequences containing no or one mismatch supported Ets-1 binding, albeit to different extents. In contrast, two mismatches seemed to compromise binding, as shown by the poor interactions detected for IL5 promoter, IL4P.1, and IL4P.2.

Collectively, these data show that the majority of the ETS sites predicted within the Th2 ECR behave as bona fide Ets-1–binding motifs in vitro, suggesting that this protein (and/or possibly other members of the ETS family) might bind at multiple locations throughout the locus, thereby participating in the concerted regulation of Th2 cytokine gene expression.

Ets-1 binds several regulatory regions in the marine Th2 locus in vivo

To examine whether Ets-1 binds to the endogenous Th2 ECRs, ChIP experiments were performed on chromatin isolated from naïve CD4+ T cells cultured under Th2-skewing conditions for 7 d. Immunoprecipitation relied upon an Ab specific for Ets-1 or normal rabbit IgG as a negative control. Real-time PCR was performed using primers that target the individual Th2 ECRs previously tested for Ets-1 binding in vitro. The promoter of Cd14, a gene that is not expressed in murine Th2 cells (data not shown), was amplified for comparison.

Fig. 5 shows that, relative to the Cd14 promoter, Ets-1–containing complexes were specifically enriched at all three Th2 cytokine promoters. The strongest occupancy was detected at the Il4 promoter, a region that contains two ETS-binding motifs. Notably, the two ETS motifs in this region failed to bind Ets-1 when tested individually in vitro, suggesting that the strong signal detected in vivo reflects cooperative binding. Ets-1 was also consistently found to bind distal Th2 regulatory regions, including RH5 of the Th2 LCR, the Il4 silencer region (HSIV), and a Th2 cytokine enhancer (CNS2). An Ets-1–specific signal was detected at IL4IE, but it barely exceeded the one generated for the Cd14 promoter. Of note, our ChIP experiments detected Ets-1 and no other members of the large ETS family, because specific enrichment was completely abrogated at all of the Th2 ECRs in Ets-1–deficient Th2 cells (data not shown). Overall, these data show that Ets-1 binds to multiple Th2 regulatory regions in vivo, supporting the possibility that Ets-1 participates in the coordinated regulation of the Th2 cytokine gene cluster.

Ets-1 is required for optimal expression of all three Th2 cytokine genes

To test the role of Ets-1 in Th2 cytokine gene expression, we compared the levels of Il4, Il13, and Il5 mRNA and protein produced by Th2 cells from Ets-1+/− or Ets-1−/− mice. Naïve CD4+ T cells isolated from these mice were cultured under Th2-skewing conditions for 7 d and stimulated for 24 h with increasing concentrations of plate-bound anti-CD3 mAb, in the presence of a constant amount of anti-CD28 Ab. mRNA levels were assessed by real-time RT-PCR. Fig. 6A shows that Ets-1−/− Th2 cells exhibited a marked decrease in Il4, Il5, and Il13 mRNA relative to Ets-1+/+ Th2 cells. In contrast, transcript levels for Il10, a cytokine gene expressed by Th2 cells and located on mouse chromosome 1, were comparable in Ets-1+/+ and Ets-1−/− Th2 cells. Impaired Th2 cytokine transcription in cells lacking Ets-1 was unlikely to reflect a defect in Th2 cell differentiation per se, because Gata3 mRNA levels in these cells were unaffected (Fig. 6B).

In parallel, we examined the impact of Ets-1 deficiency on Th2 cytokine secretion. Fig. 6C shows that, consistent with the decrease observed in Th2 cytokine transcripts, Ets-1−/− Th2 cells were also impaired in their ability to release IL-4, -13, and -5, but not IL-10. Collectively, these data demonstrate that Ets-1 acts as a global positive regulator of the expression of the Th2 cytokine gene cluster, thereby providing functional validation for our phylogenetic analysis.

Discussion

The Th2 cytokine gene locus typically acts as a unit, the distinct components of which (IL5, IL13, and IL4) are coregulated and coexpressed in response to T cell activation and differentiation.
FIGURE 4. Ets-1 binds to the majority of ETS motifs predicted in the Th2 ECR. A, Ets-1 protein/DNA interactions were characterized using a γ-[32P]-labeled Ets-1 consensus probe. Purified rhEts-1 (50 ng) was preincubated with an increasing molar excess of the indicated competitors before addition of a high-affinity Ets-1 consensus probe. B, EMSA blots were analyzed by densitometry, and the results are expressed as the percentage inhibition of the Ets-1 band relative to the uncompeeted Ets-1 complex. C, Sequences of the Ets-1 binding site core, the high-affinity Ets-1 consensus probe, and the eight conserved ETS motifs predicted within the Th2 ECRs. Bold type indicates nucleotide sites within conserved ETS motifs predicted within the Th2 ECR. 

This expression pattern is likely to be critical for effective immune responses, but its molecular underpinnings remain only partially understood. To investigate the molecular basis for coordinated regulation of Th2 cytokine genes, we combined comparative and functional analyses, and we focused on transcription factor binding sites that are highly conserved across placental mammals and common to more than one Th2 regulatory region. Our comparison of the Th2 cytokine gene locus in evolutionarily distant vertebrate species (human to chicken) revealed that although gene content, order, and orientation have been preserved for >300 million years, nearly all of the distal cis-regulatory elements known to underlie Th2 coregulation arose after the split between birds and mammals.

The main exception seems to be a single region conserved in chicken that corresponds to one of several murine DNase I HSs within the Th2 LCR (RHS5). It is tempting to speculate that this region might participate in transcriptional regulation of the chicken orthologs of mammalian type-2 cytokines.

Despite remarkable conservation of the majority of Th2 regulatory regions, a few elements were not identifiable in the opossum locus. These included a GATA-3–responsive element 5' of IL13 and the Th1-specific IL4 silencer, HSIV. The emergence of a Th1-selective IL4 silencer suggests that a fine-tuning of the Th1/Th2 dichotomy may have occurred during mammalian evolution. Resistance to certain mammalian pathogens (e.g., Leishmania spp.) requires extremely polarized Th cell responses (44, 45). Thus, regulatory mechanisms that place increasingly tighter restrictions on alternative cytokine responses might be adaptive to the host.

Perhaps most importantly, our identification of highly conserved Ets-1 binding sites in multiple cis-regulatory regions in the endogenous murine Th2 locus. Spleens from C57BL/6 mice were harvested, and naive CD4+ T lymphocytes were isolated and cultured under Th2-skewing conditions for 7 d. Chromatin was immunoprecipitated with an anti–Ets-1 Ab or normal rabbit IgG as a negative control. The precipitated DNA was amplified by quantitative real-time PCR using primers specific for the Th2 ECRs or the Cd4 promoter as a comparison. All PCR reactions were carried out in duplicate. Results are expressed as specific enrichment for Ets-1 relative to the IgG control. Shown are mean ± SE values for two independent experiments, one of which was conducted on chromatin isolated from cells pooled from four mice.
coexist in all but two of the Ets-1–binding ECRs, these sites are in close proximity only in the Il5 promoter and in CNS2, the enhancer located 3′ of Il4. This arrangement suggests that altered cooperativity between Ets-1 and GATA-3 may contribute to, but not be the only mechanism for, the locus-wide defect in Th2 cytokine expression observed in Ets-1−/− mice.

The consequences of Ets-1 deficiency are not limited to Th2 cells. Ets-1−/− Th1 cells exhibit a significant defect in the establishment of polarized cytokine expression programs, with defective IFN-γ production and inappropriate expression of Il4 (51). The latter mirrors the phenotype of CD4+ T cells lacking the Il4 silencer, HSIV (19, 51). Interestingly, our bioinformatic analysis identified a highly conserved ETS binding site within HSIV that did support Ets-1 binding in vitro and in vivo. Loss of Ets-1 binding at HSIV may play a role in the abnormal regulation of Il4 expression detected in the absence of Ets-1. Overall, our study highlighted specific functional elements that Ets proteins may target to orchestrate Th2 cytokine expression. Interestingly, our analysis also revealed a remarkable heterogeneity in the sequence of Ets-1–binding motifs, particularly their flanking regions. This heterogeneity might result in a functional hierarchy among Ets-1–binding sites, particularly under conditions of limited Ets availability, as well as in differential interaction between Ets-1 and its protein partners.

Synergy between homotypic factors that occupy distant DNA elements is a regulatory arrangement that has been appreciated since early studies conducted with phage λ (52). The highly conserved, common motifs we identified across the Th2 locus may provide a molecular basis for functional cooperation between distant Th2 regulatory regions. Indeed, data describing the three-dimensional architecture of the murine Th2 locus highlight the potential for direct communication among distant Th2 cis-elements. In naive CD4+ T cells, the Il5, Il13, and Il4 promoters are physically clustered with one another and with distant Th2 cis-regulatory regions (21). Analogous to a single regulatory element containing tandem arrays of identical binding sites, three-dimensional clustering of homotypic sites may serve as a strategy for the Th2 cytokine genes to effectively vie for limited amounts of trans-acting factors and, in turn, foster expression of the locus as a whole.

Very little is known about how long-range chromatin contacts are initiated and/or maintained, yet the earliest evidence supporting a DNA looping model pointed toward the homo-oligomerization of transcriptional activators and/or repressors bound to distant target sites (52–54). These early studies were conducted on artificial DNA templates, but more recent experiments in a native chromosomal context also link sequence-specific DNA-binding proteins to chromosomal looping and implicate distant, matching binding sites in this process. For example, the erythroid-specific Krüppel-like factor directly contributes to the formation of an active chromatin hub in the β-globin locus by interacting with multiple cis-regulatory elements distributed across the locus (55). Similarly, androgen receptor-mediated regulation of the prostate-specific Ag gene proceeds via independent recruitment of androgen receptors to a distant upstream enhancer and the proximal promoter, which subsequently form a stable chromosomal loop (56).

The distribution of the highly conserved GATA motifs across the Th2 locus seems to reflect the role that GATA-3 plays in higher-order chromatin architecture, because ectopic expression of GATA-3 in fibroblasts induces chromatin contacts between the Th2 LCR and the Il4 and Il13 promoters (21). In contrast, these associations were relatively weak, suggesting that additional T cell lineage-restricted factors may be required to reinforce and/or maintain these long-range interactions (21). Our finding that functional binding sites for Ets-1 are distributed across the Th2 locus raises the intriguing possibility that the contribution of Ets-1 to the coordinated regulation of Th2 cytokine genes may extend to the higher-order chromatin architecture of the locus.

Disclosures
The authors have no financial conflicts of interest.

References
cluster: the cluster contains functional single-copy genes for IL-3, IL-4, IL-13, and GM-CSF, a gene for IL-5 that appears to be a pseudogene, and a gene encoding another cytokine-like transcript, K334. J. Interferon Cytokine Res. 23: 600–610.


Supplemental Figure 1

ETS-1 Motifs Alignments
ETS-1 Core C(C/A)GG(A/T)G(T/C)

**IL5 Promoter**

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**RHS5**

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Pan     CATTTCAGTTCTCTGTATTTCAT
Papio   CATTTCCAGTTCTCTGTTTTTCTAT
Calli   CACTACAAGTTCTCTGTTTTTCTAT
Oto     CACTACAAGTTCTCTGTTTTTCTAT
Bos     CATTTCCAGTTCTCTGTTTTTCTAT
Canis   CATTTCCAGTTCTCTGTTTTTCTAT
Rat     CAATTTCCAGTTCTCTGTTTTTCTAT
Mus     CATTTCCAGTTCTCTGTTTTTCTAT

HSIV
Hum     TCTGCCACAGGATATGGGTFAGG
Pan     TCTGCCACAGGATATGGGTFAGG
Papio   TCTGCCACAGGATATGGGTFAGG
Calli   TCTGCCACAGGATATGGGTFAGG
Oto     TCTGCCACAGGATATGGGTFAGG
Bos     TCTGCCACAGGATATGGGTFAGG
Canis   TCTGCCACAGGATATGGGTFAGG
Rat     TCTGCCACAGGATATGGGTFAGG
Mus     TCTGCCACAGGATATGGGTFAGG

CNS2
Hum     TGGGTCAAGGAAGCCCAAGA
Pan     TGGGTCAAGGAAGCCCAAGA
Papio   TGGGTCAAGGAAGCCCAAGA
Calli   TGGGTCAAGGAAGCCCAAGA
Oto     TGGGTCAAGGAAGCCCAAGA
Bos     TGGGTCAAGGAAGCCCAAGA
Canis   TGGGTCAAGGAAGCCCAAGA
Rat     TGGGTCAAGGAAGCCCAAGA
Mus     TGGGTCAAGGAAGCCCAAGA

GATA Motifs Alignments
GAT(A/C) or GATAA

IL5 Promoter
Hum     TTCTCTATCTGTATT
Pan     TTCTCTATCTGTATT
Papio   TTCTCTATCTGTATT
Calli   cTCTCTATCTGTATT
Oto     TTCTCTATCTGTATT
Bos     TTCTCTATCTGTATT
Canis   TTCTCTATCTGTATT
Rat     TcCTCTATCTGTATT
Mus     TcCTCTATCTGTATT

RHS6.1
Hum    ATCA\text{GATA}AGGGC
Pan    ATCA\text{GATA}AGGGC
Papio  ATCA\text{GATA}AGGGC
Calli  ATCA\text{GATA}AGGGC
Oto    ATCA\text{GATA}AGGGC
Bos    ATCA\text{GATA}AGGaC
Canis  ATCA\text{GATA}AGGaC
Rat    ATCA\text{GATA}GgaGC
Mus    ATCA\text{GATA}AGGGC

\textbf{RHS6.2}

Hum    TGTAG\text{GATA}GGGATA
Pan    TGTAG\text{GATA}GGGATA
Papio  TaTAG\text{GATA}GGGATA
Calli  (sequence unavailable)
Oto    TG-\text{AGATAGGGATA}
Bos    Tgc\text{AGATAGGGATA}
Canis  caTAG\text{ATG}tGGATA
Rat    cGc\text{AGATAGGGATA}
Mus    cGc\text{AGATAGGGATA}

\textbf{IL13P (1)}

Hum    CGCTTA\text{TATCG}GGGCC
Pan    CGCTTA\text{TATCG}GGGCC
Papio  CGCTTA\text{TATCG}GGGct
Calli  CGCTTA\text{TATCG}GGGCC
Oto    CGCTTA\text{TATCG}caGCC-
Bos    CG\text{TATCG}CaGcCC
Canis  CGCTTA\text{TATCG}aGcCCC
Rat    Ct\text{TATCG}acCCC
Mus    Ct\text{TATCG}acCCC

\textbf{IL13P (2)}

Hum    CCCCT\text{TATCTCG}GCC
Pan    CCCCT\text{TATCTCG}GCC
Papio  CCCCT\text{TATCTCG}GCC
Calli  CCCCT\text{TATCTCG}CaG
Oto    CCCCT\text{TATCTCG}CaG
Bos    CCCCT\text{TATCTCG}CaG
Canis  gCCC\text{TATCTCG}GCC
Rat    CCCCT\text{TATCTCG}aCC
Mus    CCCCT\text{TATCTCG}aCC

\textbf{IL13P (3)}

Hum    TTCTA\text{GATA}GTGCC
Pan    TTCTA\text{GATA}GTGCC
Papio  TTCTA\text{GATA}GTGCC
Calli  TTCTA\text{GATA}GTGCC
Oto    TTCTA\text{GATA}GTGCC
Bos    TT\text{GATA}GTGCC
Canis  TT\text{GATA}GTaCC
Rat    TTtc\text{AGATA}aTGCC
Mus    TTtcAGATAaTGCC

CNS-1
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Pan    CCCATTAATCTTCAT
Papio  CCCATTAATCTTCAT
Calli  CCCATTAATCTTCAT
Oto    CCCATTAATCTTCAT
Bos    CtCATATCTTCATc
Canis  CtCATATCTTCAT
Rat    CtCATATCTTCAT
Mus    CCCATTAATCTTCAT

IL4P
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Pan    AGCTGATAAGATTA
Papio  AGCTGATAAGATTA
Calli  AGCTGATAAGATTA
Oto    AGCTGATAAGATTA
Bos    AGCTGATAAGATTA
Canis  AGCTGATAAGATTA
Rat    cGCTGATAAGATTA
Mus    cGCTGATAAGATTg

IL4IE
Hum    AAACAGATATTGAG
Pan    AAACAGATATTGAG
Papio  AAACAGATATTGAG
Calli  AAACAGATATTGAG
Oto    gAACAGATATTGAG
Bos    AAACAGATATTGAG
Canis  AAACAGATATTGAG
Rat    AAACAGATATTgAG
Mus    gAACAGATATTgAG

CNS-2 (1)
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Pan    TATCTGACCTGTCA
Papio  TgTCTGACCTGTCA
Calli  TgTCTGACCTGTCA
Oto    TgTCTGACCTGTCA
Bos    TgTCTGACCTGTCA
Canis  TgTCTGACCTGTCA
Rat    TgTCTGACCTGTCA
Mus    TgTCTGACCTGTCA

CNS-2 (2, 3)
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Pan    cTTCTGATAACGTTGATAAAAGTCA
Papio  cTTCTGATAACGTTGATAAAAGTCA
Calli  cTTCTGATAACGTTGATAAAAGTCA
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