Common distal elements orchestrate CIITA isoform-specific expression in multiple cell types

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Major histocompatibility class II (MHC-II) expression is critical for immune responses and is controlled by the MHC-II transactivator CIITA. CIITA is primarily regulated at the transcriptional level and is expressed from three main promoters with myeloid, lymphoid and interferon (IFN)-γ-treated non-hematopoietic cells using promoters pl, pII and pIV, respectively. Recent studies in non-hematopoietic cells suggest that a series of distal regulatory elements may be involved in regulating CIITA transcription. To identify distal elements in B cells, a DNase I hypersensitivity screen was performed, revealing a series of potential novel regulatory elements. These elements were analyzed computationally and biochemically. Several regions displayed active histone modifications and/or enhanced expression of a reporter gene. Four of the elements interacted with pII in B cells. These same four regions were also found to interact with pl in splenic dendritic cells (spDC). Intriguingly, examination of the above interactions in pl-knockout-derived spDC showed a switch to the next available promoter, pIII. Extensive DNA methylation was found at the pl region in B cells, suggesting that this promoter is not accessible in B cells. Thus, CIITA expression is likely mediated in hematopoietic cells by common elements with promoter accessibility having a part in promoter choice.

INTRODUCTION

Major histocompatibility class II (MHC-II) genes are essential for antigen presentation. MHC-II proteins form heterodimers that are expressed principally on the surface of antigen-presenting cells, thereby generating antigen-specific humoral immune responses. MHC-II expression is highly regulated at the level of transcription. The transcription factors RFX, CREB and NF-Y are necessary but not sufficient for MHC-II expression (reviewed in Choi et al.3). The MHC-II transactivator, CIITA, is required to interact with these factors and the basal transcription machinery to initiate MHC-II expression. Unlike RFX, CREB and NF-Y, which are ubiquitously expressed, CIITA expression is limiting. Thus, CIITA and the mechanisms that control its expression are responsible for regulating MHC-II gene expression and antigen processing. CIITA is regulated primarily at the level of transcription. CIITA is transcribed from three main promoters, which are used principally in a cell-type-dependent manner. Each promoter encodes a unique first exon that is spliced into a common second exon to create distinct isoforms of CIITA. Cells of the myeloid lineage, including splenic-derived dendritic cells (spDC), primarily express CIITA from the most distal promoter (promoter I or pl). Cells of the lymphoid lineage principally express CIITA from promoter III (pIII), and most cell types, including non-hematopoietic cells, will use promoter IV (pIV) in an IFNγ-inducible manner. Individual roles for these isoforms are unclear; however, they appear to be somewhat interchangeable. When CIITA is dysregulated or absent, a variety of immune defects are observed. CIITA was first identified in a study to discover the underlying gene responsible for one complementation group of bare lymphocyte syndrome, a severe combined immune deficiency disease. CIITA knockout (KO) mice lack positive selection for CD4+ T cells and do not respond well to immunization or pathogenic challenge. Thus, appropriate regulation of CIITA is key to healthy immune responses.

The proximal regulatory region for pII is well defined. A minimal unit necessary for maximal expression is contained within 319 bp of the transcription start site that contains multiple cis-regulatory elements: ARE-1 and ARE-2 elements, a nuclear factor-1-binding site and an AP1-binding site. These elements are key to healthy immune responses.

In contrast to its well-defined proximal regulatory elements, only one distal regulatory element for pII was identified previously and termed hypersensitive site 1 (HSS1). HSS1 is located ∼3 kb upstream of pl. PU.1-bound HSS1 was shown to interact directly with pII, HeLa cells, which can induce CIITA pIV expression in response to IFNγ, were found to use a network of distal elements located both upstream and downstream of the CIITA promoter regions and gene. However, it is not known whether other elements regulate CIITA expression in lymphocytes or in myeloid cell types.
To identify novel elements regulating CIITA in B cells, a PCR-based DNase I hypersensitivity assay was used and identified a number of potential regulatory regions. Four of these distal regions were found to interact with pl in B cells using a chromatin conformation capture (3C) assay. The most 3’ of these elements was found to bind the transcriptional insulator CCCTC-binding factor (CTCF). One of the 5’ elements identified was HSS1, whereas the two others were novel to B cells. These two sites were able to activate a heterologous promoter, and one displayed common histone marks of active chromatin/enhancers, as well as PU.1 binding. All four of the interacting regions were also able to interact with pl in spDC. Interactions between all distal regulatory elements and CIITA pl were reconfigured to pl in spDC derived from mice containing a genetic deletion of pl. This rearrangement of promoter choice suggested that these elements search for an accessible promoter region to drive transcription. In B cells, the pl promoter contains extensive DNA methylation and is likely to be in an epigenetic restricted state, with pl representing the first available promoter. Thus, CIITA’s regulatory regions function across hematopoietic lineages and demonstrate flexibility in choosing promoters to drive CIITA expression.

RESULTS

Multiple DNase I-hypersensitive sites are present in the CIITA locus in murine B cells

To identify regulatory regions within the CIITA locus, a PCR-based DNase I hypersensitivity assay was performed in the murine B-cell line BCL1 (CIITA-expressing). Comparisons with the CIITA non-expressing plasma cell line P3X were carried out such that B lymphocyte-specific elements might be identified. In these assays a series of 95 amplicons (1–2 kb in length) were designed to span the entire CIITA gene and the surrounding regions and encompassed ~160 kb of DNA (Figure 1a). Regions encoding repetitive DNA sequences were not screened, as it would not be possible to map any activity to the CIITA locus. Noncoding regions highly conserved between mice and humans were preferentially included as these might have regulatory function (Figure 1a). Cell lysates were incubated with increasing concentrations of DNase I, DNA-puriﬁed and used as a substrate for PCR. Analysis of the band intensities of the PCR amplicons on ethidium bromide-stained gels was used to determine a slope of sensitivity (Figures 1b and c). The absolute values of the negative slopes were plotted along the sequence (Figure 1a). Amplicons that showed no change in intensity with increasing DNase I treatments were not examined further. Regions of interest (ROIs) that displayed a decrease in amplicon intensity, corresponding to a substantial decrease in slope in BCL1 cells (for example, regions – 3, +10 and +15 kb from the pl transcription start site (TSS)) were considered as potential regulatory regions for CIITA (Figure 1b). To choose regions for additional study, sequence conservation with humans was examined, and only regions with mouse/human sequence homology were considered further. From these analyses, 13 regions were chosen (Figure 1a, horizontal bars).

To provide a higher-resolution analysis for the DNase I-hypersensitive sites, quantitative PCR (qPCR) using smaller amplicon

![Figure 1](image.png)
subsets that could be evaluated using real-time PCR was employed. As above, BCL1 and P3X cell lysates were subjected to increasing concentrations of DNase I. For analysis, amplicons of 200–600 bp in length were designed within each of the ROIs from the low-resolution screen (Figure 2a). Where possible, multiple amplicons were used for a single region. At this resolution, BCL1 cells were more accessible to DNase I than P3X at the CIITA locus (Figure 2b). The results of this analysis identified eight regions of hypersensitivity (−47, −15, −3, +10, +11, +15, +36 and +37 kb from the TSS) for further investigation, with all being more hypersensitive in BCL1 than P3X.

Four ROIs physically interact with pIII when CIITA is expressed. One mechanism through which regulatory regions exhibit activity is via physical interactions with their cognate promoters. To determine whether any ROIs were exhibiting regulatory activity by physically interacting with the lymphocyte-specific promoter (pIII), 3C assays were performed. For these experiments, ~138 kb of the CIITA locus was queried, and included nearly all hypersensitive regions identified above irrespective of whether the region was hypersensitive only in BCL1 cells or was also hypersensitive in P3X cells. In addition, regions that were found to be enriched for H3K4me1 in B cells were analyzed. Primary splenic B cells, which principally use pIII, were used for this analysis (Figure 3a). To improve resolution and to verify interactions, two different restriction enzymes were chosen for 3C analysis (Figure 3b). HindIII restriction digestion separates all the CIITA promoters from one another, and EcoRI restriction digestion refines the location of interacting regions and verifies the interactions observed in the HindIII system. The map of interactions revealed that there were three main regions that strongly interacted with pIII in splenic B cells (Figure 3c). These included regions within restriction fragments H5 and E4, which encompassed the −15 ROI; H6 and E6; and H16 and E12. Under the conditions used, interactions between pIII and HSS1 were not detected. Detection of pIII and H16/E12 interactions previously were obtained using a more sensitive 3C assay that employed a higher concentration of formaldehyde in addition to a longer crosslinking time (15 min) than used above. To determine whether such crosslinking could be replicated, 3C was repeated for the HSS1 region (H7), along with control region H4 and additional ROIs in H5 and H16. As observed, under these conditions, interactions with H7 were detected with no increase in background interactions with H4 or increased interactions at H5 and H16 (Figure 3d). Together, these data suggest that in addition to the previously identified HSS1 three new regions interact with pIII to potentially regulate CIITA expression.

CTCF binds at multiple locations across the CIITA locus. In silico sequence analyses of the H16/E12-interacting fragment suggested that CTCF might bind this region. CTCF is a transcriptional-insulator-binding protein that can block the effects of a downstream enhancer from acting on a gene promoter. CTCF is also known to have an important role in creating a three-dimensional (3D) architecture involved in gene regulation.

Analysis of CTCF chromatin immunoprecipitation (ChIP)-seq data for B cells and plasmablasts across the locus identified six CTCF sites (Figure 4a). Whereas two of these sites were in genes upstream of CIITA (Nubp1) and downstream (Clec16a), the other four were intragenic, with two of the sites (+40 and +43) residing in H16/E12. To confirm the ChIP-seq analysis, real-time coupled ChIP was performed on these regions and compared with a positive control for the H19 locus and a negative control located at +44.5 in the CIITA locus in BCL1 and P3X cells. The results confirmed CTCF occupancy at the +23, +38, +40 and +43 kb sites (Figure 4b). CTCF occupancy at +23 and +38 was increased significantly in BCL1 cells as compared with P3X plasma cells.

CIITA-hypersensitive sites are associated with active histone modifications. As regulatory regions commonly contain distinct histone modifications associated with open chromatin or enhancers, eight ROIs, the three promoters and three negative control sequences

Figure 2. Quantitative analysis of DNase I hypersensitivity shows that ROIs in BCL1 cells are generally more hypersensitive than in P3X cells. (a) Conserved ROIs were queried using qRT-PCR, with black bars indicating the amplicons screened in b. (b) BCL1 and P3X cells were treated with increasing concentrations of DNase I. Amplicons were screened by qRT-PCR using amplicons of between 200 and 600 bp in BCL1 cells and P3X cells. Error bars represent s.e.m., and *P ≤ 0.005 between untreated samples and those treated with the maximum amount of DNase I.
were assessed for the chromatin marks H3K4me1, H3K9Ac and H3K27ac in both BCL1 and P3X cells (Figure 5). H3K4me1 is associated with enhancer regions and when coupled with H3K27Ac is indicative of active enhancers. H3K9Ac is often found at active promoters and regulatory regions. For the most part, active modifications were found at many of the ROIs in BCL1 cells and these marks were reduced in P3X cells (Figure 5a). Major peaks of H3K9Ac and H3K27Ac modifications were found at pIII and pIV, as well as within the body of the gene at the +15 and +36 ROIs. Although H3K9Ac was not found at any of the upstream regions, including the −15 ROI and at pIV, it was found at each of the promoters and at the +11, +15, +36 and +37 ROIs. H3K27ac was weakly present at some of the upstream regions, including the −15 ROI and the −15 and −3 (HSS1) ROIs, but was mostly restricted to the same downstream regions as H3K9ac. H3K4me1 was present at pIII and pIV, and within the gene body, and at all of the above ROIs, including the upstream ROIs −3 and −15. Intriguingly, the presence of H3K4me1 at the −15 ROI and at pIV were similar between the BCL1 and P3X cells, suggesting that these regions may be in an open/accessible state, even though CIITA is not expressed in P3X cells.

Comparison of these data with publically available ChIP-seq data sets in primary murine B cells showed concordance of peaks of H3K9ac at HSS1, pIII and pIV promoter regions and lower levels across the body of the CIITA gene (Figure 5). H3K4me3, which signifies active or RNA Pol II-engaged promoters, was found only at pIII and pIV (Figure 5b).

PU.1 binds to many regions across the CIITA locus

The transcription factor PU.1 is known to be involved in regulation of CIITA from pIII. Analysis of the available ChIP-seq data set for PU.1 (Figure 5b) showed enrichment at five regions around the CIITA gene, including pIII, and coincided with peaks of DNase I hypersensitivity. Overall, these data indicate a potential regulatory role for four regions: −3 (HSS1), +11, +15 and +36 ROIs.

Figure 4. CTCF binds at six sites across the CIITA locus. (a) ChIP-seq was performed in primary splenic B cells and in CD138+B220int plasmablasts. Data are presented as reads per million and plotted in green (B-cell data) and blue (plasmablast data) above a schematic of the CIITA locus. Black vertical bars below the locus indicate the 3C-interacting fragment located within CIITA, as well as the amplicons generated from primers used in conventional ChIP to verify the ChIP-seq data. (b) ChIP-seq-identified CTCF sites within the CIITA gene were confirmed using conventional ChIP coupled with qPCR in BCL1 and P3X cells. Error bars represent s.e.m. for three independent replicates and *P < 0.05.
To confirm the above data sets and to determine whether PU.1 binds to any of the hypersensitive regions identified here, ChIP was performed. PU.1 occupancy peaks showed a similar distribution to the active histone modifications, with many regions of binding inside the CIITA gene body, particularly near promoters III and IV, as well as near the +15 and +36 ROIs (Figure 5b). Another PU.1 peak was observed near the \(-3\) ROI (HSS1). Thus, PU.1 binding closely mirrors the presence of chromatin marks associated with predicted enhancer regions and correlates well with previously published ENCODE data.

Two upstream ROIs and one intragenic PU.1 site display regulatory activity

The combined data collected suggest that several of the ROIs, including those that bind PU.1, contain active histone marks, or
interact with pII, and could function as independent enhancer elements capable of augmenting the expression of a reporter gene. To test for such an activity, ROIs were cloned upstream of a reporter construct. Here, 200–300 bp fragments were chosen by either cloning small segments across a 3C-interacting region of interest (for example, restriction fragment H6/E4: −8.0, −8.2 and −8.4) or by choosing conserved sequences within a restriction fragment (for example, fragment H5/E6, −15). Sites shown by ChIP-seq data to be enriched for PU.1 binding (−4, +16, +19 and +35), as well as several found through ChIP analysis (Figure 5a) to be enriched for marks of active enhancers (+36 and +37), were also examined in this context. These constructs were transfected into A20 cells and assayed for expression of the reporter. Intriguingly, only three of the constructs were able to augment the reporter and included the 3C-interacting regions H5/E4 at the −15 ROI, H5/E6 at −8.2 and within the +35 PU.1-binding site (Figure 6). Thus, three regions display the ability to enhance the expression of a heterologous reporter gene construct. The other regions were unable to function independently.

Interacting regions are shared between B cells and spDC

Given the potential for the ROIs that interact with pII to have some role in CITA expression from pl, the 3C chromatin architecture of the CITA locus was examined in dendritic cells. spDC, which principally use pl (Figure 7a), were isolated from wild-type C57BL/6 mice and 3C was performed using HindIII as the restriction enzyme. 3C analysis revealed that in wild-type spDC, restriction fragments H5, H6, H7 and H16 interact with pl (Figures 7b and c). Thus, the same ROIs that interact with pIII in B cells and dendritic cells have not been extensively studied and are poorly defined in both the human and mouse systems. To develop an understanding of the complexity and number of CITA distal regulatory elements, DNase I hypersensitivity, 3C and ChIP for the determination of active promiscuity CpGs within the three CITA promoters (Figure 8a). spDC and B cells were used as DNA sources. The results showed that the pl promoter region was differentially methylated (Figures 8b and c). The three most 5’ CpGs (−95, −69 and +38 bp) surrounding the pl TSS were unmethylated in spDC but almost completely methylated in B cells. The four downstream pl CpGs showed variable methylation in spDC and again were nearly completely methylated in B cells. In sharp contrast, pII and pIV showed low to almost no methylation in spDC and B cells. These data suggest that the ability to use pl in B cells may be compromised by the presence of CpG methylation at that promoter.

**DISCUSSION**

Despite its importance in controlling MHC-II gene expression and antigen presentation, distal cis-acting elements regulating CITA gene expression in B cells or dendritic cells have not been extensively studied and are poorly defined in both the human and mouse systems. To develop an understanding of the complexity and number of CITA distal regulatory elements, DNase I hypersensitivity, 3C and ChIP for the determination of active histone modifications were employed. Combining the results from these assays with ChIP-seq data sets and mammalian sequence conservation analyses provided a total of 21 elements as potential candidates for regulating CITA gene expression. However, as discussed below, clear regulatory potential as defined by 3C interactions with the promoter and/or gene reporter assays

![Diagram](https://example.com/diagram)

**Figure 6.** Three ROIs have regulatory activity. Regions (200–300 bp) spanning the −8.2, −15, +36, +37 ROIs and sites of PU.1 binding as determined using ChIP-seq (−4, +12, +16, +19 and +35) were cloned into a pGL3-promoter upstream of the firefly luciferase gene to test for regulatory activity. Reporter vectors and a control Renilla luciferase expression vector were cotransfected into BCL1 cells by nucleoporation and analyzed 24 h post-transfection. All data were normalized to Renilla expression and to mock transfected no DNA controls and expression plotted as fold over the pGL3-promoter empty vector, as indicated by the vertical gray line set at 1. Error bars represent s.e.m. and *P < 0.01 as determined by Dunnett’s test.
was observed for only four of these elements. This suggests that some of the features that led to an element’s inclusion in the analyses may be a consequence of transcription or participation in the architectural structure of the locus but may not have a direct or major role in the transcriptional regulation of CIITA. In addition, these assays identified four CTCF sites that may contribute to the organization of the locus.

DNase I-hypersensitive regions, which are often associated with enhancers and promoter regions, provided a first pass of potential regions that were accessible in B cells but not plasma cells and could reveal elements required for B-cell expression of CIITA. Several regions were also hypersensitive in both B cells and plasma cells, suggesting that they may be performing roles associated with the architectural features of the locus, such as the CTCF sites discussed below. Three ENCODE DNase I tracks for B cells (CD43– B cells; CD19+ B cells and A20 lymphoma cells) were available for analysis. Examination of these tracks with the regions identified in this study showed congruence at three of the

Figure 7. Distal elements interact with CIITA promoters in a usage-specific manner. (a) C57BL/6 wild-type and pl-KO spDC were assayed for CIITA promoter usage. Total RNA was collected from three independent isolations of spDC for each genotype, assayed using qRT-PCR for CIITA promoter usage, and plotted with respect to 18S RNA. Error bars represent s.d. (b) Schematic of the CIITA locus showing 3C anchor restriction fragments with pl, pl-KO and plII anchors shown as arrows. The aqua shaded box indicates the pl knockout deletion. Vertical bars indicate HindIII restriction sites. (c) 3C was performed using primers for the HindIII restriction fragments shown in Figure 3b. The relative crosslinking frequency with the indicated anchor fragments as determined with the 3C assay is shown for WT and pl-KO spDC. These experiments were performed three times. Error bars indicate s.d., and *P < 0.05 as determined by a one-tailed Student’s t-test.
DNase I sites (+15, +36 and +37), two at PU.1 sites (+16 and +19) and two at CTCF-binding sites (+40 and +43). The promoter regions at plII and plIV were also hypersensitive in the DNase I tracks. On the upstream side of plII, only one strong DNase I peak appeared in A20 cells but not within the primary B-cell populations. This broad region encompassed HSS1, which was previously identified. Thus, no hypersensitivity was revealed for −15 and −8.2 in B cells through the global genome-wide DNase I assays. This may reflect the sensitivity associated with qPCR or biases reflected in high-throughput sequencing. Alternatively, differences in DNase I hypersensitivity may reflect inherent differences between primary B cells, BCL1 and A20 tumor cell lines used in this study and the various analyses discussed above.

Long-distance regulatory regions are now thought to function through looping interactions with promoters or other elements. This looping can be mediated by transcription factors, as described at the MCP-1 locus between an upstream NF-κB element and the downstream Sp1 promoter regulatory element or via the chromosomal organizing factor CTCF as shown at the human and mouse MHC-II loci. To identify regions that would interact with plII and potentially regulate CIITA mRNA levels in B cells and plasmablasts but also most cell and tissue types examined in the ENCODE project (Supplementary Table 1; reviewed in Bernstein et al.). The conservation of CTCF binding to this region among cell types and its independence with respect to CIITA gene expression suggest that this site may function as the 3′ boundary for CIITA regulatory elements but not regulate the gene directly. This statement is further supported by experiments in which short interfering RNA depletion of CTCF did not affect CIITA mRNA levels in B cells and the observation that this region of the genome is dense, with two genes immediately 3′ to CIITA. Such boundaries may function to restrict the activity of the gene-specific enhancers in the region.

Identification of histone marks of open chromatin and active enhancers serve as useful tools for recognition of putative enhancer elements. Six of the ROIs fit with the typical profile of an enhancer element being marked by H3K4me1, H3K27Ac and H3K9Ac. The presence of these chromatin marks indicates that there are potentially a number of intragenic enhancer elements inside of the CIITA locus itself (+11, +15 and +36). However, when 1-kb segments surrounding these ROIs were cloned into a luciferase expression vector, these regions showed no enhancer activity (data not shown). Whereas this could be a result of a lack of true enhancer activity, it is also possible that these large fragments also contained repressive elements in

Figure 8. pl is differentially methylated in B cells versus spDC. (a) CpGs at indicated positions relative to their respective TSSs were queried for their methylation statuses. Numbers 1 through 27 represent the various CpGs shown. (b) A representative sample of clones collected from two independent preparations of DNA for spDC and primary B cells are shown, where open circles represent unmethylated CpGs and filled in circles represent methylated CpGs. (c) Methylation status for the queried CpGs were compiled to display the overall methylation status of each CpG at the three promoter regions in spDC and B cells compiled from two biological replicates.
addition to the putative enhancers. It is also possible that these elements only work in concert with the other elements. In contrast, the −15 ROI displayed positive regulatory activity in the luciferase assay, supporting the case for its role as an active enhancer. The −15 site is conserved between mouse and human, and is homologous to a region involved in IFNγ-mediating CIITA expression from plV in HeLa cells. The +40 CTCF-binding region was also conserved in humans and participated in IFNγ-mediated CIITA expression through plV.

In contrast, the −8.2 site, which displayed regulatory activity and interacted with pl and plII, did not possess any typical histone modifications of active enhancers. The lack of H3K4me1, H3K27Ac and H3K9Ac does not preclude the possibility of an active regulatory element as there may be yet undetermined modifications/activities at this region. The −8.2 site may therefore fall under a unique classification of cis-elements that do not fit the typical active enhancer model. In addition, the +11, +15 and +35/+36 sites appear to be marked as active enhancers, as well as having PU.1 bound, but do not physically interact with the promoter or display regulatory activity, suggesting supporting roles for these regions, or designating the observed histone modification activities as a consequence of transcription through them. Alternatively, they may simply function to maintain an open chromatin state for transcription through the gene.

The −3 site (HSS1) was previously shown to be involved in regulating CIITA expression from plII in B cells. It was found in this study to be marked with active enhancer marks, as well as verifying previous data showing PU.1 binding at this site. As PU.1 has been previously shown to mediate looping at the CIITA locus and binds at two of the four promoter-interacting restriction fragments, this is the most likely candidate for mediating the complex 3D architecture of the locus. PU.1 is highly expressed in cells of the myeloid lineage and was also shown to have a role in CIITA expression from pl in dendritic cells. Thus, PU.1 may be having identical roles in both cell types: inducing transcription and coordinating the interactions between the elements and the CIITA active promoter regions.

The findings that both pl and plII promoters interact with the same set of cis-elements is intriguing and could have implied that the elements are complex and used different factors to direct the interactions at the specific promoters. However, deletion of pl resulted in the redirection of all interactions to plII and near wild-type levels of expression. Thus, whereas some transcription factors may be different, the essential properties must be shared. The occurrence of extensive DNA methylation at the pl promoter region in B cells and nearly none in the most 5′ CpGs proximal to the TSS in spDC suggests that pl is epigenetically silenced in B cells. This is supported by previous ChIP data demonstrating a lack of open chromatin (H3 and H4 acetylation) at pl in BCL1 cells. In addition, transcription factor binding or interactions at plII may be unstable in myeloid cells because of RNA polymerase II transcription from pl through this region of DNA.

In the case of B-cell expression, a similar situation could exist where the critical elements at pl are not accessible, leaving plII as the only available element for interaction from distal elements and transcription initiation. In B cells, pl is less accessible as measured by DNase I hypersensitivity. In addition, PU.1 occupancy at plII in B cells is 18 times more than plI, which can be explained by the presence of a lower affinity PU.1-binding motif at pl versus plII. If PU.1 binding causes or is an indicator of mediating the 3D architecture of the locus, then its preference for plII could direct expression from this promoter. Together, these data suggest that
it is the first or most accessible promoter that is used. This conclusion is consistent with the experiments in which pl was deleted and both looping and transcription were redirected to pII.

Thus, the data presented here identify a host of new elements that contribute to the regulation of CIITA. Whereas some of the elements (for example, PU.1 elements +16 and +19) may simply serve as binding sites for increased accessibility to the local chromatin, other sites have independent regulatory activity and interact directly with CIITA promoters. From a disease perspective, the discovery of novel elements and their potential binding factors could be targets of microbial products aimed at reducing CIITA and MHC-II expression and avoiding immune detection. Intriguingly, one bare lymphocyte syndrome patient who exhibited profoundly reduced levels of CIITA mRNA did not have a mutation in the coding region or at pII, and pIII. The authors suggested that this represented a novel cis-element regulatory defect for CIITA expression. Unfortunately, the mutation was not mapped but provides evidence that the regulatory elements could contribute to MHC-II-associated diseases. The data also demonstrate that several of the elements are shared between cells of the myeloid and lymphoid lineages, implicating additional mechanisms, including DNA methylation and potentially other epigenetic processes as mediators of promoter choice.

MATERIALS AND METHODS

Cells, culture and mice
Murine B-cell lines BCL1 (BCL1 3B3, CRL-1669, American Tissue Type Culture (ATCC), Manassas, VA, USA) and A2O (A20, TIB-208, ATCC) and the plasma cell line P3X (P3 × 63Ag8.653, CRL-1580, ATCC) were cultured in RPMI 1640 (Mediatech Inc., Manassas, VA, USA) with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 10 mM HEPES (HyClone Laboratory, Logan, VT, USA), 1 mM sodium pyruvate (HyClone Laboratory), 1x non-essential amino acids (HyClone Laboratory) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich). CD19+ B cells were purchased from CSRN Laboratory (Bar Harbor, ME, USA) and 6- to 8-week-old mice were used to obtain primary B cells and spDC. For primary B cells, spleens were harvested and a single-cell suspension generated by forcing cells through a 40-μm nylon cell strainer before lysis of the red blood cells using ammonium-chloride-potassium-potassium chloride lysis buffer. Splenocytes were incubated with anti-CD43 antibody to capture non-B cells using MACS columns according to the manufacturer’s protocol (Miltenyi Biotech Inc., Auburn, CA, USA).

SpDC were collected as previously described from wild-type or CIITA promoter I KO mice. Briefly, 30 μg FITC-Ligand-β (Flt3-L) was injected intraperitoneally for 9 days. Flt3-L was provided by Dr R Mittler (Emory University). At the end of 9 days, mice were killed and their spleens were removed and injected with Dulbecco’s modified Eagle’s media (Mediatech Inc.) containing 10% fetal bovine serum, 1x non-essential amino acids, 10 mM HEPES, 1 mM sodium pyruvate, 0.292 mg mL⁻¹ L-glutamine, 100 units mL⁻¹ penicillin, 100 μg mL⁻¹ streptomycin (Life Technologies, Grand Island, NY, USA) and 1 mg mL⁻¹ collagenase D (Roche, Indianapolis, IN, USA). The spleens were then cut into pieces and incubated at 37 °C for 25 min. A 40-μm cell strainer was used to generate a single-cell suspension, and ammonium-chloride-potassium-potassium chloride lysis buffer was used to lyse red blood cells. CD11c⁺ dendritic cells were purified using CD11c MACS beads (Miltenyi Biotech Inc.) according to the manufacturer’s protocol. Animal experiments were conducted using protocols that were approved by the Emory University Institutional Animal Care and Use Committee.

Dnase I hypersensitivity assay
Dnase I hypersensitivity assays were performed as described previously. Briefly, 2 x 10⁶ cells were sedimented, resuspended in 1.2 mL ice-cold DNase I buffer (10 mM Hepes, pH 8.0, 50 mM KCl, 5 mM MgCl₂, 3 mM CaCl₂, 0.1% NP-40, 8% glycerol and 1 mM dithiothreitol) and incubated on ice for 10 min at 0–5 °C. Five aliquots of cells in microfuge tubes were placed in a 25 °C water bath for 3 min. Dnase I (1 U μl⁻¹, Worthington, Lakewood, NJ, USA) was added to the samples for 3 min before quenching with 20 μM EDTA. Dnase-free RNAses A and Proteinase K were then added to the samples and incubated at 65 °C overnight. Two microliters of each sample were used for PCR. For real-time PCR analysis, the DNA was purified and quantitated. Relative hypersensitivity was calculated by normalization to an insensitive region within CIITA (Y6) and the data were displayed as fold over the untreated sample. To perform semiquantitative analysis of Dnase I-treated samples, conventional PCR was used to amplify 1–2 kilobase (kb) amplicons. The resulting ethidium bromide-stained DNA bands in the agarose gel images were analyzed using iminterp3v2 (S. Edwards, Cincinnati, OH, USA) available upon request. Band intensities were compared with the untreated samples and plotted to obtain a slope to describe the change in intensity with Dnase I treatment. As Dnase I treatment either results in no change in sensitivity or an increase in sensitivity, only negative slope values were considered for further analysis.

Real-time PCR analysis
Samples were analyzed in 25 μl PCRs containing 1 x SYBR Green I (Lonza Inc., Allendale, NJ, USA) for detection by the CFX96 Real-Time PCR detection system (Bio-Rad Inc., Hercules, CA, USA). Primers used can be found in Supplementary Table 1. Data represent the average of three independent biological replicates, and error bars represent s.e.m. except where noted. Two-tailed Student’s t-tests were used to calculate P-values.

Quantitative 3C assay
The 3C assay protocol was performed as described previously. For primary splenic B cells and dendritic cells, 1 x 10⁷ cells were suspended in RPMI with 10% heat-inactivated fetal bovine serum and crosslinked for 10 or 15 min at room temperature with formaldehyde (Sigma-Aldrich) added to cells for a final concentration of 1 or 2% as noted. Glycine (Sigma-Aldrich) at a final concentration of 125 mM was used to quench the reaction. Digestion was performed overnight on nuclei collected from the crosslinked cells, and digested with either HindIII or EcoRI (New England Biolabs, Ipswich, MA, USA) as indicated at 37 °C. Overnight ligation at 16 °C with T4 DNA ligase (New England Biolabs) were performed with heat-inactivated, ~1:40 dilutions of the restriction enzyme-digested reactions. To quantitate 3C products, real-time PCR was performed against a five-point standard curve as described previously. Primers (Supplementary Table 1) were tested to determine whether they could amplify a single product on a BAP (RP23-240H17 purchased from Children’s Hospital Oakland Research Institute) digested with the appropriate restriction enzyme and re-ligated to form all possible 3C products. Data are presented as crosslinked frequency and represent an average derived from three independent biological replicates with error bars representing s.d. The Student’s t-test was used to determine significance.

Luciferase gene reporter assays
Fragments (200–300 bp) were PCR-amplified with primers containing XhoI restriction sites (see Supplementary Table 1). These fragments were cloned into the pG3 promoter vector (Promega Corporation, Madison, WI, USA) using the XhoI restriction site. Inserts were verified by restriction digest followed by DNA sequencing. Supercrossed plasmids were prepared using cesium-chloride gradients. Nucleofection was performed on A2O cells according to Amaza protocol (Lonza Inc.) using 5 μg of luciferase-containing pG3 constructs along with 200 ng of Renilla expression plasmid (pB-SLT, Promega Corporation). After 24 h, cells were harvested and dual-luciferase assays were performed according to the manufacturer’s instructions (Promega Corporation). Data were collected in at least triplicate with independent biological replicates, error bars represent s.e.m. and Dunnett’s test was used to calculate P-values.

Bisulfite sequencing
Genomic DNA (200 ng) from spDC and B cells was bisulfite-converted using the EZ DNA methylation-gold kit (Zymo Research, Irvine, CA, USA). Bisulfite-converted DNA was PCR-amplified using region-specific primers (Supplementary Table 1) and cloned using the TOPO TA cloning kit (Life Technologies). Individual clones were sequenced using an ABI3100 capillary sequencer (Beckman Coulter Inc., Brea, CA, USA). Bisulfite-converted data were analyzed as previously described. Briefly, this involved mapping the sequence back to the in silico bisulfite-converted genomic DNA sequence using the R Biostrings package. Biological duplicates were performed for all regions. Statistical significance between samples was determined using Fischer’s exact test.
Chromatin immunoprecipitation
ChiP was performed as previously described.50 In these reactions, 4 x 10^7 cells were crosslinked with 1% formaldehyde, and chromatin was purified and sonicated to ~200–600 bp fragments. In all, 30 μg of chromatin was used for each immunoprecipitation. The following antibodies were used: rabbit IgG (12–370, Millipore, Temecula, CA, USA); CTCF (07–729, Millipore), H3K9Ac (07–352 Millipore), H3K27Ac (07–360, Millipore), H3K4me1 (ab8895, Abcam Inc., Cambridge, MA, USA) and PU1 (Sp-1, T-21, sc-352, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). 1/10th of the ChiP sample was used for real-time PCR analysis using primers in Supplementary Table 1. Real-time PCR values were plotted as percent of the sample was used for real-time PCR analysis using primers in Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. 1/10th of the ChIP

Real-time RT-PCR analysis
RNA was isolated using the RNeasy mini prep kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. In all, 2 μg of RNA was used for reverse transcription with SuperScript II (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. Approximately 1/100th of the cDNA was used for each real-time PCR analysis with the primers listed in Supplementary Table 1. PCR reactions with 18S rRNA primers were used to normalize between samples. Data displayed were the average of three independent biological replicates, and error bars represent s.d.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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