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

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Abstract

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 IFN-γ treated non-hematopoietic cells using promoters pI, pIII, and pIV, respectively. Recent studies in non-hematopoietic cells suggest 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 pIII in B cells. These same four regions were also found to interact with pI in splenic dendritic cells (spDC). Intriguingly, examination of the above interactions in pI-knockout-derived spDC showed a switch to the next available promoter, pIII. Extensive DNA methylation was found at the pI 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 playing 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, such as B cells, macrophages, and dendritic cells, but are interferon (IFN)-γ-inducible in most non-immune cells [1–3]. MHC-II proteins present peptide antigens to

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CONFLICT OF INTEREST

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SUPPLEMENTAL INFORMATION

Supplementary information is available at Genes & Immunity's website (http://www.nature.com/gene).

CD4+ helper T cells [4], which upon recognition of their cognate antigen, become activated, triggering a complex immune response. Using the same MHC-II peptide/T cell receptor interaction, activated CD4+ T cells stimulate antigen-specific B cell differentiation to antibody secreting plasma 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 [5]). The MHC-II transactivator, CIITA, is required to interact with these factors and the basal transcription machinery to initiate MHC-II expression [6]. 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 [7]. *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 [8]. Cells of the myeloid lineage, including splenic derived dendritic cells (spDC), primarily express *CIITA* from the most distal promoter (promoter I or pI) [8]. 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 [2,8–11]. Individual roles for these isoforms are unclear, but they appear to be somewhat interchangeable [12]. 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 (BLS), a severe combined immune deficiency disease [13]. CIITA KO mice lack positive selection for CD4+ T cells, and do not respond well to immunization or pathogenic challenge [14]. Thus, appropriate regulation of *CIITA* is key to healthy immune responses.

The proximal regulatory region for pIII 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, an NF-1 binding site termed site A, site B, and site C [15], as well as binding sites for AP1 [9], Sp1 [16], acute myeloid leukemia (AML) 2, CREB [17], and the repressors PRDI-BF1/BLIMP-1 [18,19] and ZBTB32 [20]. PU.1, an essential B cell transcription factor, was also found to be important for positive regulation of *CIITA* pIII through site C, working in conjunction with E47 and IRF-4 [21].

In contrast to its well defined proximal regulatory elements, only one distal regulatory element for pIII was identified previously and termed hypersensitive site 1 (HSS1) [22]. HSS1 is located ~3 kb upstream of pI. PU.1 bound HSS1 was shown to interact directly with pIII [22]. HeLa cells, which can induce *CIITA* pIV expression in response to IFN-γ, were found to use a network of distal elements located both upstream and downstream of the CIITA promoter regions and gene [23]. However, it is not known if 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 pIII in B cells using a chromatin

conformation capture (3C) assay. The most 3′ of these elements was found to bind the transcriptional insulator CTCF. One of the 5′ elements identified was HSS1, while 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 pI in splenic-derived dendritic cells (spDC). Interactions between all distal regulatory elements and *CIITA* pI were reconfigured to pIII in spDC derived from mice containing a genetic deletion of pI. This rearrangement of promoter choice suggested that these elements search for an accessible promoter region to drive transcription. In B cells, the pI promoter contains extensive DNA methylation and is likely to be in an epigenetic restricted state, with pIII 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 to 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. Non-coding 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 (EtBr) stained gels was used to determine a slope of sensitivity (Figure 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 (e.g., regions −3, +10, and +15 kb from the pI 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, thirteen regions were chosen (Fig 1A, horizontal bars).

To provide a higher resolution analysis for the DNase I hypersensitive sites, qPCR using smaller amplicon 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 DNaseI 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 if any ROIs were exhibiting regulatory activity by physically interacting with the lymphocyte specific promoter (pIII), chromosome conformation capture (3C) assays [24–26] were performed. For these experiments, approximately 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. Additionally, regions that were found to be enriched for H3K4me1 in B cells [27] 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). *Hin*dIII restriction digestion separates all the *CIITA* promoters from one another, and *Eco*RI restriction digestion refines the location of interacting regions and verifies the interactions observed in the *Hin*dIII 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 [22] were not detected. Detection of pIII and HSS1 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 minutes) than used above. To determine if 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 the CCCTC-binding factor (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 [28]. CTCF is also known to play an important role in creating 3-D architecture involved in gene regulation [26]. Analysis of CTCF ChIP-seq data for B cells and plasmablasts [29] across the locus identified six CTCF sites (Fig 4A). While two sets 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 to 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 to 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 were assessed for the chromatin marks H3K4me1, H3K9Ac, and H3K27ac in both BCL1 and P3X cells (Figure 5). H3K4me1 is associated with enhancer regions [30] and when coupled with H3K27Ac is indicative of active enhancers [31]. H3K9Ac is often found at active promoters and regulatory regions [32]. 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 ROIs upstream of CIITA, 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 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 to publically available ChIP-seq datasets in primary murine B cells [27,33] 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 [22]. 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 [27,34]. Overall, these data indicate a potential regulatory role for four regions: −3 (HSS1), +11, +15, and +36 ROIs. To confirm the above datasets and to determine if PU.1 binds to any of the hypersensitive regions identified here, ChIP for PU.1 binding 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 pIII, 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 regions of interest (e.g., restriction fragment H6/E4: −8.0, −8.2, and −8.4) or by choosing conserved sequences

within a restriction fragment (e.g., fragment H5/E6, −15). Sites shown by ChIP-seq data [27] 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 pIII to play some role in *CIITA* expression from pI, the 3C chromatin architecture of the *CIITA* locus was examined in dendritic cells. SpDC, which principally use pI (Figure 7A) were isolated from wild-type C57BL/6 mice and 3C was performed using *Hin*dIII as the restriction enzyme. 3C analysis revealed that in wild-type spDC, restriction fragments H5, H6, H7, and H16 interact with pI (Figure 7B and C). Thus, the same ROIs that interact with pIII in B cells interact with pI in spDC.

A mouse (pI-KO) carrying a deletion of the pI promoter region, including ~300 bp upstream and 100 bp downstream of the TSS was created and analyzed previously [12]. SpDC from these mice expressed near wild-type levels of *CIITA* mRNA that initiated from pIII ([12] and Figure 7A), suggesting the possibility that promoter interactions would simply shift from pI to pIII. To test this, 3C was performed on spDC isolated from these mice. Indeed, in pI-KO spDC, the interacting restriction fragments containing the active ROI regulatory elements shifted their interactions to the next available promoter, pIII (Figure 7B). Together these data imply that four of the elements are commonly used to regulate *CIITA* in B cells and dendritic cells.

The proximal promoter region of pI in spDC and B cells has altered methylation that correlates with promoter use

The finding that common elements were interacting with the active promoter suggested that there may be another mechanism that helps govern promoter use. One hypothesis is that an epigenetic mechanism, such as DNA methylation contributes to *CIITA* promoter choice. Thus, clonal bisulfite sequencing [35] was performed to determine the methylation status of promoter proximal CpGs within the three *CIITA* promoters (Figure 8A). SpDC and B cells were used as DNA sources. The results showed that the pI promoter region was differentially methylated (Figure 8B and C). The three most 5′ CpGs (−95, −69, +38 bp) surrounding the pI TSS were unmethylated in spDC but almost completely methylated in B cells. The four downstream pI CpGs showed variable methylation in spDC and again were nearly completely methylated in B cells. In sharp contrast, pIII and pIV showed low to almost no methylation in spDC and B cells. These data suggest that the ability to use pI 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 *CIITA* 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 *CIITA* 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 datasets [27,29,33,34,36] and mammalian sequence conservation analyses provided a total of 21 elements as potential candidates for regulating *CIITA* gene expression. However, as discussed below, clear regulatory potential as defined by 3C interactions with the promoter and/or gene reporter assays, 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 play a direct or major role in the transcriptional regulation of *CIITA*. Additionally, 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 [34] for analysis. Examination of these tracks with the regions identified in this study showed congruence at three of the 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 pIII and pIV were also hypersensitive in the DNase I tracks. On the upstream side of pIII, 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 [22]. 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 [37,38]. 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 [26,39–41]. 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 [42] or via the chromosomal organizing factor CTCF as shown at the human and mouse MHC-II loci [26,29]. To identify regions that would interact with pIII and potentially regulate transcription, 3C was performed for >90% of the restriction fragments covering 138 kb of the *CIITA* locus. The use of two restriction enzymes in the 3C assay increased the ability to separate potential interacting elements from each other, as well as provided an independent confirmation for any observed interaction. Although the 3C analysis in this study was promoter centric, three very strong interactions

(−15, −8.2, +40/+43) with pIII were observed, and highlight the power of this approach. A fourth interaction with HSS1 and pIII, which was previously reported [22], was recapitulated. These interactions are summarized in Figure 9. CTCF was bound at the +40/+43 3C interacting region in not only B cells and plasmablasts but also most cell and tissue types examined in the ENCODE project [Supplemental Table 1; [34]]. The conservation of CTCF binding to this region among cell types and its independence with respect to *CIITA* gene expression suggests 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 siRNA depletion of CTCF did not affect *CIITA* mRNA levels in B cells [26,29] 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 [30], H3K27Ac [31], and H3K9Ac [32]. The presence of these chromatin marks indicates that there are potentially a number of intragenic enhancer elements inside of the *CIITA* gene 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). While 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 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-inducible *CIITA* expression from pIV in HeLa cells [23]. The +40 CTCF binding region was also conserved in humans and participated in IFNγ-mediated *CIITA* expression through pIV [23].

In contrast, the −8.2 site, which displayed regulatory activity and interacted with pI and pIII, did not possess any typical histone modifications of active enhancers. The lack of H3K4me1, H3K27Ac, and H3K9Ac do not preclude the possibility of an active regulatory element as there may be yet undetermined modifications/activities at this region [43–46]. 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 pIII in B cells [22]. 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. Since PU.1 has been previously shown to mediate looping at the *CIITA* locus [22] 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 [30] and was also shown to play a role in *CIITA* expression from pI in dendritic cells, 49. Thus, PU.1 may be playing 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 pI and pIII 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 pI resulted in the redirection of all interactions to pIII and near wild-type levels of expression. Thus, while some transcription factors may be different, the essential properties must be shared. The occurrence of extensive DNA methylation at the pI promoter region in B cells and nearly none in the most 5′ CpG's proximal to the TSS in spDC, suggests that pI is epigenetically silenced in B cells. This is supported by previous ChIP data demonstrating a lack of open chromatin (H3 and H4 acetylation) at pI in BCL1 cells [22]. Additionally, transcription factor binding or interactions at pIII may be unstable in myeloid cells due to RNA polymerase II transcription from pI through this region of DNA.

In the case of B cell expression, a similar situation could exist where the critical elements at pI are not accessible, leaving pIII as the only available element for interaction from distal elements and transcription initiation. In B cells, pI is less accessible as measured by DNase I hypersensitivity [22]. Additionally, PU.1 occupancy at pIII in B cells is >18 times more than pI [22], which can be explained by the presence of a lower affinity PU.1 binding motif at pI versus pIII [27]. If PU.1 binding causes or is an indicator of mediating the 3-D architecture of the locus, then its preference for pIII 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 pI was deleted and both looping and transcription were redirected to pIII.

Thus, the data presented here identify a host of new elements that contribute to the regulation of CIITA. While some of the elements (e.g., 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 pIII and pIV [48]. 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) and A20 (A-20, TIB-208, ATCC) and the plasma cell line P3X (P3X63Ag8.653, CRL-1580, ATCC) were cultured in RPMI 1640 (Mediatech Inc., Manassas, VA) with 10 % heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (HyClone Laboratory, Logan, VT), 1 mM sodium pyruvate (HyClone Laboratory), 1 x non-essential amino acids (HyClone Laboratory), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO).

C57BL/6 mice were purchased from Jackson Laboratory and 6–8 week old mice were used to obtain primary B cells and splenic dendritic cells. 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-chloride (ACK) lysis buffer. Splenocytes were incubated with anti-CD43 antibody to deplete non-B cells using MACS columns according to the manufacturer's protocol (Miltenyi Biotech, Inc., Auburn, CA).

Splenic dendritic cells were collected as previously described from wild-type or *CIITA* promoter I KO mice [12]. Briefly, 30 μg Flt3 Ligand-Ig (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 sacrificed and spleens were removed and injected with Dulbeco's modified Eagle's media (Mediatech Inc.) containing 10% FBS, 1 x non-essential amino acids, 10 mM HEPES, 1 mM sodium pyruvate, 0.292 mg/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), and 1 mg/ml collagenase D (Roche, Indianapolis, IN). The spleens were then cut into pieces and incubated at 37°C for 25 minutes. A 40 μM cell strainer was used to generate a single cell suspension, and ACK lysis buffer was used to lyse red blood cells. CD11c+ dendritic cells were purified using CD11c MACS beads (Miltenyi Biotech, Inc.) according to 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 [49]. Briefly, 2×10^7 cells were sedimented, resuspended in 1.2 ml ice-cold DNase I buffer (10 mM HEPES pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 % NP-40, 8 % glycerol, and 1 mM DTT), and incubated on ice for 5 minutes. Four aliquots of cells in microfuge tubes were placed in a 25 °C water bath for 3 minutes. DNase I (2 U/3l, Worthington, Lakewood NJ) was added to the samples for three minutes before quenching with 20 mM EGTA. DNase-free RNase A and Proteinase K were then added to the samples and incubated at 65 °C overnight. 2 μl 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 [22]. To perform semi-quantitative analysis of DNase I treated samples, conventional PCR was

used to amplify 1–2 kilobase (kb) amplicons. The resulting EtBr-stained DNA bands in the agarose gel images were analyzed using iminterp3v2 (S. Edwards, Cincinnati, OH, available upon request). Band intensities were compared to 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) for detection by the CFX96 Real-Time PCR detection system (Biorad, Inc., Hercules, CA). Primers used can be found in Supplemental Table 1. Data represent the average of three independent biological replicates, and error bars represent standard error of the mean except where noted. Two-tailed Student's *t*-tests were used to calculate *P*-values.

Quantitative Chromatin Conformation Capture assay

The chromatin conformation capture (3C) assay protocol was performed as described previously [25,26]. For primary splenic B cells and dendritic cells, 1×10^7 cells were suspended in RPMI with 10 % heat-inactivated FBS and crosslinked for 10 minutes or 15 minutes 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 125 mM was used to quench the reaction. Digestion was performed overnight on nuclei collected from the cross-linked cells, and digested with either *Hin*dIII or *Eco*RI (New England Biolabs, Ipswich, MA) as indicated at 37 °C. Overnight ligations 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 [26]. Primers (Supplemental Table 1) were tested to determine whether they could amplify a single product on a BAC (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 standard deviation. The Student's *t*-test was used to determine significance.

Luciferase Gene Reporter Assays

200–300 bp fragments were PCR-amplified with primers containing *Xho*I restrictions sites (see Supplemental Table 1). These fragments were cloned into the pGL3-promoter vector (Promega Corporation, Madison, WI) using the *Xho*I restriction site. Inserts were verified by restriction digest followed by DNA sequencing. Supercoiled plasmids were prepared using cesium-chloride gradients. Nucleofection was performed on A20 cells according to Amaxa protocol (Lonza Inc.) using 5 μg of luciferase containing pGL3 constructs along with 200 ng of Renilla expression plasmid (pRL-TK, Promega Corporation). After 24 hours, cells were harvested and dual-luciferase assays were performed according to manufacturer's instructions (Promega Corporation). Data were collected in at least triplicate with

independent biological replicates, error bars represent standard error of the mean, and Dunnett's test was used to calculate *P* values.

Bisulfite Sequencing

200 ng of genomic DNA from spDC and B cells was bisulfite converted using the EZ DNA methylation-gold kit (Zymo Research, Irvine, CA). Bisulfite converted DNA was PCR amplified using region specific primers (Supplemental 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). Bisulfite converted data was analyzed as previously described [35]. Briefly, this involved mapping the sequence back to the *in silico* bisulfite converted genomic DNA sequence using the R Biostrings package. Biological duplicates were done for all regions. Statistical significance between samples was determined using Fischer's exact test.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described [50]. In these reactions, 4×10^7 cells were cross-linked with 1 % formaldehyde and chromatin was purified and sonicated to ~200–600 bp fragments. 30 μg of chromatin was used for each immunoprecipitation. The following antibodies were used: rabbit IgG (12–370, Millipore, Temecula, CA), CTCF (07–729, Millipore), H3K9Ac (07–352 Millipore), H3K27Ac (07–360, Millipore), H3K4me1 (ab8895, Abcam Inc., Cambridge, MA), and PU.1 (Spi-1, T-21, sc-352, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). 1/10th of the ChIP sample was used for Real Time-PCR analysis using primers in Supplemental Table 1. Real-time PCR values were plotted as percent input of the chromatin added and measured against a standard curve of sonicated BCL1 genomic DNA. All ChIP assays were performed at least three times from independent preparations of chromatin.

Real Time RT-PCR Analysis

RNA was isolated using the RNeasy mini prep kit (Qiagen, Inc., Valencia, CA) according to manufacturer's instructions. 2 μg of RNA was used for reverse transcription with SuperScript II (Invitrogen, Inc., Carlsbad, CA) according to manufacturer's directions. ~1/100th of the cDNA was used for each real time PCR analysis with the primers listed in Supplemental 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 standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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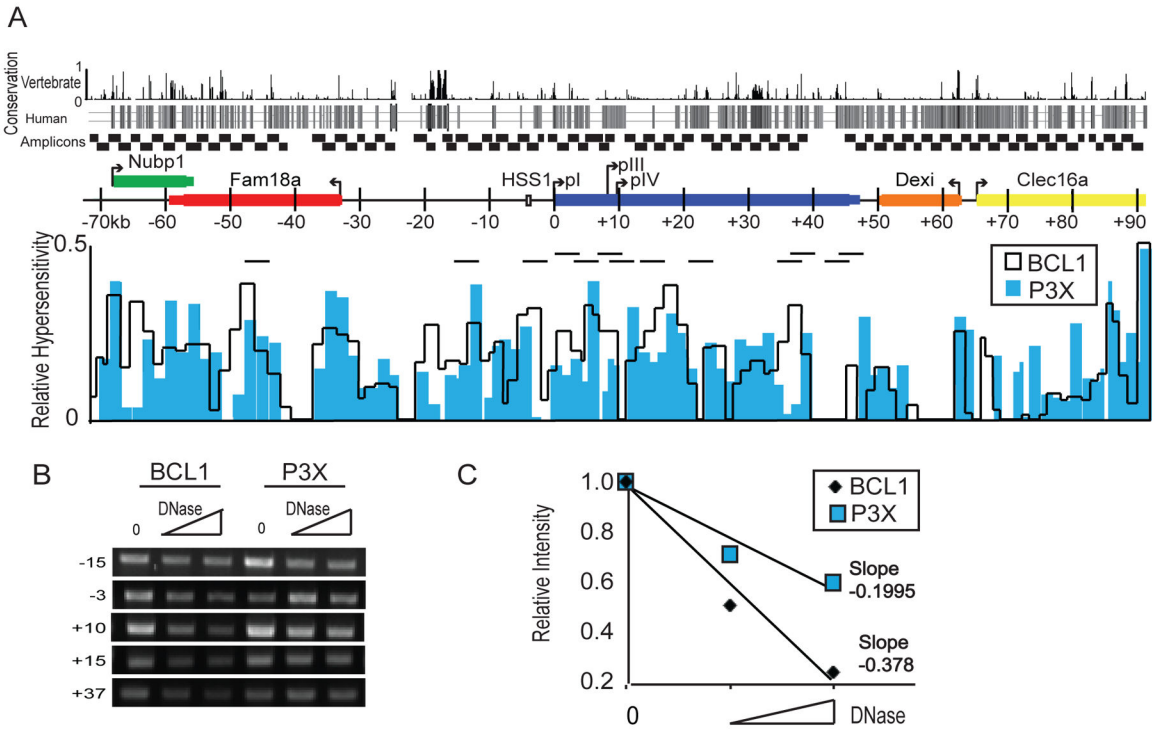

Figure 1. Analysis of DNase I hypersensitivity across the *CIITA* locus reveals 13 regions of interest

A) A schematic of the *CIITA* locus is shown along with vertebrate and human conservation downloaded from the UCSC Genome Browser [51]. The 95 amplicons across the *CIITA* locus used to screen non-repetitive regions for DNase I hypersensitivity using conventional PCR are displayed as black bars. Horizontal black lines indicate regions of interest chosen for further analysis. The relative hypersensitivity plot (bottom) derived from data shown in Supplemental Figure 1, displays the absolute value of slopes for each amplicon in both BCL1 (outlined bars) and P3X cells (gray shadow bars). B) Representative DNase I data showing amplicons found to be hypersensitive in both BCL1 and P3X cells or BCL1 cells alone. C) Semi-quantitative analysis of DNase I hypersensitivity across the *CIITA* locus of a representative region is shown indicating the intensity of PCR bands in DNase-treated samples relative to untreated samples using slope values calculated for use in the relative hypersensitivity plot in A for an amplicon screened in BCL1 and P3X cell lines.

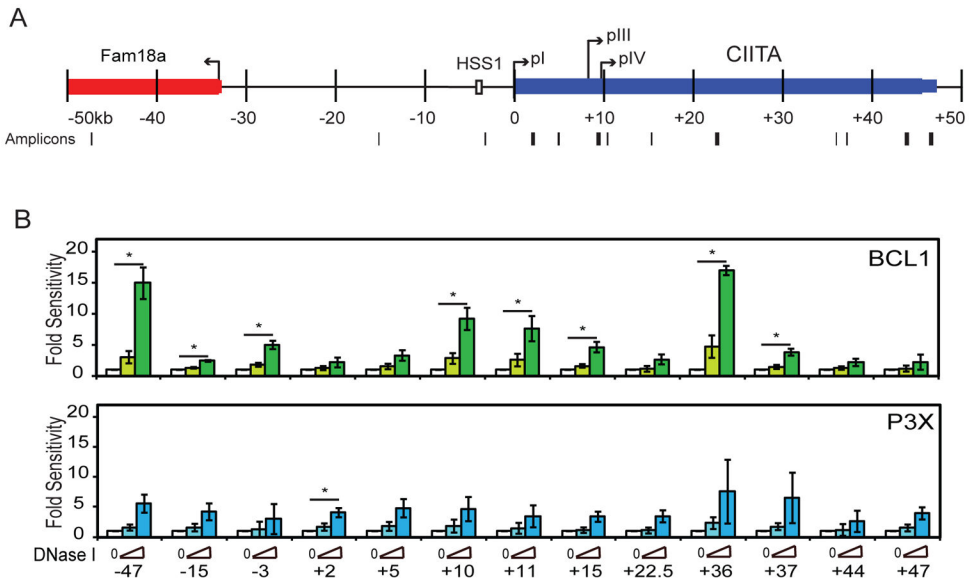

Figure 2. Quantitative analysis of DNase I hypersensitivity shows that regions of interest (ROIs) in BCL1 cells are generally more hypersensitive than in P3X cells

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. ROIs were screened by quantitative real time PCR using amplicons of between 200 and 600 bp in BCL1 cells and P3X cells. Error bars represent SEM and * indicates $p \leq 0.005$ between untreated samples and those treated with the maximum amount of DNase I.

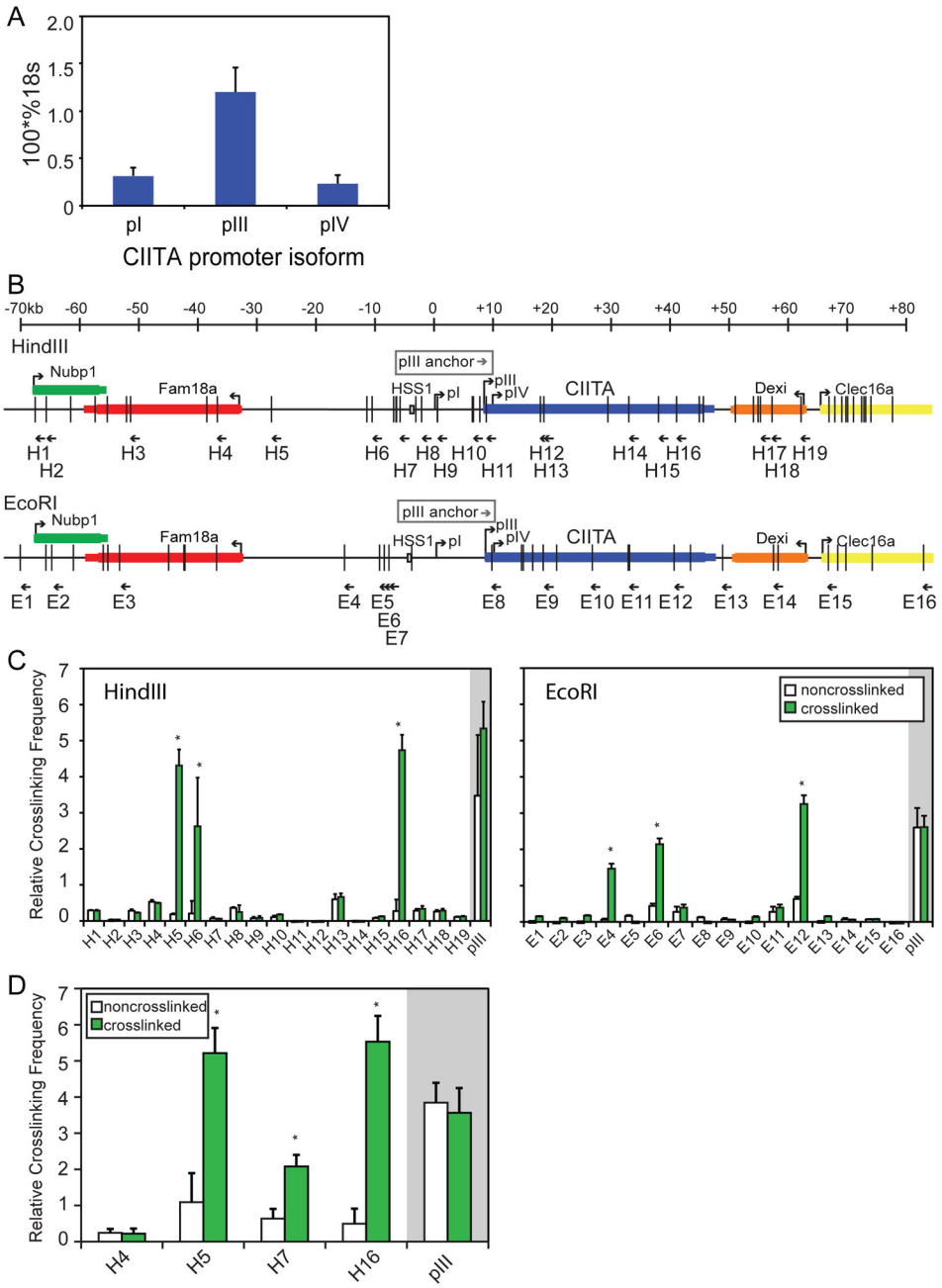

Figure 3. Four distal elements interact with promoter III in CIITA isoform III expressing B cells

A) Primary splenic B cells isolated from C57BL/6 mice were assayed for CIITA promoter usage. Three independent RNA samples were examined by qRT-PCR to query isoform-specific *CIITA* expression and were normalized to 18S RNA levels. Experimental variability is represented by SEM. B) Schematic of the *CIITA* locus showing 3C fragments queried as well as *Hind*III and *Eco*RI restriction sites. Arrows indicate 3C primers used to query fragments and vertical lines represent restriction sites. Gray arrows indicate the anchor primer for the pIII fragment. C) 3C was performed using three independent isolations of primary splenic B cells, and the relative cross-linking frequency with the anchor fragment as

determined by the 3C assay is shown using *Hin*dIII or *Eco*RI as the restriction enzyme. D) In order to detect the lower frequency interaction with the HSS1-containing fragment, a 15 minute crosslinking time with 2% formaldehyde was used in the 3C assay as previously reported [22]. Error bars represent the standard deviation across three independent replicates and * indicates $p < 0.05$.

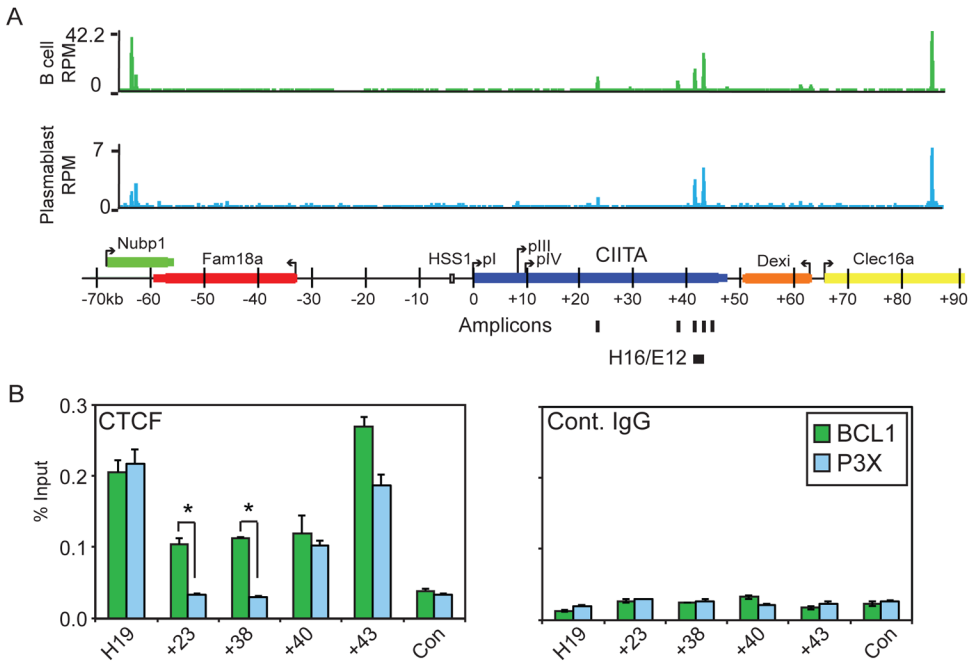

Figure 4. CTCF binds at six sites across the CIITA locus. A) ChIP-seq was performed in primary splenic B cells and in CD138^{+}B220^{int} plasmablasts

Data is presented as reads per million (RPM), and plotted in black (B cell data) and gray (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 by conventional ChIP coupled with qPCR in BCL1 and P3X cells. Error bars represent SEM for three independent replicates and * indicates $p < 0.05$.

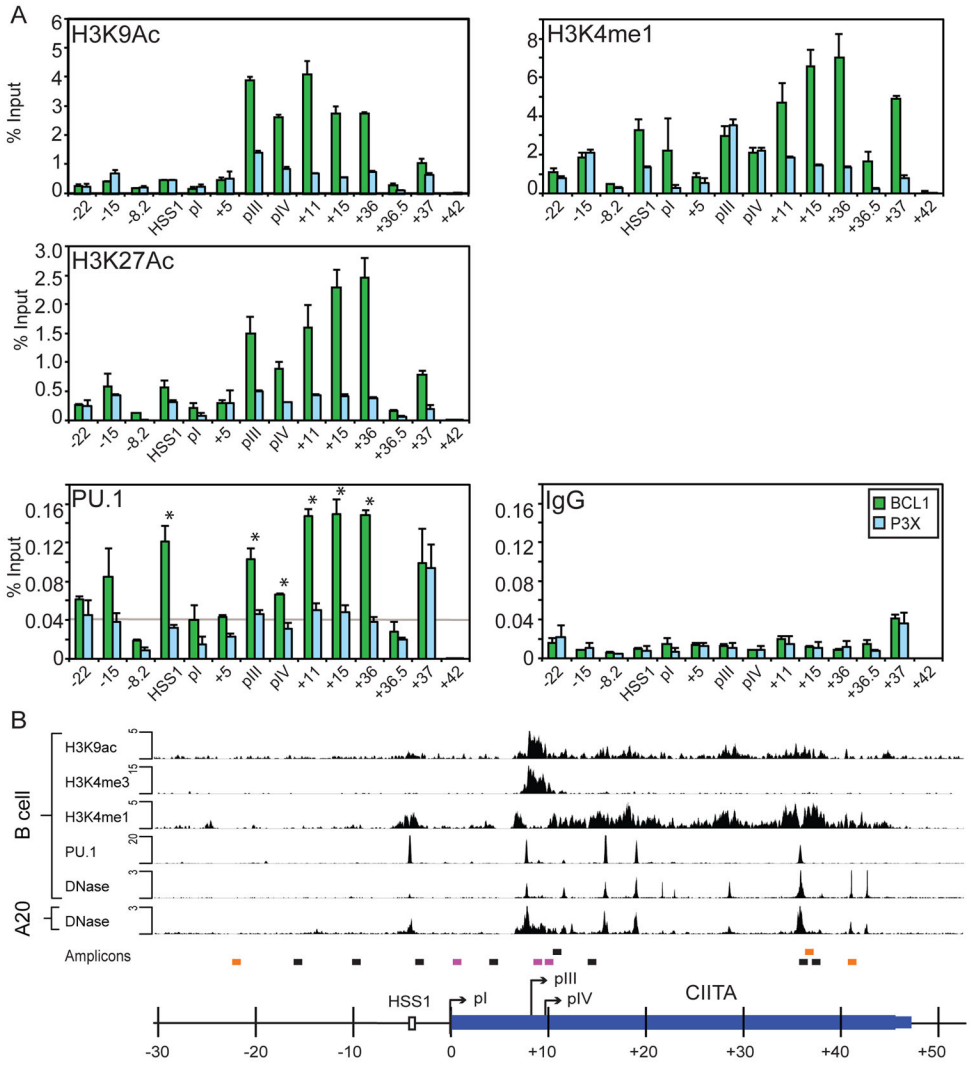

Figure 5. Open chromatin architecture, marks of active enhancers, and PU.1 binding are found at some ROIs

A) Panels of chromatin marks: H3K9Ac, H3K27Ac, H3K4me1, as well as the transcription factor PU.1 and a negative control IgG antibody. For the regions/amplicons indicated in B, the presence of active histone marks and PU.1 were determined for three independent preparations of chromatin from BCL1 and P3X cells. Real time PCR coupled with ChIP was used to quantitate percent input. Error bars represent SEM and * indicates $p < 0.05$. B) Schematic of the *CIITA* locus showing ChIP-seq (H3K9ac, H3K4me3, H3K4me1, and PU.1 from primary B cells) [27,33] and DNase I data (CD43− and A20 B cells) [34] plotted with respect to the 8 ROIs (Black), 3 *CIITA* promoters (Magenta), and 3 negative control sequences (Orange).

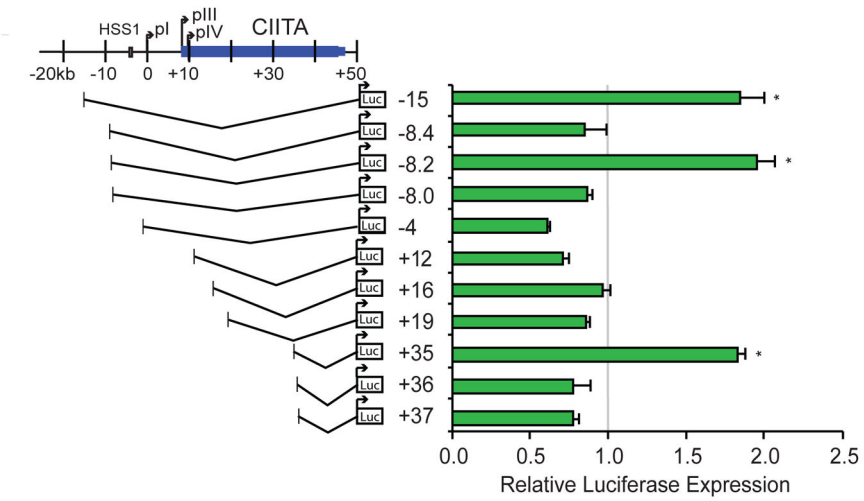

Figure 6. Three Regions of Interest have regulatory activity

200–300 bp regions spanning the −8.2, −15, +36, +37 ROIs and sites of PU.1 binding as determined by ChIP-seq (−4, +12, +16, +19, and +35) were cloned into a pGL3 promoter vector 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 hr 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 SEM and * indicates $p < 0.01$ as determined by Dunnett's test.

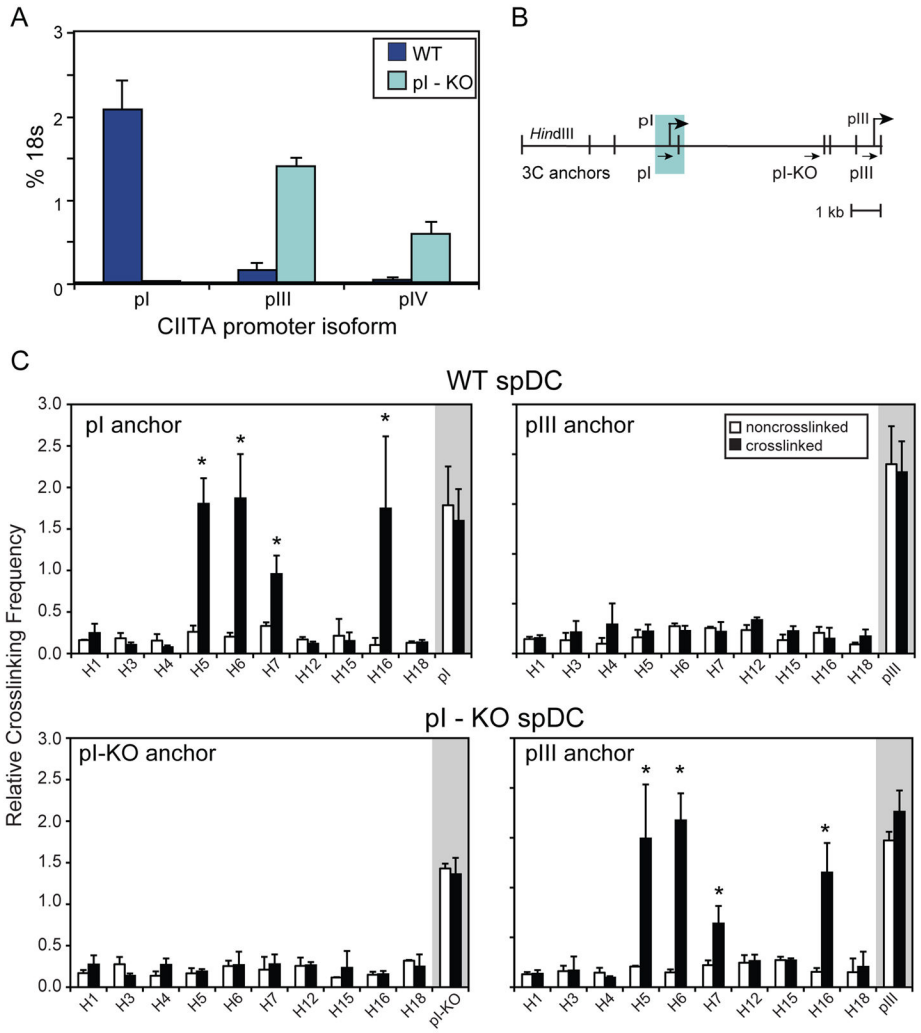

Figure 7. Distal elements interact with CIITA promoters in a usage-specific manner

A) C57BL/6 wild-type and pI-KO spDC were assayed for *CIITA* promoter usage. Total RNA was collected from three independent isolations of spDC for each genotype, assayed by qRT-PCR for *CIITA* promoter usage, and plotted with respect to 18S RNA. Error bars represent standard deviation. B) Schematic of the *CIITA* locus showing 3C anchor restriction fragments with pI, pI-KO, and pIII anchors shown as arrows. The aqua shaded box indicates the pI knock out deletion. Vertical bars indicate *Hin*dIII restriction sites. C) 3C was performed using primers for the *Hin*dIII restriction fragments shown in Figure 3B. The relative cross-linking frequency with the indicated anchor fragments as determined by the 3C assay is shown for WT and pI-KO spDC. These experiments were performed three times. Error bars indicate standard deviation, and * represents $p < 0.05$ as determined by a one-tailed Student's *t* test.

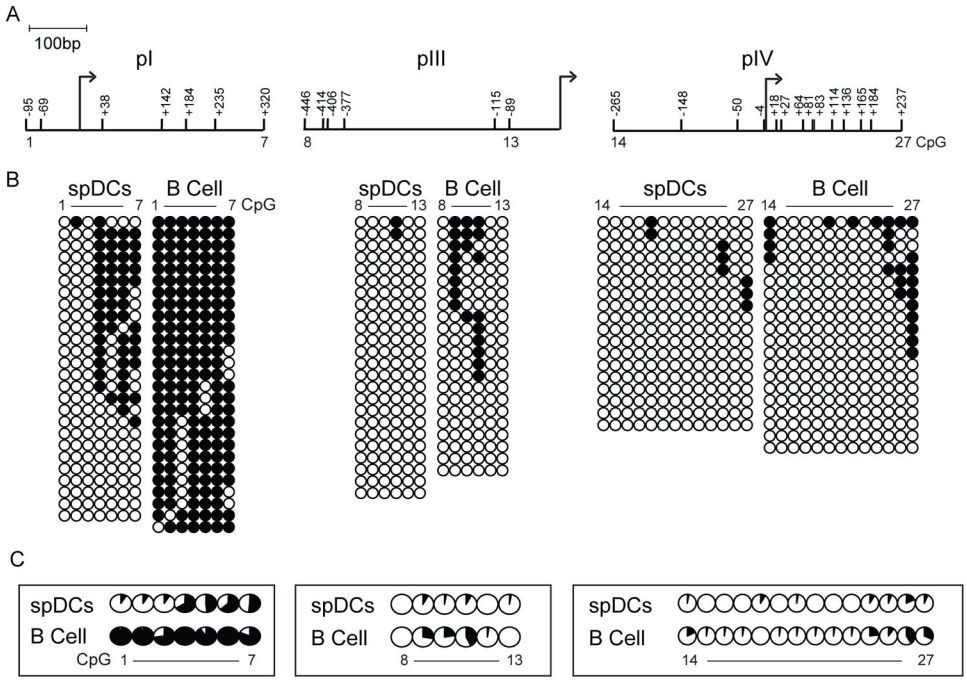

Figure 8. pI is differentially methylated in B cells versus spDC

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 2 biological replicates.

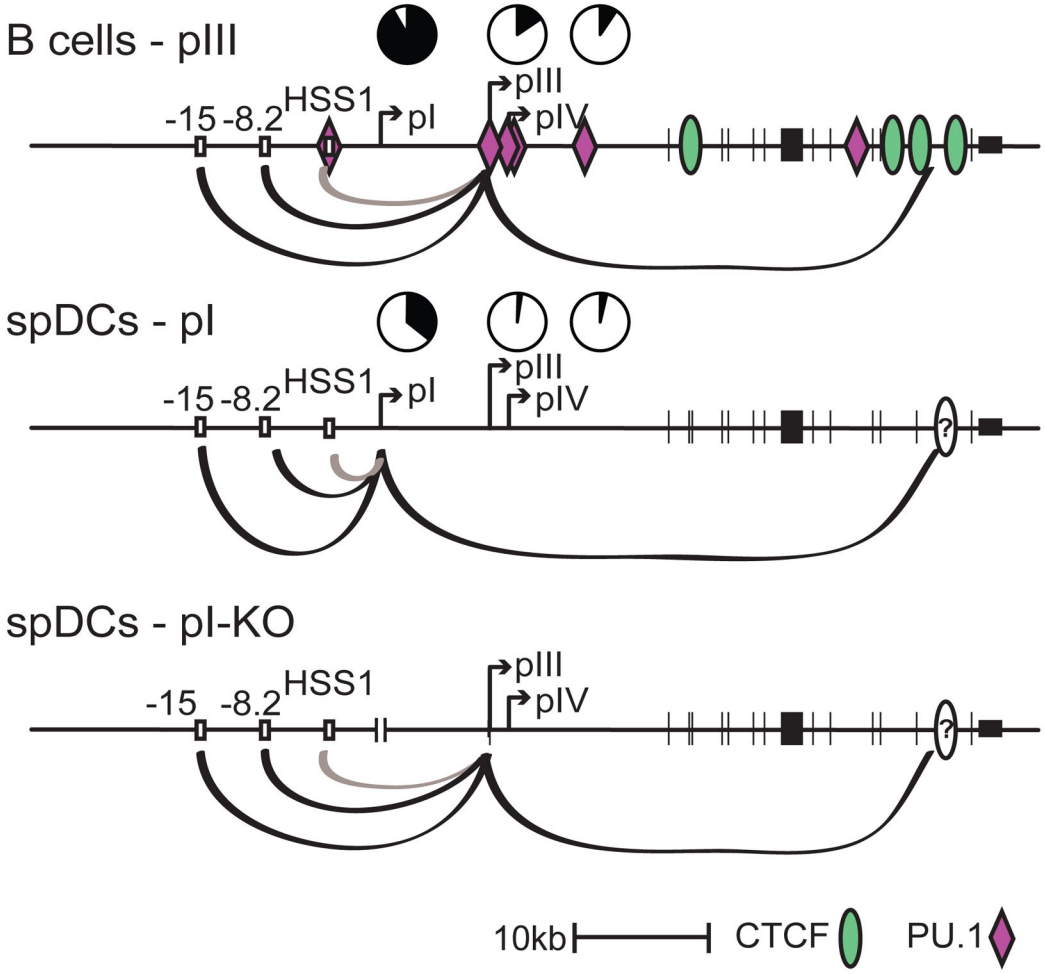

Figure 9. Schematic of the 3-D architecture of the *CIITA* locus with differential promoter usage

CIITA architecture is indicated for both B cells and spDC. Dark lines indicate strong 3-D interactions, while gray lines indicate weaker 3-D interactions. Green ovals and magenta diamonds illustrate CTCF and PU.1 sites; whereas black and open boxes represent exons and distal regulatory elements, respectively. Overall methylation at each promoter is displayed, apart from pI in the spDC pI-KO, where the CpGs were removed by the KO deletion.