Functional Dissection Identifies a Conserved Noncoding Sequence-1 Core That Mediates IL13 and IL4 Transcriptional Enhancement*§

Received for publication, July 12, 2006, and in revised form, December 1, 2006. Published, JBC Papers in Press, December 1, 2006, DOI 10.1074/jbc.M606615200

Jannine M. Strempel† and Donata Vercelli‡§¶

From the †Functional Genomics Laboratory, Arizona Respiratory Center, ‡Graduate Interdisciplinary Program in Genetics, and ¶Department of Cell Biology, College of Medicine, University of Arizona, Tucson, Arizona 85724

Conserved noncoding sequence (CNS)-1 has been shown to coordinately regulate the expression of the Th2 cytokine genes IL4, IL13, and IL5. We have used the interaction between CNS-1 and the human IL13 and IL4 promoters as a model to pursue the molecular mechanisms underlying CNS-1-dependent regulation of Th2 cytokine gene transcription. CNS-1 potently enhanced the activity of IL13 and IL4 promoter reporter vectors upon full T cell activation. Analysis of CNS-1 deletion mutants mapped enhancer activity to a short core (CNS-1–(270–337)) that contains three closely spaced cyclic AMP-responsive elements (CRE). CRE site 2 bound CRE-binding protein (CREB) and activating transcription factor (ATF)-2 in vitro and was essential for CNS-1-dependent up-regulation of IL13 transcription. Cotransfection of an IL13 reporter construct with expression vectors for wild type or mutant CREB and ATF-2 showed that CREB, but not ATF-2, regulates CNS-1 enhancer activity. Notably, chromatin immunoprecipitation analysis showed T cell activation recruits CREB and the coactivator CREB-binding protein (CBP)/p300 to the endogenous CNS-1. Moreover, CBP/p300 activity was essential for CNS-1-mediated enhancement of IL13 transcription. Collectively, these data define the region within CNS-1 responsible for enhancement of IL13 and IL4 transcription and suggest CREB/CBP-dependent mechanisms play an important role in facilitating Th2 cytokine gene expression in response to T cell receptor signaling.

Dysregulated expression of the cytokines IL2-4, IL-13, and IL-5 in CD4 T cells of the Th2 lineage plays a pivotal role in the pathogenesis of allergic inflammation. The IL4, IL13, and IL5 genes, closely arrayed within 150 kb of human chromosome 5q31 and the syntenic region of mouse chromosome 11, typically demonstrate coordinated expression (1, 2), a feature critical for the emergence of a bona fide allergic phenotype in experimental and clinical models. However, the molecular mechanisms underlying the concerted expression of Th2 cytokines remained elusive despite intense investigation.

A breakthrough came as a result of comparative genomics analyses to identify noncoding regions highly conserved (>70% identity) between humans and evolutionarily distant mammalian species (3). These elements, abundant in the human genome, display characteristics indicative of regulatory function. In particular, they tend to demonstrate higher selective constraint than genomic regions that encode translated or noncoding RNAs (4, 5) and contain short, alternating stretches of sequence with high or low divergence, a pattern typical of protein-binding sites (5).

The search for highly conserved noncoding sequences in ~1 Mb of human chromosome 5q31 identified several elements (3). The largest of these, conserved noncoding sequence (CNS)-1, mapped within the IL4/IL13 intergenic region of the Th2 cytokine locus. Deletion of CNS-1, either from a transgene or the native murine locus, led to a marked decrease in the expression of all three cytokine genes (3, 6), establishing CNS-1 as a vital regulatory element for coordinated Th2 cytokine expression. Consistent with this role, epigenetic changes in the endogenous CNS-1 chromatin, including changes in levels of histone acetylation (7) and DNA methylation (8, 9) and appearance of DNase I-hypersensitive sites (10), were found to accompany Th2 cytokine expression.

More recently, analysis of long range intrachromosomal interactions within the murine Th2 cytokine locus highlighted events that accompany the coordinated transcriptional regulation of Th2 cytokine genes and provided clues about the role CNS-1 may play in this process (11). This element was found to come in close spatial proximity with all three Th2 cytokine promoters in both T and non-T cells, suggesting it may be important for the acquisition of the initial “pre-poised” chromatin configuration of the Th2 cytokine locus. Of note, the physical interaction between CNS-1 and the Th2 cytokine promoters persisted through the T cell- and Th2 cell-specific stages of Th2 locus reorganization, pointing to an involvement of this element throughout the regulatory process. The interactions between CNS-1 and the Th2 cytokine promoters need to be...
Identification of a CNS-1 Enhancer Core

We chose the interaction between CNS-1 and the IL13 and IL4 promoters as a model to characterize the molecular mechanisms by which CNS-1 regulates Th2 cytokine gene expression in human CD4 T cells. Among the genes targeted by CNS-1, IL13 is essential to mediate Th2 effector functions critical to the pathogenesis of allergic inflammation (12–14), and IL4 is critical to initiate Th2 cell differentiation (reviewed in Ref. 15). Furthermore, expression of IL-13 and IL-4 was strongly decreased in CNS-1−/− mice (6). We show here that CNS-1 is a potent T cell activation-dependent enhancer of the human IL13 and IL4 promoters. CNS-1 enhancer activity mapped to a short (68 bp) core that bound cyclic AMP-responsive element-binding protein (CREB) and the coactivator CREB-binding protein (CBP)/p300 in activated T cells and required these factors to enhance Th2 cytokine gene transcription.

EXPERIMENTAL PROCEDURES

DNA Constructs—p2.7IL13luc was created by PCR amplification of a 2666-bp region encompassing the human IL13 promoter (−2672 to −6, relative to the IL13 ATG; GenBank™ accession numbers AC004041 and L42080) using genomic DNA as a template. We selected this region based on the analysis of a panel of human IL13 promoter reporter constructs.3 The PCR primers (IL13pro2.7F and IL13proR; all primer sequences are provided in supplemental Table 1) contained KpnI and Nhel sites that were used to clone the IL13 promoter fragment upstream of the firefly luciferase gene in pGL3Basic (Promega). p369IL13 was created by amplification of the −369 to −6 region using primers IL13pro369F and IL13proR and p2.7IL13luc as template, followed by cloning into pGL3 Basic. p800IL4 contains 800 bp of human IL4 promoter sequence (−800 to −1 relative to the IL4 ATG) amplified by PCR using primers IL4pro800F and IL4proR with the human P1 clone H11 (GenBank™ accession number AC004039) as template. The amplified fragment was cloned into the SacI and Nhel restriction sites of pGL3 Basic.

To generate the CNS-1 constructs, we initially amplified a 965-bp fragment (75604 to +6568 relative to the IL13 ATG) of the human IL4/IL13 intergenic region encompassing CNS-1, and we cloned it into the SalI site of p369IL13luc. The boundaries of the human CNS-1 element were defined based on a sequence alignment with the murine IL4/IL13 intergenic region (GenBank™ accession number AC005742). The full-length 372-bp CNS-1 element (GenBank™ accession number AC004039; nucleotides 42330–42701) was amplified by PCR (primers CNS1_1F and CNS1_372R) and cloned 3′ of the luciferase gene in p2.7IL13luc in both genomic and reverse orientations. Additionally, full-length CNS-1 was cloned downstream of the reporter gene in the Sall site of p369IL13luc and p800IL4luc.

The 372-bp CNS-1 element was dissected into three overlapping amplicons (CNS-1(1–163), CNS-1(111–262), and CNS-1(221–372)). Each fragment was cloned into the Sall site of p2.7IL13luc in genomic orientation. Further dissection of CNS-1(221–372) into three overlapping fragments (CNS-1(221–282), CNS-1(270–337), and CNS-1(319–372)) was achieved through PCR as described above. Supplemental Table 1 shows the sequences of primers used to generate these constructs. We constructed CNS-1-(270–337) cassettes containing mutations in the individual CRE-like sites by ligating double-stranded oligonucleotides, corresponding to each half of CNS-1-(270–337) and containing the mutation of interest (supplemental Table 2), to each other. These cassettes were then cloned into p2.7IL13luc. Sequence fidelity and CNS-1 orientation were determined for all constructs by sequencing.

The wild type (WT) pCMV-CREB and dominant negative pCMV-CREB133 expression constructs were purchased from Clontech. Vectors expressing activating transcription factor (ATF)-2 (amino acids 1–505; GenBank™ accession number NP_001871) or a mutant lacking the N-terminal trans-activation domain (ATF-2 Δ2–107) were generated by PCR amplification with specific primers (sequences provided in supplemental Table 1) and cloning into pcDNA3.1(−). WT ATF-2 and ATF-2 Δ2–107 were translated in vitro by using rabbit reticulocyte lysates (Promega). Both ATF-2 proteins were expressed, bound a 32P-labeled CNS-1-(270–337) oligonucleotide probe, and reacted specifically with a monoclonal anti-ATF-2 antibody (F2BR-1; Santa Cruz Biotechnology) (data not shown). The expression vectors for the WT and mutant adenoviral proteins, E1A 12S and E1A 12S Δ2–36, were kindly provided by Dr. E. Tsitsikov (CBR Institute for Biomedical Research) and have been described previously (16).

Cell Culture and Transfections—Jurkat T cells (ATCC clone E6-1) were cultured in RPMI 1640 supplemented with fetal calf serum (10%, HyClone), penicillin (100 units/ml), streptomycin (100 μg/ml), and l-glutamine (2 mM). Jurkat T cells (1 × 10⁶) in log phase of growth were transfected with endotoxin-free plasmid preparations by electroporation (1 pulse, 240 V, 50 ms). Cells were transfected with either p2.7IL13luc (20 μg) or with equimolar amounts of the indicated reporter vectors along with pRL-TK (20 ng; Promega) to control for transfection efficiency. Following electroporation, cells (5 × 10⁶) were cultured in the presence or absence of phorbol 12-myristate 13-acetate (PMA; 20 ng/ml; Sigma) and soluble anti-CD28 antibody (1.25 μg/ml; R&D Systems) and soluble anti-CD28 antibody (1.25 μg/ml; R&D Systems) for 16 h. When indicated, Jurkat cells were transfected with expression vectors for WT or mutant CREB (30 ng), ATF-2 (30 ng), and E1A 12S (1 μg) or equimolar amounts of empty pcDNA3 to control for total DNA content. Firefly and Renilla luciferase activity was determined using the dual luciferase assay system (Promega). In addition, the protein concentration for each cell lysate was quantitated with a BCA protein assay (Pierce). The relative luciferase activity (RLA) for each sample represents luciferase counts corrected for transfection efficiency and total protein content. Fold induction represents the ratio of RLA values between stimulated and unstimulated cells.

Nuclear Extract Preparation—Nuclear extracts were prepared from Jurkat T cells (1.5 × 10⁶) cultured in the presence or absence of PMA (20 ng/ml) and ionomycin (1 μM) for 3 h. Cells were resuspended in buffer A (3 mM MgCl₂, 10 mM HEPES, 40 mM KCl, 5% glycerol, 0.2% Nonidet P-40) supplemented with 3 L. Cameron, unpublished observations.
protease and phosphatase inhibitors (1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, 50 μg/ml pepstatin, 5 mM β-glycerophosphate, 1 mM NaF, 1 mM NaV, and 1 mM benzamidine) and incubated on ice for 5 min. Following centrifugation, the nuclear pellets were resuspended in buffer C (1.5 mM MgCl2, 20 mM HEPES, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA) supplemented with protease and phosphatase inhibitors as indicated above. After a 30-min incubation on ice, the nuclear lysis solution was centrifuged, and the supernatant fractions were flash-frozen in liquid nitrogen and stored at −80 °C. The protein concentration for each preparation was quantitated with a BCA protein assay (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Single-stranded complementary oligonucleotides were annealed and PAGE-purified. Annealed oligonucleotides were end-labeled with [γ-32P]ATP with T4 polynucleotide kinase. EMSA were performed with 10 μg of nuclear extract in binding buffer (100 mM NaCl, 10% glycerol, 200 ng/μl bovine serum albumin, 50 ng/μl poly(dI-dC), 10 mM HEPES (pH 7), 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.6 mM MgCl2). For competition or supershift assays, the indicated unlabeled oligonucleotide competitor (100-fold molar excess) or antibody (2 μg) was added 30 min prior to addition of radiolabeled probe. Following addition of the radiolabeled probe, the samples were incubated for 30 min at room temperature and loaded onto a 5% (w/v) polyacrylamide gel. Electrophoresis was performed at a constant 19 mA for 6 h at 4 °C, and the gels were dried prior to autoradiography. Antibodies used for supershift analysis included a polyclonal and a monoclonal anti-CREB (C-21 and X-12, respectively), a polyclonal anti-Jun (D), and a monoclonal anti-ATF-2 (F2BR-1), all from Santa Cruz Biotechnology. Normal rabbit IgG and a monoclonal anti-CREB (C-21 and X-12, respectively), a polyclonal anti-Jun (D), and a monoclonal anti-ATF-2 (F2BR-1), all from Santa Cruz Biotechnology. Normal rabbit IgG provided in Supplemental Table 3. Multiple sequence alignments were generated using the MULtiple sequence Aligner and conservation visualization tool (MULAN) (17), which uses a local alignment strategy with the threaded block set aligner and utilizes the phylogenetic relationships of the sequences provided to build the multiple sequence alignment. The alignment was exported in FASTA format to GeneDoc for visualization.

RESULTS

CNS-1 Is a Potent Enhancer of IL13 and IL4 Promoter Activity—CNS-1 is strategically positioned at the heart of the Th2 locus (Fig. 1), consistent with its ability to physically interact with the Th2 cytokine gene promoters throughout the region (11). To start defining the molecular mechanisms underlying the CNS-1-dependent regulation of Th2 cytokine transcription, the CNS-1 element (372 bp) was cloned 3’ of the luciferase reporter gene in a construct driven by a 2.7-kb fragment encompassing the promoter for human IL13. This construct (p2.7IL13/CNS1-1(1–372)) was transiently transfected into Jurkat T cells, and its activity was compared with that of an IL13 promoter construct lacking CNS-1 (p2.7IL13). Transfected cells were cultured for 16 h in the presence or absence of PMA (20 ng/ml) and ionomycin (1 μM), a combination of stimuli that results in a >100-fold increase of IL13 mRNA levels in Jurkat T cells (data not shown). Fig. 2A shows that under basal conditions the IL13 promoter and CNS-1 were essentially inactive. Stimulation with PMA and ionomycin resulted in strong (16-fold) activa-
Identification of a CNS-1 Enhancer Core

The Th2 cytokine gene locus. Location of CNS-1 within the Th2 cytokine locus and DNA sequence alignment between human (H) and mouse (M) CNS-1. Boldface type indicates the CNS-1 enhancer core identified in this study.

FIGURE 2. CNS-1 is a potent enhancer of IL13 and IL4 promoter activity. Jurkat T cells were transfected with equimolar amounts of the indicated reporter vectors. Cells were cultured with or without PMA (P; 20 ng/ml) and/or ionomycin (I; 1 μM) or anti-CD3 (2.5 μg/ml) and anti-CD28 (1.25 μg/ml) for 16 h. Plotted are the mean RLA values ± S.E. for 5 (A), 2 (B and C), or 4 (D) independent experiments. Noted above the bars are the mean fold induction values. Iono, ionomycin.

V. Identification of a 68-bp Enhancer Core within CNS-1—To identify the CNS-1 domain(s) required to mediate transcriptional enhancement of Th2 cytokine genes, three overlapping regions (CNS-1-1(1–163), CNS-1-2(111–262), and CNS-1-3(221–372); Fig. 3A) were individually cloned downstream of the luciferase reporter gene in p2.7IL13 and p800IL4. Jurkat T cells were transiently transfected with these constructs or with vectors containing the full-length CNS-1. Luciferase activity was assayed from cells unstimulated or treated with PMA (20 ng/ml) and ionomycin (1 μM) for 16 h. Fig. 3B shows that remarkably the entire enhancer activity of CNS-1 mapped to a single fragment, CNS-1-3(221–372), which encompasses the IL13 distal region of the element. We note that CNS-1-3(221–372) was nearly twice as active as full-length CNS-1, suggesting the CNS-1 enhancer core may lie within a region that constrains its activity.

To define more closely the boundaries of the CNS-1 enhancer core and guide our subsequent analysis of DNA/pro-
Identification of a CNS-1 Enhancer Core

A diagram of CNS-1-(1–372) dissection. Jurkat T cells were transfected with either p2.7IL13 or p800IL4 (20 μg) or equimolar amounts of the indicated CNS-1 reporter constructs. Cells were cultured with or without PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 16 h.

CNS-1-(221–372). Experimental protocols were the same as noted for B. Plotted are the mean RLA values ± S.E. for five and two (B, top and bottom, respectively) or four and three (D, top and bottom, respectively) independent experiments.

Identification of a 68-bp enhancer core within CNS-1. A diagram of CNS-1-(1–372) dissection. B, functional dissection of CNS-1-(1–372); Jurkat T cells were transfected with either p2.7IL13 or p800IL4 (20 μg) or equimolar amounts of the indicated CNS-1 reporter constructs. Cells were cultured with or without PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 16 h. C, diagram of CNS-1-(221–372) dissection. D, functional dissection of CNS-1-(221–372). Experimental protocols were the same as noted for B. Plotted are the mean RLA values ± S.E. for five and two (B, top and bottom, respectively) or four and three (D, top and bottom, respectively) independent experiments.

protein interactions at this region, CNS-1-(221–372) was further dissected into three overlapping fragments (CNS-1-(221–282), CNS-1-(270–337), and CNS-1-(319–372); Fig. 3C). Fig. 3D shows that virtually all of the IL13 enhancer activity resided within CNS-1-(270–337). A highly similar pattern was observed when we studied the interactions between discrete CNS-1 domains and the IL4 promoter (Fig. 3D). These results define a 68-bp region of CNS-1, encompassing nucleotides 270–337, as a potent enhancer for two distinct Th2 cytokine gene promoters.

The CNS-1 Enhancer Core Contains Binding Sites for CREB, ATF-2, and Jun Proteins—To identify the trans-acting factors involved in the enhancer activity of CNS-1-(270–337), we analyzed patterns of DNA/protein interactions by EMSA. Nuclear extracts prepared from Jurkat T cells cultured with or without PMA (20 ng/ml) and ionomycin (1 μM) for 3 h were incubated with 32P-labeled probes corresponding to nucleotides 270–303, 287–320, or 318–337 of CNS-1 (Fig. 4A).Fig. 4B shows that competition experiments with unlabeled self-related or unrelated oligonucleotides identified four specific nucleoprotein complexes (lanes 1–3 and 19–21), three of which (complex monoclonal anti-CREB antibody, which does not cross-react with other CREB family members, also altered the mobility of this complex (data not shown). Complex III appeared to contain CREB because an anti-CREB antibody (Fig. 4B, lanes 13) bound the 270–303-nucleotide region (Fig. 4B, center panel). Complex I and II, but not complex III, also bound the overlapping nucleotides 287–320. No additional interactions were detected in this region (Fig. 4B, left panel). Complex IV was detected using the 318–337 probe (Fig. 4B, right panel).

Comparative analysis of the CNS-1-(270–337) nucleotide sequence across distant species (Fig. 4C) and prediction of putative transcription factor-binding sites identified three motifs (CRE 1–3; Fig. 4B) each partially homologous to a CRE (TGACGCTCA) (20) and the related AP-1 family consensus sequence (TGACGTCA) (21). Antibody supershift experiments were therefore performed in order to test whether the complexes binding to CNS-1-(270–337) contained CRE-interacting proteins (CREB and ATF) and/or AP-1 family members. Fig. 4B shows that complex I, a faint band up-regulated by stimulation, contained ATF-2 because it was supershifted by an ATF-2-specific antibody (lane 13) but not by an anti-CREB (lane 11) or an anti-Jun antibody (lane 12). The constitutively expressed complex II was formed by CREB because addition of a polyclonal anti-CREB antibody supershifted the complex completely (Fig. 4B, lanes 4 and 11). A
Identification of a CNS-1 Enhancer Core

| Probe: | CRE 1 | CRE 2 | CRE 3 |
|-------|-------|-------|-------|
| P17   | +     | +     | +     |
| P18   | +     | +     | +     |
| P19   | +     | -     | +     |
| P20   | -     | +     | +     |
| P21   | +     | +     | +     |
| P22   | +     | +     | +     |
| P23   | +     | +     | +     |

The CNS-1 enhancer core contains binding sites for CREB, ATF-2, and Jun proteins. A, DNA sequence of the human CNS-1 enhancer core. Oligonucleotide probes used in EMSA are noted above the sequence, although the CRE motifs are underlined. B, EMSA analysis of protein/DNA interactions within the CNS-1 enhancer core. γ-32P-Labeled oligonucleotide probes, indicated above each lane, were incubated with nuclear extracts (10 μg) prepared from Jurkat T cells cultured either in the presence (+) or absence (−) of PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 3 h. Noted above each lane is the unlabeled oligonucleotide competitor (100-fold molar excess) used for analysis of binding specificity or the antibody (2 ng/ml) used for supershift analysis. C, multiple DNA sequence alignment of CNS-1-(270–337) across 9 mammalian species. Shading indicates the percent identity at each nucleotide position (black, 100%; dark gray, 90%; light gray, 80%).

The formation of distinct CREB-containing complexes (Fig. 5B). Mutation of CRE 1 resulted in the loss of complex III (Fig. 5B, lane 2). Both complex I and complex II were lost when the second CRE motif was mutated (Fig. 5B, lane 3). Despite the striking proximity of the first two CRE sites and their organization on opposite DNA strands, mutation of either one of these two sites did not appear to affect the formation of complexes on the other site. Notably, no residual binding was detected when both CRE 1 and CRE 2 were mutated (Fig. 5B, lane 4). Finally, mutation of CRE 3 abolished the binding of complex IV (Fig. 5B, lane 6). Collectively, our results show CREB interacts with each of the three CRE sites in CNS-1-(270–337), whereas Jun and ATF-2 bind selectively to CRE 1 and CRE 2, respectively (Fig. 5C).

To evaluate the contribution of each CRE motif to CNS-1 enhancer function, the CRE site mutations already characterized by EMSA were introduced in the p2.7IL13/CNS-1-(270–337) reporter construct. Fig. 6 shows that mutation of the CRE 1 and CRE 2 sites reduced CNS-1 activity substantially but only partially (54.5 and 79%, respectively), whereas mutation of CRE 2 was sufficient to abolish CNS-1-dependent IL13 enhancement. In view of the topology of the CRE motifs, these results suggest that although each CRE site contributes to CNS-1 activity, CRE 2 may be required to coordinate the formation of a supramolecular complex critical for optimal CNS-1-induced enhancement of IL13 transcription.

CREB, but Not ATF-2, Regulates CNS-1 Enhancer Activity—Because CRE 2 and, to a lesser extent, CRE 3 were essential for CNS-1-dependent transcriptional enhancement, we then investigated the role of the CRE 2/3-binding proteins CREB and ATF-2 in the regulation of CNS-1 activity. To this purpose, the p2.7IL13/CNS-1-(270–337) reporter construct was cotransfected with expression vectors encoding either WT or mutant forms of these factors. Specifically, pCMV-CREB133 encodes a dominant negative CREB variant containing a Ser → Ala substitution at position 133, a residue vital to CREB-mediated transactivation. pATF-2 Δ2–107 encodes a truncated ATF-2 protein that lacks the N-terminal trans-activation domain but retains the ability to bind CNS-1 CRE 2 (data not shown). Fig. 7 shows that expression of CREB133 markedly (47%) reduced T cell activation-dependent CNS-1 enhancer activity, whereas cotransfection of ATF-2, either WT or mutant, failed to affect IL13 transcription. CREB133-dependent inhibition was CNS-1-specific because IL13 promoter activity was virtually unaltered (data not shown). These results support a role for CREB, but not ATF-2, in the molecular events underlying CNS-1-mediated transcriptional enhancement.

T Cell Activation Recruits CREB and CBP/p300 to the Endogenous CNS-1—Our in vitro dissection identified CREB as a factor that binds functionally critical sites in the CNS-1 enhancer core and directs robust up-regulation of IL13 and IL4 transcription. Chromatin immunoprecipitation assays were therefore performed to test whether CREB-containing complexes dock onto the endogenous CNS-1. CREB protein-DNA complexes were immunoprecipitated from Jurkat T cells, resting or activated with PMA and ionomycin. Real time PCR was performed to detect a 152-bp region of CNS-1 (nucleotides 221–372) that spans the enhancer core. Although only low levels of target were immunoprecipitated with an anti-CREB antibody under basal conditions, Fig. 8 shows that T cell activation strongly increased CREB binding to CNS-1.

Activation-dependent CREB phosphorylation at serine 133 is known to foster the recruitment of the coactivator CBP (24) and its paralogue p300 (25), which augment CREB-mediated gene transcription. The inhibitory effect of the CREB133 mutant on CNS-1 activity (Fig. 7) raised the possibility these coactivators may contribute to CREB-mediated CNS-1 regulation. We there-
fore used chromatin immunoprecipitation to test whether CBP/p300 was recruited to CNS-1 in vivo. Fig. 8 shows that occupancy of CNS-1 by CBP- or p300-containing complexes was limited in unstimulated T cells. However, T cell activation resulted in robust recruitment of both factors to CNS-1.

CBP/p300 Activity Is Essential for CNS-1-dependent Transcriptional Enhancement—To assess whether CBP/p300 activity was required for CNS-1 enhancer function, p2.7IL13 or p2.7IL13/CNS-1-(270–337) was cotransfected with a construct expressing the adenoviral E1A 12S protein, a potent inhibitor of CBP/p300 function (26, 27). The E1A 12S/H90042–36 mutant, which lacks the domain necessary to interact with CBP/p300 (16), was used as a negative control. Fig. 9 shows that E1A 12S, but not E1A 12S/H90042–36, completely inhibited CNS-1-mediated enhancement of IL13 transcription, whereas the activity of the IL13 promoter was only partially reduced. Collectively, these data suggest CREB-mediated recruitment of CBP/p300 is essential for T cell activation-induced CNS-1-mediated enhancement of Th2 cytokine gene transcription.

**DISCUSSION**

Progression of naive CD4 T cells along the Th1 or Th2 differentiation pathway is a multistage process contingent upon antigenic T cell stimulation in an instructive cytokine milieu. In vivo studies defined CNS-1 as a regulatory element critical for optimal expression of Th2 cytokine genes and Th2 differentiation (3, 6) but did not dissect the molecular mechanisms underlying the role of CNS-1 in these processes. Our results characterize CNS-1 as a potent enhancer of human IL13 and IL4 transcription. Surprisingly, activity mapped to a discrete, short domain at the IL13 proximal region of CNS-1 is comparatively even more conserved across mammalian species than the enhancer core itself (data not shown). Surprisingly, the CNS-1 enhancer core does not encompass previously described binding sites for GATA3 (29) or Ikaros (28), even though ectopic overexpression of GATA3 in murine
Th1 cells was sufficient to establish DNase I hypersensitivity within the endogenous CNS-1 chromatin (29, 30). The relationship between GATA3-induced chromatin remodeling and the activity of the CNS-1 enhancer core remains to be determined.

Our experiments identified CRE site 2 and 3 and the CRE 2/3-binding protein CREB as major regulators of CNS-1 enhancer activity. This finding was somewhat unexpected because a recent analysis of mice in which CREB and ATF-1 had been deleted selectively in T cells showed IL-4 expression to be preserved (31). However, a significant proportion of spleen and lymph node T cells (24 and 12%, respectively) in the knock-out mice still expressed CREB, raising the possibility that residual CREB activity was sufficient to support IL4 mRNA expression in that experimental model. Furthermore, and perhaps more importantly, deletion of CNS-1 delayed and reduced, but did not abrogate, expression of IL-13 and IL-4 (6), suggesting CNS-1 contributes to, but is not absolutely required for, Th2 cytokine expression.

CREB is a ubiquitous transcription factor that resides constitutively in the nucleus (20). CREB activation is known to be necessary for transcriptional regulation and contingent upon phosphorylation of Ser-133, which occurs typically, although not exclusively, in response to elevated intracellular cAMP (20). In line with these requirements, our experiments showed stimulation of T cells with cAMP resulted in vigorous (4.4-fold) CNS-1-dependent enhancement of IL13 transcription (data not shown) and expression of a dominant negative CREB variant, which cannot be phosphorylated on Ser-133, reduced activity by nearly half.

Identification of a CNS-1 Enhancer Core

**FIGURE 7. CREB, but not ATF-2, regulates CNS-1 enhancer activity.** Jurkat T cells were cotransfected with p2.7IL13/CNS-1-(270–337) and expression vectors for CREB (pCMV-CREB or pCMV-CREB133) or ATF-2 (pATF-2 or pATF-2 Δ2–107) (30 ng) or a pCDNA3 control. Cells were cultured with or without PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 16 h. Plotted are mean RLA values ± S.E., normalized to the pCDNA3 control (n = 4).

**FIGURE 8. T cell activation recruits CREB, CBP, and p300 to the endogenous CNS-1.** Jurkat T cells were cultured in the presence or absence of PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 3 h. Chromatin was then isolated and immunoprecipitated with antibodies to CREB, CBP, or p300 or control IgG. For each experiment, serial dilutions of input DNA were used to generate a standard curve. Enrichment for target template was assessed by real time PCR. Results are expressed as the ratio between the number of targets immunoprecipitated with specific antibodies and the number of targets immunoprecipitated with control IgG. Plotted are the mean ± S.E. of four independent experiments.

**FIGURE 9. CBP/p300 activity is required for CNS-1-dependent enhancement of IL13 transcription.** Jurkat T cells were cotransfected with either p2.7IL13 or p2.7IL13/CNS-1-(270–337) and E1A 12S or E1A 12S Δ2–36 or pcDNA3 expression vectors (1 μg). Cells were cultured with or without PMA (P; 20 ng/ml) and ionomycin (I; 1 μM) for 16 h. Plotted are mean RLA values ± S.E. for two independent experiments.
Identification of a CNS-1 Enhancer Core

... achieved only if the antigen-dependent signal is coupled with CD28-mediated costimulation (32, 33). Consistent with the two-signal requirement for CREB trans-activation, our data revealed full T cell activation was essential for maximal CNS-1 activity. Signaling via the T cell receptor and the costimulatory pathway is required for a productive association between CREB and the transcriptional coactivator CBP (33), which recruits the RNA polymerase II holoenzyme (34). Fostering the assembly of this complex may represent a mechanism by which CREB contributes to CNS-1-dependent transcriptional enhancement. The CRE sites clustered within the CNS-1 TK cell stimulation and complements Th2-specific regulatory permissive one, which links Th2 locus regulation with antigenic pathway is required for a productive association between CREB (15). Asahara, H., Santoso, B., Guzman, E., Du, K., Cole, P. A., Davidson, I., and Montminy, M. (2001) Mol. Cell. Biol. 21, 7892–7900

REFERENCES

1. Kelly, B. L., and Locksley, R. M. (2000) J. Immunol. 165, 2982–2986
2. Santangelo, S., Cousins, D. J., Winkelmann, N. E., and Staynov, D. Z. (2002) J. Immunol. 169, 1893–1903
3. Loots, G. G., Locksley, R. M., Blankenspoor, C. M., Wang, Z. E., Looi, G. G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E. M., and Frazer, K. A. (2000) Science 288, 136–140
4. Shabalina, S. A., Ogurtsov, A. Y., Kondrashov, V. A., and Kondrashov, A. S. (2001) Trends Genet. 17, 373–376
5. Dermietzakis, E. T., Reymond, A., Scamuffa, N., Ucla, C., Kirkness, E., Rossier, C., and Antonarakis, S. E. (2003) Science 302, 1033–1035
6. Mohrs, M., Blankespoor, C. M., Wang, Z. E., Loots, G. G., Afzal, V., Hadeiba, H., Shinkai, K., Rubin, E. M., and Locksley, R. M. (2001) Nat. Immun. 2, 842–847
7. Yamashita, M., Ukai-Tadenuma, M., Kimura, M., Omori, M., Inami, M., Taniguchi, M., and Nakayama, T. (2002) J. Biol. Chem. 277, 42399–42408
8. Lee, D. U., Agarwal, S., and Rao, A. (2002) Immunity 16, 699–660
9. Guo, L., Hu-Li, J., Zhu, J., Watson, C. J., Difilippantonio, M. J., Pannetier, C., and Paul, W. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10623–10628
10. Takemoto, N., Koyano-Nakagawa, N., Yokota, T., Arai, N., Miyatake, S., and Arai, K.-I. (1998) Int. Immunol. 10, 1981–1985
11. Spilianakis, C. G., and Flavell, R. A. (2004) Nat. Immun. 5, 1017–1027
12. Wills-Karp, M., Luyimbizi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L., and Donaldson, D. D. (1998) Science 282, 2258–2261
13. Grünig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M., and Corry, D. B. (1998) Science 282, 2261–2263
14. Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., Zhang, Y., and Elias, J. A. (1999) J. Clin. Investig. 103, 779–788
15. Ansel, K. M., Dijuretic, I., Tanasa, B., and Rao, A. (2006) Annu. Rev. Immunol. 24, 607–656
16. Lee, J. S., Galvin, K. M., See, R. H., Eckner, R., Livingston, D., Moran, E., and Shi, Y. (1995) Genes Dev. 9, 1188–1198
17. Ovcharenko, I., Loots, G. G., Giardine, B. M., Hou, M., Ma, J., Hardison, R. C., Stubbins, L., and Miller, W. (2005) Genome Res. 15, 184–194
18. Ranganath, S., Ouyang, W., Bhattacharya, D., Shih, W. C., Grupe, A., Pelz, G., and Murphy, K. M. (1998) J. Immunol. 161, 3822–3826
19. Lee, G. R., Fields, P. E., and Flavell, R. A. (2001) Immunity 14, 447–459
20. Montminy, M. (1997) Annu. Rev. Biochem. 66, 807–822
21. Vogt, P. K. (2001) Oncogene 20, 2365–2377
22. Gonzalez, G., and Montminy, M. (1989) Cell 59, 675–680
23. Livingstone, C., Patel, G., and Jones, N. (1995) EMBO J. 14, 1785–1797
24. Kwok, R. P., Lundblad, J. R., Chivria, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226
25. Lundblad, J. R., Kwok, R. P., Laurance, M. E., Harter, M. L., and Goodman, R. H. (1995) Nature 374, 85–88
26. Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) Genes Dev. 8, 869–884
27. Eckner, R., Arany, Z., Ewen, M., Sellers, W., and Livingston, D. M. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 85–95
28. Grogan, J. L., Wang, Z. E., Stanley, S., Harmon, B., Loots, G. G., Rubin, E. M., and Locksley, R. M. (2003) J. Immunol. 171, 6672–6679
29. Takemoto, N., Kamogawa, Y., Lee, J. H., Kurata, H., Arai, K., O’Garra, A., Arai, N., and Miyatake, S. (2000) J. Immunol. 165, 6687–6691
30. Takemoto, N., Arai, K., and Miyatake, S. (2002) J. Immunol. 169, 4103–4107
31. Baumann, S., Kyewski, B., Bleckmann, S. C., Greiner, E., Rudolph, D., Rubay, W., Ramsay, R. G., Krammer, P. H., Schutz, G., and Mantamadiotis, T. (2004) Eur. J. Immunol. 34, 1961–1971
32. Hsieh, Y. P., Liang, H. E., Ng, S. Y., and Lai, M. Z. (1997) J. Immunol. 158, 95–93
33. Yu, C. T., Shih, H. M., and Lai, M. Z. (2001) J. Immunol. 166, 284–292
34. Vo, N., and Goodman, R. H. (2001) J. Biol. Chem. 276, 13505–13508
35. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 935–959
36. Bannister, A. J., and Kouzarides, T. (1999) Nature 384, 641–643
37. Korzus, E., Torchia, J., Rose, D. W., Xu, L., Stensland, B., McNierney, E. M., Mullen, T. M., Glass, C. K., and Rosenfeld, M. G. (1998) Science 279, 703–707
38. Yuan, L. W., and Gambee, J. E. (2001) Biochim. Biophys. Acta 1541, 161–169
39. Asahara, H., Santoso, B., Guzman, E., Du, K., Cole, P. A., Davidson, I., and Montminy, M. (2001) Mol. Cell. Biol. 21, 7892–7900