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PU.1 Binds to a Distal Regulatory Element That Is Necessary for B Cell-Specific Expression of CIITA

Hyesuk Yoon and Jeremy M. Boss

The transcriptional coactivator CIITA regulates MHC class II genes. In the mouse, CIITA is expressed from three distinct promoters (pI, pIII, and pIV) in a developmental and cell type-specific manner with pIII being responsible for B lymphocyte-specific expression. Although the promoter proximal sequences that regulate CIITA in B cells have been described, nothing is known about additional distal elements that may regulate its expression in B cells. Sequence homology comparisons, DNase I hypersensitivity assays, and histone modification analysis revealed a potential regulatory element located 11 kb upstream of pIII. Deletion of this element, termed hypersensitive site 1 (HSS1), in a bacterial artificial chromosome encoding the entire CIITA locus and surrounding genes, resulted in a complete loss of CIITA expression from the bacterial artificial chromosome following transfection into B cells. HSS1 and pIII displayed open chromatin architecture features in B cell but not in plasma cell lines, which are silenced for CIITA expression. PU.1 was found to bind HSS1 and pIII in B cells but not in plasma cells. Depletion of PU.1 by short hairpin RNA reduced CIITA expression. Chromatin conformation capture assays showed that HSS1 interacted directly with pIII in B cells and that PU.1 was important for this interaction. These results provide evidence that HSS1 is required for B cell-specific expression of CIITA and that HSS1 functions by interacting with pIII, forming a long-distance chromatin loop that is partly mediated through PU.1. The Journal of Immunology, 2010, 184: 5018–5028.

Major histocompatibility complex class II (MHC-II) genes encode proteins that present peptide Ags to CD4⁺ T lymphocytes, a process that results in the initiation of adaptive immune responses (1, 2). CIITA plays an essential role in this process by serving as the limiting transcription factor controlling MHC-II gene expression (3). Mutations in CIITA are a cause of bare lymphocyte syndrome, an inherited immunodeficiency in which MHC-II gene expression is absent (4, 5). CIITA is constitutively expressed in APCs, such as B cells, macrophages, and dendritic cells (6). CIITA and therefore MHC-II expression can be induced by IFN-γ treatment in most cell types (7). In the mouse, three distinct promoters control CIITA expression: promoters I, III, and IV (pI, pIII, and pIV) (8). Each promoter specifies the transcription of a unique exon 1, which is ultimately spliced into a downstream common exon, resulting in the production of distinct CIITA isoforms. The three CIITA promoters are activated in a cell type- or cytokine-dependent manner. Promoters I and III are used for the constitutive CIITA expression in myeloid cells and lymphoid cells, respectively (6, 8–10). Promoter IV directs CIITA expression in response to IFN-γ stimulation in nonlymphoid cells (9).

In B cells, CIITA is constitutively expressed and associates with all MHC-II promoters, recruiting histone acetyltransferases and chromatin remodeling proteins to activate MHC-II transcription (3, 11, 12). CIITA also associates with the insulator protein CCCTC transcription factor to form long-range interactions with at least some human MHC-II promoter regions (13). However, when B cells terminally differentiate into plasma cells, CIITA expression is silenced, ultimately resulting in the loss of MHC-II expression (14, 15). We previously showed that during this transition, histone modifications associated with active transcription were lost and replaced by at least one mark associated with gene silencing (15). Coupling these observations with those that show that the CIITA pIV DNA is methylated in cell types that are refractory to the induction of CIITA by IFN-γ (16–18) suggests that the locus has the potential for epigenetic regulation.

Five cis-regulatory elements located between −545 bp and +113 bp of the pII transcription initiation site were identified in B cells (8, 19). These elements, termed ARE1, ARE2, site A, site B, and site C, are occupied by the transcription factors E47, PU.1/IFN regulatory factor (IRF) 1/IRF4, SP1, CREB/CBP, and Oct1 (15, 20, 21). Site C binds PU.1, and PU.1 was found to be essential for B cell-specific CIITA expression (15, 21). Following differentiation to plasma cells, the occupancy of these sites by the above activators is lost, and the master repressor Blimp-1 binds to this region (22, 23). The histone H3 lysine 4 demethylase LSD-1 also associates with this region and removes activation-associated histone modifications (23). These latter events are likely critical to the silencing of CIITA pIII during the plasma cell differentiation process.

PU.1 is a member of the ETS domain transcription factor family and is encoded by the PU.1-Spi1-Spi2 proto-oncogene (24, 25). PU.1 expression is required for the development of common lymphoid and myeloid progenitors that give rise to B cells, T cells, NK cells, and macrophages (26–31). Targeted disruption of PU.1 in the mouse resulted in neonatal lethality (32). As a sequence-specific transcription factor, PU.1 binds at promoters and lymphoid-specific enhancer regions either by itself or interacting with other factors, like IRF4, to regulate target genes (33–35).

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Abbreviations used in this paper: ac, acetylation; BAC, bacterial artificial chromosome; 3C, chromatin conformation capture; ChIP, chromatin immunoprecipitation; HSS1, hypersensitive site 1; HSS2, hypersensitive site 2; IRF, IFN regulatory factor; me, methylation (mono, di, tri); MHC-II, MHC class II; pI, promoter I; pII, promoter II; pIII, promoter III; pIV, promoter IV; RNAi, RNA interference; shRNA, short hairpin RNA; UCSC, University of California Santa Cruz.

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Although PU.1 is required for CIITA expression, it is not known how it may coordinate interactions and activate CIITA expression. The regulation of each CIITA promoter (pl, pIII, and pIV) by its proximal promoter regulatory region has been well studied (8, 16, 20, 21). In contrast, little is known about whether there are other distal cis-regulatory elements, which are required for CIITA regulation. Recently, Ni et al. (36) described a series of distal elements within the CIITA gene that were responsive to IFN-γ and regulated expression through pIV. These results suggest the possibility that distal elements may be required for B cell-specific expression as well. To address this issue, experiments were designed to identify novel distal elements that could regulate CIITA in B cells. One element that was identified and termed hyper-sensitive site 1 (HSS1) was required for B cell-specific CIITA expression through pIII. HSS1 was occupied by PU.1 in B cells but not in plasma cells. The mechanism of action of both PU.1 and HSS1 was found to involve the ability of HSS1 to interact directly with the plI promoter region through a long-distance chromatin loop. Intriguingly, this loop was in part dependent on PU.1 expression and was significantly reduced in plasma cells, suggesting that PU.1 plays a role in loop formation and that maximum loop compression and was significantly reduced in plasma cells, suggesting that PU.1 plays a role in loop formation and that maximum loop formation is necessary for the high levels of CIITA found in B cells. Together, these results suggest that multiple elements are required to control CIITA expression in B cells and provide these cells with the ability to present Ags.

Materials and Methods

Cells and culture

The murine BCL1 3B3 B cell line (CRL-1669; American Type Culture Collection, Manassas, VA) and P3X63Ag8.653 (referred to as P3X) plasma cell line (CRL-1580; American Type Culture Collection) were cultured in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated FBS (HyClone Laboratory, Logan, UT), 10 mM HEPES (HyClone Laboratory), 1 mM sodium pyruvate (HyClone Laboratory), 1x non-essential amino acid (HyClone Laboratory), and 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, MO). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and 5-wk-old mice were used to obtain primary B cells from the spleen. Primary B cells were incubated with anti-CD16 and negatively selected using MACS paramagnetic beads and columns following the manufacturer’s protocol (Miltenyi Biotech, Auburn, CA). To induce plasma cell differentiation, purified murine primary B cells were cultured with culture media containing IL-2 (20 ng/ml; Sigma-Aldrich), IL-5 (10 ng/ml; Sigma-Aldrich), and LPS (20 μg/ml; Sigma-Aldrich) for the indicated time as previously described (15).

DNase I hypersensitivity assay

DNase I hypersensitivity assays were performed as described by Lu et al. (37). In this study, 2 × 10^6 cells were used to prepare nuclei. Preparations of nuclei were treated with increasing concentrations of DNase I (Roche) and then plotted as fold over the untreated sample. Replicates were normalized to Y6 for each concentration of DNase I used and plotted with SE. A Student’s t test was used to determine whether the differences observed were significant.

PCR primer sequences

All PCR primer sequences are provided in Supplemental Table I.

RNA isolation and real-time RT-PCR

Total RNA was extracted from the indicated cells using RNeasy mini prep kits (Qiagen, Valencia, CA). Reverse transcriptase (Invitrogen, Carlsbad, CA) was used to produce cDNA according to the manufacturer’s directions. SYBR green dye incorporation into the PCR product using ~1/100th of the cDNA as a template was measured by an iCycler with optical assembly (Bio-Rad, Hercules, CA). Parallel RT-PCR reactions were performed with 18S rRNA primers to normalize between samples. Following 18S rRNA normalization, the average of three biological replicates were plotted relative to the indicated control sample.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted as previously described (11). A total of 4 × 10^5 cells were cross-linked with formaldehyde (1%) for 15 min at room temperature. Cross-linked chromatin was purified and sonicated to generate an average DNA size of 600 bp. The cross-linked, sonicated chromatin (30 μg) was used for immunoprecipitations with the indicated Ab. The company and catalog number for each Ab used are listed in Supplemental Table II. DNA was extracted by phenol-chloroform and ethanol precipitation after cross-linking and digestion with EcoRV and BamHI (New England Biolabs, Beverly, MA). The protocol for bacterial artificial chromosome (BAC) modification followed that described previously (40, 41). The BAC encoding CIITA (RP23-240H17) was purchased from Children’s Hospital Oakland Research Institute (Oakland, CA). The shuttle vector (pLD53SCA-E-B), which was used for introduction of BAC modification, was obtained from Dr. N. Heniz (The Rockefeller University, New York, NY). The shuttle vector has an R6Kg origin, RecA gene, SacB gene, and an ampicillin-resistant gene. For deleting HSS1, the CIITA sequences between chromosome 16: 10,475,871–10,476,371 (UCSC Genome Bioinformatics) and chromosome 16: 10,477,371–10,477,870 were cloned into the Ascl and Paci restriction sites of the shuttle vector. Similarly, for deleting pIII, chromosome 16: 10,487,673–10,488,074 and 10,488,317–10,488,691 were cloned into the same sites. These sequences represent the homologous targeting arms necessary for directing the sequences to be deleted. No crossovers were observed in the recombinant events, BAC containing host bacteria were made competent and transformed with the shuttle vector by electroporation using the Bio-Rad Gene Pulser (Bio-Rad). The cells are selected and grown in 1 ml Luria broth medium containing ampicillin (50 μg/ml) and chloramphenicol (15 μg/ml). The culture was diluted 1:1000 and incubated for 16 h at 37°C. The culture was again diluted 1:5000 and incubated for an additional 8 h at 37°C. Cells were diluted and spread onto Luria broth plates containing the above antibiotics. Colonies were analyzed for integration of the targeting shuttle vector into the BAC by PCR. To resolve the recombinants (i.e., remove the shuttle vector DNA), co-integrated BAC containing bacteria were inoculated into Luria broth cultures supplemented with chloramphenicol (15 μg/ml) for 1 h at 37°C and plated on plates containing the appropriate antibiotics. Colonies were screened by PCR for homologous recombination events in BAC, and extensive restriction enzyme digestion was carried out to ensure that the recombination and resolution processes affected only the region mutated.

BAC DNAs were transfected into the human CIITA mutant cell line R2J2.2.5 by nucleofection using an Amaxa Nucleoporter with transfection kit V according to the manufacturer’s protocols (Lonza, Walkersville, MD). In each transfection, ~2 × 10^6 cells and 8 μg DNA were used. At 48 h posttransfection, RNA was isolated using RNAeasy kits (Qiagen). Real-time RT-PCR was used to analyze the murine BAC-encoded CIITA transcripts using primers specific for each CIITA isotype (Supplemental Table 1). Transcripts were normalized to 18S rRNA levels. Transfection efficiency was monitored by examining the levels of the NaBPI mRNA transcript encoded within the CIITA region of BAC used. As variation of the NaBPI transcript was ~1% between transfections, no additional normalization was conducted. The data were averaged from three independent transfections and plotted with SE. A Student’s t test was used to determine whether the differences observed were significant.

The protocol for bacterial artificial chromosome (BAC) modification followed that described previously (40, 41). The BAC encoding CIITA (RP23-240H17) was purchased from Children’s Hospital Oakland Research Institute (Oakland, CA). The shuttle vector (pLD53SCA-E-B), which was used for introduction of BAC modification, was obtained from Dr. N. Heniz (The Rockefeller University, New York, NY). The shuttle vector has an R6Kg origin, RecA gene, SacB gene, and an ampicillin-resistant gene. For deleting HSS1, the CIITA sequences between chromosome 16: 10,475,871–10,476,371 (UCSC Genome Bioinformatics) and chromosome 16: 10,477,371–10,477,870 were cloned into the Ascl and Paci restriction sites of the shuttle vector. Similarly, for deleting pIII, chromosome 16: 10,487,673–10,488,074 and 10,488,317–10,488,691 were cloned into the same sites. These sequences represent the homologous targeting arms necessary for directing the sequences to be deleted. No crossovers were observed in the recombinant events, BAC containing host bacteria were made competent and transformed with the shuttle vector by electroporation using the Bio-Rad Gene Pulser (Bio-Rad). The cells are selected and grown in 1 ml Luria broth medium containing ampicillin (50 μg/ml) and chloramphenicol (15 μg/ml). The culture was diluted 1:1000 and incubated for 16 h at 37°C. The culture was again diluted 1:5000 and incubated for an additional 8 h at 37°C. Cells were diluted and spread onto Luria broth plates containing the above antibiotics. Colonies were analyzed for integration of the targeting shuttle vector into the BAC by PCR. To resolve the recombinants (i.e., remove the shuttle vector DNA), co-integrated BAC containing bacteria were inoculated into Luria broth cultures supplemented with chloramphenicol (15 μg/ml) for 1 h at 37°C and plated on plates containing the appropriate antibiotics. Colonies were screened by PCR for homologous recombination events in BAC, and extensive restriction enzyme digestion was carried out to ensure that the recombination and resolution processes affected only the region mutated.

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RNA interference and flow cytometry

A series of lentivirus PU.1 short hairpin RNA (shRNA) constructs were purchased from Open Biosystems (catalog no. RMM4534, clone ID TRCN00000009497, TRCN00000009498, TRCN00000009499, TRCN0000009500, TRCN0000009501; Open Biosystems, Huntsville, AL). These lentivirus constructs are based on the base vector pLKO.1.1, allowing for selection by puromycin. HEK 293FT packaging cells were used as the host for transfection of the lentivirus constructs with the pseudo-envelope protein VSV-G using FuGENE 6 cationic liposomes (Roche). After 48 and 72 h posttransfection, the virus was harvested and used to infect murine B cell line BCL1 in the presence of polybrene (0.8 μg/ml). The efficiency of silencing of PU.1 was assessed by Western blot as indicated.

Lentivirus-infected BCL1 B cells were grown in the presence of puromycin (1 mg/ml) for 5 d. A total of 1 × 10⁶ cells were collected, washed with PBS containing 0.1% BSA, and stained with PE-conjugated IA/E Ab for 30 min on ice. Samples were analyzed using a FACSCalibur (BD Biosciences) cytometer, and the data were analyzed using CellQuest (CellQuest, Tampa, FL) and FlowJo 8.8.6 software (Tree Star, Ashland, OR).

Chromatin conformation capture assays

Chromatin conformation capture (3C) assays were performed essentially as described (13, 42). Briefly, 1 × 10⁷ cells were cross-linked with 2% formaldehyde for 10 min. The reaction was quenched with glycine (0.125 M). Cells were lysed in 10 mM Tris (pH 8), 10 mM NaCl, and 0.2% Nonidet P-40 followed by 15 strokes using a dounce homogenizer. The resulting nuclei were washed in NEB2 restriction enzyme buffer, resuspended in the same buffer containing SDS (0.3%), and incubated for 1 h at 37˚C. To sequester SDS, 2% Triton X-100 was added and incubated for 1 h at 37˚C. A total of 400 U HindIII was added and incubated overnight at 37˚C. To digest the DNA, 10 mM MgCl₂, 1% Triton X-100, and 0.1 M ATP) and ligated with 200 U T4 DNA ligase overnight at 16˚C. The cross-links within 3C library products were reversed and the DNA purified by overnight treatment with proteinase K at 65˚C as per assay protocol (13, 42). Quantitative real-time PCR using a standard curve was conducted to measure the frequency of the 3C products within each sample. Standard curves for 3C assays were generated using a CIITA encoding BAC (RP23-240H17) that was HindIII digested and then religated to generate all possible 3C products within the locus. A 3C frequency was determined by averaging the amount of 3C product produced for a given ampiclon (from three independent experiments) and dividing that value by the amount of 3C product determined for an irrelevant restriction fragment that does not interact. In this case, the pIII-H1 3C product served as the background control.

Results

A conserved sequence located ∼11 kb upstream of pIII is hypersensitive to DNase I

To date, only the promoter proximal sequences (within 350 bp) of CIITA pIII have been shown to regulate B cell-specific CIITA expression. To identify additional regions, a series of general assays was conducted on the sequences upstream of pIII. The sensitivity of chromatin to DNase I has been found to be associated with regulatory regions (43). Thus, as a first approach, DNase I hypersensitivity assays were employed on the murine B cell line BCL1 and the plasma cell line P3X, which represent CIITA-expressing and silenced cell lines, respectively. In BCL1 cells, an HSS appeared at ∼11 kb upstream of pIII (Fig. 1A). A strong DNase I HSS was also observed at ∼13 kb from pIII, but only in the CIITA-negative P3X plasma cell line (Fig. 1A). Thus, this region appears to be differentially accessible to DNase I, suggesting that it has some regulatory function. The ∼11 kb site specific to B cells was HSS1 and the ∼13 kb site HSS2.

A PCR-based DNase I hypersensitivity assay was used to assess HSS1 in primary murine splenic B cells (Fig. 1B). In this study,

![FIGURE 1](image-url)

A B cell- and plasma cell-specific DNase I hypersensitive sites are located in conserved regions upstream of CIITA promoter I. A, Southern blot DNase I hypersensitivity assay performed on the murine B cell line BCL1 and plasma cell line, P3X63ag8.653 (referred to as P3X). Arrowheads denote increasing DNase I concentration. A schematic diagram of the region analyzed and the probe location is shown. B, PCR-based DNase I hypersensitivity assay on purified murine splenic B cells and BCL1 cells. DNase I-treated and control samples were subjected to real-time PCR amplification over the designated regions, represented by the primer amplicon sets, which are described in Supplemental Table 1. The amount of PCR product for each sample was divided into the undigested sample, and the average of three reactions was plotted. C, The regions corresponding to HSS1 and HSS2 were analyzed for sequence conservation using the UCSC Genome Bioinformatics browser (38). The screen shot from that analysis is shown with an annotated schematic.
HSS1 was sensitive to increasing concentrations of DNase I in both primary murine B cells and BCL1 cells (Fig. 1B). Promoter III also displayed strong DNase I hypersensitivity in the primary cells. Promoters I and IV were not sensitive to DNase I in these cells, thereby providing specificity controls. A region located −14 kb from pII (represented by primer set Y6) was used as a control and showed no changes in DNase I hypersensitivity in BCL1 or primary B cells (data not shown).

Cross-species genomic DNA comparisons using the UCSC Genome Bioinformatics browser tools found that HSS1 was highly conserved, whereas HSS2 showed only low levels of sequence conservation (Fig. 1C). No micro-RNA sequences or other coding/noncoding sequence features were found within these regions. In human HeLa cells, sequences within the HSS1 region were recently shown to participate in IFN-γ induction through pIV (36), suggesting that this region may function as a complex regulatory component and may contribute to B cell-specific expression of CIITA. Thus, its characterization and function were investigated further. Southern blot-based DNase I hypersensitivity assays were also performed for the region between pl and pIII. A hyperson sensitive site was observed ~2 kb 3′ to pl in both BCL1 and P3X cells. Because this region was not differentially sensitive to DNase I, it was not explored further.

**Chromatin modifications at HSS1 reveal an accessible and active nucleosome structure**

To further characterize this element and determine if other potential regulatory regions were present within this upstream segment of the CIITA gene, a series of ChIP assays using Abs to the acetylated forms of histones H3 and H4 were conducted. These modifications are typically associated with regulatory regions and expressed genes (44). In this study, we sought to find regions that were differentially modified between B cells (BCL1) and plasma cells (P3X), as such regions could indicate a putative role in B cell-mediated expression. Twenty-three amplicons spaced at ~1-kb intervals, spanning the region between pIV and ~18 kb upstream of pIII, were used to analyze precipitated chromatin. The regions corresponding to pIII and pIV showed 47–107-fold levels of histone H3 acetylation over the control Ab in BCL1 B cells that were significantly less in the P3X plasma cell line (Fig. 2). This result was similar to that observed for human B and plasma cell lines (15). Promoter I displayed H3 acetylation levels that were 9-fold over control but not differentially modulated between the two cell lines. HSS1 displayed levels of acetylated histone H3 that were 25- and 6-fold over the control when comparing the B and plasma cell lines, respectively, suggesting that histone H3 acetylation of this region was enhanced in B cells. Similarly, pIII and pIV showed high levels of histone H4 acetylation in BCL1 cells but not in P3X cells. Compared with pIII and pIV, pl showed significantly lower levels of histone H4 acetylation that did not change between the B and plasma cell lines. In agreement with the histone H3 ChIP result, HSS1 also showed high levels of H4 acetylation in B cells (175-fold over control) and lower levels in plasma cells (29-fold). The sequences extending upstream of HSS1 to ~13 kb (HSS2) showed histone H4 acetylation levels between 54- and 98-fold over control, although the differences between B and plasma cells was not >2-fold.

Analysis of activation/silencing-associated chromatin modifications for select regions can provide a fundamental assessment of the general chromatin architecture of a region (accessibility), as well as the general gene expression activity of a region (active/inactive) (45). Additional ChIP assays were conducted on HSS1, and comparisons were made between the three promoter regions and a control region, Y4 (Fig. 3). Y4 (−16 kb from pII) represents a region with no known regulatory or promoter function. Four histone acetylation modifications were assessed: H3K9, H3K18, H4K8, and H4K16 (Fig. 3). Nucleosomes at pII were highly acetylated at histone H3K9, H3K18, and H4K8 (218- to 1600-fold over control) in BCL1 cells and substantially less in the P3X cells. Each of these marks was therefore associated with active transcription of CIITA. Although not at the level of pII, HSS1 showed differential levels (BCL1 versus P3X cells) for these three modifications as well. Promoter I showed acetylation of H3K18 and H4K8, whereas pIV displayed H3K9, H3K18, and H4K8 acetylation modifications. However, pl and pIV modifications were not indicative of CIITA expression, suggesting that they may represent a general feature of the region in the lymphocyte lineage. None of the regions displayed histone H4 K16 acetylation levels that were greater than the control region Y4.

The degree of methylation of H3 K4 is associated with gene transcription or poised RNA polymerase binding (46). Histone H3 K4 monomethylation has been associated with enhancer activity and dimethylation with gene expression and accessibility, whereas trimethylation of this residue is associated with RNA polymerase binding and active transcription (46–48). All three configurations of histone H3 lysine methylation were observed at HSS1 in B cells.
HSS1 and PU.1 regulate CIITA expression in B cells

The above data suggest the possibility that HSS1 may function in the regulation of CIITA in B cells. To examine the regulatory potential of HSS1, we employed a BAC modification system (40, 41) that would allow the introduction of a mutation into the CIITA gene and test its effect on controlling CIITA expression in the context of the locus. Thus, using the BAC system, the effect of deleting HSS1 on the expression of CIITA from each of the promoters can be assessed. The BAC (RP23-240H17) encodes the murine CIITA locus with 88,771 bp upstream of pIII and 51,386 bp downstream of CIITA’s last exon (Fig. 4A). This includes all proximal promoter regulatory regions and other regulatory regions (16, 21, 36, 50) known to regulate CIITA expression. To modify the BAC, two homologous recombination-targeting shuttle vectors were created. These vectors were designed to generate a 1-kb and 500-bp deletion of HSS1 and pII and were reduced in plasma cells (Fig. 3). Intriguingly, pl also displayed strong H3K4me1 at this residue in B cells, whereas this modification appeared at high levels at pIII and pIV in plasma cells. In contrast, H3K4me2 and K4me3 levels showed a direct relationship with CIITA expression. Promoter III showed the highest levels of these marks, as anticipated, as this promoter is predominantly used in B cells. Promoter IV also showed these two active marks, as some transcription from pIV is observed in B cells (data not shown). Promoter I did not display H3K4me2 or K4me3 modifications, suggesting that this promoter region is not transcriptionally active in BCL1 cells. RT-PCR for pl in BCL1 cells showed no detectable transcripts (data not shown). The histone variant H2AZ, which is often associated with active promoter regions (49), was observed at the highest levels in BCL1 cells at pIII and HSS1 and at lower levels at the other sites.

Repressive chromatin modifications H3K27me2 and K27me3, which are associated with the Polycomb repressor complex, were examined (Fig. 3). Low levels of H3K27me2 were associated with pl and possibly with HSS1 in BCL1 cells. By contrast, P3X plasma cells showed high levels of H3K27me3 associated with all three promoter regions, but not HSS1. Thus, the three promoters display histone code patterns that are mostly associated with gene expression, and, in general, the histone modification patterns of HSS1 also follow the transcriptional state of the locus in B cells.

**HSS1 is required for CIITA expression**

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Repressive chromatin modifications H3K27me2 and K27me3, which are associated with the Polycomb repressor complex, were examined (Fig. 3). Low levels of H3K27me2 were associated with pl and possibly with HSS1 in BCL1 cells. By contrast, P3X plasma cells showed high levels of H3K27me3 associated with all three promoter regions, but not HSS1. Thus, the three promoters display histone code patterns that are mostly associated with gene expression, and, in general, the histone modification patterns of HSS1 also follow the transcriptional state of the locus in B cells.
and pIII (ΔpIII). Primers used to assess the integrity of the BAC constructs are shown. B, PCR across the regions that were deleted in each of the mutant CIITA BAC constructs using the indicated primer pairs showed specificity of the intended deletions. C, Wild-type and mutant BACs were transfected into the CIITA-deficient human B cell line R2J2.2.5 by nucleoporation. Forty-eight hours posttransfection, the cells were harvested and the RNA analyzed by real-time RT-PCR using primers specific for each of the CIITA promoter-dependent transcripts as shown in A. Three independent transfections were performed, and the results were averaged with respect to the levels of 18s rRNA and plotted as relative expression with SE. Student t tests showed that the differences between wild-type and ΔHSS1 and ΔpIII were highly significant (*) for pl and pIII (p < 0.04). No differences were observed at pIV.

real-time RT-PCR. Mock-transfected cells showed that the primers are specific for murine CIITA as they did not detect any of the mutant CIITA transcripts expressed in R2J2.2.5. The wild-type BAC expressed CIITA from pIII and pl at levels of -370-fold and 27-fold over the mock-transfected cells, respectively (Fig 4C). The ΔHSS1 mutation resulted in nearly a complete loss of pIII expression, suggesting that the HSS1 element is critical to pIII-mediated expression. The ΔHSS1 mutation had a similar influence on pl. As anticipated, the ΔpIII mutation caused a complete loss of pIII-directed CIITA expression. Surprisingly, the ΔpIII mutation also affected expression from pl. This result suggests that the B cell-specific elements contained within the ΔpIII deletion were responsible for expression from pl. In contrast, no expression was detected from pIV irrespective of the CIITA BAC constructs assayed. Low levels of both pl and pIV CIITA mRNA isoforms were detected in Raji cells, but these levels were 43–64-fold lower than the pIII isoform (data not shown). Thus, with the exception of pIV, the BAC mimics to a large extent the properties of the CIITA locus, and HSS1 plays a critical role in expression from pIII.

PU.1 binds to HSS1 and CIITA pIII

To predict the identity of factors that may bind HSS1 in B cells, the DNA sequence of HSS1 was analyzed using the Genomatix software program. The analysis found that HSS1 had two highly significant DNA sequence spaced at intervals of ~1 kb between −11 kb and +4 kb relative to CIITA pIII. B, Purified splenic B cells were used for PU.1 ChIP assays either immediately (black bars) or ex vivo differentiated in LPS, IL-2, and IL-5 for 1, 3, or 5 d (gray bars) as indicated. The regions analyzed by ChIP are stated. In all of these assays, three independent replicates were carried out, averaged, and plotted versus the nonspecific control with SE. All real-time PCR values were determined as above by comparison with a standard curve for the amplicon generated from genomic DNA. Student t tests were used to determine the significance of the differences from undifferentiated cells. HSS1, pl, and pIII showed statistical significance of p < 0.05 between untreated and all days posttreatment (*). C, Western blot of murine splenic B cells (day 0) ex vivo differentiated to plasma cells (days 1, 2, and 3) that was stained for PU.1 and actin.

FIGURE 4. HSS1 is required for B cell expression of CIITA from pIII. A, A schematic representation of the murine CIITA encoding BAC and the surrounding loci is shown with the indicated deletion mutations that were created in HSS1 (ΔHSS1) and pIII (ΔpIII). Primers used to assess the integrity of the BAC constructs are shown. B, PCR across the regions that were deleted in each of the mutant CIITA BAC constructs using the indicated primer pairs showed specificity of the intended deletions. C, Wild-type and mutant BACs were transfected into the CIITA-deficient human B cell line R2J2.2.5 by nucleoporation. Forty-eight hours posttransfection, the cells were harvested and the RNA analyzed by real-time RT-PCR using primers specific for each of the CIITA promoter-dependent transcripts as shown in A. Three independent transfections were performed, and the results were averaged with respect to the levels of 18s rRNA and plotted as relative expression with SE. Student t tests showed that the differences between wild-type and ΔHSS1 and ΔpIII were highly significant (*) for pl and pIII (p < 0.04). No differences were observed at pIV.

FIGURE 5. PU.1 binds to HSS1 and CIITA pIII. A, ChIP assays were conducted to assess the binding of PU.1 to HSS1 and other sites across the locus using chromatin prepared from BCL1 and P3X cells. The binding of PU.1 was analyzed on the CIITA gene using primers that represent sequences spaced at intervals of ~1 kb between −11 kb and +4 kb relative to CIITA pIII. B, Purified splenic B cells were used for PU.1 ChIP assays either immediately (black bars) or ex vivo differentiated in LPS, IL-2, and IL-5 for 1, 3, or 5 d (gray bars) as indicated. The regions analyzed by ChIP are stated. In all of these assays, three independent replicates were carried out, averaged, and plotted versus the nonspecific control with SE. All real-time PCR values were determined as above by comparison with a standard curve for the amplicon generated from genomic DNA. Student t tests were used to determine the significance of the differences from undifferentiated cells. HSS1, pl, and pIII showed statistical significance of p < 0.05 between untreated and all days posttreatment (*). C, Western blot of murine splenic B cells (day 0) ex vivo differentiated to plasma cells (days 1, 2, and 3) that was stained for PU.1 and actin.
locus as above, using chromatin prepared from BCL1 and P3X cells (Fig. 5A). The result showed that PU.1 bound to HSS1 in BCL1 cells (Fig. 5A). Robust PU.1 binding was also detected at pIII in BCL1 cells, which was in agreement with previous studies that showed PU.1 binding to pIII (15). Low levels of PU.1 binding were detected in BCL1 cells at pl and at a region located 2 kb upstream of pIII. No binding of PU.1 at any site was detected in P3X plasma cells. This result is consistent with the very low levels of PU.1 that are found in P3X cells (data not shown). Thus, recruitment of PU.1 to at least two and possibly four sites may be important for the B cell-specific expression of CIITA.

To examine the dynamics of PU.1 occupancy at HSS1 during the B cell to plasma cell transition, chromatin preparations from primary murine B cells ex vivo stimulated with IL-2, IL-5, and LPS to induce plasma cell differentiation (15) from day 0 through day 5 were examined. This treatment results in the silencing of CIITA expression (15). Similar, robust levels of PU.1 binding were observed at HSS1 and pIII in the untreated freshly isolated murine B cells. PU.1 occupancy was lost rapidly during the differentiation process (Fig. 5B). This correlated with the loss of PU.1 protein that occurs during the differentiation process (Fig. 5C).

**FIGURE 6.** PU.1 is required for CIITA expression in BCL1 cells. A. Five different shRNA lentiviral expression vectors were used to knock down the expression of PU.1 in BCL1 B cells. Infected cells were selected using puromycin for 5 d. Lysates prepared from the resistant cells were assayed by immunoblotting for PU.1 and actin protein. B. RNA from PU.1-shRNA–depleted BCL1 cells was purified and analyzed by real-time RT-PCR for the levels of the indicated transcripts. These experiments were performed three times from independent shRNA infections. The results of each experiment were averaged and plotted with respect to the parent lentiviral construct (pLKO.1)-infected cells. *Significance was determined using a Student t test and comparisons between control and PU.1 shRNA with p values < 0.05. C. Flow cytometry was used to assess the level of MHC-II surface expression on pLKO.1 and shPU.1 vector 5-infected BCL1 B cells.
equally accessible to restriction enzymes irrespective of cell type or conditions of the assay (Supplemental Fig. 1). Additionally, the ability of a digested fragment to self-ligate and form a 3C product was also evaluated (Fig. 7B, primers H4 and H5) and demonstrated that these sites were equally accessible between B cells and plasma cells.

To determine the role of PU.1 in the ability of pIII to interact with HSS1 and pl, 3C assays were performed on BCL1 cells infected with the PU.1 shRNA lentivirus vector 5. Compared with pLKO.1 lentivirus-infected cells, the shPU.1 lentivirus showed a 52% reduction in the 3C product formed between HSS1 and pIII. Intriguingly, interactions between pIII and pl were unchanged by PU.1 knockdown. No interaction was observed with the control region (primer H7-H6). Another way to deplete PU.1 from the system is to ex vivo differentiate primary B cells to plasma cells with LPS, IL-2, and IL-5 as above. In concordance with the loss of PU.1 occupancy at HSS1 and pIII, ex vivo differentiation resulted in a 65% decrease in the frequency of 3C product formation between HSS1 and pIII and a 62% loss of the interaction between pl and pIII. These results suggest that PU.1 plays a role in HSS1 interacting with pIII but that interactions between pl and pIII are dependent on another set of factors.

**Discussion**

In the mouse, CIITA expression is regulated from three promoter regions, with each region initiating the transcription of a unique isoform of CIITA (8). The three promoters are regulated in a cell-type–dependent manner, with pIII functioning in lymphoid cells. In B cells, pIII is expressed constitutively but is silenced as B cells transition to plasma cells. Several groups have shown that the proximal region 5’ to the pIII transcription start site encodes a complex set of binding sites that is critical to B cell-specific expression (20, 51). However, no other regions associated with pIII expression in B cells have been described. Using a number of

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**FIGURE 7.** A long-distance interaction occurs between HSS1 and CIITA pIII. A, A schematic of CIITA region analyzed by 3C assays is shown with HindIII sites, 3C primers, and relative position of HSS1 and the three CIITA promoters is shown. Note that one of the pIII region-specific primers has been termed PIII. B, Schematic representation of 3C products for pIII interacting with HSS1 (primer H2) and pl (primer H3) are shown. These 3C products were cloned and sequenced to verify their location. Agarose gel electrophoresis of 3C reactions was carried out with the indicated primer pairs using BCL1 and P3X cells. C, Real-time PCR analysis of 3C products formed between pIII and the H7 downstream region, HSS1, and pl in BCL1 and P3X cells. A relative cross-link frequency was determined by calculating the amount of 3C product between pIII and the experimental restriction fragment and dividing it by the amount of 3C product formed for pIII and H1 (an irrelevant upstream restriction fragment; see B). The average of three independent experiments is shown with SE and a Student t test evaluation of the significance. No significant interactions were observed between pIII and pI in the assay conditions. Relative 3C cross-link frequency for pIII interactions in BCL1 cells infected with pLKO.1 and PU.1 shRNA lentiviral vector 5 (D) and undifferentiated and day 3 ex vivo-differentiated murine splenic B cells (E) are presented as in C.
tools, we identified a region located upstream of pIII and pI that is critical for B cell-specific expression. This region, termed HSS1, is highly conserved, hypersensitive to DNase I treatment, and displayed chromatin marks that were associated with an active and accessible locus. A key parameter showing that HSS1 was critical to B cell-specific expression of CIITA was the deletion of this element from a BAC construct. Due to the multiple promoters that control CIITA, this experiment provided an in-context evaluation of the role of HSS1 with respect to all the CIITA promoters. Although pIII-dependent transcripts were robustly expressed in the BAC system, low levels of pl but no levels of pIV-specific CIITA expression were observed. The pl transcripts were affected by the presence of HSS1 and pIII, suggesting that transcription from pl was likely controlled by factors bound at these elements. Analysis of CIITA transcript utilization from the human B cell line Raji, which is the parent of RJ2.2.5, showed that pl and pIV produced minimal levels of steady-state transcripts compared with pIII. In contrast, no pl or pIV transcripts could be detected from the murine BCL1 cells (data not shown). Thus, there is some variation between the human and murine systems or cell lines.

DNase I hypersensitivity Southern blots also identified a robust HSS that was present in the plasma cell line P3X but not in B cells. Cross-species conservation of HSS2 was not high, and HSS2 displayed significant levels of histone H4 acetylation but oddly near background levels of histone H3 acetylation. Intriguingly, the region just upstream of HSS2 showed no histone modifications associated with an active locus. Although such demarcations can sometimes suggest that the element functions as a boundary element, no binding of the insulator factor CCCTC transcription factor to HSS2 was found (data not shown). Thus, at this time, the function of HSS2 is not known. DNase I HSSs were not detected at pl or at the PU.1 site located between pl and pIII (Fig. 1B and data not shown). Although this is inconsistent with the ChIP data for the low levels of binding of PU.1, it is consistent with the ChIP data for active histone modifications that were observed in these regions. Thus, the DNase I hypersensitivity assays correlate more closely with the histone modifications and chromatin conformation rather than the binding of a factor.

In a previous study (15), we found that chromatin histone modifications that were in general associated with active gene expression were lost at the human CIITA proximal promoter regions when human B cell lines were compared with plasma cell lines. This suggested that there were global changes that controlled chromatin accessibility and the overall architecture of the locus. In this study, similar findings were observed for the murine B cell line BCL1 and plasma cell line P3X. Intriguingly, in the plasma cell line P3X, chromatin marks associated with Polycomb gene silencing (45, 56) were observed at all three CIITA promoters, suggesting that there is an active mechanism that results in the recruitment of Polycomb factors to silence CIITA expression.

The polycomb H3K27me3 methyltransferase EZH2 was found to play a role in regulating pIV in uveal melanoma cells (18). Preliminary data showed that EZH2 binds to pII in plasma cells (H. Yoon and J.M. Boss, unpublished observations).

Genome-wide studies have helped identify the global locations to various histone marks (47, 48). Histone H3 K4 methylation marks are mostly indicative of the gene expression state of a locus. H3K4me3 is most often associated with promoter regions of actively transcribed genes (46). For CIITA in B cells, histone H3K4me3 was most associated with pIII, but was also found at lower levels at pIV and HSS1. Only background levels of histone H3K4me3 were observed in plasma cells, and thus, this mark is clearly associated with CIITA expression. Histone H3K4me2 is more broadly associated with gene expression and can be found covering large segments of the promoter and upstream regulatory regions of genes (46) and perhaps is a mark of accessibility. HSK4me2 was lower in plasma cells, suggesting that the region may be less accessible, perhaps due to silencing marks associated with Polycomb. Although histone HSK4me1 was reported to be associated with enhancer regions (48), the patterns for CIITA promoters and HSS1 correlated with transcription initiation in that pIII and pIV showed high levels of this modification in plasma cells, whereas the opposite was observed for HSS1 and pl in B cells. The finding of multiple modifications within a region may reflect the number of nucleosomes assessed in the ChIP assays, as well as the dynamic nature of the marks that is likely to occur.

For B cells, CIITA expression and the interaction between HSS1 and pIII was in part dependent on PU.1. Depletion of PU.1 by RNAi or through the use of plasma cells or differentiation of B cells to plasma cells showed significant reduction in CIITA mRNA levels and in HSS1 interactions with pIII. The inability to completely deplete PU.1 levels in the RNAi and ex vivo differentiation systems is likely to underplay PU.1’s role in transcription of CIITA. Conservatively, we conclude that PU.1 is necessary for full activity of HSS1 and B cell-specific expression of CIITA, but that other factors are likely to be involved. The finding of PU.1 at both HSS1 and pIII was intriguing and may suggest a role for PU.1 to help bridge these regions together, as its reduction by shRNA resulted in a decrease in the 3C product. We suggest that the ChIP data reflect direct in vivo binding of PU.1 to both sites and that this is not due to 3C interactions between these sites. This conclusion is based on the equal and robust ChIP results for primary B cells (Fig. 5B) and the fact that the conditions used to fix the cells have not shown indirect ChIP binding due to 3C interactions at other sites that are known to interact (13). Whether PU.1 interacts with other DNA binding proteins or whether it interacts with a set of coactivators to stabilize the interactions between pIII and HSS1 can only be speculation at this point.

Recently, Ni et al. (36) identified a series of sequences within the CIITA locus of human HeLa cells that bound STAT1 and IRF1 in response to IFN-γ signaling. Some of these elements were required for the IFN-γ expression through pIV, the CIITA promoter responsible for IFN-γ expression in nonhematopoietic cells (36). A role for the IFN-γ elements in B cells was not determined. We found that HSS1 is the murine homolog to the human region termed −16 (36), as referenced from the pIV transcriptional start site. In IFN-γ–treated HeLa cells, −16 interacted with pIV in a BRG1-dependent manner. BRG1 is the ATPase of the Swi/Snf chromatin-remodeling complex that is associated with the expression of many genes and likely facilitates nucleosome movement to allow the interactions to occur (57).

With the discovery that HSS1 functions in CIITA regulation through pIII in B cells and that there were multiple elements found to fully orchestrate pIV regulation by IFN-γ in HeLa cells (36), two questions arise. How many total elements will be required to regulate CIITA in a tissue-specific manner, and how will promoter usage be derived? Five elements were required for IFN-γ signaling through pIV in HeLa cells (36). Our current findings imply that at least HSS1 responds to multiple signals, controlling both B cell- and IFN-γ–inducible expression. It is possible that like HSS1, other elements that were discovered in the IFN-γ system will specify expression from the different CIITA promoters. However, whereas pl was found to interact with pII in B cells, it was not found to interact with pIV in the IFN-γ system (36). Thus, it may be that only a limited set of these elements may function as master regulators of CIITA and that a set of promoter-specific elements will also exist. HSS1 has the potential to be one such master regulatory element for CIITA. For such elements, the transcription factors that bind to the
master element could determine which promoter is used. For IFN-γ treatment of HeLa cells, STAT1/IRF1 are the key factors that likely target pIV transcription, whereas, in B cells, PU.1 is, at the very least, a key component of HSS1 and pII B cell regulatory mechanism. Although little is known about even the proximal promoter elements for pI, it is possible that PU.1, which is also expressed in myeloid-derived dendritic cells (30), also plays a role in this cell lineage for CITIA expression. Considering that HSS1 has the potential to bind PU.1, it is possible that it will also function in the regulation of pI. Nonetheless, the current data suggest that the HSS1/−16 element may function in multiple cell-type contexts with its use dependent on the specific set of transcription factors that are expressed or induced.

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