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Distal elements coordinate CIITA isoform-specific expression in myeloid and lymphoid cell lineages

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Distal elements coordinate CIITA isoform-specific expression in myeloid and lymphoid cell lineages

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B.A., Agnes Scott College, 2007

Advisor: Jeremy Boss, Ph.D.

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Abstract

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By Sarah Lohsen

The major histocompatibility class II (MHC-II) molecules present antigens acquired from the local immune environment to CD4 helper T cells, which is essential for the generation of humoral immunity. Peptide-antigen complexes are presented by professional antigen presenting cells (pAPCs) of the lymphoid (B cells) and myeloid (macrophages and dendritic cells) lineages. MHC-II expression on pAPCs is highly regulated at the level of transcription, and the MHC-II transactivator, CIITA, is obligate for its expression. *CIITA* itself is also principally regulated at the level of transcription, and can be expressed from three main promoters. In cells of the myeloid lineage, *CIITA* is primarily expressed from promoter I, and in cells of the lymphoid lineage *CIITA* is highly expressed from promoter III (corresponding to MHC-II expression). The proximal promoter regions of *CIITA* have been characterized for all three of the principle promoters. Recent studies suggest that a series of distal regulatory elements may be involved in regulating transcription from promoter IV, but only one such distal element has been characterized in the context of promoter III in B cells. By screening the *CIITA* locus in lymphoid cells for DNase-I hypersensitive sites, a series of potential novel distal regulatory elements were identified. These elements were analyzed computationally to identify key regions of cross-species homology and transcription factor binding. Characterization of histone marks, transcription factor binding, luciferase assays to determine enhancer activity, and elucidation of the 3-D chromatin architecture around these sites further point to putative functional regulatory elements. At least two of the four elements involved in interactions with the active promoter III are shared in regulation of *CIITA* from promoter IV, and all are shared by promoter I-utilizing splenic dendritic cells. Examination of DNA methylation at promoters I and III reveal a potentially multifaceted role in the context of facilitating promoter choice from these promoters in both a direct and indirect fashion. This study has identified a series of novel distal regulatory elements involved in the regulation of *CIITA* and its promoter choice in pAPCs, allowing for a better understanding of the control of antigen presentation in the immune system.

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Chapter 1: Introduction

This chapter presents a review of antigen processing and presentation and the regulation of *MHCII* and *CIITA*.

This chapter was written by S. Lohsen.

In this introduction a summary of the current field of knowledge regarding *CIITA* regulation is presented. This begins with a general introduction of antigen presentation, the major histocompatibility complexes, and the function of CIITA. A more detailed examination of the regulation of each *CIITA* promoter in addition to a brief comparison of the functional differences between the CIITA isoforms follows. The well-established regulation of these promoters by the sequences proximal to the promoter is described, as well as the more complex distal *cis*-regulatory network involved in the regulation of *CIITA* isoform IV, and the possibility of a similar network involved in the regulation of the other isoforms is described. The introduction culminates with a discussion regarding the alteration of *CIITA* expression in both cancers and diseases, underscoring the importance of a thorough understanding of *CIITA* regulation.

I. Antigen Processing and Presentation

To elicit immune responses, foreign pathogens must first be recognized by the immune system. This is made possible through the process of antigen processing and presentation (Townsend et al., 1985). Proteins in the cytosol of cells are presented to T cells on Major Histocompatibility Class (MHC) I molecules (Ackerman and Cresswell, 2004). MHCI molecules present peptide antigens to CD8 T cells, and recognition of this MHCI antigen complex triggers an adaptive response that primarily results in cell death of the presenting cell (Grommé and Neefjes, 2002). Protein antigens acquired from the local immune environment are presented primarily by professional antigen presenting cells (pAPCs) or by most other cell types in an interferon (IFN) γ-inducible manner on MHCII molecules to CD4 T cells, and recognition of this MHCII antigen complex results in

activation or suppression of adaptive immune responses (Benacerraf, 1981; Germain, 1986) initiating the process that leads to antibody generation.

II. Major Histocompatibility Class I Molecules and Antigen Presentation

MHCI molecules are synthesized and retained in the endoplasmic reticulum (ER) until they bind peptide. MHCI molecules are maintained in a partially folded state in the ER by chaperones (Bouvier, 2003), such as calnexin, until β_2-microglobulin (β_2-m) binds to create the MHCI: β_2-m complex (Townsend et al., 1989) (shown in Figure 1A below). This complex then dissociates from calnexin and associates with the MHCI loading complex, which is comprised of chaperone proteins (calreticulin and Erp57), as well as tapasin, forming a bridge between MHCI and the transporter associated with antigen processing (TAP) (Bouvier, 2003). Intracellular proteins are degraded into peptide fragments by proteasomes (reviewed in (Tanaka and Kasahara, 1998)) and delivered to the ER via TAP (Koopmann et al., 1997) where they are modified for loading onto MHCI molecules by ERAAP (ER aminopeptidase associated with antigen processing) (Serwold et al., 2002). These peptides can then bind MHCI, causing the release from the MHC class I loading complex, which allows MHCI:peptide complex transport to the cell surface (reviewed in (Williams et al., 2002)).

III. Major Histocompatibility Class I Regulation

Family members of the nucleotide-binding domain, leucine-rich repeat (NLR) gene family play important roles in regulation of inflammation and predominantly innate immune responses (Ye and Ting, 2008). A member of this family, the MHC class II transactivator, CIITA, is known to play a role in regulating *MHCII* gene expression, and also mediates

MHCI upregulation through a *cis*-regulatory region termed site α (Gobin et al., 1997; Martin et al., 1997). Despite this role, it is not critical for *MHCI* expression or IFNγ-induced up-regulation (Williams et al., 1998).

It is another member of the NLR family, NLRC5, which is the transcriptional regulator of genes important in the MHC class I pathway, including *MHCI* itself, as well as β_2-m (Meissner et al., 2010). NLRC5 mediates *MHCI* expression through the removal of the repressive chromatin mark histone H3 lysine 27 trimethylation (H3K27me3) at *MHCI* promoters (Robbins et al., 2012).

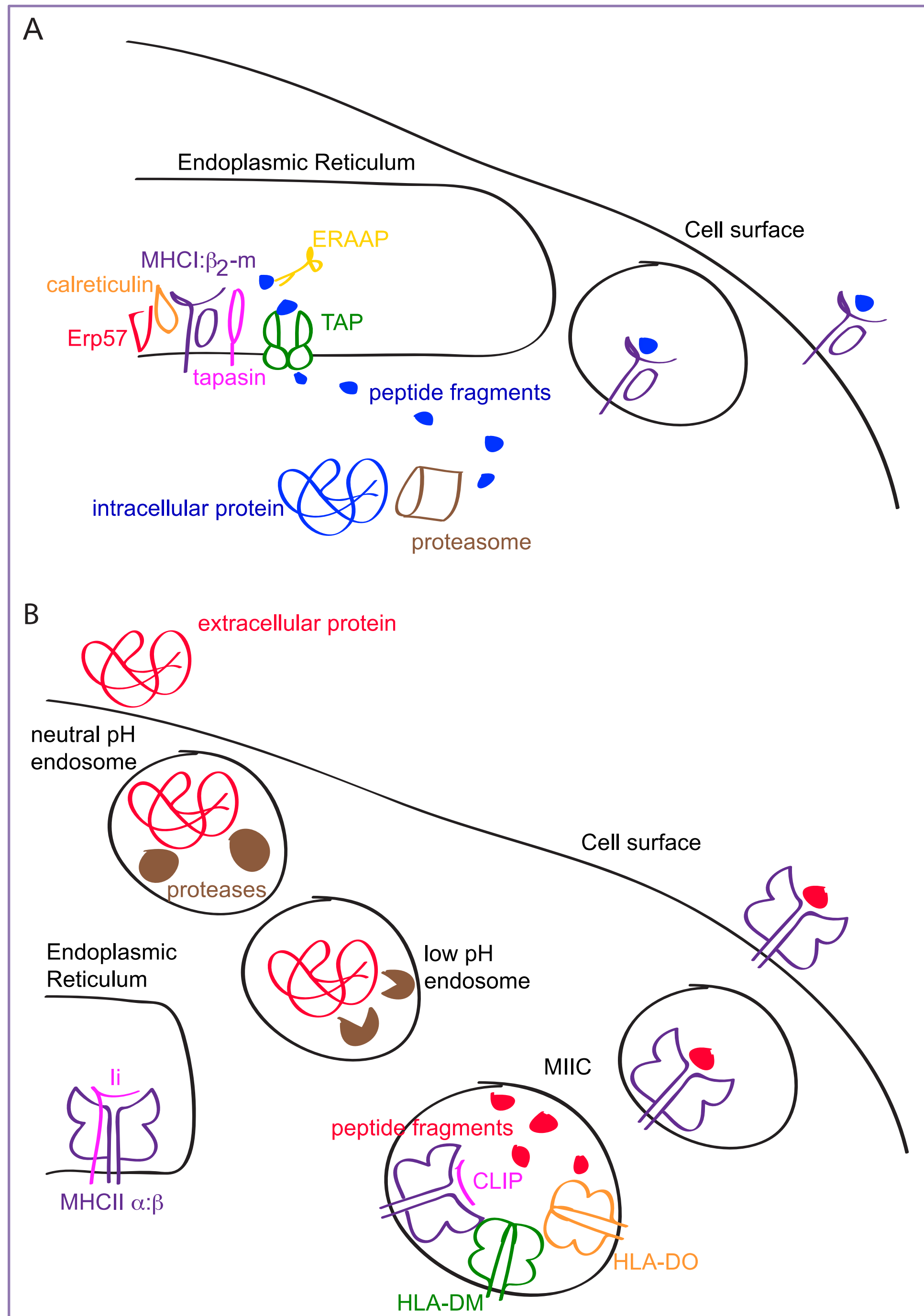

Figure 1 - Antigen processing and presentation via MHCI and MHCII molecules. A) Intracellular proteins (blue) are degraded into peptides and loaded onto MHCI molecules in the ER. Following loading, peptide:MHCI complexes are transported to the cell surface. B) Extracellular proteins (red) are endocytosed into cells and processed in the endosomal compartment before loading onto MHCII molecules and transport to the cell surface.

IV. Major Histocompatibility Class II Molecules and Antigen Presentation

Figure 1B above provides an overview of MHCII antigen processing and presentation. Endocytosis of proteins from the extracellular space places these proteins in intracellular vesicles. These proteins are delivered to early endosomes, which contain inactive endosomal proteases and are of a neutral pH. As the vesicles mature, they progress into the cell and become acidified, activating various proteases (Vyas et al., 2008). These proteases act upon the protein antigens to generate peptide fragments that can be presented by MHCII molecules (Buus and Werdelin, 1986).

MHCII α and β chains form a heterodimer in the ER upon synthesis (reviewed in (Cresswell, 1994)). To prevent binding of self peptides within the ER to MHCII molecules, the MHCII-associated invariant chain (Ii) forms a trimer with the MHCII α and β chains and blocks the peptide binding groove (Koch et al., 1989). Ii targets these complexes either directly to compartments for antigen processing (Warmerdam et al., 1996) or to the surface of the cell (reviewed in (Hiltbold and Roche, 2002)). The Ii is cleaved several times primarily by cathepsins S (Riese et al., 1996) and L (Chapman, 1998). With the action of other proteases (Villadangos et al., 1997) a peptide fragment of Ii, termed CLIP (*cl*ass II-associated *i*nvariant-chain *p*eptide), is bound in the MHCII binding groove (reviewed in (Cresswell, 1996)). Ii also serves to target MHCII to a low-pH endosomal compartment to allow peptide loading (Odorizzi and Trowbridge, 1994), termed MIIC, or MHC class II compartment (Peters et al., 1991).

The release of CLIP and the loading of a peptide from the endosomal compartment is facilitated by the MHC class II-like molecule HLA-DM, which is found principally in the MIIC (Harding and Geuze, 1993). HLA-DM is responsible for loading stably-bound peptides to MHCII (Denzin and Cresswell, 1995; Sanderson et al., 1994; Sloan et al., 1995) in

a process termed peptide editing. HLA-DM forms a stable complex with HLA-DO (Liljedahl et al., 1996) and is negatively regulated by HLA-DO in B cells and several other cell types (Douek and Altmann, 1997). A negative regulatory factor, HLA-DO together with DM serves to stabilize MHCII complexes more effectively than just DM (Kropshofer et al., 1998). HLA-DO prevents the unloading of CLIP by HLA-DM and thus the binding of antigenic peptide to MHCII molecules (Denzin, 1997; van Ham et al., 1997). Some evidence suggests that HLA-DO may also play an as-yet-undefined role in the immune system (Fallas et al., 2007). Once loaded with the best fitting peptide, MHCII:peptide complexes can then travel to the cell surface for presentation (Harding and Geuze, 1993).

V. Lack of MHCII Leads to a Severe Combined Immunodeficiency – Bare Lymphocyte Syndrome

Bare Lymphocyte Syndrome (BLS) patients lack expression of MHCII not through a defect in MHCII proteins, but due to mutations in proteins regulating *MHCII* expression (Durand et al., 1994; Masternak et al., 1998; Steimle et al., 1993, 1995). Patients with BLS lack humoral immune responses and as such are acutely sensitive to a variety of pathogens, leading to diagnosis at a very young age due to recurrent infections (Klein et al., 1993). Four complementation groups of BLS exist, elucidated by cell-fusion experiments (Bénichou and Strominger, 1991). Each of the groups define transcription factors necessary for *MHCII* expression: RFXB (also known as RFXANK), RFX5, RFXAP, and the MHCII transactivator CIITA (Durand et al., 1997; Masternak et al., 1998; Steimle et al., 1993, 1995).

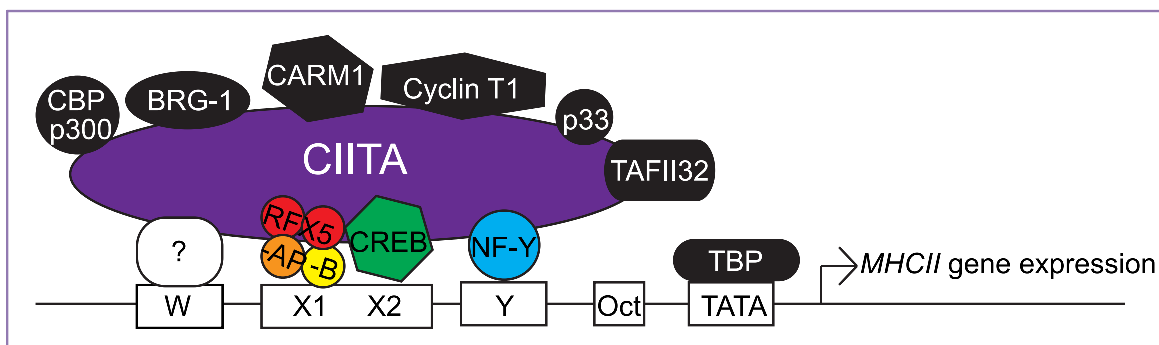

Figure 2 – *MHCII* promoter showing the CIITA-enhanceosome complex. CIITA interacts with transcription factors bound at the *MHCII* promoter and coordinates recruitment of histone modifying enzymes and components of the transcriptional machinery.

VI. Major Histocompatibility Complex Class II Regulation

MHCII is primarily regulated at the level of transcription (reviewed in (Choi et al., 2011)). Key to this regulation is a WXY box common to the promoter region of *MHCII* genes (Benoist and Mathis, 1990), which is similarly occupied whether *MHCII* is being expressed constitutively or in an IFNγ-inducible manner (Kara and Glimcher, 1993) as shown in Figure 2 above. Also contributing to regulation are X box-like sequences, some of which are found intergenically in the class II locus as enhancers, resulting in an increase in chromatin accessibility at these regions (Gomez et al., 2005) A number of transcription factors bind constitutively to the WXY boxes but are not sufficient to allow transcription to occur.

The RFX multimeric phosphoprotien complex (Moreno et al., 1997) binds at the X box (Reith et al., 1988) and is composed of a 2:1:1 ratio of RFX5:RFXAP:RFXB, which can associate with another RFX5 dimer (Garvie et al., 2007). The RFX5 subunit drives recognition of the DNA sequence at the promoter, and the –AP and –B subunits together

contribute to the binding specificity of the complex (Burd et al., 2004). All subunits are critical for RFX function in *MHCII* regulation, as exemplified by the fact that RFX-B is the most common subunit of the RFX complex mutated in BLS (Nagarajan et al., 1999). Importantly, lack of any of the three components results in no *MHCII* expression (Reith and Mach, 2001). The RFX complex binds together with the X2 box-binding protein (X2BP) (Hasegawa et al., 1991), which has subsequently been identified as CREB, and works with CIITA to facilitate MHCII expression (Moreno et al., 1999). NF-Y binds at the CCAAT-box (Mantovani et al., 1992; Zeleznik-Le et al., 1991) located within the Y box. The binding of RFX, CREB, and NF-Y to DNA creates a very stable complex (Louis-Plence et al., 1997).

As stated above the binding of these factors is not sufficient for *MHCII* expression. The binding of CIITA to constitutively bound transcription factors activates transcription from the *MHCII* promoters, and a quantitative relationship between *CIITA* and *MHCII* expression exists (Otten et al., 1998). The importance of CIITA is best seen in its identification through an expression cloning approach to discovering the underlying gene responsible for BLS complementation group A (Steimle et al., 1993). In the case of inducible-MHCII expression in many cell types, CIITA appears to effectively stabilize the protein complex formed at the proximal promoter region (Villard et al., 1999; Wright et al., 1998). CIITA interacts via the X-element (Riley et al., 1995) through interactions with RFX5 (DeSandro et al., 2000; Scholl et al., 1997) and its action is also dependent on the presence of the W-box (Brown et al., 1998; Zhou and Glimcher, 1995). CIITA and RFX will interact without the presence of DNA, along with CREB (Burd et al., 2004). CIITA's induction of *MHCII* is dependent on the spacing between the X and Y boxes. It requires stereospecifically aligned WXY elements in that X and Y must be separated by complete helical turns of the DNA (Vilen et al., 1991; Zhu et al., 2000). Part of the W box, also

termed the S box, is critical to allow CIITA tethering, and the exact 16-bp spacing between the W/S and X boxes must be preserved (Fontes et al., 1997a; Muhlethaler-Mottet et al., 2004).

VII. CIITA Protein Structure and Posttranslational Modifications

CIITA was originally described a member of the CATERPILLER (CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats) family, which is contained within the NLR superfamily of proteins. CIITA spans two subfamilies of the CATERPILLER family, as one of its isoforms contains a CARD (*ca*spase *r*ecruitment *d*omain, typically an apoptotic signaling motif (Hofmann and Bucher, 1997)) domain (isoform I), and all of its isoforms contain a transactivation domain (Harton et al., 2002) as shown in Figure 3 below. Initial studies to determine important domains of CIITA examined cDNAs from mouse and human and found two carboxy-terminal regions, an N-terminal transactivating domain (AAD), as well as a proline-rich collagen-like region (P/S/T) that potentially serves as a spacer that were highly conserved, and therefore likely important for function (Riley et al., 1995).

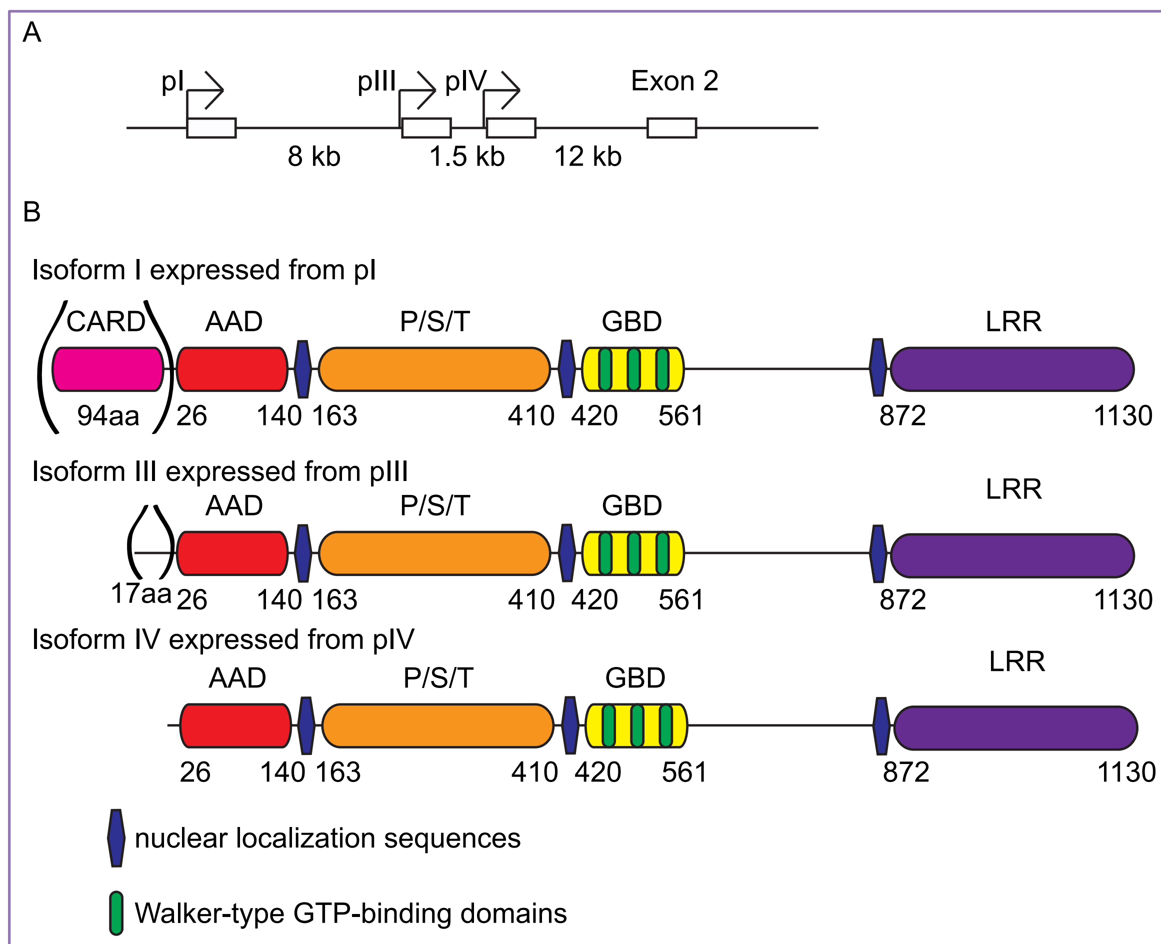

Figure 3 – Protein structure of CIITA isoforms. A) The murine *CIITA* locus showing the three principle promoters and their relative locations. B) CIITA domains are shown for the three CIITA isoforms: isoform-I specific caspase recruitment (CARD) domain, and the shared transactivating domain (AAD), proline-rich collagen-like region (P/S/T), GTP-binding domain (GBD), leucine rich repeats (LRR), nuclear localization sequences and GTP-binding domains.

The CATERPILLER family has much diversity in its N-terminal sequences (Harton et al., 2002), and the N-terminus of CIITA contains a GTP-binding domain (GBD) which regulates CIITA's intrinsic acetyltransferase activity (Raval et al., 2001), but also a

proline/serine/threonine-rich region, which together with the GTP-binding domain make up critical domains for CIITA function, as mutants in either are transdominant-negative molecules (Chin et al., 1997b). Mutations in the GTP-binding domain also fail to localize to the nucleus (Harton, 1999) due to a nuclear localization signal (NLS) adjacent to the GTP-binding domain (NLS2) (Cressman et al., 2001). The N-terminal portion of CIITA is critical for orchestrating interactions with transcription factors that bind the *MHCII* promoter. RFXB interacts with amino acids (AAs) 1-335, RFX5 with AAs 335-612, NF-YC with AAs 218-335, NF-YB with AAs 518-612, and lastly, AAs 1-793 necessary for optimal CREB binding (Zhu et al., 2000). The N-terminal 36 amino acids of CIITA have also been shown to bind CBP (Zhu and Ting, 2001), and the N-terminal 140 amino acids interact with a component of the SWI/SNF complex, BRG-1 (Mudhasani and Fontes, 2002), further demonstrating the importance of the N-terminus of CIITA in mediating interactions with other transcription factors.

The C-terminal 41 amino acids are critical for a majority of CIITA's transactivation capability, though the reason is unclear, as this highly conserved region does not contain any well-known motifs or structures (Chin et al., 1997a). Intriguingly, 72% of the CIITA protein, consisting of the C-terminal 813 residues were found to be necessary and sufficient for directing transcriptional activation activity to the DRα promoter (Zhou and Glimcher, 1995). The C-terminus also contains at least four leucine-rich repeats (LRRs), and binding to the *MHCII* promoter is dependent on their presence (Hake et al., 2000). Nuclear localization (Cressman et al., 1999) and interactions with a 33kDa novel protein (p33) are also dependent on the LRRs (Hake et al., 2000). The critical importance of the LRRs is demonstrated in one BLS patient whose mutation mapped to deletion of part of the LRR (Peijnenburg et al., 2000).

The LRR along with the GTP-binding domains are also important for the self-association of CIITA (Linhoff et al., 2001). This self-association can occur via homo- or heterotypic interactions, and differing associations appear to be recognized selectively by nuclear import machinery to impact CIITA's nuclear import (Kretsovali et al., 2001). In addition, self-association appears to be important for CIITA's transactivation potential, and is speculated to be a result of interaction with CBP (Sisk et al., 2001).

Several groups have shown the importance of posttranslational modifications to maintenance of CIITA levels and CIITA action. Acetylation of CIITA by PCAF and CBP occurs within one of CIITA's nuclear localization signals (NLS3), and this acetylation serves to increase the nuclear accumulation of CIITA (Spilianakis et al., 2000). CIITA is shielded from proteasomal degradation by deacetylation of CIITA by SIRT1 in macrophages (Wu et al., 2011).

VIII. CIITA Function

CIITA serves to recruit a number of histone modifying enzymes to facilitate histone acetylation and methylation (Choi and Boss, 2012; Masternak and Reith, 2002), as well as other coactivators that facilitate the recruitment of RNA polymerase II and thus serves as a transcriptional integrator (Fontes et al., 1999). Histone acetylation contributes to an environment of open chromatin, allowing transcription factors to bind and transcription to occur from these open regions. CIITA facilitates the interactions of various transcription factors with the MHCII promoter region. CIITA binds OBF-1 (Bob1, OCA-B) (Fontes et al., 1996), Cyclin T1 of P-TEFb (Kanazawa et al., 2000), CBP (Fontes and Kanazawa, 1999; Kretsovali and Agalioti, 1998), the coactivator-associated arginine methyltransferase 1 (CARM1) (Zika et al., 2005), and the general transcription complex (Mahanta et al., 1997),

including a subunit of TFIID, $TAF_{II}32$ (Fontes et al., 1997b). CIITA also interacts with a component of the nucleosome remodeling complex SWI/SNF, BRG-1, whose presence is required to allow MHCII expression (Mudhasani and Fontes, 2002). All of these proteins work together to facilitate transcription at CIITA-regulated genes: *MHCII* genes in particular.

CIITA's ability to facilitate histone acetylation at the *MHCII* locus correlates with the presence of the CBP/p300-interactive region of the activation domain (Beresford and Boss, 2001). In the IFNγ -inducible system of *MHCII* expression, CIITA's presence is correlated with a first phase of more global histone H4 acetylation (at lysines 5 and 8), and a second phase of more local histone H3 acetylation (at lysines 9 and 14) (Beresford and Boss, 2001). Other histone modifications, such as methylation of the histone tail of histone H3 (including di- and trimethylation of lysine 4, demethylation of arginine 17, and trimethylation of lysine 9) are transcription-coupled (Rybtsova et al., 2007), and therefore require the presence of CIITA to be introduced to the chromatin architecture at the *MHCII* genes.

In addition to its role as a master regulator of *MHCII* transcription, CIITA is also known to be involved in the activation and repression of a number of other genes. CIITA regulates HLA-DMα and DMβ (Kern et al., 1995), DOβ (Nagarajan et al., 2002a), DOα (Taxman et al., 2000), as well as Ii (Kern et al., 1995), and therefore shows a global role in regulation of antigen presentation genes (N. H. Chang, Rayner, and Boggs 1995). Microarray analyses to examine the extent of CIITA gene targets have found conflicting evidence for CIITA's involvement in regulation of genes outside of antigen presentation. One study suggests that the involvement in processes apart from antigen presentation is at best only indirect (Otten et al., 2006), and another examining B cells and dendritic cells, only found antigen-presentation-related genes to be regulated by CIITA (Krawczyk et al., 2008). On the other hand, a number of CIITA targets, both related to and unrelated to antigen

presentation, were found via microarray in another analysis of B cell lines (Nagarajan et al., 2002b). In contrast to some of these microarray studies, a non-antigen presentation gene shown to be upregulated by CIITA was plexin-A1 in dendritic cells (Wong et al., 2003). CIITA was also shown to increase HIV-1 LTR promoter activity (Saifuddin et al., 2000).

CIITA has been shown to be involved in the negative regulation of IL-4 in T cell differentiation (Gourley et al., 1999) by competing with an activator of IL-4, NF-AT, for binding with CBP/p300 (Sisk et al., 2000). CIITA works through a similar mechanism of CBP/p300 binding to repress the transcription of the Fas ligand in T cells (Gourley and Chang, 2001). This repression occurs by squelching CBP from the target gene. CBP squelching is mediated through the CBP-interacting N-terminal 36 amino acids of CIITA, and results in the suppression of a variety of genes, including collagen α2, thymidine kinase, and cyclin D1 (Zhu and Ting, 2001). This mechanism of action has also been postulated to explain CIITA's repression of other genes, such as a number of muscle-specific genes (MyoD and Myog) which are repressed in an IFNγ-dependent manner (Londhe and Davie, 2011). The N-terminal P/S/T domain has also been shown to mediate the negative regulation of cathepsin E through a similar mechanism (through CBP), but also through inhibition of PU.1 activity via indeterminate means (Yee et al., 2004).

Apart from the well-characterized instances of individuals with null mutations in CIITA suffering from BLS, the most vivid demonstrations of the importance of CIITA's function have been seen in several mouse models in which CIITA has been knocked out. One of the first knock out mice had a deletion in downstream exons, which resulted in a CIITA null mouse (Chang et al., 1996). These mice showed no MHCII expression apart from the interdigitating reticular cells in the thymus. Atypical *MHCII* genes were also downregulated, though splenocytes still showed expression of H2Oα and β (murine DO

genes), demonstrating that CIITA alone is not responsible for their expression (Chang et al., 1996). Due to a lack of MHCII, these mice do not show positive selection of T cells, leading to a large reduction in CD4 T cell numbers, as well as a defect in response to immunization/pathogenic challenge in those T cells that remain (Chang et al., 1996). A second CIITA null mouse, lacking the first three exons of *CIITA*, showed similar phenotypes to the first CIITA null mouse, as CD4 T cell numbers were greatly diminished, but showed trace levels of MHCII on B cells and dendritic cells (Williams et al., 1998).

IX. General *CIITA* Regulation

Like *MHCII*, *CIITA* itself is also highly regulated at the level of transcription. It is expressed in a highly cell-type specific manner from four promoters (three promoters in the mouse) (Muhlethaler-Mottet et al., 1997). These promoters are regulated in unique ways (Pai et al., 2002). Each promoter has a unique first exon that is spliced into a common second exon. This study focuses murine *CIITA* system. Isoform I, expressed from promoter I, is expressed primarily in macrophages and dendritic cells, cells of the myeloid lineage. Isoform III, expressed from promoter III, is expressed primarily in B cells, and other cells of the lymphoid lineage (Muhlethaler-Mottet et al., 1997), such as activated human T cells (Holling et al., 2002). While most dendritic cells use promoter I, plasmacytoid dendritic cells (pDCs) primarily use the lymphoid promoter III (LeibundGut-Landmann et al., 2004). Lastly, isoform IV, expressed from promoter IV, is expressed in most cell types in response to IFNγ (Chin et al., 1994; Muhlethaler-Mottet et al., 1997; Rigaud et al., 1996; Steimle et al., 1994; Waldburger et al., 2001a). The individual roles of these unique isoforms are unclear, but they appear to some extent to be interchangeable (Zinzow-Kramer et al., 2012).

In examining *CIITA* mutations in BLS patients, only one showed what was postulated to be a mutation in a regulatory region resulting in no CIITA expression, and while the exact region remains unidentified, it is the only *cis*-regulatory CIITA dysfunction described (Dziembowska et al., 2002). Apart from the BLS model, much work has been done to define the *cis*-regulatory regions and architectures responsible for the various CIITA isoform expressions, as CIITA is a good target for modulation of immune responses via *MHCII* genes and antigen presentation in general. As such, pathogens (Abendroth et al., 2000; Ghorpade et al., 2013; Li et al., 2009; Miller and Rahill, 1998; Srisatjaluk et al., 2002), as well as some cancers (van der Stoep et al., 2002a; Yazawa et al., 1999), dysregulate *CIITA* expression in order to evade immune system surveillance. Below is a detailed overview of the unique regulation of each isoform of CIITA, culminating in how disease, pathogens, and cancers alter this regulation.

X. *CIITA* promoter I Regulation

In cells of the myeloid lineage, the predominant form of CIITA expressed is isoform I from promoter I (Muhlethaler-Mottet et al., 1997). This is not exclusive, as splenic dendritic cells (spDC) express small amounts of CIITA from promoter III and macrophages express small amounts of isoform IV in addition to isoform I (Zinzow-Kramer et al., 2012).

The mechanism of regulation of CIITA isoform I is the least well characterized, but some recent studies have elucidated the importance of PU.1 and IRF4/8 in isoform I expression (Kitamura et al., 2012; Smith et al., 2011). In vivo footprint analysis of promoter I revealed PU.1, Ap1, Sp-1, NF-κB, E2A and an Ets family member binding sites, as well as an Ets/IRF composite element (EICE) (Smith et al., 2011). EMSA confirmed binding of PU.1, as well as IRF8 and IRF4 (stronger binding of IRF8 than of IRF4) to promoter I

sequences, and ChIP confirmed binding of PU.1, IRF8, p65 (a subunit of NF-kB), and Sp1 (Smith et al., 2011). PU.1, EICE and NF-κB sites in particular were found to be critical for transcriptional activity. PU.1's importance in promoter I regulation was demonstrated by siRNA knockdown of PU.1, resulting in a reduction of isoform I *CIITA* expression (Kitamura et al., 2012). siRNA against PU.1 resulted in a decrease in general histone acetylation at promoter I (Kitamura et al., 2012), suggesting a role for PU.1 in recruiting histone acetyltransferases to facilitate *CIITA* expression.

Conflicting evidence for the involvement of several different Signal Transducer and Activator of Transcription (STAT) proteins exists. Initial work examining the inhibition of Jak2/STAT3 led to downregulation of *CIITA* from promoter I in myeloid dendritic cells, pointing to a role for STAT3 in promoter I regulation (Nefedova et al., 2005). Subsequent studies using a conditional knockout of STAT3 in dendritic cells showed no reduction in MHCII expression, pointing to the possibility that STAT3 does not have an effect on *CIITA* expression (Melillo et al., 2010). A potential role for STAT5 in promoter I regulation was determined in examining the downregulation of MHCII on dendritic cells of cancer patients (Choi et al., 2009). Tumor-conditioned media was found to contain IL-10, which was found to be responsible for the inhibition of *CIITA* expression, and thus the downregulation of MHCII (Choi et al., 2009). This study revealed that STAT5 binding was correlated with histone H3 and H4 acetylation at promoter I (Choi et al., 2009), most likely mediated through STAT5's known interaction with p300/CBP (Pfitzner et al., 1998). This does not discount a role for STAT3, as IL-10 activates STATs 1, 3, and 5 (Wehinger et al., 1996), and only STAT5 binding was queried (Choi et al., 2009).

Maturation of dendritic cells leads to a decrease in *CIITA* expression that correlates with global histone deacetylation at all of the *CIITA* promoters (Landmann et al., 2001).

Deacetylation is most likely mediated through the recruitment of HDAC2 to *CIITA* promoter I (Smith et al., 2011) and is triggered by signaling through ERK and p38 MAPK (Yao et al., 2006). There is conflicting evidence that maturation of dendritic cells also results in an eviction of bound activators (Landmann et al., 2001; Smith et al., 2011); however, maturation is coincident with the binding of the repressor PRDM1/Blimp-1 (Smith et al., 2011). Blimp-1 has been shown to recruit the histone H3 methyltransferase G9a to mediate transcriptional silencing (Gyory et al., 2004), and this is a likely mechanism of silencing at promoter I, as G9a is also found to bind at promoter I during dendritic cell maturation (Smith et al., 2011). Figure 4 below details the regulation of promoter I in the mouse.

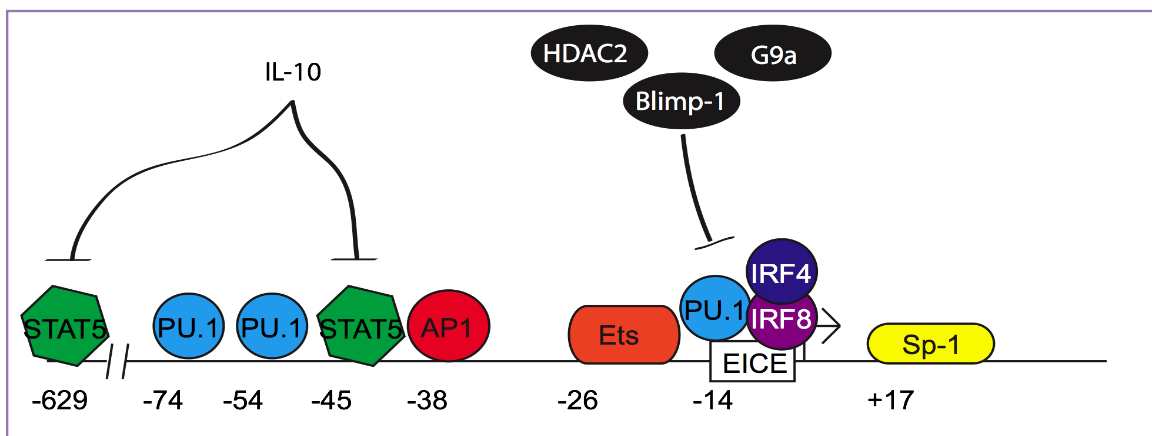

Figure 4 – Murine *CIITA* promoter I. Transcription factor binding with distances from the transcriptional start site are displayed. Black colored transcription factors indicate factors involved in repression of expression from the promoter.

In addition to the regulation mediated at the proximal promoter described above, there appears to be an upstream enhancer region that acts on promoter I in myeloid cells to allow transcription to occur from this promoter (Zinzow-Kramer et al., 2012), but it has not yet been identified.

XI. *CIITA* promoter II Regulation

Initial studies to understand the various isoforms of *CIITA* using RACE-PCR to examine the 5' ends of *CIITA* found that there were four different 5' sequences in human, while there were only three in the mouse (Muhlethaler-Mottet et al., 1997). The sequence found uniquely in the human samples corresponded to the isoform expressed from promoter II, which shares an initiation codon with promoter IV (Muhlethaler-Mottet et al., 1997). Beyond this initial study, very little is known about isoform II of CIITA.

Activated human T cells, despite typically expressing *CIITA* from promoter III (Holling et al., 2002), were found to have acetylated histones H3 and H4 across all four of the promoters, as well as the presence of RNA-Pol II at all of the promoters (van Eggermond et al., 2011), though transcript levels were not determined. Some melanoma cell lines have demonstrated constitutive and IFNγ-inducible expression of isoform II of CIITA (in addition to showing expression of most other CIITA isoforms) (van der Stoep et al., 2007), but its regulation or significance have not been examined further.

XII. *CIITA* promoter III Regulation

In the initial identification of CIITA as a causal component of BLS isoform III was the first identified form of CIITA. A cell line derived from Raji, an MHCII positive Burkitt lymphoma B cell line, RJ2.2.5 (Accolla, 1983) is negative for MHCII expression, and was used to identify the locus containing *CIITA* which is deleted at one allele, and contains a 1.8 kb deletion in the other *CIITA* allele. The locus was initially called the "activator of immune response genes-locus 1" (alr-1) (Accolla et al., 1986). RJ2.2.5 was used to screen a library of cDNAs for restoration of MHCII expression resulting in the isolation of the lymphocyte-

specific isoform of *CIITA*, which was found to be expressed in a manner coincident with MHCII expression (Steimle et al., 1993). A summary of the factors and elements known to control CIITA promoter III prior to this dissertation is shown in Figure 5 below.

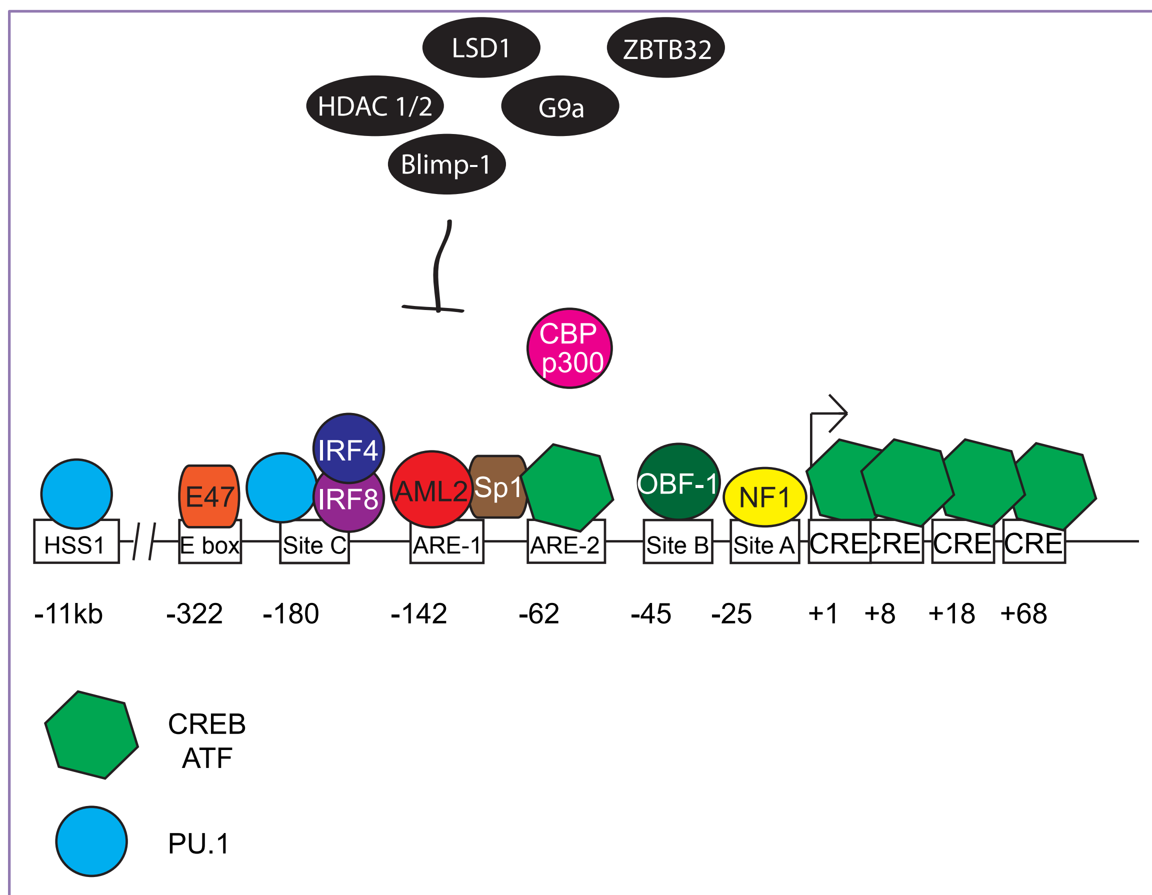

Figure 5 – *CIITA* promoter III. Regulatory elements and transcription factor binding at promoter III is shown with indicated distances from the transcriptional start site. Transcription factors colored in black indicate repressive factors.

Activation of promoter III

Characterization of the B-cell specific promoter for *CIITA* began by identifying a 1.8kb fragment containing several putative binding sites for transcription factors such as AP1, ETS-1, Sp1, MZF1, GATA-2, as well as GAS sites that induced expression in a B cell

system (Lennon et al., 1997). Regions required for B cell expression were narrowed down to within 668 bp of the transcription start site (TSS) (Piskurich et al., 1998), and then even further to within 319 bp of the TSS (Ghosh et al., 1999). In vivo genomic footprinting showed two activation response elements, ARE-1 (bound by Sp1 (Green et al., 2006) and acute myeloid leukemia (AML) 2 (Holling et al. 2002)) and ARE-2 (bound by CREB/activating transcription factor (ATF) family members (Holling et al. 2002)), primarily responsible for promoter III activity, as well as Site A (bound by NF-1), Site B (very weakly bound by OTF-1/Bob1), and Site C (Ghosh et al. 1999). Site C contains an Ets-IRF composite element (EICE) that binds PU.1 coordinately with IRF4/8 (Nienke van der Stoep et al. 2004). Upstream from Site C, 2 E-box motifs bind E47 (Nienke van der Stoep et al. 2004). The proximal promoter region and 5' UTR contain cAMP responsive elements (CREs) binding CREB-1/ATF-1, which boost activity from promoter III (Nienke van der Stoep, Quinten, and Van den Elsen 2002). IRF-4's importance in regulation of promoter III was implied through inhibition of IRF-4 leading to a decrease in MHCII expression (Nienke van der Stoep et al. 2004). PU.1's significance was elucidated in a similar manner, this time using shRNA against PU.1, which resulted in decreased *CIITA* expression (H. Yoon and Boss 2010). PU.1's importance was reiterated in a mast-cell model of promoter III expression (Nakano et al. 2011).

A regulatory element 11kb upstream from promoter III bound by PU.1 was the first distal-acting regulatory site found to be involved in promoter III regulation (H. Yoon and Boss 2010). This study revealed the beginnings of a role for unique chromatin architecture in regulating CIITA expression from promoter III, mediated by PU.1, as PU.1 is partially responsible for mediating 3-D interactions between this upstream regulatory region and the promoter (H. Yoon and Boss 2010).

In cell types where CIITA is not constitutively expressed, regulation of promoter III is slightly altered. Fibrosarcoma cells transfected with 7kb of upstream sequence from promoter III revealed that promoter III can be IFNγ-inducible, and that this is dependent on STAT1 (Piskurich et al. 1999). Activated human T cells show much more similarity to B cells in their regulation of promoter III. ARE-1 (bound by AML-2 as in B cells, as well as AML-3), and ARE-2 (bound by CREB/ATF as in B cells) are also critical for promoter III activity in T cells (Holling et al. 2002). Site C is also occupied in T cells as in B cells, but by an unidentified Ets family member (Holling et al. 2002).

The epigenetic landscape of histone marks surrounding promoter III is similar between B and T cells expressing CIITA isoform III. Acetylation of histones H3 and H4 is found across all of the CIITA promoters, in addition to trimethylation of histone H3 at lysine 4 (H3K4me3) (van Eggermond et al., 2011; Green et al., 2006).

Repression of promoter III

MHCII is expressed on B cells, but is silenced during their differentiation to plasma cells (Latron et al., 1988) due to a silencing of *CIITA* expression (Silacci et al., 1994) by a trans-acting repressor (Sartoris et al., 1996), and this suppression is specific to promoter III (Lennon et al., 1998). The trans-acting repressor BLIMP-1 binds at an interferon-stimulated response element (ISRE) -180 to -171bp upstream of human promoter III (Piskurich et al., 2000). BLIMP-1 mediates silencing of *CIITA* through its zinc finger and a proline-rich (PR) domain, and this silencing is not related to BLIMP-1's ability to recruit HDACs (Ghosh et al., 2001). Blimp1 is known to interact with lysine-specific demethylase 1 (LSD1) (Su et al., 2009), and therefore could be mediating repression of *CIITA* through its recruitment. Early repression of *CIITA* from promoter III (before BLIMP-1 exerts its influence) is mediated by

the repressor of GATA3, also known as ZBTB32 (Yoon et al., 2012). ZBTB32 appears to work cooperatively with BLIMP-1 however, as they coimmunoprecipitate (Yoon et al., 2012).

Epigenetic modifications play an important role in the silencing of *CIITA* promoter III in several different cell types. In murine T cells, promoter III is methylated, accounting for its lack of expression (Schooten et al., 2005). In unactivated human T cells, the entire *CIITA* promoter region is enriched for marks of closed/repressive chromatin, such as H3K27me3 and H4K20 trimethylation (H4K20me3) (van Eggermond et al., 2011). Examination of epigenetic marks associated with *CIITA* silencing in the B cell system revealed that silencing occurs in a step-wise manner, with a loss of histone acetylation followed by a loss of transcription factor binding, ending with the acquisition of repressive histone marks, such as demethylation of histone H3 at lysine 9 (Green et al., 2006). Apart from histone modifications, a trophoblast non-coding RNA (TncRNA) acts near the ARE-1 element to repress promoter III expression of *CIITA* when transfected into a B cell line (Geirsson et al., 2003a, 2004).

XIII. *CIITA* promoter IV Regulation

The regulation of the IFNγ-inducible promoter IV is perhaps the best characterized of the *CIITA* promoters, and much of what is known is shown in Figure 6A below. As with other isoforms of *CIITA*, *MHCII* expression induced by IFNγ is mediated by CIITA (Chin et al., 1994; Rigaud et al., 1996; Steimle et al., 1994), and a quantitative relationship between CIITA and MHCII expression exists in the IFNγ-inducible system (Otten et al., 1998). Isoform IV is similarly regulated in most cell types when induced by IFNγ, including B cells (Piskurich et al., 2006). Isoform IV is critically important for positive selection of CD4 T

cells, as it is the isoform expressed by thymic epithelial cells (Waldburger et al., 2003, 2001a).

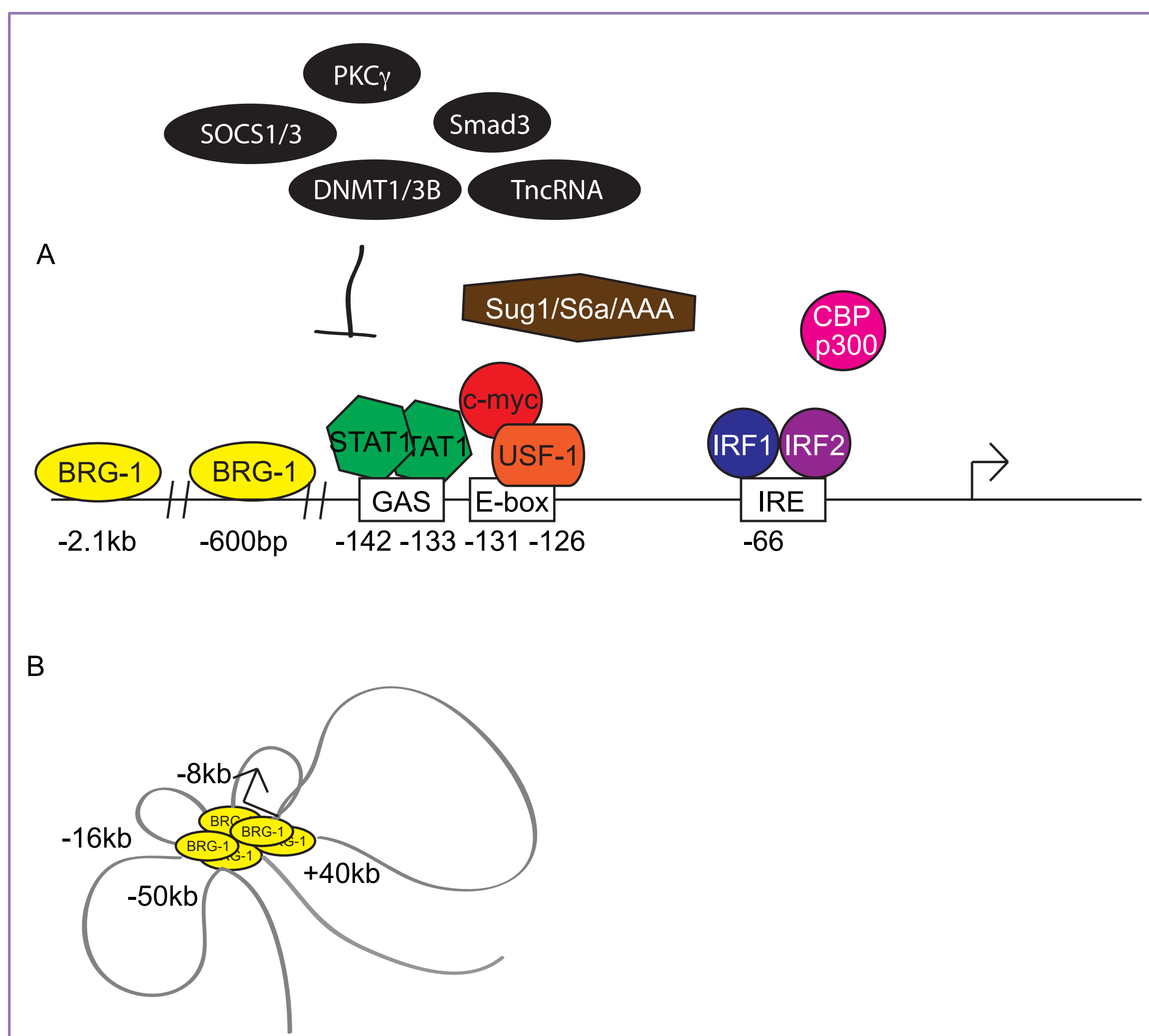

Figure 6 – *CIITA* promoter IV. A) Regulatory elements and transcription factor binding with distances from the transcriptional start site are indicated. Transcription factors colored in black are repressive transcription factors. B) BRG-1-mediated 3D architecture of the *CIITA* locus in pIV-expressing HeLa cells.

Activation of promoter IV

All events in the regulation of promoter IV expression of *CIITA* have a requirement for an ATPase subunit of the SWI/SNF complex, BRG1 (Pattenden et al., 2002). BRG1 binds

constitutively at low levels to promoter IV, and the binding is increased with IFNγ treatment (Ni et al., 2005).

Induction of *CIITA* by IFNγ generally does not require new protein synthesis, but does require a functional Jak1 (Chang et al., 1994) and STAT1α (Lee and Benveniste, 1996; Meraz et al., 1996). Jak1 is one of the Jak family kinases activated by the IFNγ receptor, activation of which leads to phosphorylation of STATs, such as STAT1α (Darnell Jr. et al., 1994). These STATs translocate to the nucleus where they bind sites like the IFNγ activation site (GAS) and direct transcription (Darnell Jr. et al., 1994). STAT1 is involved in *CIITA* expression from promoter IV as STAT1-defective cells lose IFNγ-inducible *CIITA* expression (Piskurich et al., 1998).

Identification of the intergenic promoter for the IFNγ-inducible isoform IV (Lennon et al., 1997) facilitated identification of IRF-1 binding sites (interferon response elements, IRF-Es) at promoter IV (Sims et al., 1997). The presence of binding sites coincident with defective CIITA isoform IV expression in an IRF-1 KO demonstrates a partial dependency on new protein synthesis of IRF-1 (Hobart et al., 1997; Morris and Beresford, 2002). In addition to IRF-1, IRF-2 co-occupies the IRF-E, and works synergistically with IRF-1 to activate *CIITA* expression from promoter IV (O'Keefe et al., 2001; Xi et al., 1999), as knockout of IRF-2 shows only partial reduction in CIITA expression (Xi et al., 2001).

STAT1 binds at a GAS (Muhlethaler-Mottet et al., 1998) located approximately 140bp upstream of the TSS adjacent to an E box, and about 90bp upstream from the IRF-1 binding site (Piskurich et al., 1999). GAS occupancy by STAT1 is dependent upon USF-1 binding at the E box (Dong et al., 1999), which is constitutive at low levels, but increased upon IFNγ treatment (Morris and Beresford, 2002). A 1 bp space between the GAS and E box is essential for their function (Muhlethaler-Mottet et al., 1998). STAT1 and USF-1 first

occupy their binding sites, followed by c-myc and IRF-1 at the IRE to form a complex allowing maximal binding of RNA polymerase II (Ni et al., 2005).

Epigenetic modifications also play a role in the regulation of *CIITA* from promoter IV. The requirement for BRG1 for all events in the regulation of promoter IV may be due to BRG1's role in creating a unique 3-D architecture between the promoter and a series of 4 distal regulatory elements (Ni et al., 2008), as shown in Figure 6B above. Some basal architecture exists without BRG1 present, but the change in architecture induced by IFNγ is dependent on BRG1 (Ni et al., 2008). The early binding of STAT1 is accompanied by an increase in histone H3 and H4 acetylation (Morris and Beresford, 2002), and this increase appears to be due to the presence of BRG1 (Ni et al., 2005). Early epigenetic events also include the recruitment of p300 and CBP, acetylation at lysine's 9 and 18 of histone H3, lysine 8 on histone H4, as well as the acquisition of di- and trimethylation of histone H3 at lysine 4 (Ni et al., 2005). Several of these histone modifications (H3K18ac, H3K4me3, as well as H3R17me2) are somewhat dependent on the 19S proteasomal ATPase Sug1 (Koues et al., 2009). Sug1 also appears to play a role in recruiting subunits of the MLL/COMPASS complex responsible mediating the trimethylation of H3K4, such as the UTX subunit responsible for removing the repressive trimethylation of H3K27 (Crawford and Hess, 2006; Koues et al., 2009, 2010). Additional 19S proteasomal subunits, S6a (S6'/Tat-binding protein 1) and AAA (*A*TPases *a*ssociated with diverse cellular *a*ctivity), appear to play an important role upstream in the regulation of *CIITA* from promoter IV, as a decrease in these subunits leads to reduced histone acetylation at promoter IV and diminished recruitment of transcription factors (Truax et al., 2010).

Repression of promoter IV

Transforming growth factor (TGF)- β has been thoroughly established as a repressor of *MHCII* and *CIITA* (Lee et al., 1997; Nandan and Reiner, 1997; Piskurich et al., 1998). It has been postulated that this repression is mediated at the level of transcription (Lee et al., 1997). This repression does not occur via the Jak/STAT pathway as its function is unaltered during the inhibition of MHCII (Nandan and Reiner, 1997). TGF-β functions through the Smad family of transcription factors (reviewed in (Massague and Wotton, 2000)), and Smad3 appears to be a mechanism through which the inhibition of *CIITA* occurs (Dong et al., 2001). This inhibition is dependent on a region -50 to -70 bp upstream from the TSS. It is not mediated through direct binding of Smad3 itself, but indirectly by the binding of an unknown protein at this region (Dong et al., 2001). Apart from TGF-β, IL-1β has also been shown to inhibit IFNγ-inducible *CIITA* expression via a region of DNA located within the 154 bp upstream of the TSS, through unknown mechanisms (Rohn et al., 1999).

Diminished phosphorylation of STAT1 due to protein kinase Cδ (PKCδ) has been observed in conjunction with reduced levels of histone acetyltransferase (CBP and p300) recruitment at promoter IV during IFNγ-induced *CIITA* expression in B cells as one mechanism of repression of *CIITA* (Kwon et al., 2007). This mechanism of inhibition due to diminished STAT1 phosphorylation is also seen as part of the negative feedback loop of IFNγ signaling. IFNγ induces the expression of the suppressors of cytokine signaling (SOCS)-1 protein (Starr et al., 1997), which not only reduces the phosphorylation of STAT1, but also reduces expression of STAT-1, and reduces binding of STAT-1 and IRF-1 at promoter IV (O'Keefe et al., 2001).

Repression of *CIITA* from promoter IV has been best characterized in trophoblasts. The initial observation in trophoblasts found that MHCII expression is absent from these cells (Faulk et al., 1977; Sunderland et al., 1981) despite the presence of the MHCII genes

and IFNγ receptors (Peyman and Hammond, 1992). Lack of MHCII expression in these cells is due to lack of CIITA, and MHCII expression can be restored by addition of CIITA (Murphy and Tomasi, 1998). The Jak/STAT signaling pathway was intact in these cells, and IRF-1 was expressed (Morris et al., 1998). An epigenetic mode of repression was proposed as promoter IV is methylated in trophoblast-derived cells, and correlates with a lack of transcription factor binding at the promoter (van den Elsen et al., 2000; Morris et al., 2000). This promoter methylation not only correlates with a lack of USF-1, STAT1 and IRF-1 binding, but also with a general lack of histone acetylation at the promoter (Morris and Beresford, 2002). Looking in primary cytotrophoblasts, this methylation was not found, but further evidence for epigenetic regulation was observed as treatment with a histone deacetylase inhibitor, TSA, showed some weak activation of *CIITA* (Holtz et al., 2003).

Beyond epigenetic mechanisms, evidence for a trans-acting repressor was revealed through trophoblast-B cell fusions that silence MHCII and lack CIITA expression (Coady et al., 1999). This trans-acting repressor was identified as a TncRNA and acts on promoter IV through the -117 to -87 region (Geirsson et al., 2003b). This TncRNA acts independently of DNA methylation as a mechanism of *CIITA* silencing (Geirsson et al., 2003b). Beyond TncRNAs, several microRNAs (miRs) have been identified to play a role in inhibition of *CIITA* expression from promoter IV in HeLa cells (Asirvatham et al., 2008). The 3'UTR of *CIITA* contains target sites for two miRs, miR-145 and miR-198 (Asirvatham et al., 2008). miR-145 appears to play a role in repressing translation of CIITA, and miR-198's role is unclear (Asirvatham et al., 2008).

XIV. Functional differences of CIITA Isoforms

While *CIITA*'s promoters appear to function independent of one another and do not appear to engage in crosstalk (LeibundGut-Landmann et al., 2004), little is known about the functional differences between the isoforms of CIITA. CIITA isoforms translocate into the nucleus with similar efficiencies (Barbieri et al., 2002). Knockout of isoforms III and IV together result in a nearly null phenotype with a lack of positive selection of CD4 T cells and no antigen presentation from cells of non-hematopoietic and lymphoid lineages (LeibundGut-Landmann et al., 2004). In mice lacking isoform IV of CIITA, professional APCs retain MHCII expression, but in cells of non-hematopoietic lineage, including thymic epithelial cells necessary for CD4 T cell positive selection, no MHCII is present (Waldburger et al., 2003, 2001b). Despite the unique presence of the CARD domain in isoform I and a purported increased potency in activation of MHCII (Nickerson et al., 2001), this potency has not been recapitulated (Butticè et al., 2006), perhaps due to a shorter half-life for isoform I mRNA (Pai et al., 2002). Its knockout appears to have no phenotype (Zinzow-Kramer et al., 2012). Without isoform I, isoforms III and IV take its place, and this does not appear to affect CIITA's activation of MHCII and thus downstream immune responses, as well as T cell selection (Zinzow-Kramer et al., 2012).

XV. Alteration of CIITA Expression Through Disease and Infection

One tactic by which pathogens evade immune surveillance is to downregulate MHCII. This downregulation is seen in many cases of autoimmune diseases, as well as in viral and bacterial infections. CIITA, as the master regulator of MHCII transcription is a relatively common target for its downstream affect on *MHCII* transcription. In addition to

this, as more and more genome-wide association studies (GWAS) studies have been performed, a number have revealed disease associations with the *CIITA* locus.

In the autoimmune disorder of multiple sclerosis (MS), it has been established that IFNγ-induced antigen presentation by astrocytes facilitates the disease process in the central nervous system (Olsson, 1994). It is therefore unsurprising that several SNPs in the *CIITA* locus have been found to be associated with MS, including one missense mutation (rs4774G/C, the functional consequences of which are not known) especially when associated with the MHCII variant HLA-DRB1*1501 (Bronson et al., 2010), as well as one polymorphism in the 3'UTR of CIITA which showed a weak association with primary progressive MS (Rasmussen et al., 2001). There is some uncertainty between studies as to whether the 168A->G promoter III variation (which results in differential MHC molecule expression) is associated with MS susceptibility (Swanberg et al., 2005) or not (Bronson et al., 2010). Looking in mouse models of MS, overexpression of MHCII molecules is also associated with the acquisition of autoimmune disease, particularly in the case of experimental autoimmune encephalitis (EAE) (Massa et al., 1987). Hyperexpression of MHCII in astrocytes via *CIITA* promoter IV was found to make mice more susceptible to EAE (Nikcevich et al., 1999). This effect is not restricted to astrocytes, as CD11c+ dendritic cells upregulate *CIITA* via promoters I and IV in mice with EAE (Suter et al., 2000). Studies of another experimentally induced autoimmune disorder in mice, experimental autoimmune myocarditis (EAM, a model of the human T cell-mediated autoimmune disease myocarditis), showed the damaging effects of *CIITA* upregulation by knocking down *CIITA* expression to reduce morbidity and mortality associated with EAM (Cai et al., 2005).

Conflicting evidence for an association between rheumatoid arthritis (RA) and *CIITA* exist. The earliest association between RA and *CIITA* with the same SNP found to

be associated with MS, 168A/G (Swanberg et al., 2005), conflicted with an earlier study showing no associations (Sartoris et al., 2000). Subsequent work shows no association between *CIITA* and RA (Bronson et al., 2011a; Ronninger et al., 2012). Yet one study maintains that it is a population dependent effect of two different SNPs, which show association with developing RA (Eike et al., 2012).

Other diseases found to be associated with SNPs in *CIITA* (or the *CIITA* locus) include systemic lupus erythematosus (Bronson et al., 2011b), oral lichen planus (Wu et al., 2013), celiac disease (Dubois et al., 2010), autoimmune Addison's disease (Skinningsrud et al., 2008), and an increased susceptibility to myocardial infarction (Swanberg et al., 2005). Insulin-dependent diabetes mellitus and myasthenia gravis were both found at one point to be associated with SNPs at the *CIITA* locus, but these associations have subsequently been disproved (Ramanujam et al., 2010; Sartoris et al., 2000).

Figure 7 below describes a number of infectious agents' effects on *CIITA* expression. As previously discussed, downregulation of *CIITA* is a common mechanism used to avoid immune surveillance, and as a result, a number of pathogens have a variety of mechanisms through which this is accomplished. This inhibition is typically targeted to the IFNγ inducible promoter IV.

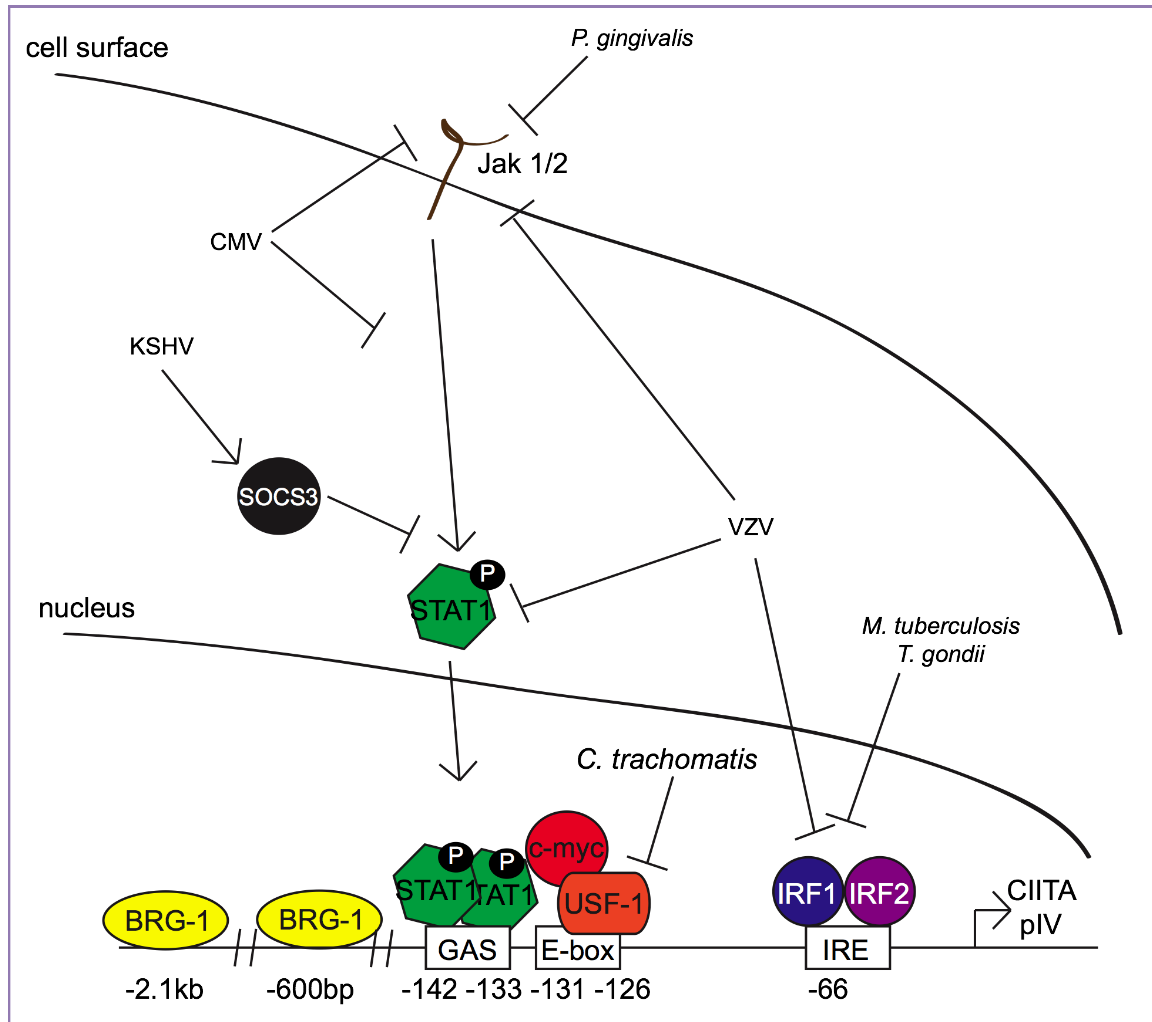

Figure 7 – Mechanisms of alteration of *CIITA* expression from promoter IV by pathogenic agents. *CIITA* promoter IV and the Jak/STAT pathway leading to its activation are shown. Lines indicate modes of influence by the indicated pathogens.

Beginning at the first step in the signaling cascade, *Porphyromonas gingivalis* (cause of adult periodontitis) disrupts IFNγ signaling transduction at the level of Jak1 and Jak2 (Srisatjaluk et al., 2002). Along these same lines, Cytomegalovirus (CMV) targets Jak1 for degradation (Miller and Rahill, 1998), and Varicella-Zoster Virus (VZV) inhibits Jak2 at the level of transcription (Abendroth et al., 2000). In the next step down the signaling pathway,

STAT1α transcription is inhibited by VZV gene products (Abendroth et al., 2000). STAT1α is also a target for inhibition by Kaposi's sarcoma-associated herpesvirus (KSHV) via a negative regulator of the JAK/STAT pathway, Suppressor of Cytokine Signaling 3 (SOCS3), which inhibits phosphorylation of STAT1α (Butler et al., 2012). Inhibition of STAT1α phosphorylation is a common viral target, as CMV also inhibits this phosphorylation (Roy et al., 1999). Another frequent target for viral modulation of CIITA from promoter IV is IRF-1. VZV inhibits IRF-1 transcription (Abendroth et al., 2000). *Toxoplasma gondii* inhibits of IRF-1 binding to the CIITA promoter (Schneider et al., 2013), as does *Mycobacterium tuberculosis* (Pai et al., 2003). Another transcription factor targeted to cause inhibition of CIITA expression from promoter IV is USF-1, which is targeted for degradation by *Chlamydia trachomatis* (Zhong et al., 1999).

Promoters beyond IV are also targets for pathogenic inhibition. In the case of the Epstein-Barr Virus (EBV), a virally encoded protein Zta binds to Zta-response elements located within promoter III resulting in downregulation of CIITA (Li et al., 2009). KSHV encodes a protein with homology to the IRFs, one of which, vIRF3, inhibits CIITA expression from both promoters III and IV (Schmidt et al., 2011). A second KSHV-encoded protein, LANA, also suppresses expression from promoters III and IV through sequestration of IRF4 (Cai et al., 2013). The bacteria *Mycobacterium bovis* initially showed the ability to reduce CIITA and MHCII levels in macrophages (Wojciechowski et al., 1999). Later the mechanism was determined to be through the actions of KLF4 recruitment to the CIITA promoter, allowing epigenetic modification by EZH2, as well as KLF4-mediated upregulation of the miRNA miR150, which targets the 3'UTR of CIITA (Ghorpade et al., 2013).

Beyond alterations of *CIITA* at the level of transcriptional regulation, two viruses have found ways to modulate the action of CIITA at the protein level. In infections with the Human Immunodeficiency Virus (HIV), the viral protein Tat squelches CIITA's activity by out-competing it for binding to Cyclin T1 to reduce CIITA's ability to transactivate *MHCII* expression (Kanazawa et al., 2000). The adenoviral protein E1A binds the N-terminus of CIITA, blocking its ability to bind CBP, and again reducing CIITA's ability to transactivate *MHCII* expression (Kretsovali and Agalioti, 1998).

XVI. Alteration of CIITA Expression in Cancers and Cancer Treatments

In addition to pathogens, cancers also have reason to attempt immune system evasion. As a result, CIITA dysregulation is seen in many different types of cancer in order to alter MHCII expression. It is because of this that artificial upregulation of CIITA has been explored as a therapeutic treatment for some cancers.

Early attempts at gene therapy showed that achieving a low level of CIITA expression in tumors to induce tumor immunity may be beneficial (Martin et al., 1999). Turning to a tumor vaccine model, several groups have had some success in mouse models with immunization of animals with *CIITA*-transfected tumor cells either on their own or loaded onto dendritic cells to induce immunity in the form of memory or in the form of T cell activation (Fan et al., 2013; Frangione et al., 2010).

A general silencing of *CIITA* has been shown to occur in several different cancer types at the transcriptional level. Small cell lung cancer (SCLS) cells show strongly reduced levels of MHCII expression due to a lack of *CIITA*, even with the addition of IFNγ (Yazawa et al., 1999). The cause of the silencing of *CIITA* in this case was postulated to be through DNA methylation (van den Elsen et al., 2003). In a screen of developmental tumor cell lines,

many were shown to have no IFNγ-inducible *CIITA*, and that treatment with the demethylating agent 5-azacytidine restored *CIITA* expression, further supporting the idea of *CIITA* silencing in some cancers through DNA methylation (van der Stoep et al., 2002a). A mouse fibrosarcoma cell line demonstrated a defect in transcription initiation of *CIITA* (Naves et al., 2002).

At the protein level, a mouse adenocarcinoma cell line expressed *CIITA* transcript, but no protein, therefore displaying a defect in either translation, nuclear export, or protein stability (Naves et al., 2002). In human adenocarcinoma cells, IFNγ stimulation revealed expression of a CIITA variant missing exon 7 which produced lower levels of MHCII molecules versus the wild-type CIITA protein.

A general alteration in the regulation of promoter I of *CIITA* is observed in cancer patients, whose dendritic cells express less MHCII (Choi et al., 2009). Tumor-conditioned media elucidated the role for IL-10 in its inhibition of histone acetylation at promoter I, most likely through a blockade of STAT5 binding (Choi et al., 2009), which normally would interact with p300 to facilitate transcription from the promoter (Pfitzner et al., 1998).

More specific instances of alterations in promoter III expression of *CIITA* are seen in patients with melanomas and to some extent in breast cancer or gliomas (Deffrennes et al., 2001a; Shi et al., 2006; Soos et al., 2001). In contrast to previously discussed cancers in which *CIITA* is downregulated, in melanomas constitutive expression of MHCII is associated with rapid progression of the cancer (Baton et al., 2004). This constitutive expression of MHCII is due to *CIITA* expression from promoter III, and the expression of *CIITA* in these cancers is dependent upon a 1-kb enhancer element about 5kb upstream from promoter III, mediated by an unknown trans-acting factor (Deffrennes et al., 2001b), as well as the promoter III upstream regulatory region (PURR) (van der Stoep et al., 2007).

Malignancy in glioma cells is associated with an upregulation of CIITA expression from not only promoter III, but promoter IV as well (Soos et al., 2001). In contrast, highly metastatic breast cancer cell lines display a downregulation of CIITA expression from promoters III and IV (Shi et al., 2006). In these cases, silencing of CIITA can be reversed by treatment with 5-azacytidine, which coupled with IFNγ treatment allows for normal transcription factor occupancy of the promoters (Shi et al., 2006).

Methylation of promoter IV is a relatively common means of silencing MHCII expression in cancers, and is seen in uveal melanoma, some neuroblastomas, colorectal and gastric cancer cells, and many T-cell and myeloid leukemia cell lines, as well as in acute myeloid leukemia (Croce et al., 2003; Morimoto et al., 2004; Radosevich et al., 2007; Satoh et al., 2004). In addition to methylation at promoter IV, some neuroblastomas appear to have secondary mechanisms through which CIITA is repressed, proposed to be a post-transcriptional defect in CIITA translocation (Croce et al., 2003). Still other cancer types, such as squamous cell carcinoma, silence promoter IV through unknown methylation-independent mechanisms (Kanaseki et al., 2003), while in breast cancer cells, EZH2 recruitment to promoter IV results in an increase in histone K27 trimethylation and a decrease in transcription factor recruitment (Truax et al., 2012).

XVII. Summary

It can therefore be seen that as a result of CIITA's key role in regulating MHCII expression and thus antigen presentation it is a valuable target for dysregulation by pathogens and cancers in myriad ways in order to escape the immune system's typical surveillance. By gaining a clear picture of *CIITA* regulation, these mechanisms can be better understood, providing more ways in which to counter pathogenic or cancerous evasion of

the immune system. In pursuing a further understanding of the regulation of *CIITA*, insight can be gained into the fundamental question of promoter choice, as well as the more basic model of gene expression and silencing. More generally, understanding the regulation of *CIITA* gives a better understanding of the control of antigen presentation in the immune system.

With this background, the goals of this dissertation were to define the regulatory architecture of *CIITA* in B cells, determine if this architecture is shared across hematopoietic lineages to answer the question of whether or not 3-D chromatin structures are critical in regulating *CIITA* in professional APCs, and finally to begin to understand the mechanisms by which promoter choice is made at the *CIITA* locus. A novel set of *cis*-regulatory elements are defined for the lymphoid promoter in Chapter 3, and a subset are shown to not only interact with pIII, but also interact with the active promoter I in myeloid cells. Chapter 4 provides a glimpse into a potential mechanism of *CIITA* promoter choice, showing that DNA methylation plays potentially both a direct and indirect role in guiding promoter choice in B cells. Chapter 5 integrates the results presented in these studies and shows how these data can be combined with current knowledge of *CIITA* regulation to begin to further explain the complex role of regulatory regions in the *CIITA* locus and how promoter choice is made in APCs.

Chapter 2: Materials and Methods

This chapter was written by S. Lohsen with B. G. Barwick contributing the bisulfite sequencing 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 (Zinzow-Kramer et al., 2012). Briefly, 30 mg 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 (DMEM) (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 mg/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 mM 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.

Peritoneal macrophages were collected four days after intraperitoneal injection of 2.5ml of a 3% solution of thioglycolate medium (Sigma-Aldritch). Peritoneal cells were plated at approximately $1x10^6$ cells/ml in DMEM with previously described supplements. After allowing the cells to adhere for 2 hours, non-adherent cells were washed off, and the adherent macrophages were supplied with fresh media.

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 (Oestreich et al., 2008). 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/μl, 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 (Yoon and Boss, 2010). 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 (Majumder et al., 2008; Tolhuis et al., 2002). For primary splenic B cells and dendritic cells, 1 x 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 (Majumder et al., 2008). 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 mg 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, macrophages, A20 cells 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 (Scharer et al., 2013). 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)

ChIP was performed as previously described (Beresford and Boss, 2001). 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.) 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.

Demethylation Treatments

5-Azacytidine (Sigma-Aldritch) dissolved in RPMI or Decitabine (5-Aza-2'-deoxycytidine, Sigma-Aldritch) dissolved in DMSO at a concentration of 50mg/ml was used to treat A20 and primary splenic B cells at the indicated concentrations every 8 hours. After 72 hours, cells were collected. For RNA collection, cells were pelleted and lysed directly with lysis buffer. For genomic DNA, cells were pelleted and washed in PBS before snap freezing and DNA isolation.

Table 1: List of Primers and ENCODE accession numbers used. Primer sequences for conventional and qRT-PCR, cloning, 3C, and bisulfite sequencing as well as ENCODE accession data. (This table is referred to as "Supplemental Table 1" in this dissertation)

Conventional PCR Dnase I Hypersensitivity Primers

-70.7 Forward	GAGCCAGGAAAATAGGATTGCC
-70.7 Reverse	GTGGGATAACAGACCTGAAAGG
-69.3 Forward	GCTGGCATGATCACAATCTG
-69.3 Reverse	GTCGCCAACGAACGTTATTC
-67.5 Forward	GTAACAGAATCTAGGGTAGGGC
-67.5 Reverse	CGGTCACTACCAACCTGAAAAG
-65.9 Forward	CTGGGTTTGGTTGTTGAGTC
-65.9 Reverse	CTGGACTGCATACTGAAAGG
-64.3 Forward	CTTAGCTGTGTAACACCAGC
-64.3 Reverse	AGATCTTGTGGGGACTTAGG
-62.6 Forward	CTTGATTGGTGATCTGGTGG
-62.6 Reverse	GTGAGCTTCCTATTTGCTGG
-60.9 Forward	ATGATCCTCATGGGAGAGAG
-60.9 Reverse	ACCCACACTTGCTCTTTGCA
-59.1 Forward	TGTTGACTTGTTGCTGAGGC
-59.1 Reverse	CAGTCCTAGGGACACATAAC
-57.4 Forward	TGTGTGCACAGCCATCTTAG
-57.4 Reverse	CCCTAAGGGATAGGTTCAAG
-55.3 Forward	TCTGCATCCAGGTGCCATTCAT
-55.3 Reverse	TGATTGCTGGCATTTCCCTCCA
-54.0 Forward	GGAGCTAGTGTCTAAAGGAC
-54.0 Reverse	GTCACTCTAGCAGCTTGATG
-52.2 Forward	ATGGTGAATGTACCATGGGG
-52.2 Reverse	TGTGACTGGTTCAGCAAGAG
-50.5 Forward	AGAGGAAGCCTTACCTTCAC
-50.5 Reverse	CTTGAAATGGTAGGGACACC
-48.8 Forward	AGAGTCAGAGTCACATCACC
-48.8 Reverse	GTGAGAGGGGAATTTCACTG
-46.9 Forward	GAAACAGCTGTTACCTCAGG
-46.9 Reverse	AGCTGATGCCTTTAGTTGGG
-45.0 Forward	TGTTTGGTCCTTTGTTCTGCCC
-45.0 Reverse	GGCGCCCAATCAATGGGTTATT
-43.1 Forward	CACTGTTTGCTGGGCTTAG
-43.1 Reverse	CATGGAAAAGAGCTTGGCTG
-41.6 Forward	AACATAGACATCTCCCCCAG
-41.6 Reverse	CTATCTGGGTGTTTAGCCAC
-36.0 Forward	GGAGTTTCCTACAGCAAACTGC
-36.0 Reverse	GTCAAGACTGAAGGGTGAAG
-35.0 Forward	GATTATACCTCCGAGGTGTC
-35.0 Reverse	TGTAGGAGAGGAGTCAGTCTG
-32.6 Forward	CAGGCCTCTAAGTATGCTAG
-32.6 Reverse	CACTTGCTTGTGCATATGCC
-31.0 Forward	CTGCACACACCACTCTTTAG
-31.0 Reverse	CAAAGGCCTGGAATAGACTC

-29.5 Forward	CCTGTGACTAAGTCACCTGTTG
-29.5 Reverse	CAGGAGGAAAGAGCATTGCT
-28.0 Forward	TGGGACCTGTGCAAATTCTG
-28.0 Reverse	CAGTAGTGCTTCTGTGTTGC
-26.6 Forward	GCACCCACTGACAAGAAAGG
-26.6 Reverse	TCAGGACCAGGACCAGAGG
-25.1 Forward	CCTGGCTTTGGAGGCTTT
-25.1 Reverse	GGCTAGCCTGGCTTACACAT
-20.4 Forward	TGGACACCTTGGTTTATTTATC
-20.4 Reverse	ACACTGGTCCCTCTGGACAC
-18.8 Forward	ATCCAGGACTTCTTAGCACTC
-18.8 Reverse	GCCCACTCCCACTTCTTG
-16.2 Forward	TGAACTGTTTCTGCCCTTGT
-16.2 Reverse	CCAAGGATAAACGAAGCTCTG
-15.6 Forward	GCTCACCTCAGCAGTCTTGG
-15.6 Reverse	GCAGAAGTTCCTTGCTCCAG
-14.0 Forward	GGACTGGATTAACCAAAAGGAAG
-14.0 Reverse	CCTAAGGGTTGGTGAGATGG
-11.9 Forward	TCACTGAGCCATCTCACCAAC
-11.9 Reverse	CAGTGAAGGACATCTGTGCTG
-10.1 Forward	ATGTGTTTCAGGTGACTGCC
-10.1 Reverse	TCCAAATCCGAGCATAGCAG
-8.9 Forward	TGTGGTCTCAAAAGCCACTG
-8.9 Reverse	CCACAGGGATTTCCAGTCAT
-7.7 Forward	GCAAACTGAATTCTGGGTAAATG
-7.7 Reverse	GTGAGGTGAGCTTCGGAGAC
-6.0 Forward	CAGCTACTGTCTGAAGCTTG
-6.0 Reverse	TCATTCTCTGGGAGCTACTTCA
-4.5 Forward	AAAATGCGCTTCAAGTGGAC
-4.5 Reverse	CCCCTGTGTGTGTCTCTCTG
-3.1 Forward	GGGGGAGGCTCAGAGTTCTA
-3.1 Reverse	CCTGCACGGAAGCTTGTAGT
-1.8 Forward	TACAAACCCACAGCTGGACCAT
-1.8 Reverse	TGCATGTGAAAGTCCTTCCCGA
0.0 Forward	GAGGCGACTCTGCAATTTAC
0.0 Reverse	CCTGTCTTCCCAATAGATGG
+1.6 Forward	CCTGCTTCCAACAGACACAC
+1.6 Reverse	ATACAAGGTCTGGGGTGCAG
+3.2 Forward	GATTTCCATCAGGGTGCAAG
+3.2 Reverse	TGGCTGGGAAAATTATCTGG
+4.2 Forward	TGGAAGTGGGTAGGCTTCAG
+4.2 Reverse	AAATCCAGTGTTGGCTGCTC
+6.4 Forward	CATCTTCCCAATTCCCAAATAGG
+6.4 Reverse	GGCAACACTGACTCTTCTCC
+8.0 Forward	TTTTCTGTATACCACCCACAC
+8.0 Reverse	CCGCAGGCTTGCTTATAGTC

+8.8 Forward	ATAAGAAGCTGTGGTGGCTAAGGG
+8.8 Reverse	CAACCCAAGTTTGCTTGGACTGAG
+12.0 Forward	GTGGAAACTGAGCCCAGAGA
+12.0 Reverse	TGGCTCAAATATGCTTATCTTACA
+13.6 Forward	TGTGTAGGGTAGAGGGCCTAAA
+13.6 Reverse	GGCACTGCTTTCCTGTCATT
+14.9 Forward	GGAAGGGGTTGGGATCTAGT
+14.9 Reverse	TCCAGATCAGTGCTCACCTCT
+16.6 Forward	GCTCAGTGGGTGTTTCCATT
+16.6 Reverse	AAGGCAGCAGCTGAGAAGAC
+18.0 Forward	TGTGCATGCAGATTCAAGAG
+18.0 Reverse	TGGTTTCTTCAGCTCCTGGT
+19.5 Forward	CATCACACAGGCAAAGCAGT
+19.5 Reverse	TGCTGTGAATGGAAGTGCTC
+20.8 Forward	CCCGGCTTTGACAACTTACA
+20.8 Reverse	GGCAAAGGCAGAGAGTGAAC
+24.0 Forward	GAAATTGAGCAGTTTGACAAGG
+24.0 Reverse	TATTACAGGGCTTGGGGATG
+25.3 Forward	CACCCTTCAAAGCCATTAGC
+25.3 Reverse	GCTTCCTGTGCTTTGAGTCC
+26.8 Forward	CCCAGAGCAGTTGGCTCTAATAGT
+26.8 Reverse	GGACACTCAAGTCTCCAGTCTCTT
+28.4 Forward	TCAAACACAAAGCAAAGCAAA
+28.4 Reverse	GTACAAGAAGGCTGCCCAAG
+29.7 Forward	ACTGGGGCAGAGAGTGTGAC
+29.7 Reverse	ATAGCTCACAGCTGCCCATT
+31.3 Forward	CTCCCCTCTCAAAACCCATA
+31.3 Reverse	GTGTGCAACATGCTCCCATA
+32.7 Forward	AGACAAGGAGCTGCCACAGT
+32.7 Reverse	TCTGCACCTTGGCTCTGTTA
+34.2 Forward	GAGGCAAGGGGTCAGAGTTA
+34.2 Reverse	TCCTGCTACCCTGGCTACAC
+35.6 Forward	TCAGAGCAGCAGAGAATTGG
+35.6 Reverse	AAGCCAGCTCTCGAGCCTA
+37.2 Forward	GGGGGCTATTTTAGGGTGTC
+37.2 Reverse	CTGTCTGCCCTTTGTGGTTT
+38.5 Forward	CACACTGGCAAAGATCCTTC
+38.5 Reverse	GACAACTTCCACCGCTCAGT
+45.9 Forward	CCTGCCAGATAGGATGTGGT
+45.9 Reverse	GGGCTCCTCACTTTCTCCTC
+47.5 Forward	CTGTCTGGGAGCCCTGAGT
+47.5 Reverse	CCTGCATTGAGCTGTGTGTT
+48.8 Forward	AGTTGAAGCCAATGCCTGG
+48.8 Reverse	CTACAACAGGTGTGAGTGCTTG
+50.5 Forward	CGGGGCTACAACCGTATTCT
+50.5 Reverse	AGCAGGAAATGACAGGCAAT

+51.8 Forward	CCCTGCTCTCACCCTTAGGT
+51.8 Reverse	GGGAAGCAGCTTACTGTGGA
+53.5 Forward	CTCTGTCCGTTTTCCAGAGC
+53.5 Reverse	CGACCACCTTCACCTTCTTT
+54.8 Forward	CGCACCTTGACCTAGGTTTC
+54.8 Reverse	GGTCACTTGGAGGGAAACTG
+56.3 Forward	CAGTTTCCCTCCAAGTGACC
+56.3 Reverse	CCTTAGAGTTTTAGTGCTCCTTGG
+57.7 Forward	CCAAGGAGCACTAAAACTCTAAGG
+57.7 Reverse	AGCCTGCAGTGTCAACAGAA
+59.1 Forward	GGCCTGGAGACCTTTTCTTC
+59.1 Reverse	GCAGAAACATTCCTGCTTCC
+60.6 Forward	CTCTGGGACTTGCCTTTCTG
+60.6 Reverse	AACAAGGGAAAGGGAACAGC
+62.3 Forward	AGCATTCCCCAGCTATAGGG
+62.3 Reverse	CAATAAATACGGGCGAGCAT
+63.8 Forward	ACTCGCTTTGTACGCTGC
+63.8 Reverse	CATTCTGGTTTGTCCTTACAACCTT
+65.1 Forward	AATCTTGCTTCCCACCGAGGTT
+65.1 Reverse	TCAAGACAACTCCAGCCCACAA
+66.5 Forward	TGGACCACCTGAAGTGAGTG
+66.5 Reverse	ACATGCAGCCCAGTCAAAAG
+68.0 Forward	TTGGGACTGTAGCCCCTTTA
+68.0 Reverse	AACTAAGCACCCGCTCTCTG
+69.5 Forward	TTGATGGTTCACTGCCAGAC
+69.5 Reverse	CACCCCAAAGACATTAAACCA
+71.1 Forward	GAGCTGTGGCGTTTTCTAGG
+71.1 Reverse	AGACCAAACCAAACTTTGTGC
+72.4 Forward	TGCTCTCATAAGTCCATGCAA
+72.4 Reverse	AGAGATCCTTGCTTACGTTAAGTT
+73.8 Forward	AAGACATGGGAGGCAACATC
+73.8 Reverse	CCAAAGAAAGCACAAAGAAACA
+75.6 Forward	TCCTGGGAGCTCTTAGCATC
+75.6 Reverse	TCCCTTCTGGAGAGGCAGTA
+76.9 Forward	CCTCCCCTTCTCTCCTCCT
+76.9 Reverse	CAGGTCAGGGAGAAGCAGTC
+78.5 Forward	GCAGCAAGGGCAGTCTATTC
+78.5 Reverse	GATCAAAAGGAGGTGGCTCA
+80.1 Forward	GGAAAAAGGAAAAAGAAAACTGG
+80.1 Reverse	GCTGGCCCTCAGTAAATACG
+81.5 Forward	TTTACTGAGGGCCAGCAAGAGT
+81.5 Reverse	AAGTCTGCTGGTTGACTCCGTT
+83.3 Forward	ACCTCTGCTGTCATTCAGCATC
+83.3 Reverse	CTTCTTGGCCTTCTAAATCTTAAGCC
+84.4 Forward	CCTTCTGCTGCTTCAACTCACAGT
+84.4 Reverse	AGCTGCACATGTCGTTTCCAGT

+85.9 Forward	AACGACATGTGCAGCTTTTG
+85.9 Reverse	AAGCACCTAGATGCCCACAG
+87.2 Forward	GCAGACACACACCTTTGGCTTT
+87.2 Reverse	TCAGGTTAACCACCTCAGGTCCTA
+88.7 Forward	CCAAGAGCTGCCTCAGGTAT
+88.7 Reverse	CGCAGCTGCCATTTGTACT
+90.2 Forward	TCTGCTCCTTGCAGTGTGAC
+90.2 Reverse	AGGGAGGCAGAGGGTCTTAG

Real Time DNase I Hypersensitivity Primers

Y6 Forward	TAGCACATAACAGCGGTAGATTAC
Y6 Reverse	CCCAAGTTTGAGACAAGCAGAC
-47 Forward	TGACCCCTTGGCTGCATCTC
-47 Reverse	ACGTAGGTGGGTCTCGGGCT
-15 Forward	GGTCAAGGGCACACACTGCCAT
-15 Reverse	CCCAGCAGGTGGGAAGGCTCA
-3 Forward	ACGTGGGGCCAGAGACCACT
-3 Reverse	GCCCCACCATGGCCTTGACA
+3 Forward	ATTAGGTTGGCCGCTCCTGTAT
+3 Reverse	AACACAGGCATTGTGCTCCTCT
+5 Forward	CAGCGTCATCCTTCTGGGCGC
+5 Reverse	TGTGCTGTTACCTGGTCTCCCCA
+10 Forward	ACTGGCTCAAATCTGTCGTCCT
+10 Reverse	TGCTGTCTGACTTCGGGCAAAT
+11 Forward	GGTTTTCCTCCTCCGTAGCCTGGT
+11 Reverse	CCCGTCATCAACGGCCTCCCA
+15 Forward	GCCTCTGCTCCACTGGCTGC
+15 Reverse	GCCTCCTCCACCTGGGCTCT
+22.5 Forward	AGCCAATGAGCCGCCATGCA
+22.5 Reverse	GGGTGTTAGCCAGAGGTGGGC
+36 Forward	AGAGGCTGCGGGGTGGAACA
+36 Reverse	GGGAACATGGGGGTGGCTGC
+37 Forward	ACCTTGGCTGGTGTGGGGACT
+37 Reverse	GGACCACCCAGAGGCCTGCT
+44 Forward	AGTTCAACAAGTTCACGGCTGC
+44 Reverse	TTGAGTGCTTTGTGTGGCAACG
+47 Forward	AACCCAGAAGTCAGGCTGGAAT
+47 Reverse	TGTAGCCAAGGCTGACCAATGT

RT-PCR Primers

m18srRNA(FF)	GTAACCCGTTGAACCCCATT
m18srRNA(RR)	CCATCCAATCGGTAGTAGCG
mCIITA Ia Fwd	AAGAGCTGCTCTCACGGGAAT
mCIITA IIIa Fwd	TCTTACCTGCCGGAGTT
mCIITA Iva Fwd	GAGACTGCATGCAGGCAGCA
mCIITA Reva	GGTCGGCATCACTGTTAAGGA

IRF8 For	GAGCGAAGTTCCTGAGATGG
IRF8 Rev	TGGGCTCCTCTTGGTCATAC
Stat1 For	ACAACATGCTGGTGACAGAGCC
Stat1 Rev	TGAAAACTGCCAACTCAACACCTC
IRF4 FF	GCAGCTCACTTTGGATGACA
IRF4 RR	CCAAACGTCACAGGACATTG
PU.1 FF	ATCTGACCAACCTGGAGCTC
PU.1 RR	GTTGGTATAGCTCTGAATCG

3C Primers

promoter III anchor (H)	GTGCTGTACTTCTAGGAGACC
H1	CGGGGTTAGCCTCTTAAATGC
H2	AATCTGAGTAAGTCTGTCTGTTATG
H3	GGTGGACAATATGGCTCAGG
H4	CCATCCCCAGTGCGTTCC
H5	ACACTAATGGGCATACCTCAC
H6	TAGCATCTGGCCCAAATGTG
H7	CCTACATAAGCAGTGTGAATCC
H8	CCTCTGTGGACCTGTCATC
H9	ATTGTCGATGGTGCTCAGG
H10	TAGACCCATTCCCACAAGAAC
H11	TAGATACACCGCAGGCTTG
H12	TAGGCTTTGTAGTGAGACTCTG
H13	CCCAACTCACTAGAGGAAGG
H14	CCTTCCACGACCACATCAG
H15	TCTGCCCTTTGTGGTTTCG
H16	CTCACTCACCTCAGCCTTAG
H17	TTGGAGCTTGCGAGATGG
H18	ACTTTGCTCTCTGGCTTTATTC
H19	ATAGTAGTGTTTGAAGAGATTTCCC
H20	CTGCTGCTCGCTCATATTTAAG
promoter III anchor (E)	GGTGTAGATGAGGTGGCTTAC
E1	ATGTTGGTGAGACTGAGTGTAG
E2	CTGGAGATATTGTGCTTCTGACC
E3	CTGCTGACAGAAGTGTTGGG
E4	GGTTACCAAGGATAAACGAAGC
E5	CTTGCTTGATTGATTGATGATTGG
E6	CCCTTTGTCCCTCTCTTTGC
E7	AGAAACTTGCTTTGGACTTTGG
E8	CAGGAGGAGAGGACGACAG
E9	TCTGTTAAATAAGAGAGTTGTAGTAGG
E10	TTTCCTTCCTCCCTCCTTTCC
E11	GAGAGGCACAGGGAGTCC
E12	TGCTTTATCATCACTATGTTCTCAG
E13	TGAAAGAAAGGCTGAGAACTCC
E14	AGGATTCCATTCAGGTGATGTG

E15	AGGTGCTACTGCTGACTATCC
E16	GGGCAGAGGCAGGAAAGC
promoter I anchor (H)	GGCAACAATGTATGACCAACC
KO-promoter I anchor (H)	TACTGAGTTCTGCTGCTTATAGG

CTCF Real Time Screening Primers

H19 CTCF FWD	ACCTCAGTCGCCAAATGGTTGT
H19 CTCF REV	AGGCAAACTGCACCTCCAAACT
CIITA+23 CTCF FWD	TTCTAACGTTGCTGCCCACCT
CIITA+23 CTCF REV	TGCCACTGGGCTGAGTTCAAAT
CIITA+38 CTCF FWD	ATGGTGTGTGGCCAGCTGAAT
CIITA+38 CTCF REV	AGGTGGGATCAAATGCAGCCAT
CIITA+40 CTCF FWD	GCTGCTCTTTGTGGCACATCACC
CIITA+40 CTCF REV	CAGCCCAGAAGCCAGCACTGC
CIITA+43 CTCF FWD	AGAACACTGGTTGCTCTTGCAG
CIITA+43 CTCF REV	TGGATGGCTGTGCTTCATAGGT
CIITA.control FWD	AGCGATGTTTGTTGTCCAGCAC
CIITA.control REV	AGCTGTGCCTTCCCTCTTTCT

ChIP Primers

-22 ChIP Forward	GATGCTATCTTAGGAAGTGATGG
-22 ChIP Reverse	GAGAGTGAGACAGGAAGTAGG
-15 ChIP Forward	GCCTTCCCACCTGCTGGGGA
-15 ChIP Reverse	TCGTCTCTCTGTGGCTCCTGCC
-8.2 ChIP Forward	GGTCAAGGGCACACACTGCCAT
-8.2 ChIP Reverse	CCCAGCAGGTGGGAAGGCTCA
HSS1 ChIP Forward	ACGTGGGGCCAGAGACCACT
HSS1 ChIP Reverse	GCCCCACCATGGCCTTGACA
pI ChIP Forward	TCTTATTGCTGTCCAAGTCACC
pI ChIP Reverse	GTTGTCAGTGTAGCCTTCTCC
+5 ChIP Forward	CAGCGTCATCCTTCTGGGCGC
+5 ChIP Reverse	TGTGCTGTTACCTGGTCTCCCCA
pIII ChIP Forward	CCACAGCCGCGGTAGGTGTC
pIII ChIP Reverse	TCCGGCCCTGCTGTCTGACT
pIV ChIP Forward	GGATCTTGGACGGACTGTATGC
pIV ChIP Reverse	CGCCACTTGCCTTCACTACC
+11 ChIP Forward	GGTTTTCCTCCTCCGTAGCCTGGT
+11 ChIP Reverse	CCCGTCATCAACGGCCTCCCA
+15 ChIP Forward	GCCTCTGCTCCACTGGCTGC
+15 ChIP Reverse	GCCTCCTCCACCTGGGCTCT
+36 ChIP Forward	AACAAGACCCAGCCCCCGGT
+36 ChIP Reverse	AGGAAAGTGAGGGCCAGCGTG
+36.5 ChIP Forward	GCAGCACCTGGAGTAAGC
+36.5 ChIP Reverse	GCATAGAGTTAGTATTGAGATAAAGAAC
+37 ChIP Forward	ACCTTGGCTGGTGTGGGGACT
+37 ChIP Reverse	GGACCACCCAGAGGCCTGCT

+42 ChIP Forward	TAGGAAGTAGACTGGATAGGAAG
+42 ChIP Reverse	CCCAAAGCATGGAAATTATAAGG

Luciferase Reporter Cloning Primers

-15 Luciferase Forward	GACTCGAGCACACTGCCATAGAGAAG
-15 Luciferase Reverse	GACTCGAGCATTAGGATTCCTCCACTG
-8.4 Luciferase Forward	GACTCGAGGAATAAACTCACCTCTTCTCT
-8.4 Luciferase Reverse	GACTCGAGTTCTCCCTTTCCTCCTAC
-8.2 Luciferase Forward	GACTCGAGTGACTGCTGAACTCTTTAC
-8.2 Luciferase Reverse	GACTCGAGATGGCTGTAACTCAACAC
-8.0 Luciferase Forward	GACTCGAGCCAGGTGTTGAGTTACAG
-8.0 Luciferase Reverse	GACTCGAGGGGATTCAAGTCTGCTATT
-4 Luciferase Forward	GACTCGAGGTTCCAGTCTCACAGTGGGG
-4 Luciferase Reverse	GACTCGAGCCTGGGATCTGCCTGTCTCT
+12 Luciferase Forward	GACTCGAGACCATTGCTACCTCCCTTT
+12 Luciferase Reverse	GACTCGAGTCTCTGCGATTGTGTTCAGG
+16 Luciferase Forward	GACTCGAGGGTGTCACAGTTGGGTGGAG
+16 Luciferase Reverse	GACTCGAGTCGGAATTCCTGTGCTGGTC
+19 Luciferase Forward	GACTCGAGTCTTGTGATCACCTGAAGCCC
+19 Luciferase Reverse	GACTCGAGGTCCAACAGCTCATGGAAGGT
+35 Luciferase Forward	GACTCGAGACACTCGCTGCCAGCTTC
+35 Luciferase Reverse	GACTCGAGGGTACCCAGAGTGCAGTTCC
+36 Luciferase Forward	GACTCGAGCCCGCTTTCTCCTGAAACAA
+36 Luciferase Reverse	GACTCGAGTGAGACCGGCATGGTAGAA
+37 Luciferase Forward	GACTCGAGTCATGCAATCTGGTGTGACC
+37 Luciferase Reverse	GACTCGAGCCCTGACTCCCTAGTGTCTTAT

Bisulfite Sequencing Primers		Primer Start/End Coordinates (UCSC mm9)
CIITA.pI.bis.fwd	GTAATAATTTGTTTATATGTGATAGT	10479921 / 10479947
CIITA.pI.bis.rev	CCTTAAAATTAATCATACATTATTACC	10480545 / 10480572
CIITA.pIII.bis.fwd	GAATTAGAGTTAATATTTTGGGTGAA	10487896 / 10487922
CIITA.pIII.bis.rev	CAAAACCTACCAAACTCAACTAACATAC	10488292 / 10488321
CIITA.pIV.fwd.bis	GGGGTGTAGATGAGGTGGTTTATAAAT	10489538 / 10489565
CIITA.pIV.rev.bis	CTCCCTACTCCCAAATCCTACATACAA	10490114 / 10490141

ENCODE Data

Tissue	Assay type	DCC_Accession	Resource Provider Lab	Institution
A20	DNase-seq	wgEncodeEM001733	Stamatoyannopoulous	Univ Washington
B-cell (CD43-)	DNase-seq	wgEncodeEM001734	Stamatoyannopoulous	Univ Washington
Bone Marrow	ChIP-seq (CTCF)	wgEncodeEM001687	Ren	LICR*
Bone Marrow Derived Macrophage	ChIP-seq (CTCF)	wgEncodeEM002663	Ren	LICR*
Cerebellum	ChIP-seq (CTCF)	wgEncodeEM001689	Ren	LICR*
Cortex	ChIP-seq (CTCF)	wgEncodeEM001690	Ren	LICR*
Heart	ChIP-seq (CTCF)	wgEncodeEM001684	Ren	LICR*
Kidney	ChIP-seq (CTCF)	wgEncodeEM001685	Ren	LICR*
Liver	ChIP-seq (CTCF)	wgEncodeEM001696	Ren	LICR*
Lung	ChIP-seq (CTCF)	wgEncodeEM001697	Ren	LICR*
MEF	ChIP-seq (CTCF)	wgEncodeEM001698	Ren	LICR*
MEL	ChIP-seq (CTCF)	wgEncodeEM002661	Ren	LICR*
Olfactory Bulb	ChIP-seq (CTCF)	wgEncodeEM002585	Ren	LICR*
Small Intestine	ChIP-seq (CTCF)	wgEncodeEM002591	Ren	LICR*
Spleen	ChIP-seq (CTCF)	wgEncodeEM001700	Ren	LICR*
Testis	ChIP-seq (CTCF)	wgEncodeEM002593	Ren	LICR*
Thymus	ChIP-seq (CTCF)	wgEncodeEM002587	Ren	LICR*
CH12	ChIP-seq (CTCF)	wgEncodeEM001954	Snyder	Stanford Univ

* Ludwig Institute for Cancer Research

Chapter 3: Common distal elements orchestrate CIITA isoform-specific expression in multiple cell types

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S. Lohsen isolated and prepared primary splenic B cells, performed DNase assays, conventional and Real Time PCR and RT-PCR analyses, luciferase gene reporter, and ChIP assays.

P. Majumder performed 3C and associated PCR assays with cells isolated by S. Lohsen and C.D. Scharer.

W.M. Zinzow-Kramer and C.D. Scharer isolated and prepared splenic dendritic cells.

C.D. Scharer and P. Majumder performed the CTCF-seq, and conventional CTCF-ChIP was performed by C.D. Scharer.

B.G. Barwick, and J.W. Austin performed bisulfite sequencing and B.G. Barwick performed analysis of the bisulfite data.

This manuscript was written by S. Lohsen and J.M. Boss

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 IFNγ 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 (Collins et al., 1984; Pai et al., 2002; Steimle et al., 1994). MHC-II proteins present peptide antigens to CD4+ helper T cells (Benacerraf, 1981), 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 (Choi et al., 2011)). The MHC-II transactivator, CIITA, is required to interact with these factors and the basal transcription machinery to initiate MHC-II expression (Masternak and Reith, 2002). 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 (Silacci et al., 1994). *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 (Muhlethaler-Mottet et al., 1997). Cells of the myeloid lineage, including splenic derived dendritic cells (spDC), primarily express *CIITA* from the most distal promoter (promoter I or pI) (Muhlethaler-Mottet et al., 1997). 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 (Chin et al., 1994; Lennon et al., 1997; Muhlethaler-Mottet et al., 1997; Rigaud et al., 1996; Steimle et al., 1994). Individual roles for these isoforms are unclear, but they appear to be somewhat interchangeable (Zinzow-Kramer et al., 2012). 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 (Steimle et al., 1993). CIITA KO mice lack positive selection for CD4+ T cells, and do not respond well to immunization or pathogenic challenge (Chang et al., 1996). 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, a nuclear factor-1-binding site termed site A, site B, and site C (Ghosh et al., 1999), as well as binding sites for AP1 (Lennon et al., 1997), Sp1 (Green et al., 2006), acute myeloid leukemia (AML) 2, CREB (Holling et al., 2002), and the repressors PRDI-BF1/BLIMP-1 (Ghosh et al., 2001; Piskurich et al., 2000) and ZBTB32 (Yoon et al., 2012). 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 (van der Stoep et al., 2004).

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) (Yoon and Boss, 2010). HSS1 is located ~3 kb upstream of pI. PU.1 bound HSS1 was shown to interact directly with pIII (Yoon and Boss, 2010). 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 (Ni et al., 2008). 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 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, 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 (Majumder et al., 2008; Naumova et al., 2012; Tolhuis et al., 2002) 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 (Heinz et al., 2010) 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 (Yoon and Boss, 2010) 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 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 (Bell et al., 1999). CTCF is also known to play an important role in creating 3-D architecture involved in gene regulation (Majumder et al., 2008). Analysis of CTCF chromatin immunoprecipitation (ChIP)-seq data for B cells and plasmablasts (Majumder et al., 2014) 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 (Heintzman et al., 2007) and when coupled with H3K27Ac is indicative of active enhancers (Creyghton et al., 2010). H3K9Ac is often found at active promoters and regulatory regions (Karmodiya et al., 2012). 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 (Heinz et al., 2010; Revilla-I-Domingo et al., 2012) 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 (Yoon and Boss, 2010). 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 (Heinz et al., 2010; The ENCODE Project Consortium, 2012). 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 (Heinz et al., 2010) 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 (Zinzow-Kramer et al., 2012). SpDC from these mice expressed near wild-type levels of *CIITA* mRNA that initiated from pIII ((Zinzow-Kramer et al., 2012) 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 (Scharer et al., 2013) 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 (Heinz et al., 2010; Majumder et al., 2014; Ochiai et al., 2013; Revilla-I-Domingo et al., 2012; The ENCODE Project Consortium, 2012) 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 (The ENCODE Project Consortium, 2012)

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 (Yoon and Boss, 2010). 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 (Benjamini and Speed, 2012; Cheung et al., 2011). 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 (Austin et al., 2014; Gheldof et al., 2010; Kulaeva et al., 2012; Majumder et al., 2008). 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 (Teferedegne et al., 2006) or via the chromosomal organizing factor CTCF as shown at the human and mouse MHC-II loci (Majumder et al., 2008, 2014). 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 these interactions highlight the power of this approach. A fourth interaction with HSS1 and pIII, which was previously reported (Yoon and Boss, 2010), 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; (The ENCODE Project Consortium, 2012)]. 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 (Majumder et al., 2008, 2014) 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 (Heintzman et al., 2007), H3K27Ac (Creyghton et al., 2010), and H3K9Ac (Karmodiya et al., 2012). 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 IFNg-inducible *CIITA* expression from pIV in HeLa cells (Ni et al., 2008). The +40 CTCF binding region was also conserved in humans and participated in IFNγ-mediated *CIITA* expression through pIV (Ni et al., 2008).

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 (Chen et al., 2013; Pekowska et al., 2011; Zentner et al., 2011; Zhu et al., 2013). 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 (Yoon and Boss, 2010). 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 (Yoon and Boss, 2010) 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 (Heinzman et al., 2007) and

was also shown to play a role in *CIITA* expression from pI in dendritic cells (Smith et al., 2011). 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 (Yoon and Boss, 2010). 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 (Yoon and Boss, 2010). Additionally, PU.1 occupancy at pIII in B cells is >18 times more than pI (Yoon and Boss, 2010), which can be explained by the presence of a lower affinity PU.1 binding motif at pI versus pIII (Heinz et al., 2010). 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 (Dziembowska et al., 2002). 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.

FIGURE LEGENDS

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 (Miller et al., 2007). 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-I-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 (Yoon and Boss, 2010). 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 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 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) (Heinz et al., 2010; Revilla-I-Domingo et al., 2012) and DNase I data (CD43- and A20 B cells) (The ENCODE Project Consortium, 2012) plotted with respect to the 8 ROIs (Black), 3 *CIITA* promoters (Magenta), and 3 negative control sequences (Orange).

Figure 6. Three ROIs 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.

Supplemental Figure 1: Representative (1 of 3) medium throughput DNase I Hypersensitivity screen of BCL1 and P3X cells. 95 amplicons across the *CIITA* locus were used to screen non-repetitive regions for DNase I hypersensitivity. Murine B cell line BCL1 and murine plasma cell line P3X cells were treated with increasing concentrations of DNaseI. Conventional PCR was used to amplify 1-2 kilobase (kb) amplicons.

Figure 1

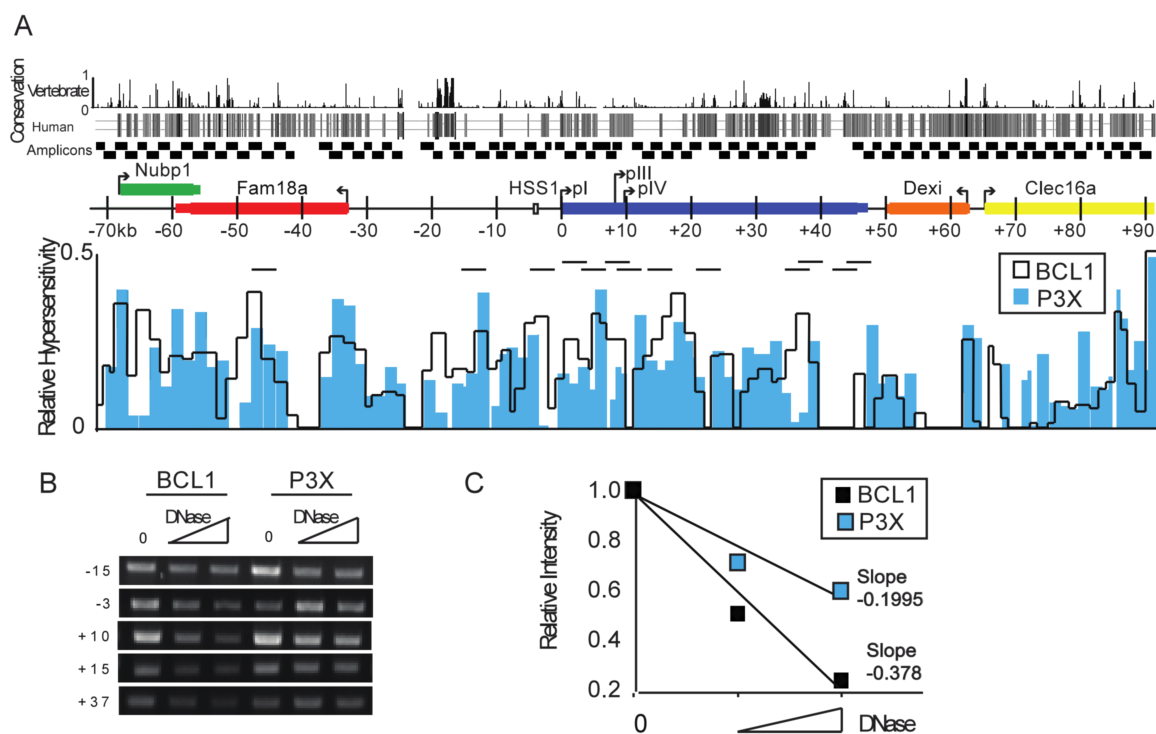


Figure 2

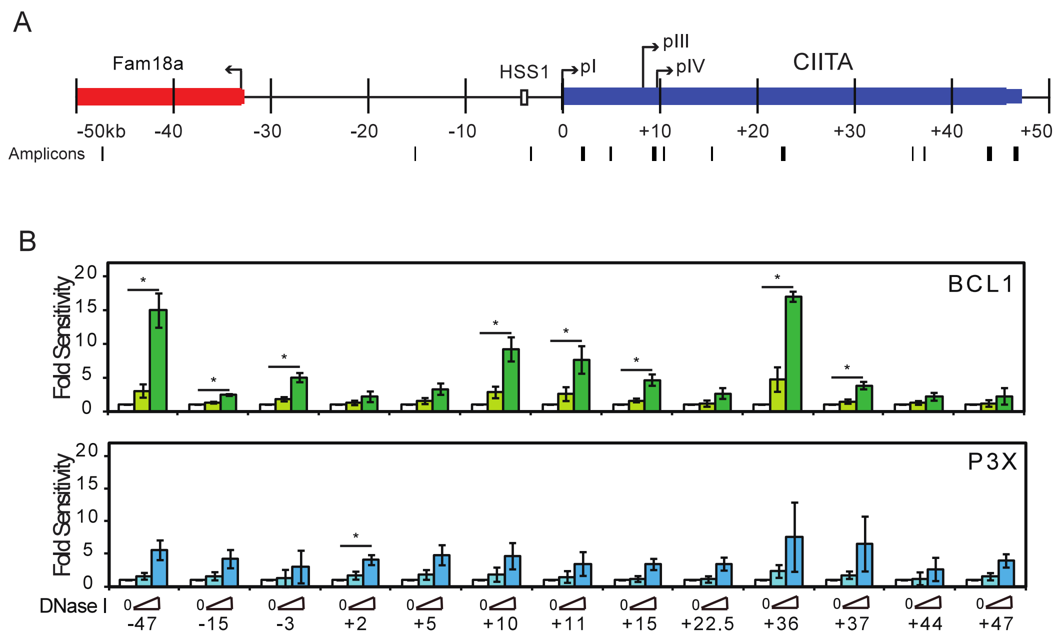


Figure 3

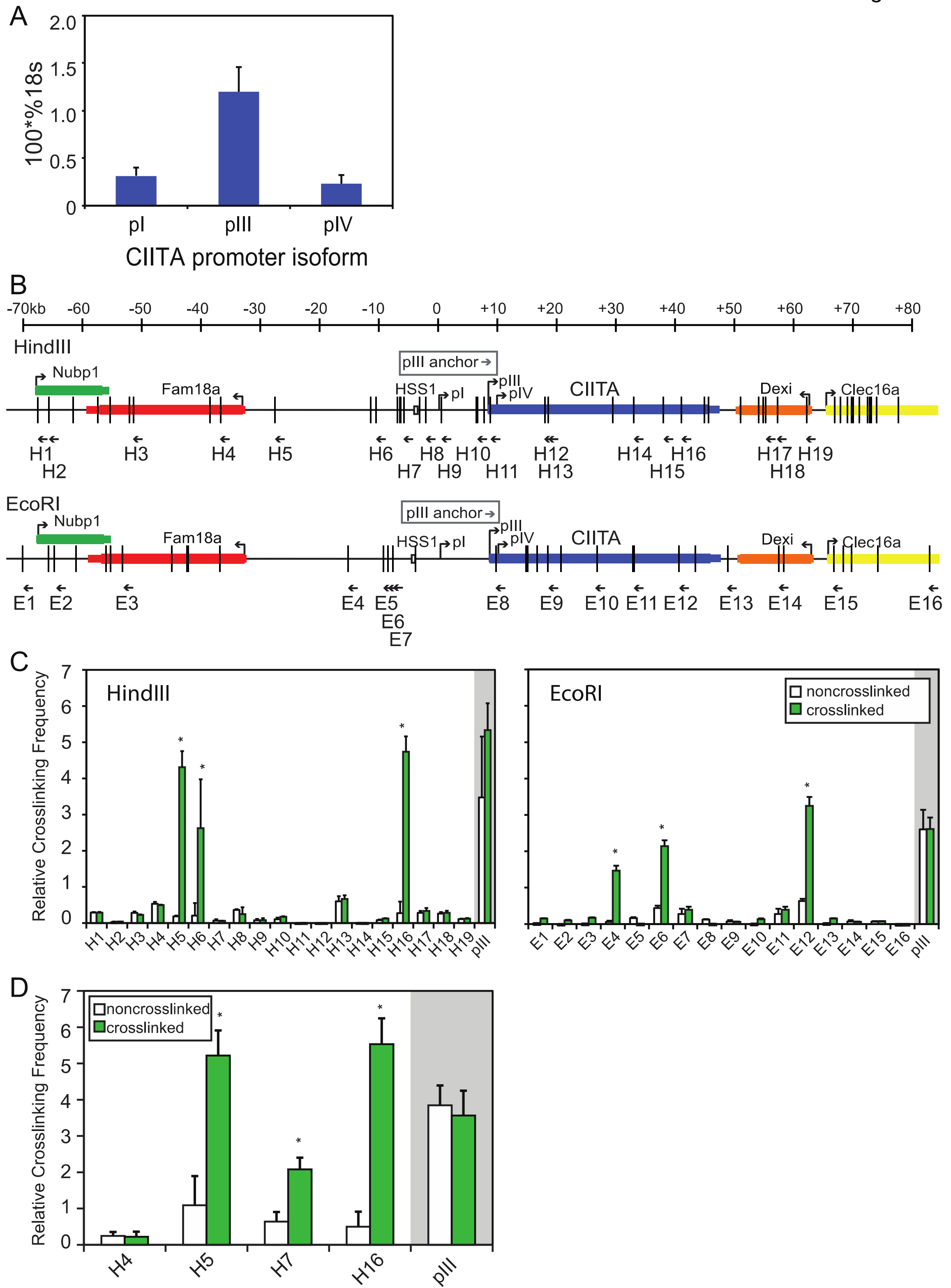


Figure 4

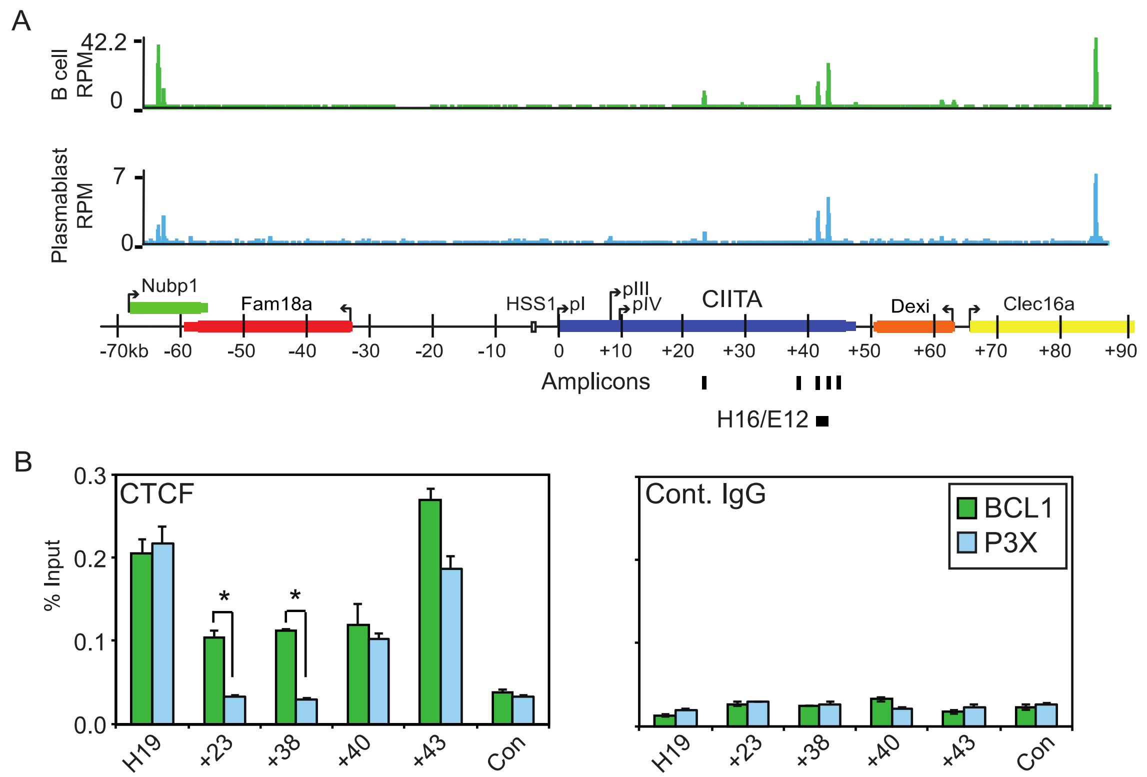


Figure 5

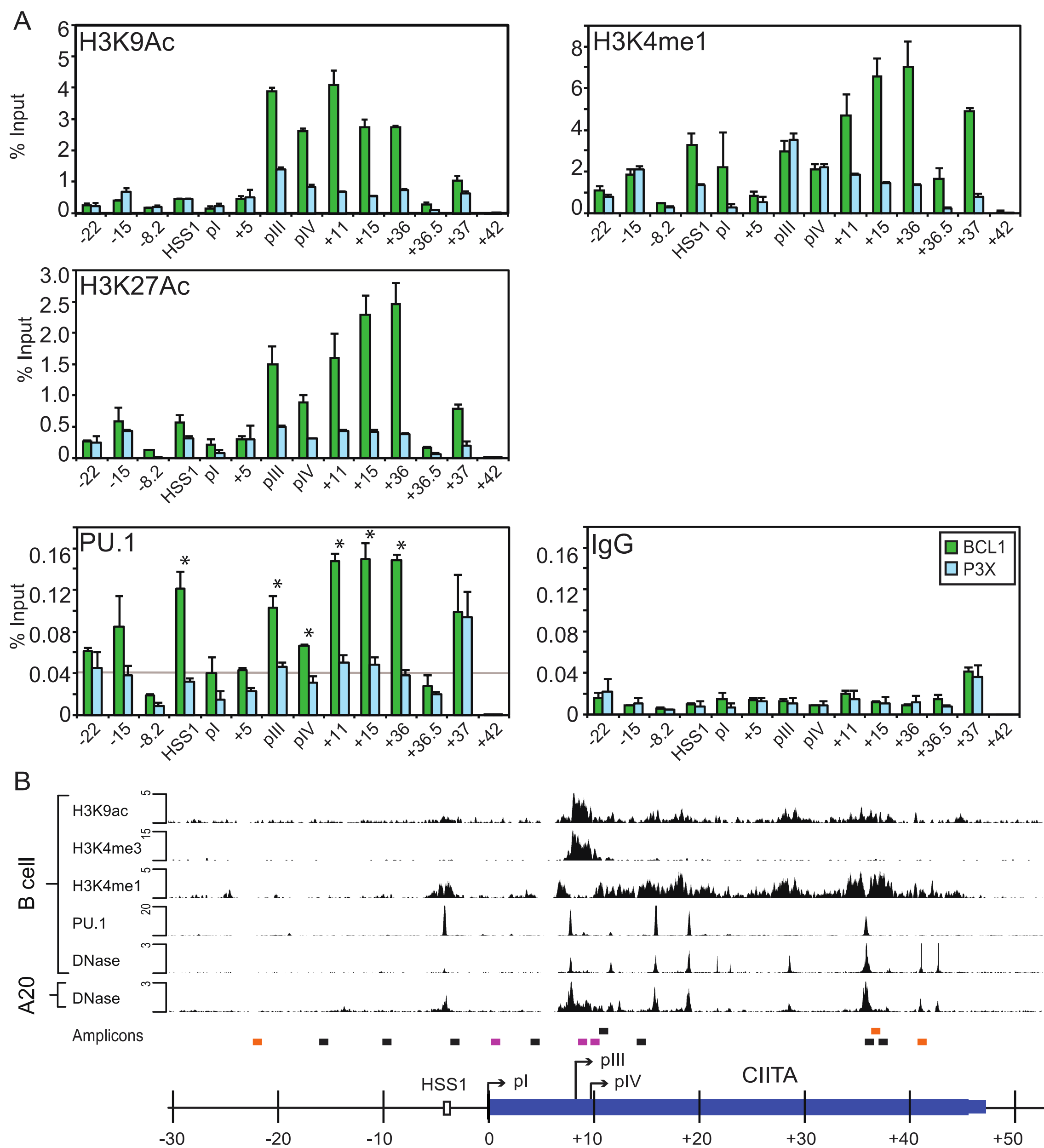


Figure 6

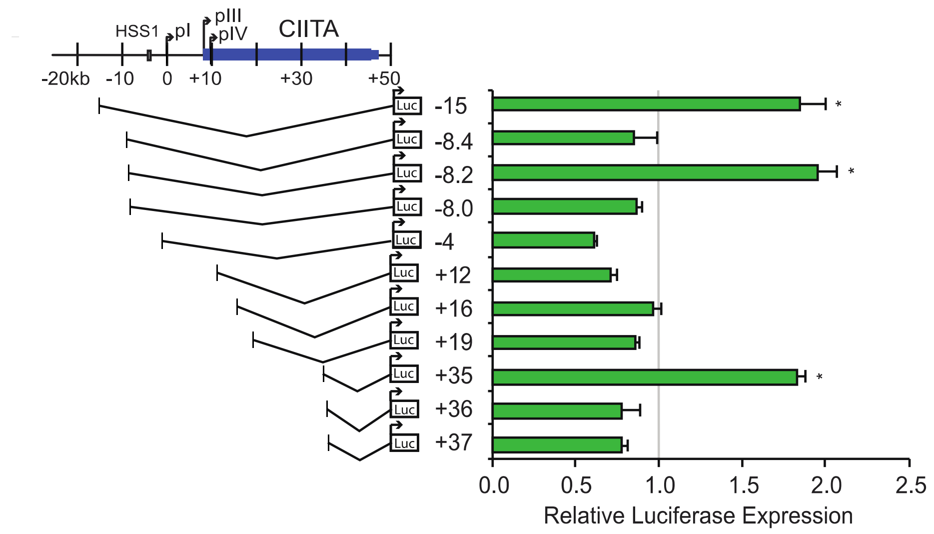


Figure 7

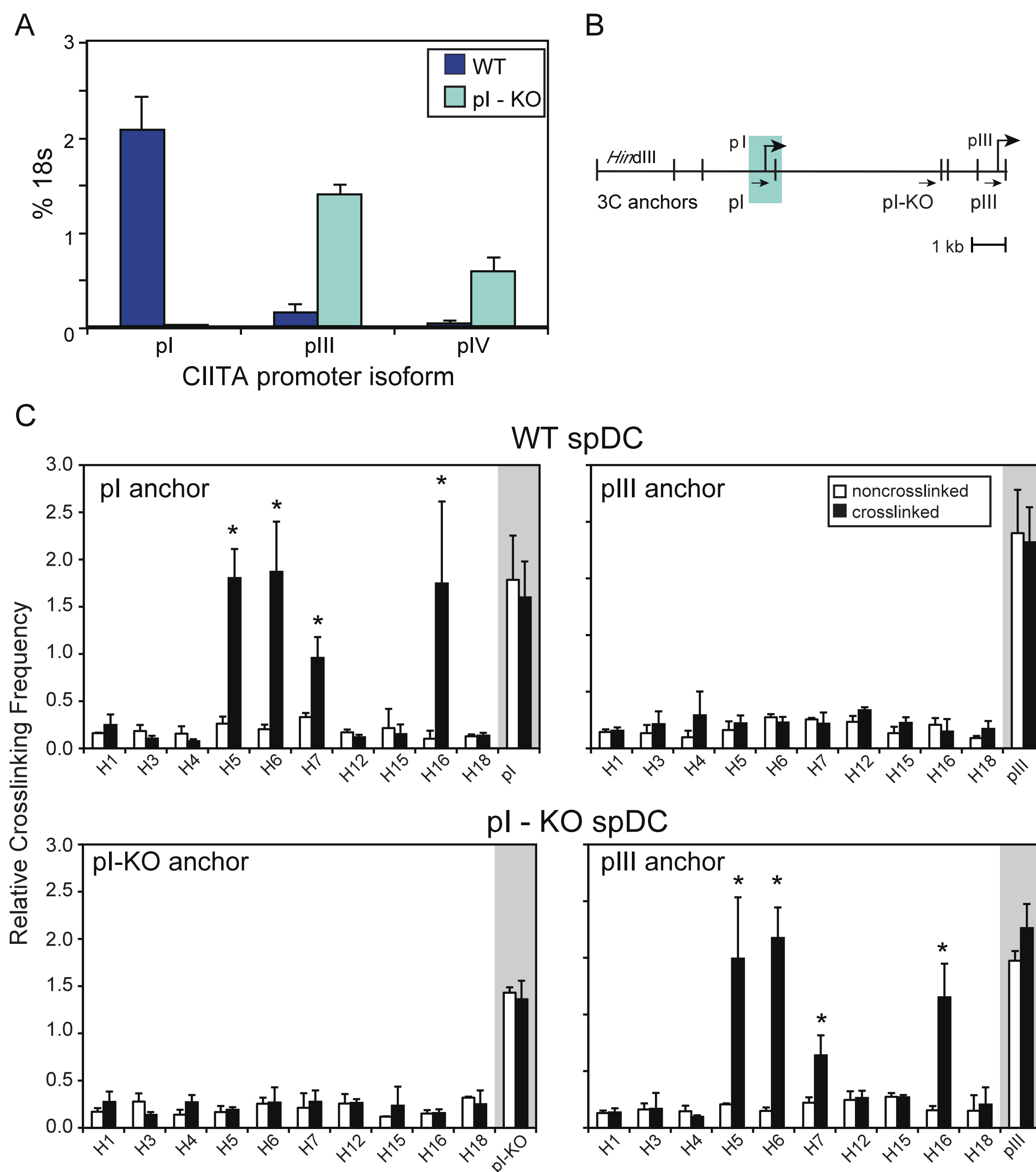


Figure 8

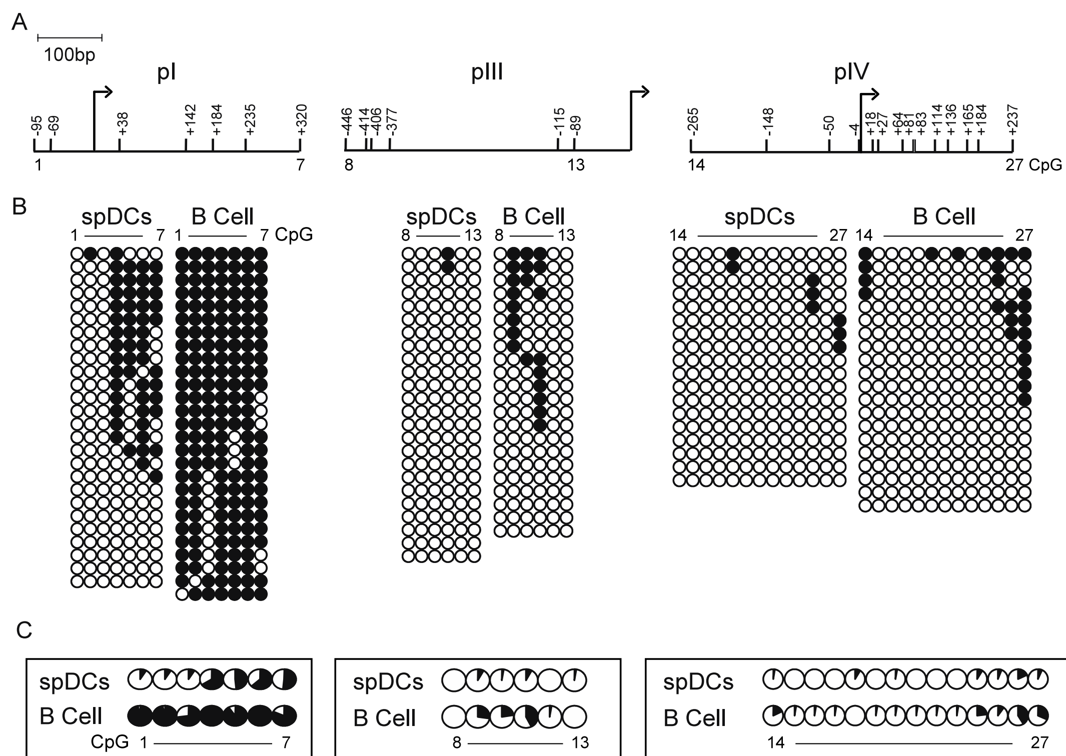
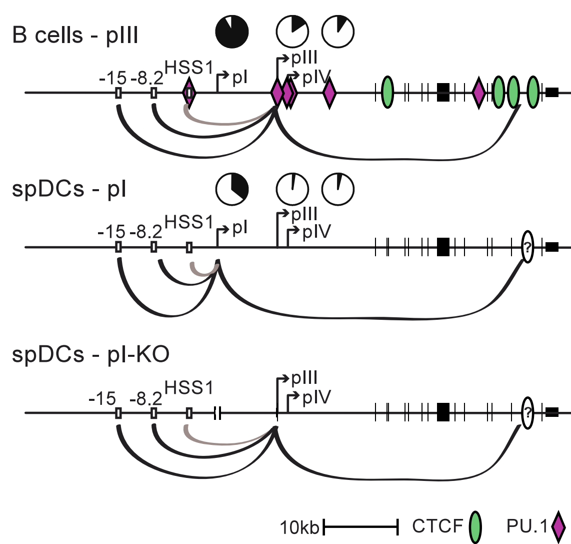
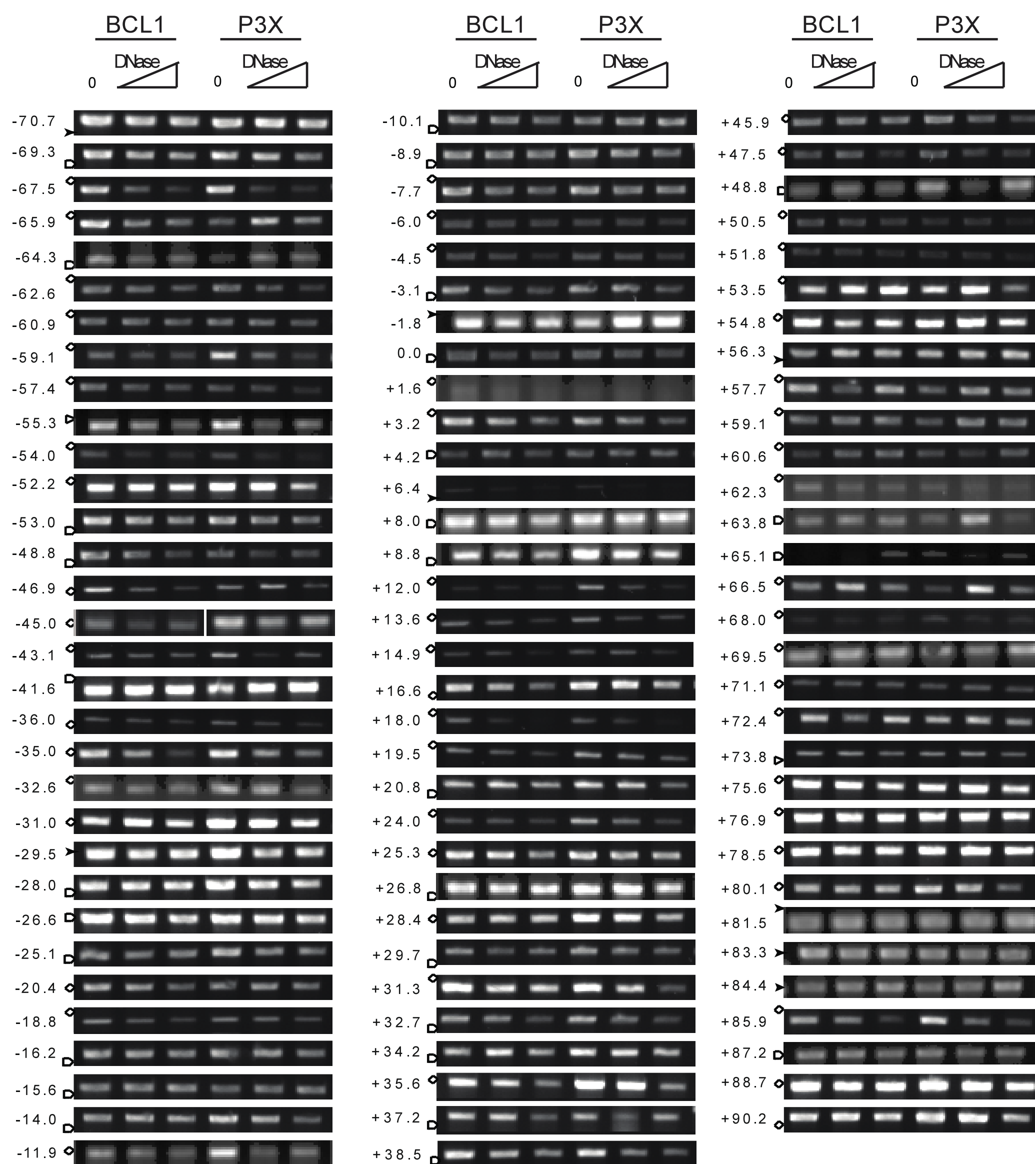


Figure 9



Supplemental Figure 1



Molecular Weight Markers: 2.5kb 2kb 1.5kb 1kb

Chapter 4: Global demethylation alters *CIITA* promoter choice in B cells

This chapter presents current knowledge of the influence of DNA methylation on CIITA promoter choice.

This chapter was written by S. Lohsen.

Data for this chapter were contributed by: S. Lohsen, W.M. Zinzow-Kramer, Benjamin G. Barwick, and James W. Austin.

S. Lohsen isolated and prepared primary splenic B cells, did Decitabine treatments, and qRT-PCR analyses.

Splenic dendritic cells and primary peritoneal macrophages were isolated, prepared, and analyzed by qRT-PCR by W.M. Zinzow-Kramer.

S. Lohsen and W.M. Zinzow-Kramer did 5-azacytidine treatments.

B.G. Barwick and J.W. Austin performed bisulfite treatment, and B.G. Barwick performed analyses.

Introduction

Major histocompatibility (MHC) class II molecules serve to display peptides acquired from the local immune environment to CD4+ T helper T cells (Benacerraf, 1981; Germain, 1986). In this fashion, foreign pathogens can be recognized by the immune system and trigger the complex adaptive immune response necessary to fight infection. Expression of *MHCII* is regulated primarily at the level of transcription by the class II transactivator, CIITA (reviewed by (Choi et al., 2011)). CIITA functions as a master regulator, orchestrating interactions in an enhanceosome centered over a network of transcription factors bound at the WXY box, including the RFX complex (Moreno et al., 1997; Reith et al., 1994; Steimle et al., 1995) binding together with CREB (Moreno et al., 1999; Reith et al., 1994), and NF-Y (Mantovani et al., 1992). CIITA interacts with these constitutively bound transcription factors and serves to facilitate the recruitment of chromatin-modifying enzymes such as BRG-1 (Mudhasani and Fontes, 2002), CARM1 (Zika et al., 2005), and CBP/p300 (Fontes and Kanazawa, 1999; Kretsovali and Agalioti, 1998), as well as members of the basal transcription machinery TAFII32 (Fontes et al., 1997b) and Cyclin T1 (Kanazawa et al., 2000). Without the transcriptional integrator activity of CIITA (Fontes et al., 1999), *MHCII* expression cannot occur. Mutation of *CIITA* leads to an absence of antigen presentation and adaptive immune responses, resulting in a severe combined immunodeficiency (Reith and Mach, 2001; Reith et al., 1995).

CIITA itself is similarly regulated at the transcriptional level. Its three principle promoters are chiefly regulated in a cell-type specific manner (Muhlethaler-Mottet et al., 1997). Various transcription factors are known to orchestrate the expression of each promoter (as detailed in Chapter 1), with some factors shared between promoters. In cells of the myeloid lineage, STAT5 (Choi et al., 2009), IRF4/8, and PU.1 (Smith et al., 2011)

directly bind to and regulate CIITA expression from promoter I (pI). In lymphoid cells, IRF4/8, PU.1, E47, Sp1, CREB-1, and Ets family members bind to and regulate expression from promoter III (pIII) (Green et al., 2006; Lennon et al., 1997; van der Stoep et al., 2002b, 2004; Yoon and Boss, 2010). Expression from promoter IV (pIV), occurring in most cell-types when induced by IFNγ, is regulated by the binding of USF-1, IRF-1/2, STAT1, c-myc, and the proteasome complex (Dong et al., 1999; Koues et al., 2009; Lee and Benveniste, 1996; Lennon et al., 1997; Meraz et al., 1996; Morris and Beresford, 2002; Muhlethaler-Mottet et al., 1998; Ni et al., 2005; O'Keefe et al., 2001; Piskurich et al., 1999, 1998; Truax et al., 2010; Xi et al., 1999).

In addition to transcription factor binding, epigenetic mechanisms have been implicated in *CIITA* regulation at all three of its principle promoters. Histone acetyltransferases, such as p300 create open chromatin architecture via acetylation of the histone tails of H3 and H4 to facilitate gene expression from all three promoters (Choi et al., 2009; van Eggermond et al., 2011; Green et al., 2006; Ni et al., 2005). BRG1 has been shown to orchestrate 3D chromatin interactions associated with pIV regulation (Ni et al., 2008), and a series of distal regulatory elements interact with the active promoter and are shared between pI and pIII (Chapter 3).

Cytosine methylation at promoter-proximal CpGs is responsible for shutting down expression of CIITA from pIV in fetal cells and some cancers (van den Elsen et al., 2003; Morimoto et al., 2004; Morris and Beresford, 2002; Radosevich et al., 2007; Satoh et al., 2004; van der Stoep et al., 2002a). Cytosine methylation is correlated with a lack of transcription factor binding at the promoter, as well as a lack of the activating marks of histone acetylation (Morris and Beresford, 2002). This epigenetic mechanism of gene regulation has not been explored in the context of the other promoters.

In this study, treatment with demethylating agents of isoform III expressing B cell lines revealed a de-repression of expression from pI. This was recapitulated in primary B cells, and treatment with 5-azacytidine led to a small but significant loss of methylation at the CpGs proximal to pI. This is evidence for a direct role for DNA methylation in controlling the choice of promoter use at the *CIITA* locus. Conflicting evidence shows that demethylation also leads to an increase in expression of some of the transcription factors responsible for CIITA expression from pI and points to an indirect role for DNA methylation on the expression of CIITA isoform I. It is therefore likely that DNA methylation's role in CIITA promoter choice is multifaceted, playing both direct and indirect roles in regulating CIITA promoter choice, specifically expression from pI.

RESULTS

pIII is unmethylated regardless of promoter usage while pI is only unmethylated in pI-expressing cell types.

To determine basal methylation status of the *CIITA* promoters, bisulfite sequencing was employed. pI-expressing spDC and macrophages and pIII-expressing splenic B cells were queried. All cell types examined were unmethylated at pIII-proximal CpGs (Figure 1B). Intriguingly, at pI, while four of the 7 promoter proximal CpGs displayed methylation in all cell types, the three 5' most TSS-proximal CpGs displayed differential methylation correlating with promoter usage (Figure 1A). The splenic B cells which use pIII displayed methylation at these CpGs, whereas the two pI-expressing cell types both displayed a significant reduction in methylation.

Induced demethylation in a pIII-expressing B cell line results in increased expression of pI.

Given the correlation between a lack of methylation at CpGs proximal to pI in CIITA isoform I-expressing cells, demethylating agents were used to determine if removal of methylation from the normally-methylated pI in isoform III-expressing B cells would result in expression of *CIITA* from pI. Murine B-cell line A20 cells were treated with 5-azacytidine for 48 hours, and the change in *CIITA* isoform mRNA expression was calculated as fold over untreated cells. While no appreciable change in expression was observed for isoforms III and IV, a large increase was seen in isoform I mRNA expression relative to untreated samples (Figure 2). While total *CIITA* expression was not appreciably altered, this was not unexpected, as isoform I expression continued to make up a proportionally small amount of *CIITA* expression relative to the dominate isoform III.

Primary B cells recapitulate the increase in isoform I expression, and are significantly demethylated at pI following 5-azacytidine treatment.

To ascertain if the phenotype observed in a B cell line could be recapitulated in primary cells, primary B cells were treated with 5-azacytidine for 48 hours. As in Figure 2, isoform-specific and total *CIITA* expression was plotted as fold-over untreated samples. Again, a significant increase in *CIITA* expression was observed for *CIITA* isoform I, while isoforms III, IV, and *CIITA*-total expression were not appreciably altered (Figure 3A). Primary B cells therefore display a similar pattern to the 5-azacytidine treated A20 cell line.

Bisulfite treatment of mock and 5-azacytidine treated primary splenic B cell samples at pI revealed a small but significant decrease in methylation at pI. Thus a correlation

between the increase in *CIITA* isoform I expression and methylation status at pI exists (Figure 3B).

Demethylation via Decitabine treatment reveals a concomitant increase in CIITA isoform I expression with IRF4 and Stat1 expression.

5-azacytidine is incorporated into both DNA and RNA. This incorporation might confound interpretation of RNA data. To remove this confounding factor, Decitabine (5-Aza-2'-deoxycytidine), which is only incorporated into DNA, was used as a demethylating agent. A20 cells treated with Decitabine for 48 hours were examined for alterations in *CIITA* isoform expression. As with 5-azacytidine treatment, no alterations in isoform III or IV expression were observed (data not shown), but I soform I was again significantly increased (Figure 4A). While the fold-increase in isoform I expression was highly variable between samples, 4A shows that all samples show consistent increases in isoform I expression.

To determine if an increase in isoform I expression was correlated with an increase in expression of transcription factors responsible for expression from pI, a small panel of transcription factors were queried. The expression levels of *IRF4*, *IRF8*, *Stat1*, and *PU.1* mRNAs were examined. Coincident with the upregulation of *CIITA* isoform I mRNA, *IRF4* and *Stat1* expression was also increased with Decitabine treatment, supporting a potential indirect role for DNA methylation and *CIITA* expression.

DISCUSSION

While previous studies have outlined a role for DNA methylation in regulating the expression of *CIITA* from pIV, it was unclear as to what, if any, role DNA methylation played at *CIITA*'s other promoters. In cells of the myeloid lineage, the three most TSS-proximal CpGs are unmethylated, and the 5'-most promoter, pI is utilized. In cells of the lymphoid lineage, such as B cells, the myeloid promoter is methylated and the next available promoter is chosen, pIII. In examining the effect of global DNA demethylation in B cells, it was observed that demethylation resulted in a small but significant increase in expression from the myeloid promoter, pI, and that this correlated with a loss of methylation at this promoter. This data points to a direct role for DNA methylation to play in promoter choice. When the levels of transcription factors known to play a role in *CIITA* expression were examined upon global demethylation, evidence supporting a potential indirect role for DNA methylation in promoter choice was found. The mRNA expression levels of the transcription factors *IRF4* and *STAT1* were found to be upregulated upon DNA demethylation in B cells, pointing to a potential role for the increased levels of these transcription factors in facilitating expression from pI. These direct and indirect effects most likely work in conjunction in the facilitation of *CIITA* promoter choice in cells of the myeloid and lymphoid lineages.

While the data in this study support the theory that DNA methylation does play a role in *CIITA* regulation, some confounding factors are present. Relative to the immortalized B cell line A20, a reduced induction of pI was observed in primary splenic B cells. This is most likely due to a lack of division in culture relative to A20 cells. Figure 3 shows that in the culture of primary B cells, there are a few cells that were completely demethylated, while many others were not. Primary B cells exist in a resting state, and are

induced to divide and proliferate upon exposure to antigen leading to differentiation. Attempts were made to encourage division without inducing differentiation by treating the primary B cells with small quantities of LPS. Large quantities of LPS in conjunction with cytokines are used in culture to *ex vivo* differentiate B cells (which causes the silencing of *CIITA*), and it was anticipated that a small dose of LPS would facilitate division without differentiation. An increase in cell division was observed without the loss of the B cell marker B220, a surface protein that is lost during plasma cell differentiation. Induction of expression from pI upon 5-azacytidine treatment was inconsistent in these samples (data not shown). Several explanations for this exist. The first is that while LPS was not present at a concentration sufficient to induce full differentiation as seen by loss of B220, these cells were beginning along the differentiation pathway, resulting in the alteration of *CIITA* expression independent of the demethylation treatment. A second possibility is that the mechanism of action of 5-azacytidine confounded the RT results. As discussed above, 5-azacytidine is incorporated into both DNA and RNA. It is unclear if this has an effect on either the stability of the RNA generated during 5-azacytidine treatment or the subsequent RT reaction.

For the initial experiments in this study, 5-azacytidine treatment led to an observable increase in isoform I expression. When additional transcription factors' expression levels were queried, however, no change was observed. Given the inconsistency in some of the subsequent 5-azacytidine data, and this unexpected result, the demethylating agent Decitabine (5-aza-2'-deoxycytidine) was used. Decitabine is only incorporated into DNA, and therefore is unlikely to confound RNA data. The use of Decitabine not only recapitulated the increase in isoform I expression observed with 5-azacytidine, but also revealed the increase in expression of the transcription factors STAT1 and IRF4. It is

therefore possible that this points to a more profound alteration of isoform I expression versus the other transcription factors, as its alteration in expression was observed despite the confounding factor of 5-azactyidine incorporation into RNA.

While the use of Decitabine allowed for a more precise view of gene expression changes during global demethylation, it appeared to demonstrate that there was a large variation in the levels of induction of *CIITA* isoform I between samples. This could reflect inconsistent treatment of samples (drug not dissolved completely etc), or variability between the cultures in terms of cell division (where more division would lead to more incorporation and thus more observed effect). Steps were taken to mitigate these factors by treating samples in parallel with the same master mixture of Decitabine solution, and it is therefore unclear as to what caused the variability in *CIITA* isoform I induction.

The bisulfite sequencing used to examine DNA methylation status at *CIITA* promoters in this study is a powerful tool in that it reveals the methylation status of specific CpGs in individual cells at specific loci. To examine the DNA methylation of the promoter-proximal CpGs at the *CIITA* locus, this technique was ideal. This focused approach does have the disadvantage of biasing the search, which was in part alleviated by examining gene expression data for a variety of additional transcription factors. A more global examination of DNA methylation and gene expression data through sequencing of Reduced Representation Bisulfite Sequencing and RNA-seq libraries would provide a more comprehensive view of DNA methylation's affect on not only *CIITA*, but also the network of transcription factors involved in *CIITA* regulation.

Despite these additional factors, it is clear that DNA methylation is playing a role in regulating *CIITA* expression. To determine the extent to which methylation plays a part in directly affecting promoter choice, specifically the choice of pIII over pI in cells of the

lymphoid lineage due to the methylation pI, a more *in vitro* approach is required. A *CIITA* locus Bacterial Artificial Chromosome (BAC) construct is present in the lab, including the nearest upstream and downstream genes. By amplifying this BAC in a bacterial system where CpG DNA methylation is lacking, this unmethylated BAC can be introduced into a B cell system, and expression levels of isoform I can be determined. If methylation is the primary mechanism by which pI is bypassed in B cells, this BAC should reveal a significant increase in isoform I expression of *CIITA*.

To explore the potential indirect effect of DNA methylation on *CIITA* expression, the transcription factors found to be upregulated upon global demethylation, STAT1 and IRF4, can be overexpressed in the B cell system. This can be done in the context of the native *CIITA* B cell locus (containing methylated CpGs at pI), as well as in the BAC system described above. If overexpression of these factors results in an increase in *CIITA* isoform I expression in the native B cell locus system, or augmentation of isoform I expression in the BAC system, it can be determined what role these transcription factors play in facilitating promoter choice, whether it be wholly indirect, wholly direct, or a combination of both.

The region displaying altered methylation surrounding the pI promoter extends from the TSS up through -95, which encompasses PU.1, STAT5, AP1, Ets, and IRF4/8 binding sites. The affected CpGs, however, are not found within any of the transcription factors' binding sites. One CpG in particular, however, is located 69 bp upstream from the TSS within 10 bp of two potential PU.1 binding sites. It is possible that CpG methylation at this location facilitates a closed chromatin state, as there is a decrease in H3 and H4 acetylation at pI in B cells where this CpG is methylated (Yoon and Boss, 2010). Examination of PU.1 binding also showed lower levels of PU.1 at pI versus pIII (Yoon and Boss, 2010). It has been proposed that DNA methylation and closed chromatin conformation at this site in B

cells facilitates promoter choice by leading to a bypass of pI in B cells (Chapter 3). Determination of 3-D chromatin structure of the promoters in demethylated B cells would help support the role for methylation in promoter choice if it revealed switching of the foci of interactions from pIII to pI.

Thus, the data here show a potentially multifaceted role for DNA methylation in the context of *CIITA* promoter choice. The CpGs proximal to the TSS at pI are unmethylated in myeloid cells as well as in B cells where expression from pI is induced. Transcription factors necessary for *CIITA* expression, IRF4 and STAT1, are also upregulated in response to global demethylation. It is therefore most likely that DNA methylation plays both a direct and indirect role in facilitating expression of *CIITA* from pI.

FIGURES

Figure 1. Methylation status at *CIITA* promoters I and III reveal correlation between pI methylation and isoform I expression. SpDC, primary peritoneal macrophages, and splenic B cells were isolated from wild-type C57BL/6 mice, genomic DNA isolated and subjected to bisulfite conversion. Analysis of methylation status at the CpGs proximal to pI (A) and pIII (B) were performed and plotted with respect to the TSSs. Circles represent percent methylated CpGs observed at a given cytosine, with black indicating methylation and white indicating demethylation. spDC and B cell data shown here are also presented in the dissertation of W. M. Zinzow-Kramer (Zinzow-Kramer, 2012) and Chapter 3.

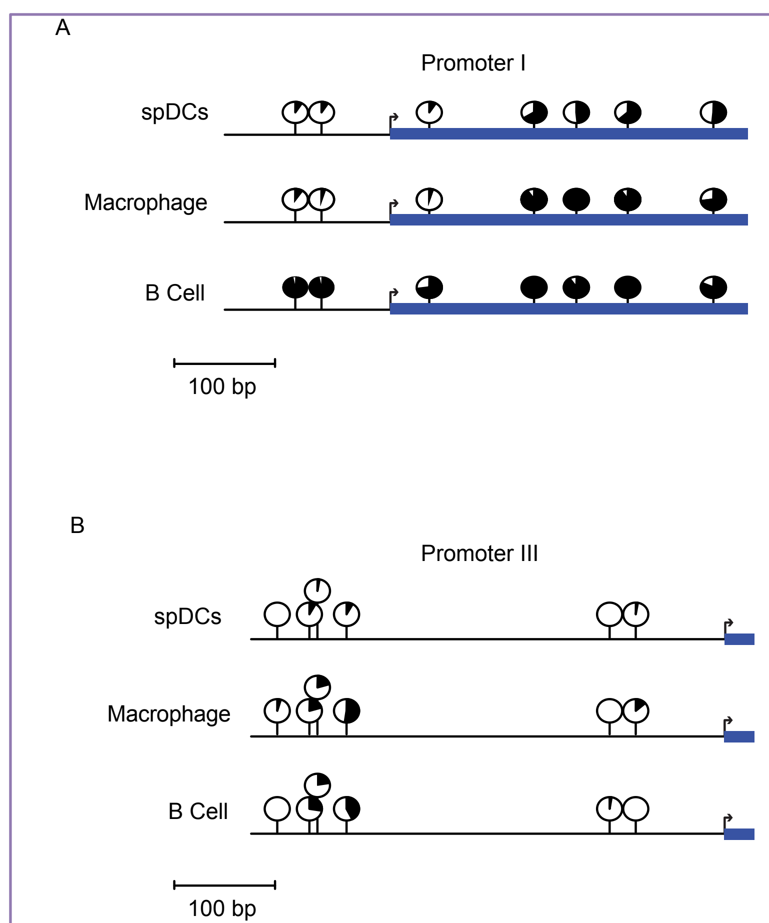


Figure 2. 5-azacytidine treatment of A20 cells results in a relative increase in expression of *CIITA* isoform I. Four independent cultures of the B cell line A20 were treated with increasing concentrations of 5-azacytidine every eight hours. After 48 hours, RNA was collected and analyzed by qRT-PCR for isoform-specific *CIITA* expression. Data represent fold change in expression relative to untreated A20 cells. As these data were collected with W.M.Z-K, they also appear in her dissertation (Zinzow-Kramer, 2012).

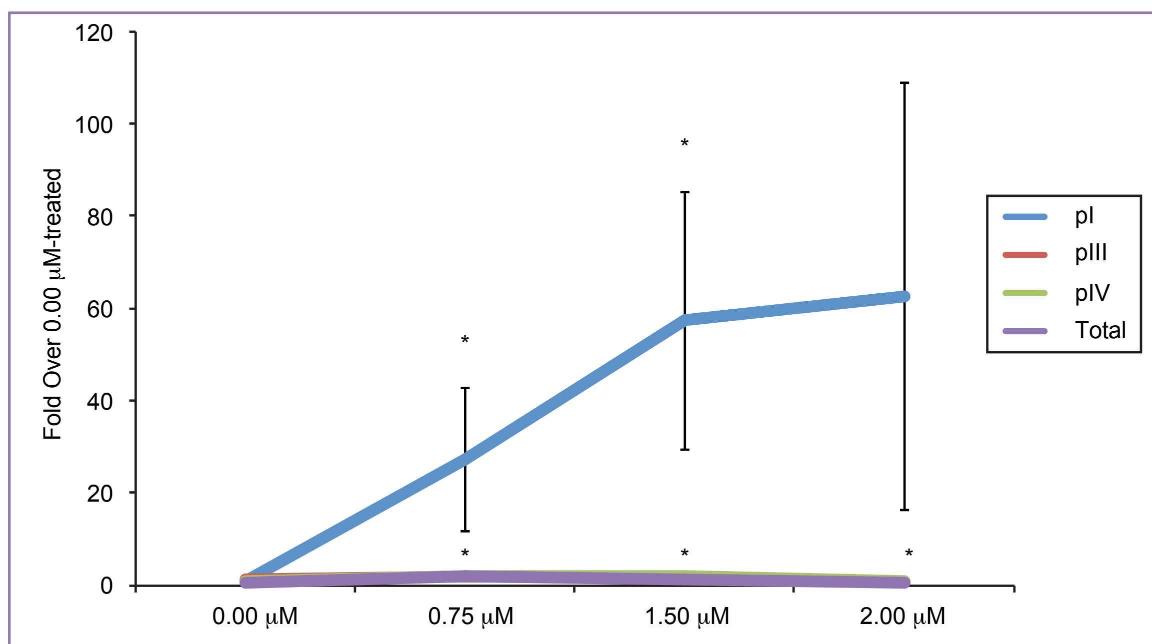


Figure 3. 5-azacytidine treatment of primary splenic B cells also leads to a relative increase in expression of *CIITA* isoform I, and pI displays a significant decrease in methylation. Primary splenic B cells isolated from wild-type C57BL/6 were maintained in culture and treated with increasing concentrations of 5-azacytidine every 8 hours. After 48 hours, cultures were divided into two. A) RNA was prepared from half of the sample, and analyzed by qRT-PCR for *CIITA* isoform-specific expression. B) Genomic DNA was prepared from the other half of the sample, and was subjected to bisulfite treatment and analysis of CpG methylation status at pI.

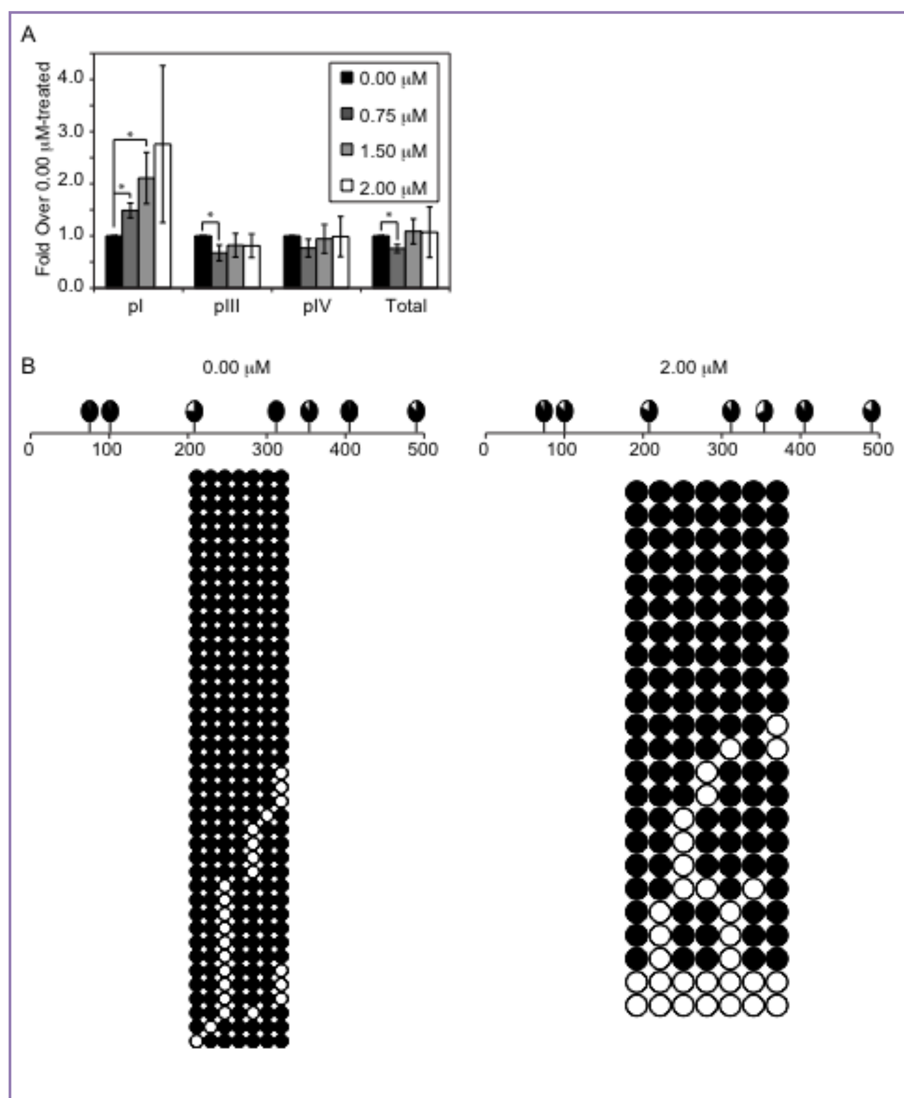
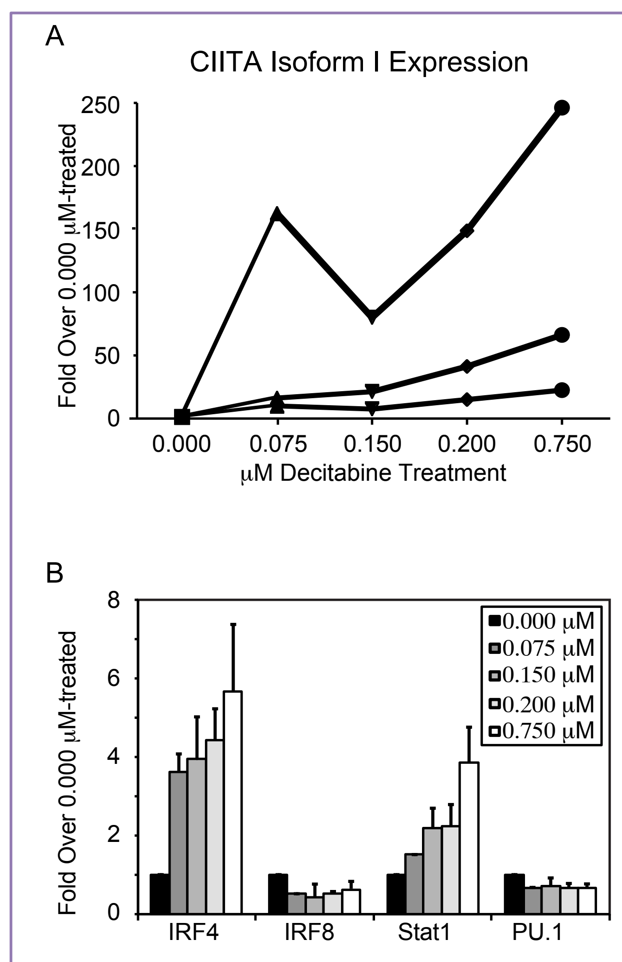


Figure 4. Decitabine treatment of A20 cells de-represses the expression of *CIITA* isoform I and the transcription factors *IRF4* and *STAT1*. A20 cells were treated with increasing concentrations of Decitabine every 8 hours. After 48 hours of treatment, RNA was collected and analyzed by qRT-PCR for *CIITA* isoform I expression (A) and expression of *IRF4*, *IRF8*, *Stat1*, and *PU.1* (B).



Chapter 5: Discussion

This chapter was written by S. Lohsen.

Despite the well-defined role the proximal promoter regions play in the regulation of *CIITA* expression, few reports have described distal elements or the complex actions that they may have. One report outlined the IFNγ-induced pIV network of distal *cis*-regulatory elements involved in *CIITA* regulation in HeLa cells (Ni et al., 2008). A second identified one distal element HSS1 (Yoon and Boss, 2010) which functions in lymphoid cells. This study sought to answer the question of whether or not 3-D chromatin structures were critical in regulating *CIITA* in professional APCs (posed in (Reith and Boss, 2008)). Working in the murine system through a combination of large-scale screening and site-specific analyses, a network of *cis*-regulatory elements was established as playing a role in the regulation of *CIITA* from both the myeloid pI and the lymphoid pIII. In doing so, this work has provided unique insight into the question of *CIITA* promoter choice in cells of the lymphoid and myeloid lineages. As these are the cells that ultimately present antigen to initiate immune responses, this work lays the foundation for initiating this process.

In general, the assays used in this study fall into two general categories: broad screening approaches and site-specific queries. The broad screening approaches to discover potential regulatory regions were three-fold: DNase I hypersensitivity assays to asses the general state of open or closed chromatin across the *CIITA* locus, 3C to determine the 3D conformation of the locus, and ChIP to query for the presence of histone marks associated with regulatory regions. These three approaches provided a large number of candidate regions that were then pared down through further DNase I hypersensitivity assays to isolate the regions of interest coupled with a selection for sites found to be highly conserved across vertebrates, with a focus on conservation between mouse and human sequences. Upon completion of the general screening approaches, three different site-specific approaches were employed to

further define and characterize the potential regulatory regions. These included luciferase assays to directly assess regulatory activity in a cell culture setting, further ChIP assays, which included queries for potential transcription factor binding, and finally DNA methylation assays to begin to gain an understanding of the mechanism of promoter choice facilitated by the newly-characterized regulatory regions.

The initial focus of this study was the DNase I hypersensitivity assay. By ascertaining the general open or closed state of chromatin around the *CIITA* locus in the isoform III expressing murine lymphoma cell line BCL1 and the CIITA-negative murine plasmacytoma line P3X, a landscape of DNase I hypersensitivity was established. This broad region included two of the 5' neighboring genes, as well as most of the two 3' neighboring genes and spanned ~160 kb. To avoid potentially selecting regulatory elements for neighboring genes, the search for sensitive sites was trimmed down to approximately 35 kb upstream and 50 kb downstream of *CIITA*'s pI. While the DNase I large-scale assay provided a good starting point, there were some drawbacks to this approach. Each cell line examined displayed varying overall sensitivities to DNase I. This is seen through the use of BCL1, P3X, and primary splenic B cells in that different concentrations of DNase I were required to provide consistent data across known sensitive and insensitive controls. When comparing the data obtained herein to DNase-seq data sets from mouse CD43- primary splenic B cells as part of the ENCODE project (see Chapter 3, Figure 5), it can be seen that one principal difference is that the primary cell ENCODE data does not reveal HSS1 and in fact does not reveal any upstream hypersensitive sites. It is therefore likely that the concentrations of DNase I used in this study allow for the detection of more subtly hypersensitive sites, such as HSS1, that may have been overlooked had the ENCODE DNase-seq data been used exclusively for screening purposes. Even so, there is still a need for multiple assays to

determine the presence of regulatory regions, as even this more sensitive DNase I screen did not reveal the presence of the -8.2 regulatory site.

The second broad screening assay used in this study was 3C. Interactions between potential regulatory regions and the cognate promoter provide some of the most convincing evidence for regulatory regions. This approach, like the DNase I hypersensitivity assay, has the advantage of being mostly unbiased, though it is promoter-centric. Unfortunately, at this time, the less biased global HiC (Lieberman-Aiden et al., 2009), which provides a view of all interactions in the genome, lacks the resolution to consider the regulation of a single locus. As a result, this promoter-centric approach remains one of the most powerful tools for determining regulatory potential at this scale. The power of this approach was revealed when the -8.2 region, which was not picked out of the DNase I hypersensitivity assay, was revealed in B cells. Using a unique approach to 3C by combining the data obtained using two different restriction enzymes, this study demonstrates the power of independent confirmation and the ability to narrow down regions of interest that two separate restriction enzymes provide. The importance of the regions characterized by 3C was underscored when the 3C assay was performed in spDC. Primarily expressing isoform I of *CIITA*, the four regions shown to interact with the active pIII in B cells were found to also interact with the active myeloid promoter in spDC. Most intriguingly, in pI-KO-spDC, in which expression shifts from isoform I to III, the interactions also shift to follow the active promoter. The question of promoter choice will be considered further below.

The last assay used that was broad in scope was in the consideration of ChIP data in the context of lymphoid cells (Chapter 3, Figure 5). This included ChIP-seq data for the histone marks H3K9Ac and H3K4me1 (Heinz et al., 2010; Revilla-I-Domingo et al., 2012). These data showed that these marks were present at many locations inside of *CIITA* (specifically

the +11, +15, +36, and +37 kb regions), as well as at the previously established *cis*-regulatory element HSS1. They did not appear at any other upstream locations, whereas conventional ChIP analysis revealed the presence of low levels of these marks at the -15 upstream regulatory site, in addition to H3K27Ac. ChIP-seq data has caveats, including biases as a result of the high-throughput sequencing used (Benjamini and Speed, 2012; Cheung et al., 2011), which can account for some of the differences seen in ChIP-seq data as compared to the conventional ChIPs performed as part of the study in Chapter 3.

These three broad assays therefore provided a general starting place from which novel distal regulatory elements for pIII were identified in both B cell and plasma cell lines, as well as in primary splenic B cells. As this study was conducted in murine cell lines, consideration must be given to the mouse-centric versus the human-centric approach. In this study, the data suggest that there are regions that are mouse-specific in regulation (the -8.2 site), as there is no known homology to the human *CIITA* locus. Figure 1 shows a comparison between the mouse and human UCSC genome browser views at the *CIITA* locus. Apart from the -8.2 site revealed in the 3C assay, the other three interacting regions do contain homology to sequences in the human *CIITA* locus. While this lack of homology could be an artifact of the methods used to determine the homology (Miller et al., 2007), it is slightly unclear as to how this will translate for regulation in the human system as we did not determine how the locus would respond and be regulated without -8.2. It is anticipated that future determination of the mechanism of action of these 3D interactions will reveal more clearly how this system is translated in the human context, especially in the case of the -8.2 region. Currently unidentified transcription factors key to mediating the 3D structure of the locus may have less stringent binding motifs that do not meet the criteria for displaying mouse-human sequence homology.

The first site-specific assay used, the luciferase assay, represents a convincing approach by which the functionality of regulatory sites can be queried. However, when a luciferase assay does not reveal regulatory potential, it doesn't guarantee that there is no genuine regulatory activity. The use of a heterologous promoter makes the system a very artificial one, as well as the fact that the region being queried is being taken completely out of its locus' context. The first caveat can be worked around by exchanging the heterologous promoter for the native one, but this is not without its own complications. In the human system, the minimal promoter construct for maximal expression was determined to be a 319 bp region (Ghosh et al., 1999); however, when the homologous region in the mouse was cloned into the luciferase expression vector, it was only expressed at a minimal level in the A20 B cell line relative to the other *CIITA* promoter constructs, and therefore were not a viable system in which to test regions for regulatory potential. Sites that work in conjunction with other regulatory regions must also be considered. In the native locus, several regions may work together to exert their regulatory function, and thus isolating individual components for testing in this system would not reveal their mechanism of action. Thirdly, the luciferase system also removes any role that DNA methylation may play in regulation. By creating the luciferase constructs in bacterial cells, no DNA methylation is present, and therefore any effect that DNA methylation might have on gene expression would be missed. In addition, there is also inherent variability in cell lines when using the luciferase system. B cell lines such as A20 appear to have lower basal levels of activity as well as lower levels of activation seen when compared to even another lymphocyte cell line, EL4 T cells ((Austin et al., 2014), Chapter 3). This could be a result of the B cells being simply harder to transfect, although 90% GFP positive cell populations upon nucleofection with a control pmax-GFP vector argues against this. Considering the example of the HSS1 site, this regulatory region

has been well established as an important regulatory element, but performs relatively poorly in the luciferase system when the 1 kb region surrounding this region (-2.6) was cloned into the luciferase system (data not shown). The data are therefore not fully conclusive when the results are negative in stating that a region has no regulatory activity or potential. When the HSS1 site was queried in the context of a Bacterial Artificial Chromosome (BAC) system, it was apparent that it was playing a important role in the locus (Yoon and Boss, 2010). The *CIITA* BAC system contains ~160 kb of the *CIITA* locus (including the neighboring upstream and downstream genes), and a deletion of HSS1 in this context led to a decrease in 3D chromatin interactions between this site and the promoter, as well as a profound decrease in expression from pIII. The BAC system is also dependent on successful nucleofections and production of the BAC in a bacterial system, and therefore an alternative would be ideal. One possibility would be to delete potential regulatory regions directly from the *CIITA* locus in a cell line, and determine what effect, if any, the deletion has. This could now be possible through use of the CRISPR-CAS 9 system, in which guide RNAs can be used to target DNA cleavage at precise loci, where repair results in a deletion of a particular region (Cong et al., 2013; Mali et al., 2013). By making alterations of the native locus, most of the drawbacks of the luciferase system are avoided. This system also has the advantage of being functional in multiple different cell types, regardless of nucleofection potential, allowing for examination of the effects of multiple regulatory regions across cell types that express all three of the different *CIITA* isoforms. The difficulty is in screening for successful targeted deletion and the potential issues of off-target effects.

The second site-specific assay used was conventional ChIP. In addition to the histone marks discussed above, ChIP was performed for the transcription factor PU.1. As discussed in Chapter 2, PU.1 is known to play an important role in *CIITA* locus in both lymphoid

(Yoon and Boss, 2010) and myeloid (Smith et al., 2011) cells. Given its role in orchestrating the 3D architecture between HSS1 and pIII in the B cell system, it has been proposed to play an important role in promoter choice. Figure 2 shows that PU.1 is bound at multiple locations across the *CIITA* locus in both myeloid and lymphoid cells. Considering the context of promoter choice, sharing of PU.1 between multiple promoters allows flexibility in promoter usage. This flexibility is seen in the case of pI-KO-spDC, where usage is switched to pIII without the addition of novel transcription factors. This flexibility could be mediated by the varying degrees of consensus of binding sites for PU.1, directing binding to the higher consensus site at pIII over the lower consensus site at pI. Therefore in B cells, if PU.1 is expressed relatively less than in spDC, the higher fidelity site at pIII will be chosen. If PU.1 is expressed more highly in spDC, this will allow occupation of the lower-consensus binding site at pI to facilitate expression there. While PU.1 might still be bound at pIII in this case, the passage of the transcription machinery initiated from pI through the site would dislodge the PU.1 bound. PU.1 is therefore a tempting candidate for orchestrating this complex 3D architecture, but PU.1 is not found at all four of the interacting sites. In order to obtain a clearer picture of the factors involved in promoter choice, other transcription factors shared between the interacting regions need to be identified and examined further.

While much of this study has focused on identification of regions involved in expression of *CIITA* from pI and pIII, only preliminary work has been done examining the mechanism of promoter choice. To begin to gain a mechanistic perspective of promoter choice, a site-specific approach was used: bisulfite sequencing. Bisulfite sequencing of isoform I and III-expressing cell lines first revealed the correlation between methylation and promoter choice (Chapter 4, Figure 1). While pIII and pIV were unmethylated in all cell types examined, the three CpGs most proximal to the TSS at pI were only unmethylated in isoform I expressing

cells. Further study using demethylating agents revealed a potential direct role for methylation (Chapter 4, Figures 2 and 3) via a correlation between demethylation at pI and increased expression from pI in normally isoform III-expressing cells. This study also revealed a potential indirect role for DNA methylation on promoter choice (Chapter 4, Figure 4B) via an increase in mRNA expression of *CIITA*-affecting transcription factors that accompanied this increased expression from pI.

Thus, from a mechanistic perspective, this study only examined two main sets of experiments. The bisulfite sequencing coupled with treatment by demethylating agents revealed interplay between direct and indirect effects of DNA methylation, and the 3C data provided a glimpse into another potential mechanism. The previously discussed differential binding of PU.1 based on expression levels and binding motif consensus provides a third potential mechanism of promoter choice. 3D architecture coupled with differential PU.1 binding, preference for the 5'-most promoter, and the presence of closed chromatin at unused upstream promoters provide the beginnings of an understanding of mechanism, as detailed in Figure 3.

Promoter choice in myeloid and lymphoid cell lineages appears to be distinct from pIV regulation as revealed in the IFNγ-inducible HeLa system. A side-by-side comparison in both the murine and human systems is necessary to explore this further. It is possible that if these same experiments were repeated in human B cells and spDC, more overlap with the set of distal *cis*-regulatory regions indentified in the HeLa system would be present. Alternatively, if the same sets of experiments were conducted in a murine pIV system, it is possible that the same set of four regulatory elements would engage with the active pIV. Currently, only the -8.2 site is completely novel to the murine system, and as discussed above, the lack of homology could be the result of a less stringent binding motif of the transcription

factor responsible for orchestrating regulation or 3D chromatin structure from this site. Regulation in the HeLa system revealed the integral role for the chromatin-modifying enzyme BRG1 in regulating expression isoform IV of *CIITA* (Ni et al., 2005, 2008). BRG1 was found to orchestrate factor assembly, histone modification, and the complex set of 3D interactions involved in pIV regulation, and maybe it is playing a similar role at pI and pIII. Beginning with ChIP assays to determine if it binds at the regions of interest in myeloid and lymphoid cells, siRNA of BRG1 coupled with determination of expression levels of *CIITA* and 3C assays to observe alterations in chromatin structure would determine BRG1's role in the context of the other promoters.

3C and luciferase assays have emphasized the importance of the novel -8.2 regulatory site. It remains unclear; however, how it is functioning as an enhancer. While it is not marked as a typical enhancer, the -8.2 site could be marked with less conventional indicators of a regulatory region, including H3.3, H3K36me3, or H3K4me3 that also indicate potential enhancer activity, and the presence of enhancer RNAs may also be queried (Chen et al., 2013; Pekowska et al., 2011; Zentner et al., 2011; Zhu et al., 2013). Further studies to identify factors that bind at this site to facilitate the observed enhancer activity will undoubtedly be invaluable to understand *CIITA* regulation by the -8.2 site.

Other putative regulatory regions within the *CIITA* gene display a phenotype opposite to the -8.2 site in that they lack activity in the luciferase assay, do not physically interact with the promoter, but are epigenetically marked by modifications characteristic of active enhancers. The +11, +15, +36, and +37 sites were all found to be marked as active enhancers, containing both H3K4me1 and H3K27Ac modifications (Chapter 3, Figure 5). Similarly, ChIP in B cells also reveals that these putative intergenic regulatory elements are occupied by PU.1, furthering the case for their potential role in *CIITA* gene expression.

Intergenic regulation is postulated to occur via looping (Hoffmann et al., 2013; Moabbi et al., 2012; Nagpal et al., 2014), but this is obviously not the case at the *CIITA* locus. ChIP-seq data shows that H3K4me1 is high throughout the gene body of *CIITA* (Chapter 3, Figure 5), and it is possible that as it is a highly transcribed gene in this cell type, these intergenic regulatory regions serve simply to hold the locus open in an open chromatin state that facilitates elongation and the high levels of *CIITA* expression.

This study introduces the concept of DNA methylation playing a role in regulating *CIITA* promoter choice at pI through a combination of indirect and direct means. Examination of the *CIITA* locus in murine T cells reveals that there is silencing of *CIITA* in T cells in the mouse versus human system as a result of DNA methylation at pIII in the mouse (Schooten et al., 2005). This difference in methylation at the *CIITA* locus between mouse and human in the context of pIII demonstrates the need to verify that the differential DNA methylation at pI translates over to the human system from the mouse.

If DNA methylation is playing a direct role in regulating expression from pI, it is unclear as to whether it is a cause or consequence of the action of other transcription factors or histone marks present at the promoter. Determination of the repressive landscape at pI in lymphoid cells will aid the dissection of this order of events. The repressive histone mark H3K27me3 is found at pI in B cells (Yoon and Boss, 2010). G9a, an enzyme responsible for methylation of H3K27 as well as H3K9 (Tachibana et al., 2001) has been shown to be recruited to pI in myeloid cells (Smith et al., 2011), and has been independently shown to interact with DNMT1 in the context of maintenance of DNA methylation (Estève et al., 2006). Another possibility is silencing via either the polycomb repressive factor EZH2 or the master transcriptional regulatory Blimp-1. EZH2 has been shown to downregulate *CIITA* expression in the pIII (Yoon et al., 2012) as well as pIV systems (Truax et al., 2012). If it is

also present in the pI system, it is possible that it is recruiting DNA-methyltransferases to facilitate DNA methylation (Viré et al., 2006). Blimp-1 is also known to function across multiple CIITA promoters, and is present in both the pI (Smith et al., 2011) and pIII systems (Yoon et al., 2012). Blimp1 has been shown to interact with the histone lysine demethylase LSD1 (Su et al., 2009), which demethylates H3K4, which can then recruit DNMT3L (Ooi et al., 2007). There are therefore several mechanisms by which the repressive chromatin state at pI leads to the recruitment of DNA-methyltransferases, pointing to the likelihood that DNA methylation's presence at pI is a result of the repressive chromatin landscape.

Beyond examination of the regulation of *CIITA* itself, further studies into CIITA's role in the regulation of other genes must be considered. When examining the *CIITA* locus from the human perspective (Figure 1) the *CIITA* locus itself has been identified as a super-enhancer (Hnisz et al., 2013). This raises questions as to a potential role in a more global context of chromatin structure (which could be queried by HiC), and if this importance varies between APCs versus non-APCs. Additionally, this super-enhancer function could play a role beyond CIITA's integral role in the regulation of the MHC locus. Previously, the CIITA-negative RJ2.2.5 cells have been used to examine CIITA's role in the regulation of other genes (Nagarajan et al., 2002b). Additional studies in a variety of cell types have showed an inconsistent role of CIITA in the regulation of genes outside of the antigen presentation pathway (Krawczyk et al., 2008; Nagarajan et al., 2002b; Otten et al., 2006). RJ2.2.5 cells contain one completely deleted *CIITA* allele and the other allele with a 1.8kb internal deletion (Brown et al., 1995) and are effectively CIITA null. While the most profound alterations in gene expression are seen in the genes of the antigen presentation pathway, perhaps the genes outside of the antigen presentation pathway that demonstrate

inconsistent regulation by CIITA and are only slightly effected are contradictory as a result of loss of only some of (or in the cases of other cell types, none of) the super-enhancer effect of CIITA. The effect of CIITA regulation on these genes outside of the antigen presentation pathway might be elucidated by examination of a cell line completely null for the *CIITA* locus at both alleles, and then perhaps the super-enhancer effect could be determined. If the super-enhancer effect of the CIITA gene is playing a role in regulation of these genes, and not the gene product of this locus, an explanation for previously inconsistent data regarding CIITA's role in regulation of genes outside of the antigen presentation pathway might be generated.

In conclusion, in this study a number of new elements contributing to the regulation of *CIITA* were identified. Some of these elements appear to serve as transcription factor binding sites to allow increased accessibility to the local chromatin, while other sites have independent regulatory activity and interact with *CIITA* promoters. Most intriguingly, several of the elements are shared between cells of the myeloid and lymphoid lineages, defining a role for these elements as mediators of promoter choice through mechanisms including DNA methylation and other epigenetic processes in facilitation of this choice. DNA methylation appears to play a multifaceted role in the context of *CIITA* regulation, both in a direct and indirect fashion. Thus, while this study introduces new findings on the subject of promoter choice at the *CIITA* locus, it also provides a foundation upon which further studies can be conducted and synthesized into the body of existing knowledge.

FIGURES

Figure 1 – Comparison of the murine and human *CIITA* loci. The *CIITA* gene and its neighbors are shown for the mouse and human genomes. Alignment tracks from the UCSC genome browser show where genomic regions are conserved between mouse and human. Black boxes indicate ROIs from chapter 3 and pIV-interacting regions.

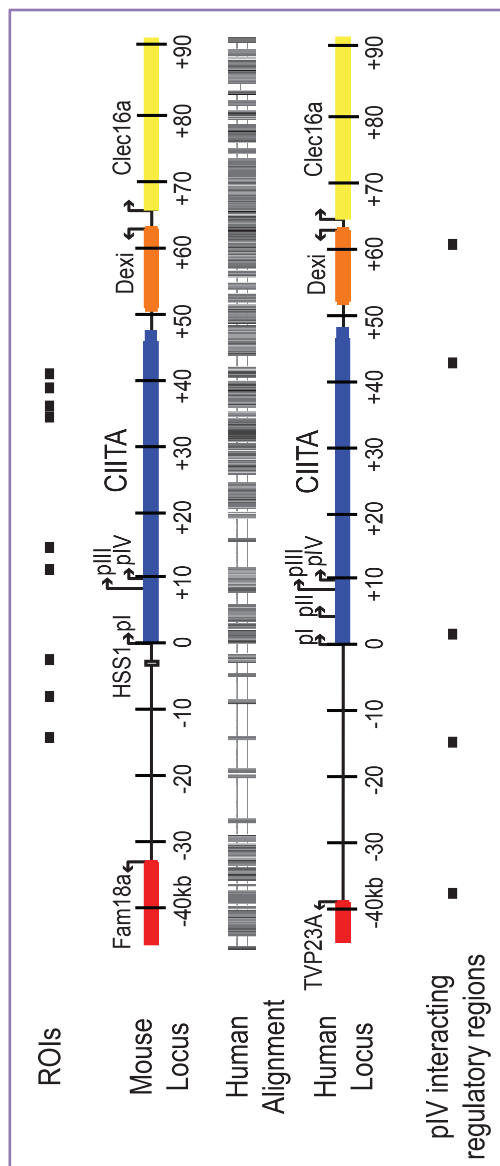


Figure 2 – Comparison of PU.1 binding between B cells and spDC. ChIP-seq data for PU.1 in B cells and macrophages at the *CIITA* locus (Heinz et al., 2010).

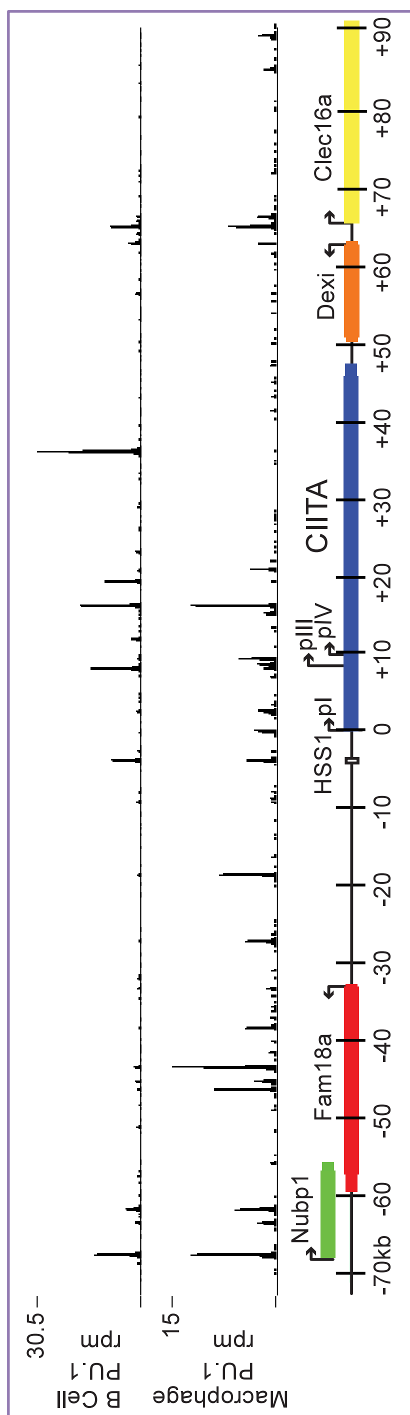
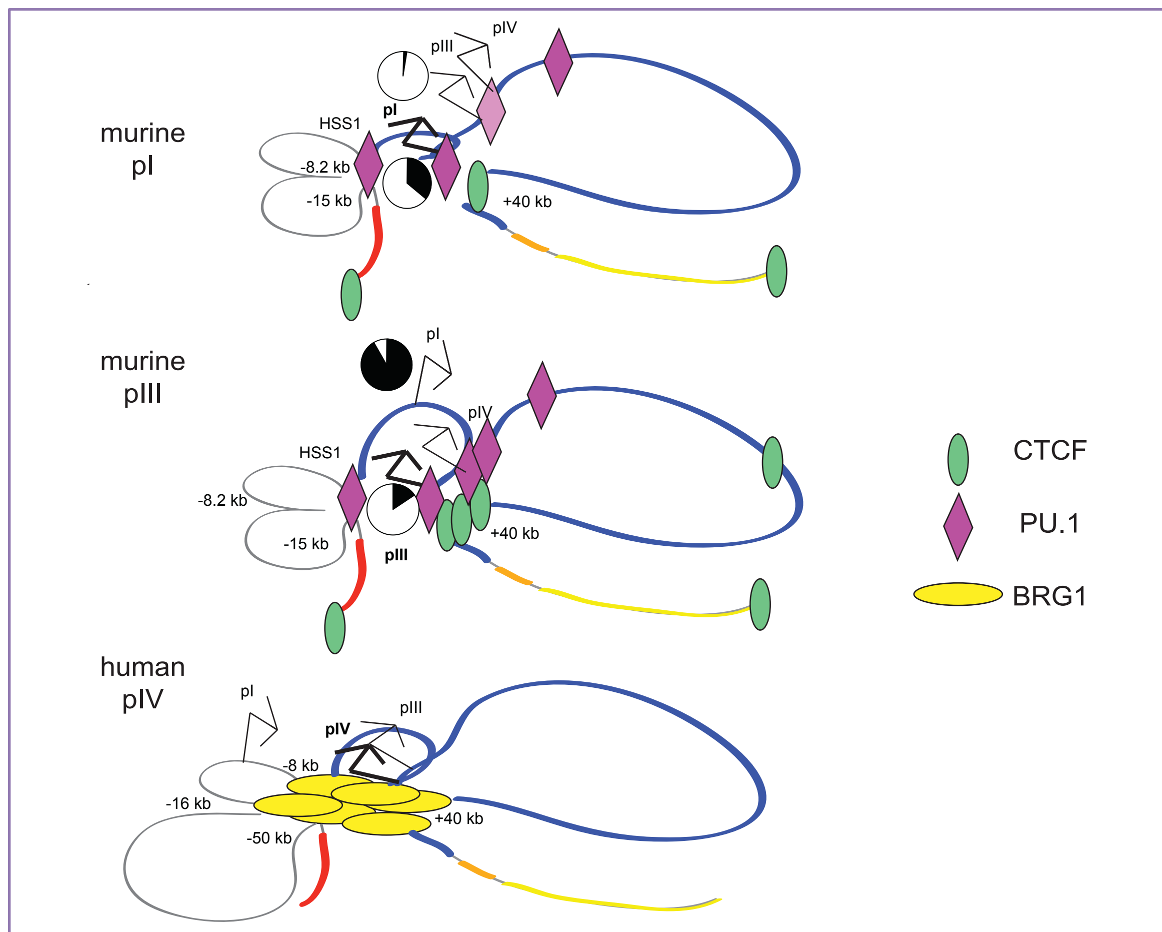


Figure 3 – Looping models for *CIITA* expressed from pI and pIII. Promoters are indicated by arrows, and red lines indicate *Fam18a*, blue *CIITA*, orange *Dexi*, and yellow *Clec16a*.



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