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## The Role of S7, A Subunit of the 19S Proteasome, in the Transcriptional Regulation of MHC II.

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**The Role of S7, a Subunit of the 19S Proteasome, in the Transcriptional Regulation  
of MHC II Genes**

by

Dawson Gerhardt

Under the Direction of Susanna Greer

**ABSTRACT**

Induction of an adaptive, or antigen specific, immune response is critical for eliminating most infections. Pathogen clearance is accomplished primarily, by the actions of CD4+ T cells through their ability to recognize foreign antigens presented at the cell surface by major histocompatibility class II (MHC II) molecules. Consequently, the capacity to regulate expression of MHC molecules is essential to control the adaptive immune response. MHC molecules are regulated at the level of transcription by a master regulator, the class II transcriptional activator, CIITA. Thus, the expression of MHC II is directly related to proper CIITA activity. This thesis focuses on the novel role of S7, an ATPase subunit of the 19S proteasome, in the transcriptional regulation of CIITA and MHC II molecules.

**INDEX WORDS:** MHC II, S7, proteasome, CIITA, adaptive immunity

**THE ROLE OF S7, A SUBUNIT OF THE 19S PROTEASOME, IN THE  
TRANSCRIPTIONAL REGULATION OF MHC II GENES**

by

DAWSON GERHARDT

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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in the College of Arts and Sciences

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### List of Abbreviations

<b>AAA</b>	ATPase Associated with Various Cellular Activities
<b>APC</b>	Antigen Presenting Cell
<b>ATP</b>	Adenosine Tri-phosphate
<b>ATPase</b>	Enzyme that catalyzes breakdown of ATP
<b>BCR</b>	B Cell Receptor
<b>BLS</b>	Bare Lymphocyte Syndrome
<b>bp</b>	Base Pair
<b>BRG</b>	Brahma-related Gene
<b>CBP</b>	Creb Bindind Protein
<b>cDNA</b>	Complementary DNA
<b>CIITA</b>	Class II Trans-activator
<b>CIITA PIV</b>	Class II Trans-activator Promoter IV
<b>CREB</b>	cAMP Response Element-Binding
<b>FAM</b>	6-carboxyfluorescein
<b>GAS</b>	Gamma Activted Sequence
<b>H2A</b>	Histone 2A
<b>H3</b>	Histone 3
<b>H4</b>	Histone 4
<b>HAT</b>	Histone Acetyltransferase
<b>HDAC</b>	Histod Deacetylase
<b>HLA</b>	Human Leukocyte Antigen
<b>IB</b>	Immunoblot
<b>IFN</b>	Interferon
<b>Ii</b>	Invariant Chain
<b>IP</b>	Immunoprecipitation
<b>IRE</b>	Interferon Response Element
<b>IRF</b>	Interferon Regulatory Factor
<b>JAK</b>	Janus Acitvated Kinase

<b>MHC I</b>	Major Histocompatibility Complex I
<b>MHC II</b>	Major Histocompatibility Complex II
<b>mRNA</b>	Messenger RNA
<b>MSS1</b>	Mammalian Suppressor of Sgv1
<b>NF-<math>\kappa</math>B</b>	Nuclear Factor kappa Beta
<b>NFY</b>	Nuclear Factor Y
<b>pCAF</b>	GCN5 Histone Acetyltransferase
<b>PCR</b>	Polymerase Chain Reaction
<b>PSMC2</b>	Gene encoding S7
<b>qPCR</b>	Quantitative PCR
<b>RFX</b>	Regulatory Factor X
<b>RNA Pol II</b>	RNA Polymerase II
<b>RNAi</b>	RNA Interference
<b>rRNA</b>	Ribosomal RNA
<b>RT-PCR</b>	Reverse Transcription Polymerase Chain Reaction
<b>SAGA</b>	Spt-Ada-Gcn5-acetyltransferase
<b>SDS-PAGE</b>	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<b>siRNA</b>	Small Interfering RNA
<b>STAT</b>	Signal Transducer and Activator of Transcription
<b>TAMRA</b>	6-carboxy-tetramethyl-rhodamine
<b>TBP</b>	TATA Binding Protein
<b>TCR</b>	T Cell Receptor
<b>TF</b>	Transcription Factor
<b>USF</b>	Upstream Regulatory Factor

## **Introduction**

### **The Vertebrate Immune System**

Driven by encounters with countless pathogens, the vertebrate immune system has evolved as a complex system of defense against infection (1, 2). The immune system of higher vertebrates is composed of two “arms” termed the innate and adaptive immune systems, with each arm consisting of distinct cell subsets and functions (1). The innate immune system, present in some form in all multicellular organisms, provides the first line of defense against the entry of pathogens and infection (1, 3). Conversely, the adaptive immune system has evolved only in higher vertebrates and provides a specific, albeit delayed, response to pathogens (3, 4). Also, unlike the innate immune response, cells of the adaptive immune response are able to establish memory to pathogens they have encountered, allowing for long term protection against previously encountered pathogens (3, 4). It is this specificity and immunological memory, the hallmarks of the adaptive immune response, that have made the advent of vaccines possible (3).

The innate immune system is composed of the physical barriers to pathogens including the skin and epithelial linings of internal organs, antimicrobial compounds on the skin, complement proteins in the blood, and specialized effector cells (3). Effector cells of the innate immune system, such as dendritic cells, macrophages, natural killer cells, mast cells, basophils, and eosinophils, function to recognize pathogens and contain infections (3, 5, 6). These effector cells are able to recognize a broad range of infectious agents via germline encoded cellular receptors, resulting in a rapid induction of an innate immune response (3).

A typical innate immune response occurs almost immediately after pathogen recognition and involves rapid recruitment of effector cells and inflammation mediated by the production of inflammatory cytokines (3). Although prompt, the innate immune response is not always sufficient to contain infection, making the activation of the adaptive immune response necessary (3). Induction of an adaptive immune response is facilitated by the innate immune system as recognition of harmful pathogens by innate immune cells triggers expression of costimulatory molecules and cytokines, both of which are needed to activate effector cells of the adaptive immune system (3, 4).

Clearly, by serving as the initial response to pathogens and through interactions with adaptive immune cells, the innate immune system is an essential component of host defense (6). It is, however, the adaptive, antigen specific immune response that is absolutely critical for eliminating pathogens and transformed cells, and without it humans would be susceptible to a range of diseases (4, 7). Underscoring the importance of the adaptive immune system, its existence has been suggested to be a key factor in the evolutionary development of humans (6).

The need for an adaptive immune response is partly due to the limited specificity for antigen recognition and lack of immune memory conferred by an innate immune response (4). Evolutionarily conserved receptors on innate immune cells recognize patterns that are common to pathogenic microorganisms, thus making them effective at distinguishing self from non-self (1). Cells of the adaptive immune system, on the other hand, contain non-germline encoded antigen receptors that, through a series of gene rearrangements, are able to recognize an almost unlimited amount of antigenic structures (1). Once receptors of adaptive immune cells recognize a specific antigen, they are able

to clonally divide, creating a set of immune cells with identical antigen specificity and an amplified response against pathogens (3). Of equal importance to the diversity of antigens they can recognize, cells of the adaptive immune system provide for a mechanism of long term protection from any pathogens they have previously encountered (8). This trait, termed immunological memory, enables adaptive immune cells to establish a memory of encountered antigens, thus allowing them to rapidly and specifically respond to subsequent encounters with the same antigen (3).

The adaptive immune system effects its protective functions via specialized effector cells following antigen driven activation (8). In a disease free context, activation of effector cells occurs only after recognition of a specific antigen by the effector cell receptors and appropriate costimulatory signals (8). Effector cells of the adaptive immune response are referred to as lymphocytes and include antibody secreting B cells and effector T cells such as cytotoxic CD8 T cells, regulatory T cells, and CD4 helper T cells (2). T lymphocytes are defined as CD4 or CD8 based on the presence of the CD4 or CD8 receptors (9). CD4 T cells can be further subdivided into Th1 and Th2 helper cells, based upon their cytokine secretion profile (2). Both B and T lymphocytes have membrane bound receptors, respectively called B cell receptors (BCR) and T cell receptors (TCR), that recognize specific antigens (4, 8). This wide range of antigen recognition by BCRs and TCRs is achieved by mutations and rearrangement of the genes encoding the receptors (1).

Although similar mechanisms give rise to the structural variety of lymphocyte receptors, they have different modes of antigen recognition (10). BCRs can recognize intact macromolecules and proteins, but TCRs are only able to recognize antigen

fragments complexed with major histocompatibility complex (MHC) molecules on self cells (10). There are two classes of MHC molecules, class I and II, present on human cells (4). These different classes of MHC molecules, which are present on different types of cells, vary in the antigens they are able to present and the type of T cell they bind (4). Recognition of antigen by a BCR or TCR-MHC complex, in addition to costimulatory signals, is necessary to activate the lymphocytes and hence the adaptive immune response (10). Activation of a B or T lymphocyte induces an intracellular signaling cascade leading to transcription of multiple genes that, ultimately, results in proliferation of the activated T or B cell and secretion of appropriate cytokines (8, 10).

Activation of B and T lymphocytes, both the CD4 and CD8 subsets, is needed to effect an adaptive immune response, but each of these lymphocytes have very different functions (8, 10). B cells are specialized to produce soluble antibodies that specifically bind to extracellular pathogens (8, 10). CD8, or cytotoxic, T cells directly kill virally infected and transformed cells after recognition of MHC I-complexed intracellular antigen fragments presented on the target cell surface (10, 11). CD4 helper T cells are able to recognize extracellular antigens complexed to MHC II molecules and function mainly to stimulate proliferation and activation of B cells and CD8 T cells (8, 10). CD4 T cells also play a role in establishing immunological memory by promoting the generation of CD8 memory T cells (2).

As CD4 T cells play large roles in the activation of both B cells and CD8 T cells, and in the generation of CD8 memory cells, their function is central to the induction and maintenance of adaptive immunity (2). For instance, children with the bare lymphocyte syndrome (BLS), a hereditary immunodeficiency, succumb to opportunistic infections

solely because their CD4 T cells cannot be activated (12). The importance of CD4 T cells in the adaptive immune response is additionally illustrated by the extensive immunosuppression following CD4 T cell depletion by HIV infection (13). The extreme cases of immunodeficiency described above highlight the fact that proper activation and function of CD4 T cells is absolutely necessary for the development of an adaptive immune response (7).

Along with being key players in the control of infections, CD4 T cells are emerging as important components of the anti-tumor response (2). Recently, abundant evidence supporting the concept that a host immune system can suppress tumor growth has been put forward (11, 14). Historically, CD8 T cells were thought to be singularly responsible for eliminating tumor cells, but anti-tumor therapies limited only to CD8 T cell activation have had limited success (2, 11). A plausible explanation for this is the necessity for CD4 T cell activation to enhance the proliferation of CD8 T cells and induce immunological memory (2). Interestingly, studies not only suggest that activation of CD4 T cells is necessary to stimulate the anti-tumor effector functions of CD8 T cells, but also that activated CD4 T cells are able to recruit other immune cells with anti-tumor activity (15, 16).

Given the findings highlighted above, the following three points regarding the adaptive immune response should become evident. The first is that the adaptive immune response is absolutely essential to survival, both by controlling infections and growth of tumors that evade the innate immune response (2, 5). The second is that CD4 T cells play an indispensable role in activating and maintaining an adaptive immune response (2). The third is that, by virtue of the dependence of lymphocytes on stimulation by CD4 T cells,

an effective adaptive immune response is initiated only when CD4 T cells are activated by the recognition of a specific antigen (8).

### **Activation of CD4 T cells**

Activation of CD4 T cells is mediated by recognition of specific antigen fragments on antigen presenting cells (APC) by the T cell receptor (TCR) (13). Antigen presenting cells include B cells, dendritic cells (DC), macrophages, and thymic epithelial cells (13). Critical for APC mediated antigen presentation to CD4 T cells are major histocompatibility class II (MHC II) molecules, as the TCR of CD4 T cells can only recognize antigen fragments complexed to MHC II molecules on the surface of antigen presenting cells (17). MHC II molecules are heterodimeric, transmembrane, glycoproteins constitutively expressed on the surface of antigen presenting cells and inducibly expressed on all nucleated cells during an immune response. (12)

Encoded by the polymorphic human leukocyte antigen (HLA) genes on chromosome 6, the function of MHC II molecules is to present exogenously derived peptides on the surface of cells (4, 18). The three main isotypes of MHC II molecules encoded by these genes and expressed on the surface of human cells are HLA-DR, HLA-DP, and HLA-DQ (18). These different isoforms of MHC II molecules vary in the peptide antigens they are able to present to CD4 T cells, thus increasing the variety of antigens presented (18). HLA-DM and HLA-DO are also encoded by the HLA genes, but they are cytoplasmic and involved in peptide loading on MHC II molecules(4).

In order for MHC II to properly present antigens, co-expression of two additional and closely related genes, invariant chain (Ii) and HLA-DM, is also needed (19). The invariant chain plays an integral role in antigen presentation by MHC II by preventing

loading of endogenous peptides in the endoplasmic reticulum (ER) and HLA-DM promotes loading of foreign peptides(19). The other class of MHC molecules on human cells, MHC class I, are present on all nucleated cells and are specialized to present intracellular antigen fragments to cytotoxic CD8 T cells (4) .

MHC II mediated presentation of antigen to the TCR of CD4 T cells leads to the activation and proliferation of T cells, initiating an immune response against the presented antigen (7). Additionally, ligation of MHC II molecules and CD4 TCRs increases expression of cytokines and co-stimulatory molecules needed to effect an immune response and clear infection (20). Due to the fundamental role of MHC class II molecules initiating the adaptive immune response, defects in MHC class II expression lead to severe consequences including an inability to respond to foreign antigens, increased incidence of autoimmune disease, and impaired anti-tumor responses (2, 12). Specific examples of diseases resulting from defective MHC II expression include diseases resulting from a lack of MHC II expression as in the Bare Lymphocyte Syndrome and diseases resulting from aberrant or excessive MHC II expression which include insulin-dependent diabetes mellitus and multiple sclerosis (4, 12).

### **Regulation of MHC II**

The range of diseases associated with abnormal expression of MHC II molecules necessitates the tight regulation of their expression (12). MHC class II molecules are constitutively expressed on the surface of antigen presenting cells, thymic epithelial cells and activated T cells (12). Expression of MHC II molecules can also be induced in all nucleated cells by stimulation with the cytokine interferon-gamma (IFN- $\gamma$ ) during an immune response (21). IFN- $\gamma$  is a type II interferon that, through binding to the type II

IFN receptor, activates transcription of target genes such as MHC II (22). Inducible expression of MHC class II allows for enhanced antigen presentation and induction of a localized immune response (12).

MHC class II expression, both constitutive and IFN- $\gamma$  induced, is tightly controlled at the level of transcription by a regulatory region located 139 base pairs upstream of the transcription initiation site on the MHC II gene (4, 12). Initiation of MHC II gene transcription, similar to other eukaryotic genes, is a particularly complex event due to the vast array of proteins that must interact with DNA in a spatially and temporally restricted manner (23). In order to be contained in the eukaryotic nucleus, DNA must exist in a the highly compact structure of chromatin, which is achieved by DNA being wrapped around nuclear histone proteins (23-25). For transcriptional machinery to access DNA, the histone proteins of chromatin must be modified by enzymes to produce an “open” chromatin state (23-25). A typical chromatin modification associated with “open” chromatin is histone acetylation, but many other chromatin modifications have been identified (23-25).

MHC II transcriptional control is mediated by many transcription factors and chromatin remodeling enzymes that bind to a conserved regulatory region in the MHC II gene(4, 12). This regulatory region consists of the conserved sequences designated as the S box, X1 box, X2 box, and Y box (4). These sequences are respectively recognized by the ubiquitously expressed DNA binding factors regulatory factor X (RFX), c-AMP responsive element-binding protein (CREB), and nuclear factor Y (NF-Y) (4). Both RFX and NF-Y are trimeric, with the RFX trimer consisting of RFX-5, RFX-AP, and RFX-ANK. The NF-Y trimer consists of NF-YA, NF-YB, and NF-YC (4).

Believed to be initiated by binding of RFX5, the nucleoprotein complex formed upon binding of RFX, CREB, and NF-Y to the DNA sequences in the MHC II regulatory region is referred to as the MHC class II enhanceosome (20). Enhanceosome formation is necessary, but insufficient for initiation of MHC class II transcription (26, 27). Instead, the enhanceosome serves as a platform for the recruitment and binding of the class II transactivator (CIITA) (7, 27). CIITA is referred to as the Master Regulator of MHCII expression, as it has been found to be responsible for driving the activation and transcription of class II MHC, Ii (invariant chain), and HLA-DM genes (4, 19). Furthermore, expression of MHC II directly parallels expression of CIITA, and lack of functional CIITA results in absence of MHC II expression, even in the presence of all other requisite transcription factors (12, 28).

CIITA, like MHC II, is constitutively expressed in APCs and exhibits IFN- $\gamma$  inducible expression (4). CIITA is not a DNA binding protein, but instead binds to the assembled enhanceosome complex via protein-protein interactions and functions to stabilize binding of CREB, NFY, and RFX, over their MHC class II proximal promoter elements (29, 30). In addition to stabilizing the enhanceosome, CIITA recruits general transcriptional machinery, promotes chromatin remodeling of the MHC II promoter, and facilitates transcriptional elongation (4, 29). Specifically, CIITA has been shown to interact with TF<sub>II</sub>D components, TF<sub>II</sub>B, TATA-binding protein (TBP), and TAF<sub>II</sub>32 to initiate transcription, and with the positive transcription elongation factor b (P-TEFb) (4, 31). CIITA binding to the MHC II enhanceosome also promotes transcriptionally conducive chromatin architecture of the MHC II promoter through association with chromatin remodeling proteins such as the histone acetyl transferases (HATs) pCAF and

CBP/p300, histone deacetylases (HDACs), and the ATP-dependent BRG-1(4, 21, 32). These various functions of CIITA allow for precise regulation of MHC II expression at the levels of enhanceosome stabilization, efficient recruitment of transcriptional machinery, and accessibility of the MHC II promoter. Each of these functions is critical for constitutive and cytokine inducible MHC II expression and thus the initiation and maintenance of an adaptive immune response.

### **Regulation of CIITA**

In order to maintain tight regulation of cell specific and cytokine inducible MHC II expression, CIITA expression is also tightly regulated. Like MHC II, CIITA is constitutively expressed in professional APCs and can be induced by stimulation with IFN- $\gamma$  (28). As CIITA is the master regulator of MHC II, IFN- $\gamma$  induced transcription of MHC II follows that of CIITA, with CIITA mRNA present 2 hours after IFN- $\gamma$  stimulation and MHC II transcripts appearing 12-16 hours later (28, 33).

CIITA is encoded by the gene *MHC2TA* and is initially regulated at the transcriptional level (4). Transcription of CIITA is regulated in a cell specific manner by four different promoters termed PI, PII, PIII, and PIV (28). By virtue of different transcriptional start sites, each promoter drives expression of a unique first exon (28, 34). Subsequent translation of these transcripts yields three CIITA isoforms (4, 28, 35). CIITA promoter I drives expression of CIITA in dendritic cells, while CIITA PIII regulates CIITA expression in B cells (28). IFN- $\gamma$  inducible expression of CIITA is regulated by CIITA PIV (28).

IFN- $\gamma$  induced transcription of CIITA, which allows for enhanced antigen presentation by all nucleated cells, is achieved by binding of appropriate transcription

factors to specific DNA sequences of CIITA PIV (28). These sequences include a gamma-activated sequence (GAS) element, an E-box, and an interferon regulatory factor 1 (IRF-1) binding element (IRE) (28). These elements are bound, respectively, by the transcription factors signal transducer and activator of transcription (STAT1), upstream stimulating factor 1 (USF-1), and IRF-1(4, 28, 36). Activation of the inducible CIITA PIV is initiated by binding of  $\text{INF-}\gamma$  to the type II IFN cell surface receptor and occurs through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal transduction pathway (36).

Owing to the inducible nature of CIITA PIV activation, transcription only occurs in the context of an active immune response (3, 28). Typically, this  $\text{INF-}\gamma$  is produced by dendritic cells during the innate immune response (3). Dendritic cells, similar to other cells of the innate immune system, have receptors that recognize patterns common to pathogens and, following pathogen recognition, rapidly transcribe  $\text{INF-}\gamma$  (11).  $\text{INF-}\gamma$  secreted by innate immune cells binds to receptors on the adaptive immune lymphocytes and initiates transcription of interferon responsive genes in the following sequence (11). First,  $\text{INF-}\gamma$  binding results in receptor dimerization, with activation of the JAK1 and JAK2 tyrosine kinases following (36). Activated JAK1 and JAK2 phosphorylate STAT1, causing STAT1 to translocate to the nucleus(36, 37). In addition to binding to the GAS box of CIITA PIV, translocation of STAT1 to the nucleus induces the expression of IRF-1(4). Binding of both STAT1 and USF-1 to CIITA PIV have previously been shown to occur within minutes of  $\text{INF-}\gamma$  stimulation while IRF-1 binding exhibited a one hour delay (38).

Transcriptional regulation of IFN- $\gamma$  induced CIITA expression is also regulated by the chromatin state of CIITA PIV (36). In addition to promoting binding of transcription factors to CIITA PIV, IFN- $\gamma$  has been shown to induce acetylation of histones within the promoter, thus making the promoter more accessible (38). Further evidence indicates that IFN- $\gamma$  induced expression of CIITA is dependent on the remodeling activity of the ATP-dependent chromatin remodeling protein, BRG-1 (36, 38).

Regulation of CIITA expression at the transcriptional level serves as an initial step in regulating CIITA and, consequently, MHC II expression, but the function of CIITA is also regulated by various post-translation modifications (4). Post-translation modifications to CIITA, such as phosphorylation, acetylation and ubiquitination, affect the localization and activity of CIITA (4, 39). Modifications to CIITA are particularly important for transcription of MHC II, as it has been found that unmodified CIITA is not recruited to the MHC II enhanceosome (30). Although specific post-translational modifications to CIITA have been identified, the exact mechanism of regulation of CIITA binding to MHC class II promoter has not been elucidated (30). It has, however, been shown that the monoubiquitination, the covalent attachment of a single ubiquitin molecule to lysine residues, of CIITA may increase CIITA activity (4, 30).

### **Ubiquitin as a Transcriptional Regulator**

The 76 amino acid protein, ubiquitin, was first discovered as a modifying protein that was covalently attached to lysine residues of target molecules (40). Historically, linkage of ubiquitin molecules to target proteins was thought to mark proteins for degradation through the ubiquitin-proteasome pathway (40). Currently, however, many

studies have implicated ubiquitin and components of the proteasome as transcriptional regulators, independent of their roles in proteolysis(40).

Monoubiquitination is achieved in an ATP dependent reaction through a covalent linkage of a single ubiquitin moiety to lysine residues on a target protein (41, 42).

Ubiquitin molecules themselves contain several lysine residues, allowing for formation of ubiquitin chains, or polyubiquitination (41). It has been well established that polyubiquitination of a protein resulting in the addition of four ubiquitin groups targets the protein for proteasomal destruction by the 26S proteasome. Recent studies however have demonstrated that ubiquitination also regulates other cellular processes such as DNA repair, endocytosis, protein interactions, histone modifications, and nuclear export (41). Additionally, although ubiquitin targets many transcription factors for proteasomal destruction, there is also an emerging role for ubiquitin in transcriptional regulation that is independent of proteasomal protein destruction (40).

One of the earliest observations tying ubiquitin to transcriptional regulation was the identification of histone H2A as the first ubiquitinated protein, consequently linking ubiquitination to chromatin modifications (43). It is well known that chromatin, the complex formed by association of DNA with nuclear histone proteins, is subjected to modifications such as acetylation, methylation, ubiquitination and phosphorylation in order to become accessible or “open” for transcription (44). Thus, in the context of chromatin, monoubiquitination could regulate transcription via chromatin structure.

Ubiquitin also influences transcription through regulation of transcription factors(44). Monoubiquitination can effectively serve as a protein modifier as it allows for a specific method for recognition of modified proteins, and removal of ubiquitin by

deubiquitinating enzymes allows for a reversible reaction (41). In these ways, ubiquitination resembles phosphorylation, possibly playing similar regulatory roles (45). Through the ubiquitin-proteasome pathway, ubiquitination can affect transcription factor location, as in the case of NF- $\kappa$ B where proteasomal degradation of the NF- $\kappa$ B inhibitor, I $\kappa$ B $\alpha$ , allows NF- $\kappa$ B to translocate from the cytoplasm to the nucleus, or levels of transcription factors (43). Relevant to this work is the recent observation that ubiquitination also affects the activity of CIITA in a manner independent of proteolysis (30). In these studies by Greer et al., monoubiquitination enhanced CIITA activity and recruitment to the MHC II promoter (30).

Given the findings of the non-proteolytic relationship of ubiquitin to transcriptional regulation and the ability of the 26S proteasome to specifically recognize ubiquitinated proteins; recent work has focused on the possibility that components of the 26S proteasome may contribute to a nonproteolytic role in transcription (46). In fact, many of these studies do indicate that subunits of the 26S proteasome play non-proteolytic roles in transcription ranging from transcription initiation, recruitment of activators, mediating modifications to chromatin, and promoting transcriptional elongation (47).

### **Proteasomal Subunits as Transcriptional Regulators**

The 26S proteasome is believed to be the principal intracellular eukaryotic protease and is responsible for degrading polyubiquitinated cellular proteins (48, 49). This proteolytic role of the 26S proteasome makes it an essential regulator of the cell cycle and also contributes to its previously demonstrated roles in antigen presentation by degrading intracellular, ubiquitinated substrates for presentation on MHC class I

molecules (8, 49). The 26S proteasome is a 2000 kD, ATP-dependent enzyme and is assembled from two complexes: the 20S core particle and the 19S regulatory particle (RP) (49, 50).

The 20S core, which catalyzes proteolysis of substrate proteins, is flanked on either end by the 19S regulatory particle (25, 49). The 20S core can exist as a separate entity from the 26S proteasome, although it cannot degrade ubiquitinated proteins unless associated with the 19S (25). The 20S proteasome is comprised of 4 stacked rings, with each ring containing 7  $\alpha$ -type of  $\beta$ -type subunits (51). Accordingly, these 20S subunits are named  $\alpha$ 1-7 and  $\beta$ 1-7 (51). In the presence of ATP, the 20S catalytic core associates with the 700 kD 19S regulatory particle to form the 26S proteasome, allowing for the recognition of ubiquitinated substrates (51).

The 19S regulatory particle, which channels polyubiquitinated substrates to the 20S core, is composed of at least 18 subunits and can itself dissociate into a “lid” and “base” (49, 51). Six of the 19S subunits are ATPases contained in the “base” of the 19S RP, named Rpt1-Rpt6 in yeast, and are members of the AAA (ATPases associates with a variety of cellular activities) protein family (52). Other proteins that belong to the AAA protein family are involved in functions such as cell-cycle control, proteolysis, and transcription (52).

The mammalian 19S ATPases are designated S7, S4, S6a, S10b, S6b, and S8 and correspond, respectively, to yeast homologs Rpt1-Rpt6 (51). Although these ATPases are considered to be significantly homologous, studies in yeast have shown that deletion mutants of any single subunit is lethal, indicating non-redundant functions (51). The 19S ATPases are believed to recognize ubiquitinated proteins and act as chaperones to unfold

substrates, but they also have motifs suggesting an ATP-dependent RNA/DNA helicase activity (51, 52). These potentially diverse functions of the 19S ATPase subunits indicate that they may be involved in cellular processes independent of proteolysis. Interestingly, before they were identified as proteasomal subunits, the ATPases S7, S8 and S10b were thought to function as transcription factors (53, 54). Despite these initial findings, once they were identified as proteasomal subunits, the only role in transcription ascribed to these ATPases was a proteolytic one (55-57). However, in light of accumulating data demonstrating a non-proteolytic role for the 19S ATPases in transcriptional regulation, this view appears to be changing again (25, 47, 55, 58).

A majority of the current data implicating 19S ATPases as non-proteolytic transcriptional regulators comes from experiments in yeast. Notable among these yeast studies are those performed by Johnston et. al, which show 19S ATPases associated with general transcription factors, RNA polymerase II, activated promoters, and transcriptional elongation (59-62). Key to these studies was the finding that only 19S ATPases, not components of the 20S proteasome, were linked to transcriptional activation (59-62). Providing additional support to a non-proteolytic function of 19S ATPases in transcriptional regulation are findings tying the ATPases to modulation of chromatin structure (63). Two important observations are that S8 and S10b are necessary for an activating modification to chromatin and that 19S ATPases can enhance the recruitment of the yeast histone acetyltransferase, SAGA, to gene promoters (63, 64).

## Specific Aims

Precise regulation of MHC II expression is necessary to achieve an effective immune response when confronted with harmful pathogens, to limit the occurrence of autoimmune diseases, and to mount an optimal anti-tumor response (2, 12, 65). Consequently, the control of MHC II expression to better treat disease is an attractive endeavor. Being that MHC II expression is regulated entirely at the level of transcription, modulating its expression demands a thorough understanding of events at the MHC II promoter (65, 66). Recent findings such as the enhancement of CIITA, the master regulator of MHC II, activity by monoubiquitination and the ability of 19S proteasomal subunits to function non-proteolytically as transcriptional regulators suggest a link between the ubiquitin-proteasome system and the transcriptional regulation of MHC II (30, 53, 60, 62, 67).

Although abundant data supporting non-proteolytic functions of 19S proteasomal subunits in regulating transcription in yeast cells are emerging, there remains a deficiency of similar studies involving mammalian cells (59, 60, 62-64). We hypothesize that S7, a mammalian ATPase subunit of the 19S proteasome, regulates MHC II transcription in a proteolytically independent manner. The experiments detailed below were designed to examine the novel, non-proteolytic function of proteasomal subunits in the transcriptional regulation of mammalian genes. Specifically, studies were directed at elucidating the non-proteolytic function of S7 in the transcriptional regulation of MHC II. The function of S4, an 19S ATPase that forms a heterodimer with S7, in regulating MHC II transcription was also studied (67).

## **Materials and Methods**

### **Cells and Tissue Culture**

Hela cells obtained from ATCC (Manassas, VA) were maintained using high-glucose Dulbecco modified Eagle (DMEM) medium (Mediatech Inc., Herndon, VA) supplemented with 10% FCS, 50U/ml of penicillin, 50µg/ml of streptomycin and 2mM of L-glutamine. The cells were grown in conditions of 37°C with 5% CO<sub>2</sub>.

### **Plasmids and Reagents**

HLA-DRA luciferase, pCDNA3 and Flag tagged CIITA, RFX5, and NFY-A expression vectors were previously prepared by SFG (68). Monoclonal antibodies anti-S7, Anti-S4, Anti-α4, and Anti-S10b were obtained from Biomol (Plymouth Meeting, PA) and Anti-IRF1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody anti-CIITA was generated as previously described (30).

### **Construction and Transfection of Transient Small Interfering RNA (siRNA)**

The siRNA sequence (5'-AACTGCGAGAAGTAGTTGAAA-3') (Qiagen, Valencia, CA) targeting S7 (PSMC2) corresponded to nucleotides 627-647. The sequence was designed to have GC content of 35-55 % and was blasted with NCBI nucleotide BLAST to ensure limited off-target sequence homology. Positive-silencing control siRNA (Quiagen) targeting the nuclear membrane protein Lamin was used to test for transfection efficiency. As a negative control to test for the non-specific effects of siRNA transfection, a non-silencing, scrambled sequence siRNA was used (Quiagen). For transfections in 6-well plates, cells were first trypsinized and seeded at a density of  $4 \times 10^4$  cells per well. 24 hours after cells were plated, siRNA (.675 µg) targeting S7 or

control non-silencing siRNA was transfected using RNAifect transfection reagent (Quiagen). The RNAifect transfection reagent was used at a ratio of 6  $\mu$ l transfection reagent to 1  $\mu$ g siRNA. Cells were lysed 72 hours after siRNA transfection with 100  $\mu$ l of 1% Nonidet P-40 buffer ( NP-40:1M Tris pH8.0, 1M KCl, 10%NP40, 0.5M EDTA, 5M NaCl, 1M DTT, dH<sub>2</sub>O) with protease inhibitor (Roche). The cell lysates were analyzed by western blot for S7 knockdown. For transfections in 10 cm plates, cells were first trypsinized and seeded at a density of  $4 \times 10^5$  cells per plate. 24 hours after cells were plated, siRNA (6  $\mu$ g) targeting S7 or control non-silencing siRNA was transfected using RNAifect transfection reagent (Quiagen). Cells were lysed 48 hours following siRNA transfection.

Transfection of S4 siRNA was performed in exactly the same manner as S7. The sequence targeted by the S4 specific siRNA was 5-AATCCCTGTTCCCACTGATTT

#### **Luciferase Reporter Assay for CIITA Activity in the Presence of the S7 Knockdown**

Hela cells were plated in 6 well plates at  $4 \times 10^4$  cells per well and were incubated at 37°C. 24 hours after cells were plated, transfection of siRNA with .675  $\mu$ g of S7 siRNA, negative control siRNA, or transfection reagent alone (NT) was performed according to the Qiagen protocol. 48 hours following siRNA transfection, transfection of the HLA-DRA-Luc (100 ng), CIITA (500 ng) and pCDNA3 (500 ng) plasmids was carried out using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. 24 hours following transfection of HLA-DRA-Luc and CIITA, and 72 hours after transfection of siRNA, cells were lysed with 100 $\mu$ l of 1x cell culture lysis reagent for 15 minutes (Promega, Madison, WI). Cells were then scraped and the cell suspension was centrifuged for 2 minutes at 12,000 rpm at 4°C (Thermo electron 851, Thermo INC,

Needham Heights, MA). Approximately 80 % of the cell lysates were frozen at -80 C and used for quantification of protein concentration and western blot analysis. 20 µl of the supernatant was added to a 96-well luciferase assay plate, the luminometer was primed with 1200 µl of luciferase reagent (Promega) and 100 µl of reagent was injected into reaction wells and the corresponding luciferase reading was recorded.

### **Western Blotting**

Cells were lysed on ice with 1% NP40 with protease inhibitors. To ensure equal loading of protein, protein concentration was quantified using Bradford reagent (Pierce). Concentrations were measured using a Spectrophotometer (BioRad). Following addition of Laemmli buffer, lysates were boiled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following separation by SDS-PAGE, gels were transferred to nitrocellulose membrane and incubated with the indicated primary and HRP conjugated secondary antibodies. HRP conjugates were detected with Supersignal West Pico Chemiluminescent substrate (Pierce).

### **Co-Immunoprecipitations**

For detection of endogenous S7 or S4 associations with products from transfected plasmids,  $8 \times 10^5$  HeLa cells were plated in 15cm tissue culture plates. 24 hours later, cells were transfected with 5µg of pCDNA3, Flag-CIITA, Flag-RFX5 or Flag-NFY-A using Fugene 6 (Roche) according to the manufacturer's instructions. Approximately 6hrs following transfection, cells were stimulated with IFN- $\gamma$  (25 µg/ml) at indicated time points. Cells were scraped, washed with PBS and lysed in 1% Nonidet P-40 lysis buffer with protease inhibitors (Roche) for 30 minutes on ice. Lysates were centrifuged, normalized for protein concentration, and pre-cleared for 30 minutes with 25µl mouse

IgG (Sigma-Aldrich, Saint Louis, MO). 20  $\mu\text{g}$  of lysates were frozen at  $-80\text{ C}$  to be used as a loading control. The remaining lysate was then immunoprecipitated with 5  $\mu\text{g}$  of mAb against S7 (Biomol), S4 (Biomol) or  $\alpha 4$  (Biomol). Control samples were immunoprecipitated with 50  $\mu\text{l}$  protein G (Pierce) beads alone. At least 2 hours following addition of immunoprecipitating antibody, 50  $\mu\text{l}$  of protein G beads was added to isolate immune complexes. The non-immunoprecipitated lysates and immune complexes were then denatured with Leammli buffer, boiled and separated by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose and individually immunoblotted with HRP conjugated anti-Flag monoclonal antibody (Sigma-Aldrich). HRP conjugates were detected with Supersignal West Pico Chemiluminescent substrate. Equal loading was determined by western blot of the non-immunoprecipitated lysates.

For co-immunoprecipitation of endogenous proteins, the above protocol was used with the following alterations:  $2 \times 10^6$  HeLa cells were plated in 15cm plates and lysed 24 hours later with 1% NP40 alone or in combination with 1% RIPA buffer. Control samples were immunoprecipitated with Protein G beads only. Specific primary and secondary antibodies were used for immunoblots as indicated.

#### **Detection of mRNA Expression Levels in S7 and S4 Knockdowns**

$4 \times 10^5$  HeLa cells were plated in 10cm tissue culture plates. 24 hours later, cells were transfected with 1.5  $\mu\text{g}$  specific siRNA, non-silencing siRNA, or transfection reagent alone. 24 hours following siRNA transfection, cells were stimulated with IFN- $\gamma$  (25  $\mu\text{g}/\text{ml}$ ) for 16 hours and 20 hours. Approximately 15% of the cells were then lysed with 1% Nonidet P-40 buffer with protease inhibitor (Roche) and analyzed as above by western blot for S7 knockdown. RNA was extracted from the remaining cells. Cells

were washed with cold PBS, centrifuged at 3,000 rpm at 4°C for 5min and resuspended in 1ml of Trizol reagent (Invitrogen, Carlsbad, CA). RNA was extracted according to the manufacturer's instructions, reconstituted in 30µl DEPC water (MP Biomedical, LLC, Aurora, OH) quantified and stored at -80°C. Using the Omniscript reverse transcription kit (Qiagen), RNA (1µg) was then used to make 20µl of cDNA. Gene specific antisense primers (Sigma, Saint Louis, MO) were used for reverse transcription (RT). RT reactions were performed in an ependorf microcycler with a 10 minute incubation at 65°C followed by a 60 minute incubation at 37°C according to the manufacturer's instructions (Qiagen). Real-time PCR reactions were performed on an ABI prism 7900 (Applied Biosystems, Foster City, CA). MHC class II and CIITA promoter IV probes were labeled with 6-carboxyfluorescein (FAM) reporter dye at the 5' end and with N,N,N,N-tetramethyl-6-carboxyrhodamine (TAMRA) quencher dye at the 3' end. The primer and probe sequences are as follows: MHC class II sense sequence, 5'-AA GCCAACCTGGAAATCA-3'; antisense sequence, 5'-GGCTGTTCGTGAGCAC AGTT-3'; probe sequence, 5'-6 FAM-CTCCGATCACCAATGTACCTCCAGA-TAMRA-3'; human CIITA pIV sense sequence, 5'-GGGAGAGGCCACCAGCAG-3' ; antisense sequence, 5'-GCTCCAGGTAGCCACCTTCT-3' ; probe sequence, 5'-6 FAM-CTGTGAGCTGCCGCTGTTCCC-TAMRA-3'.

18S ribosomal mRNA was generated and used as a control for real-time PCR reactions. 18S rRNA cDNA samples were diluted 1:500 in DEPC-treated water (MP Biomedical, Solon, OH). 18S rRNA probes were labeled with tetrachloro-6-carboxyfluorescein (TET) reporter dye at the 5' end and with TAMRA quencher dye at the 3' end. Primer and probe sequence for 18S rRNA are as follows: sense sequence, 5'-

GCTGCTGGCACCAGACTT-3'; antisense sequence, 5'- CGGCTACCACATCCA AGG-3'; probe sequence, 5'-6 TET-CAAATTACCCAC TCCCGACCCG-TAMRA-3'. Real-time reactions were performed in triplicate. Standard curves amplifying 18S rRNA, MHC class II and CIITA pIV were used. Standard curves were generated by amplifying genomic DNA with gene specific primers. These target DNA sequences were then inserted into plasmid. Data obtained from real-time PCR was analyzed using the SDS 2.0 program.

### **Chromatin Immunoprecipitation**

$2 \times 10^6$  HeLa cells were plated on 15cm tissue culture plates. Approximately six hours after cells were plated; they were stimulated with IFN- $\gamma$  (25 $\mu$ g/ml) for 18, 4, or 2 hours. Cells were then crosslinked with 1% formaldehyde for 10 minutes at room temperature. To stop crosslinking, 0.125M glycine was added for at least 5 minutes at room temperature. Cells were lysed in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris pH8.0, dH<sub>2</sub>O, Protease inhibitor added fresh) for 30min on ice. Following lysis, cells were sonicated at constant pulse to generate an average of 500-750bpr sheared DNA. ChIP assays were performed as previously described (30). Briefly, the sonicated lysates were pre-cleared with 30 $\mu$ l salmon-sperm coated agarose beads for 1 hour (Upstate Biotechnology, Lake Placid, NY). 5% of the lysate was stored at -80°C to be used as input controls. Half of the lysate was then immunoprecipitated overnight at 4°C with 10 $\mu$ g of antibody to CIITA or S7. The remaining half of the lysate was used as a control and was immunoprecipitated with salmon-sperm coated agarose-beads alone. Following immunoprecipitation with antibody, samples were immunoprecipitated for an additional 2 hours with 60 $\mu$ l of salmon-sperm coated agarose-beads. All samples were then washed

with 1mL of Low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH8.0, 150mM NaCl, dH<sub>2</sub>O), High salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH8.0, 500mM NaCl, dH<sub>2</sub>O), LiCl buffer (0.25M LiCl, 1% NP40, 1% DOC, 1mM EDTA, 10mM Tris pH8.0, dH<sub>2</sub>O) and 1xTE buffer. DNA was then eluted two times with SDS elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>, dH<sub>2</sub>O). Protein-DNA crosslinks were reversed with addition of 5M NaCl overnight at 65°C.

Immunoprecipitated DNA was isolated using phenol:chloroform:isopropanol mix (Invitrogen) according to the manufacturer's instructions. Real-time PCR using primers spanning the W-X-Y box of the MHC class II *HLA-DRA* promoter was performed as above on the isolated DNA. MHC II promoter primers and probe sequences are as follows: MHC-II promoter probe, 5'-6 FAM-CTGGACCCTTTGCAAGAACCCTTCCC-TAMRA-3'; sense primer, 5'-TCCAATGAACGGAGTATCTTGTG T-3'; and antisense primer, 5'-TGAGATGACGCATCTGTTGCT-3'. Values were calculated based on standard curves specific for MHC II. All values were normalized to total DNA input and the amount of MHC II promoter DNA immunoprecipitated in bead immunoprecipitation controls was subtracted from the antibody samples.

## Results

### **S7 associates with CIITA and components of the MHC II enhanceosome**

The mammalian 19S ATPase S7, also called MSS1, is encoded by the gene PSMC2 (69). Prior to being identified as a subunit of the 26S proteasome S7, was shown to positively regulate the transcription of HIV genes by stimulating a viral transcriptional

activator (53). Additional studies arguing for a role of S7 in transcriptional regulation of mammalian genes showed association of S7, but not 20S proteasomal subunits, with general transcription factors, RNA polymerase II, and TBP (67, 70).

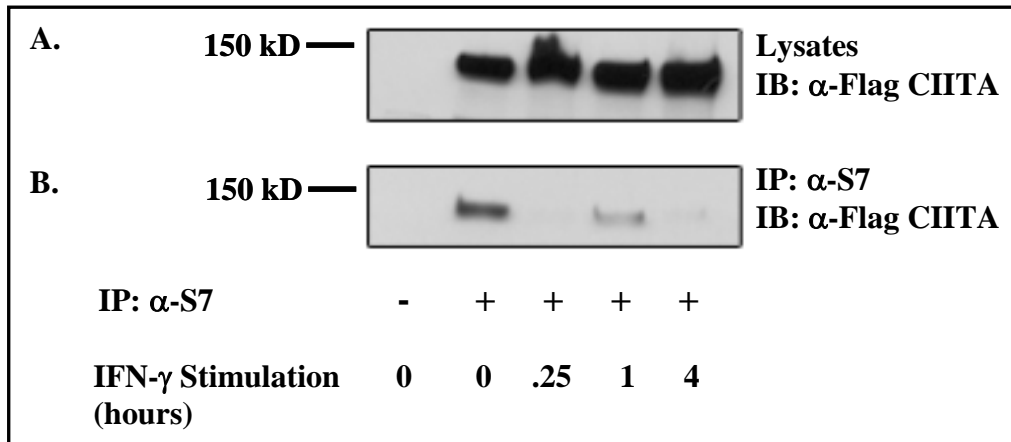
Studies of the non-proteolytic function of S7 in regulating the transcription of yeast genes augment those completed in mammalian cells. The yeast homolog of S7, referred to as Rpt1, Cim5, or YTA3, has been shown to associate with the promoter of the yeast GAL1/10 gene and with promoters of stress induced yeast genes (61, 71). Suggesting a proteolytic independent function in transcription, subunits of the 20S proteasome did not associate with the promoters described above with the same pattern as S7 (61, 71).

The potential of S7 to act as a transcriptional regulator of mammalian genes prompted experiments to examine the role of S7 in the transcriptional regulation of MHC II. To determine this, it was first necessary to find if S7 associated with CIITA and the components of the MHC II enhanceosome, NFY-A and RFX-5. This was accomplished by performing co-immunoprecipitation experiments where endogenous S7 was immunoprecipitated as associations with enhanceosome subunits was examined. IFN- $\gamma$  stimulation was used to establish if associations between S7 and the enhanceosome components were constitutive or inducible upon cytokine stimulation.

Briefly, HeLa cells were transfected with 5  $\mu$ g of Flag-tagged CIITA, Flag-NFY, Flag-RFX5, or an empty vector (pcDNA3). Control samples were transfected with 5  $\mu$ g of pcDNA3. Following transfection, cells were either stimulated with IFN- $\gamma$ , at time points ranging from 15 minutes to 20 hours, or not treated. Cells were then lysed and immunoprecipitated (IP) with a monoclonal antibody against S7 (Biomol).

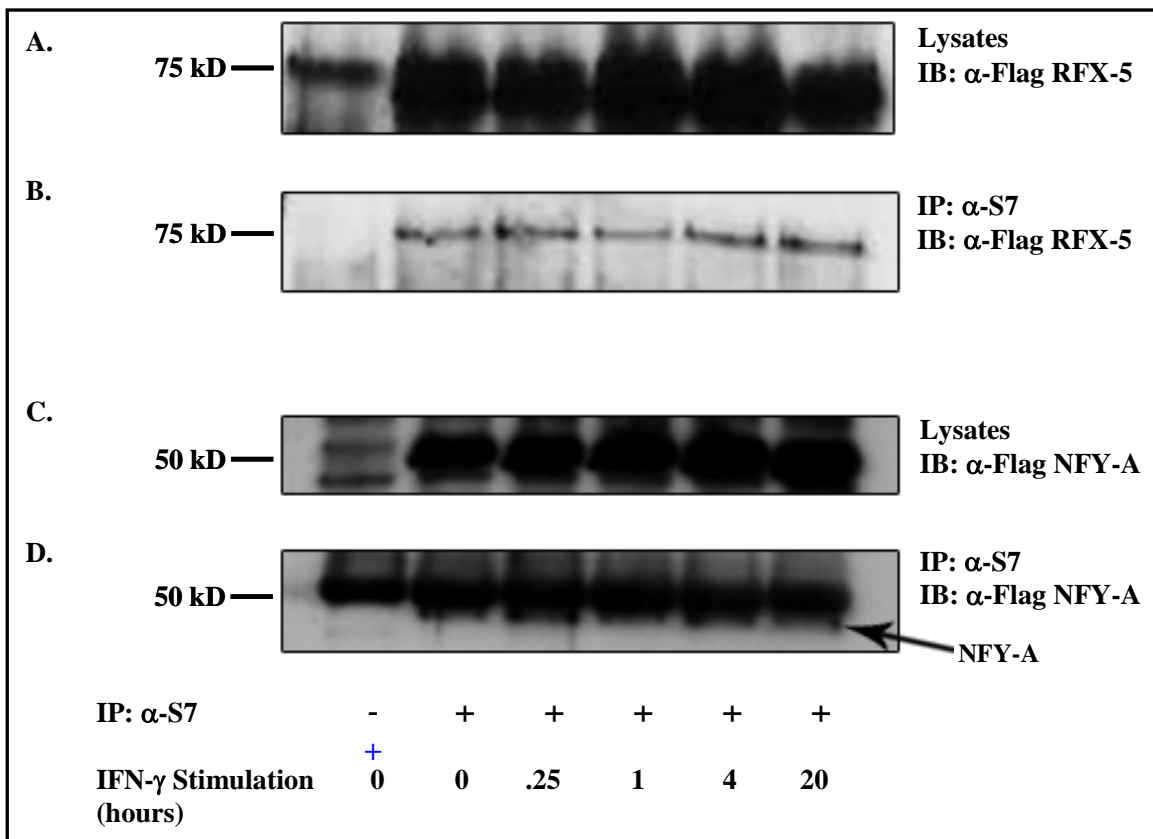
Approximately 20  $\mu$ g of the lysate samples were used as loading controls and received no immunoprecipitating antibody. The samples were then analyzed by immunoblotting (IB) for the Flag tagged proteins, Flag-CIITA, Flag-NFYA, or Flag-RFX5. . Samples immunoprecipitated with S7 antibody were labeled with a “+”, samples not treated with IFN- $\gamma$  were labeled as “0.” In order to treat all samples with equal amounts of DNA, the 3 control samples ( **Figure 1 and 2, lane 1**) were transfected with pcDNA3.

The S7 co-immunoprecipitation results showed association of S7 with CIITA, RFX-5, and NFY-A, although association of S7 with CIITA seemed to be more robust than association with either RFX-5 or NFY-A. The association pattern of S7 with the various enhanceosome components differed as well. It appeared that S7 associates with CIITA in unstimulated cells, but association decreases within 15 minutes of IFN- $\gamma$  stimulation (**Figure 1**). Interestingly, the association between S7 and CIITA was restored following one hour of cytokine stimulation and disappeared after prolonged (4hr) stimulation. This could indicate that S7 helps to recruit CIITA to IFN- $\gamma$  activated gene promoters such as MHC II.



**Figure 1. S7 associates with CIITA in a biphasic pattern.** HeLa cells were transfected with 5 $\mu$ g Flag-CIITA or pCDNA3. No immunoprecipitating antibody was added to lysate samples, which were used as loading controls. CIITA expression in loading controls was assayed by immunoblot (**A**). Remaining cell lysates were used for co-immunoprecipitation (**B**). Samples were immunoprecipitated for S7 and immunoblotted for Flag-CIITA. The negative control (**lane 1**), transfected with pCDNA3, showed no CIITA expression. Association between S7 and CIITA was seen in unstimulated cells (**lane 2**), but association disappeared 15 minutes after stimulation with the cytokine, IFN- $\gamma$  (**lane 3**). Association reappeared following 1 hr. of cytokine stimulation (**lane 4**), but was absent after prolonged stimulation (**lane 5**). Western blot is representative of 3 separate experiments.

Conversely, S7 constitutively associated with both RFX-5 and NFY-A, with no change in the association pattern after cytokine stimulation (**Figure 2**). Expression of both RFX-5 and NFY-A was analyzed by immunoblot (**2A and 2C**) and showed that equal amounts of protein were loaded. Immunoprecipitation with anti-S7 antibody revealed weak association between S7 and the enhanceosome components, with association appearing to be more robust with RFX-5 (**2B**).



**Figure 2. S7 constitutively associates with RFX-5 (B) and NFY-A (D).** HeLa cells were transfected with 5 $\mu$ g Flag-RFX-5, NFY-A, or PCDNA3. No immunoprecipitating antibody was added to lysate samples, which were used as loading controls. Expression of RFX-5 (A) or NFY-A (C) in loading controls was assayed by immunoblot. Remaining cell lysates were used for co-immunoprecipitation (B and D). Samples were immunoprecipitated for S7 and immunoblotted for with anti-flag antibody. The negative control (lane 1), transfected with PCDNA3, showed no RFX-5 or NFY-A expression. Constitutive association between S7 and RFX-5 (B) or NFY-A (D) was seen in unstimulated cells (lane 2) and cells stimulated with IFN- $\gamma$  (lanes 3-6). Western blots are representative of 3 separate experiments.

### S7 siRNA targeting construct effectively reduces S7 expression

Sequence specific gene silencing, or RNA interference (RNAi), by double stranded RNA (dsRNA) has proven to be an effective method of suppressing expression of target genes(72, 73). Thought to have evolved as an anti-viral defense mechanism, RNAi functions by inducing degradation of complementary target mRNA(74). Although

introduction of dsRNA into mammalian cells can result in a cytotoxic response, use of small interfering RNA (siRNA) allows for reduced expression of target genes (73).

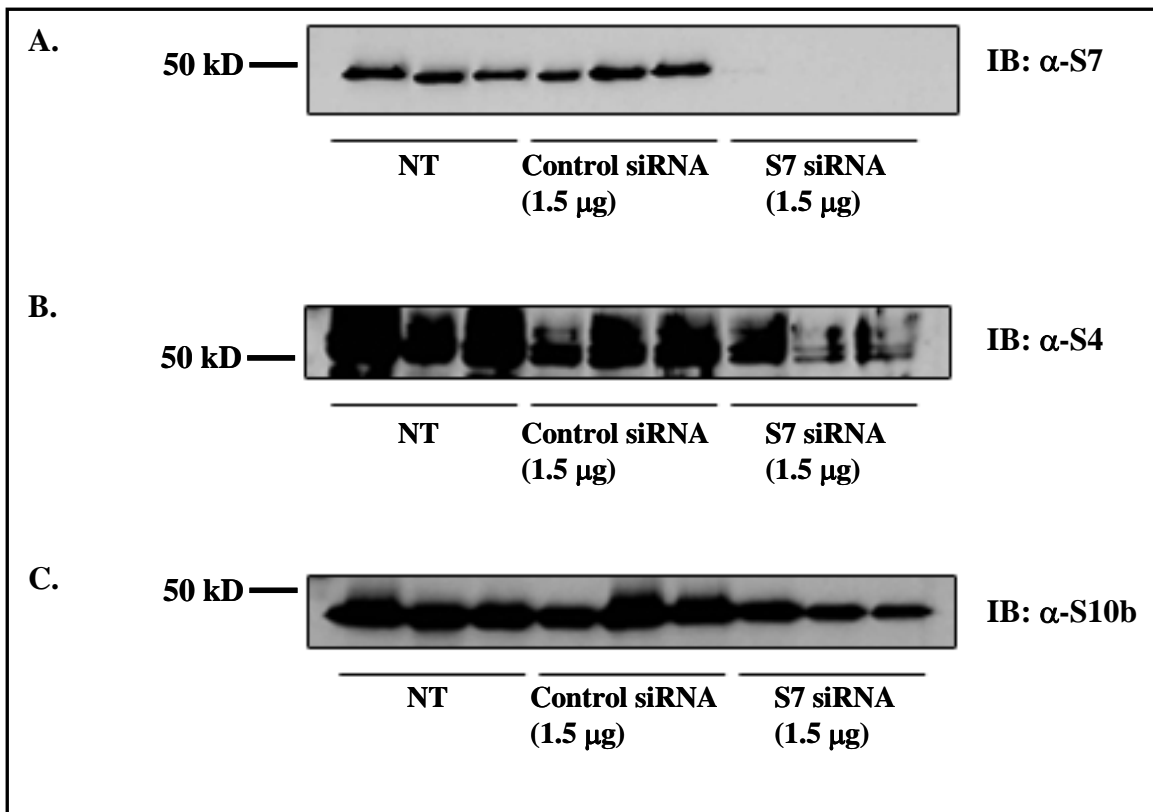
siRNAs are dsRNAs that are approximately 19-22 nucleotides long and silence expression of target genes by inducing cleavage of complementary mRNA sequences(74).

Because S7 associated with CIITA, we were interested in determining if S7 played a role in endogenous MHC II transcription. To accomplish this, S7 expression was silenced and the effect on MHC II transcription was observed. In order to reduce expression of S7, a siRNA targeting construct with sequence complementarity to S7 mRNA, was chemically synthesized (Quiagen) and transfected into HeLa cells using a liposomal transfection reagent. As addition of siRNA could result in knock down of off target genes, a BLAST search was performed on the sequence of the S7 siRNA construct (75). Results of the BLAST search revealed complementarity to mammalian S7, but no significant sequence homology was found to any other mammalian genes.

The efficiency of the S7 siRNA construct at various concentrations and time points was determined. It was found that S7 siRNA at a concentration of .65 $\mu$ g reduced S7 expression after 72 hours, while 1.5 $\mu$ g of S7 siRNA reduced S7 expression in 48 hours (**Figure 3A**). Specificity of the siRNA construct for S7 was confirmed by observing protein expression of S7 and expression of the additional 19S proteasomal ATPases, S4 and S10b, after S7 siRNA addition (**Figure 3B and 3C**). Also, to ascertain the effect of exogenous siRNA addition on S7 protein expression, a non-silencing, negative control siRNA construct was transfected at concentrations identical to S7 siRNA.

As determined by western blot, transfection of 1.5 $\mu$ g of S7 siRNA dramatically reduced expression of S7, while transfection of non-silencing siRNA or treatment with transfection reagent alone did not affect S7 expression. The protein concentration of each sample was determined by Bradford assay to ensure that equal amounts of protein were loaded and that reduced S7 expression could be attributed to the efficiency of the S7 siRNA.

In addition to efficiently reducing S7 expression, the targeting construct did not significantly reduce expression of the closely related 19S proteasomal ATPases, S4 and S10b (**Figure 3A and 3B**). Expression of S4 and S10b were assayed by western blot, using the same lysates and equal amounts of protein as the S7 western blot. These two proteins were chosen as controls as they are both ATPases of the 19S proteasome and therefore share some sequence similarity with S7 (49). Although there does seem to be some reduction in expression of S4, reduction of S7 expression is greatly decreased in comparison. This could be due to the sequence similarity of S4 and S7, but expression levels could differ due to use of a polyclonal antibody for S4 detection. Expression of S10b, however, was not reduced upon transfection of S7 siRNA. Also, transfection of the non-silencing control siRNA did not seem to reduce expression of any of the proteasomal ATPases in this experiment.



**Figure 3. S7 siRNA targeting construct efficiently reduces S7 expression.** HeLa cells were transfected with reagent only (**lanes 1 and 2**), 1.5 $\mu$ g of negative control siRNA (**lanes 3 and 4**), or 1.5  $\mu$ g of the S7 siRNA targeting construct (**lanes 5 and 6**) and assayed by immunoblot (IB) for expression of S7 (**A**). The S7 siRNA construct did not significantly reduce expression of the control 19S ATPases, S4 or S10B as assayed by immunoblot (**B and C**).

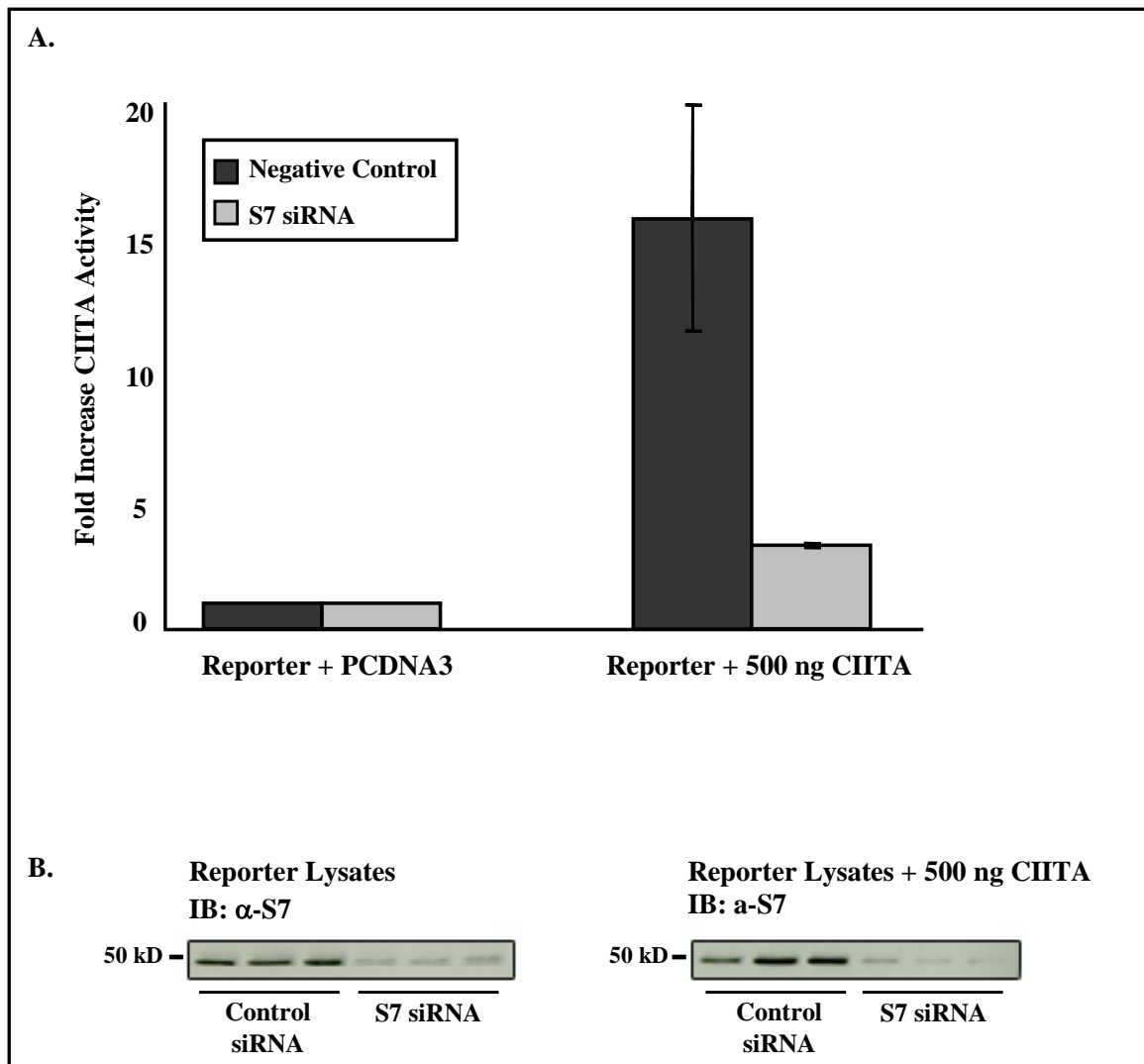
### **S7 is Required for Efficient CIITA Activity**

Although co-immunoprecipitation experiments showed association of S7 with CIITA and MHC II enhanceosome components, they did not establish a clear link between the transcriptional regulation of MHC II and S7. As CIITA is the master regulator of CIITA transcription, has increased activity when mono-ubiquitinated, and associates with S7, the next set of experiments was designed to elucidate the effect of S7 on CIITA activity (4, 30). Using a luciferase reporter assay, CIITA mediated activation of

the HLA-DRA promoter, fused with a luciferase (Luc) reporter gene, was measured. The reporter construct was designed to have a functional MHC II promoter fused upstream of the luciferase gene, with transcriptional activation of MHC II resulting in production of the enzyme, luciferase. This cannot occur, however, without binding of CIITA to the MHC II promoter. Therefore, quantifying the luciferase product after silencing expression of S7 provided a measurement of CIITA activity.

HeLa cells were transfected with S7 siRNA (.65  $\mu$ g), non-silencing control, or reagent only and S7 expression was assayed by immunoblot (**Figure 4B**). Samples treated with transfection reagent only were labeled as “No Treatment”, those treated with non-silencing siRNA were labeled as “Negative Control” and those treated with S7 siRNA were labeled as “S7 siRNA.” 48 hours following siRNA transfection, the HLA-DR-Luciferase construct (100 ng) and CIITA (500 ng) were transfected. Cells were lysed 72 hours after siRNA transfection, luciferin, the substrate of luciferase, was added, and luciferase activity was measured. The transcriptional activation of the MHC II promoter by CIITA was proportional to luciferase activity.

As HeLa cells do not constitutively transcribe CIITA, control samples transfected with HLA-DR-Luc had no observable luciferase activity (**Figure 4A**). In cells with normal S7 expression, co-transfection of the HLA-DR-Luc and CIITA resulted in a large increase of luciferase activity. This activity decreased when S7 expression was silenced by S7 specific siRNA (**Figure 4A**). Although transfection of non-silencing siRNA moderately reduced luciferase activity as compared to samples not treated with siRNA (**data not shown**), luciferase activity was still markedly increased as compared to S7 specific siRNA (**Figure 4A**).



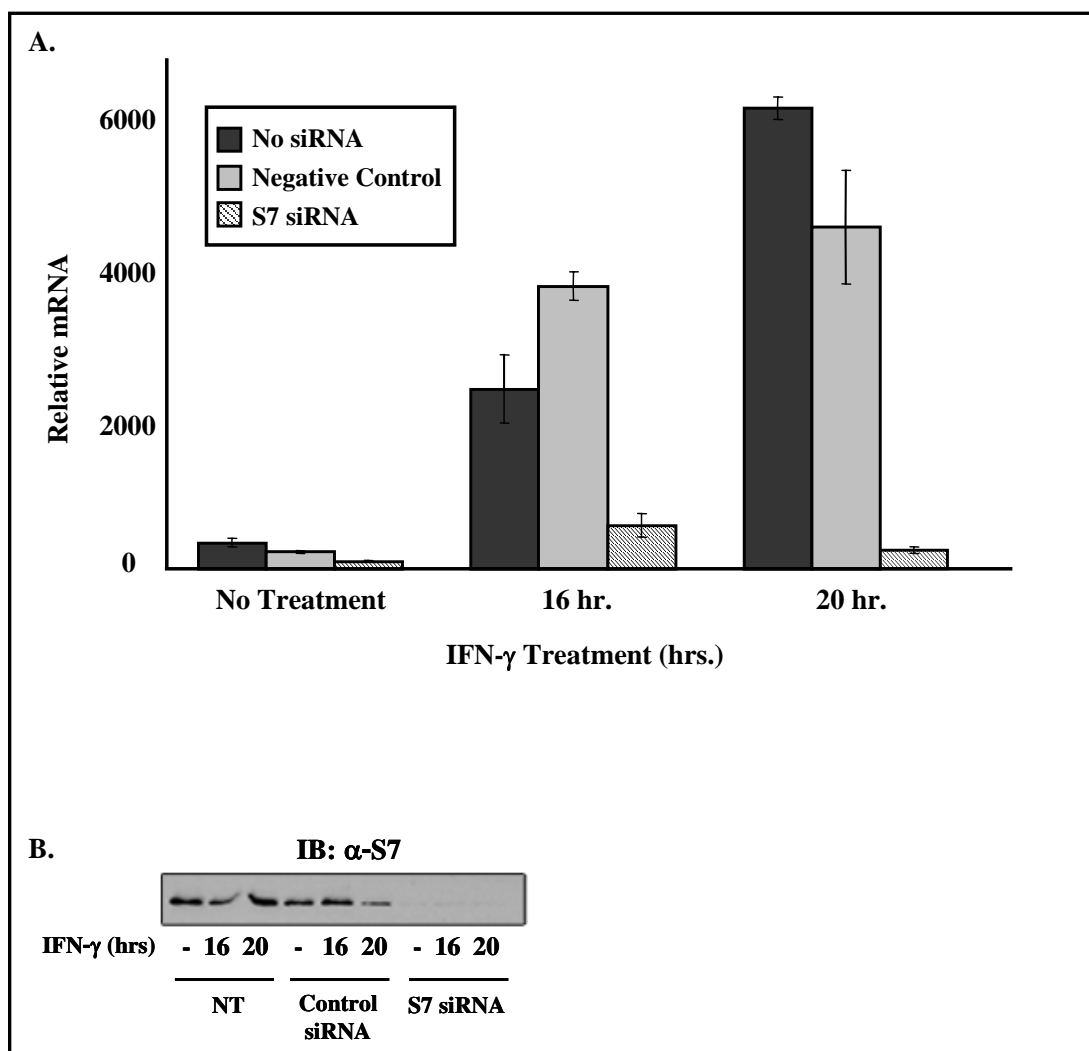
**Figure 4. S7 is required for optimal MHC II activation by CIITA.** S7 expression was silenced using S7 specific siRNA (.65  $\mu$ g). Non-silencing siRNA (.65  $\mu$ g) was used as a control. After transfection of siRNA, cells were transfected with HLA-DR-Luc (100 ng) and PCDNA3 (500 ng), or HLA-DR-Luc (100 ng) and CIITA (500ng). Samples not transfected with CIITA had minimal luciferase activity in comparison to samples transfected with CIITA (**A.**). Silencing S7 expression resulted in decreased luciferase activity (**A.**) S7 expression was assayed by immunoblot. S7 expression was reduced by S7 siRNA, but not by non-silencing siRNA (**B.**) Values and western blot are representative of 3 separate experiments.

### **S7 is Required for Optimal Endogenous Transcription of MHC II HLA-DRA Genes**

The previous data indicate that S7 is necessary for optimal activation of the MHC II promoter by CIITA, but, due to the limitations of the promoter-reporter system, the particular part played by S7 in MHC II transcription was not apparent. The luciferase assay employed an artificial promoter system, which does not reflect the chromatin structure of the MHC II promoter. To ascertain the role of S7 in chromatin remodeling, S7 expression was silenced using siRNA and endogenous mRNA transcripts of both MHC II and CIITA were measured.

To reduce S7 expression in a 48hr. time period, the S7 siRNA construct (1.5 $\mu$ g), non-silencing control (1.5 $\mu$ g), or reagent only was transfected into HeLa cells. Samples treated with transfection reagent only were labeled as “No Treatment”, those treated with non-silencing siRNA were labeled as “Negative Control” and those treated with S7 siRNA were labeled as “S7 siRNA.” Following siRNA transfection, cells were stimulated with IFN- $\gamma$  for 16 hours or 20 hours to induce transcription of CIITA and to promote accessibility of the MHC II promoter (30). Approximately 20% of the cells from each sample were lysed in 1% NP40 and used to determine efficient knockdown of S7 (**Figure 5B**). From the remaining cells, RNA was extracted and quantified. Using RT-PCR, RNA (1 $\mu$ g) was reverse transcribed to make MHC II or 18S cDNA. To quantify the molecules of cDNA, and thus the number of mRNA transcripts, real time PCR was used to amplify MHC II cDNA and 18S cDNA. Primers and probes specific for MHC II HLA-DRA or 18S were used to amplify the cDNA. MHC II transcripts were normalized to 18S transcripts, as the amount of 18S transcripts should not change with cytokine stimulation.

Samples that did not receive IFN- $\gamma$  treatment had negligible amounts of MHC II mRNA (**Figure 5A**). As expected, the relative number of transcripts was dramatically increased in samples that were treated with IFN- $\gamma$  for 16hours (**Figure 5A**), and decreased expression of S7 resulted in decreases in the levels of MHC II mRNA (**Figure 5A**). Transcription of MHC II after 20 hours of IFN- $\gamma$  treatment exhibited a greater increase as compared to 16 hours of treatment. Again, reduced S7 expression almost completely abolished MHC II transcription (**Figure 5A**). Decreased MHC II transcription can be largely attributed to reduced S7 expression as the non-silencing, negative control siRNA did not significantly affect the relative amount of MHC II mRNA. This data shows that, in addition to being necessary for CIITA mediated activity, S7 is necessary for endogenous MHC II transcription.



**Figure 5. S7 is required for endogenous transcription of MHC II Genes.** To induce MHC II transcription, HeLa cells were stimulated with IFN- $\gamma$  at the indicated time points. Relative mRNA was obtained by normalizing the molecules of MHC II mRNA to 18S mRNA. With normal S7 expression, MHC II transcription markedly increased after IFN- $\gamma$  treatment at both 16 and 20 hours (**A. and B. lanes 1-6**). Reduction in S7 expression resulted in a drastic decrease of MHC II transcripts at both 16 and 20 hours (**A. and B. lanes 7-9**). Values and western blot are representative of 3 separate experiments.

### **S7 Exhibits Inducible Association with the MHC II Promoter**

The previous data indicating decreased levels of MHC II transcripts in the absence of S7 strongly suggest a role for S7, independent of proteolysis, in the regulation

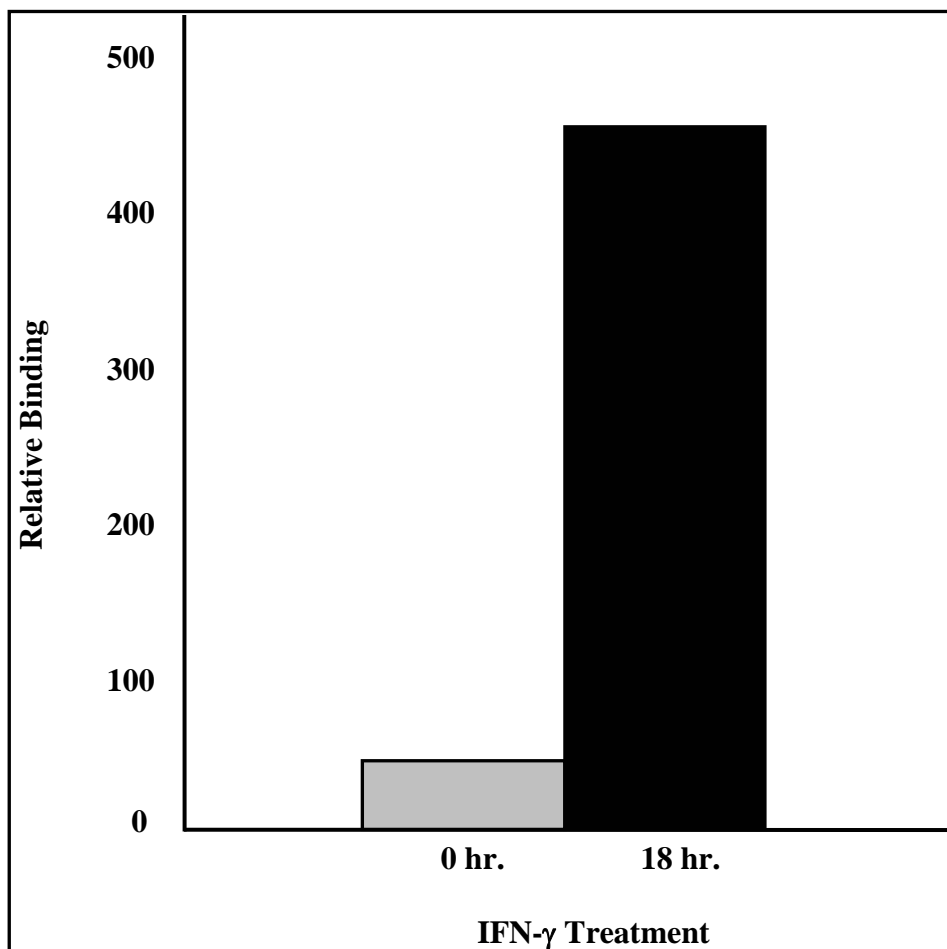
of MHC II transcription. One mechanism by which S7 may regulate transcription at the MHC II promoter may through direct interaction with the promoter where S7 may stabilize the enhanceosome complex, recruit chromatin remodeling enzymes or alter the levels of ubiquitination of promoter associated factors. To test whether S7 is recruited to the endogenous MHC II promoter, HLA-DRA chromatin immunoprecipitation (ChIP) assays were performed. Chromatin immunoprecipitation, coupled with real-time PCR, allows quantification of levels of proteins with specific DNA sequences to be observed (76).

To determine if S7 binds at the MHC II promoter, formaldehyde was used to crosslink DNA to proteins in live cells antibodies specific to S7 were then used to immunoprecipitate S7 and associated DNA. DNA bound to S7 was then eluted and subjected to real-time PCR with primers and probe specific to the HLA-DRA promoter. Using this method, the amount of MHC II promoter DNA associated with S7 at various time points of cytokine stimulation could be quantified. To account for non-specific binding of DNA to S7, DNA immunoprecipitated by control samples was subtracted from the amount of immunoprecipitated MHC II promoter sequence DNA. This quantity of DNA was then normalized to total input DNA.

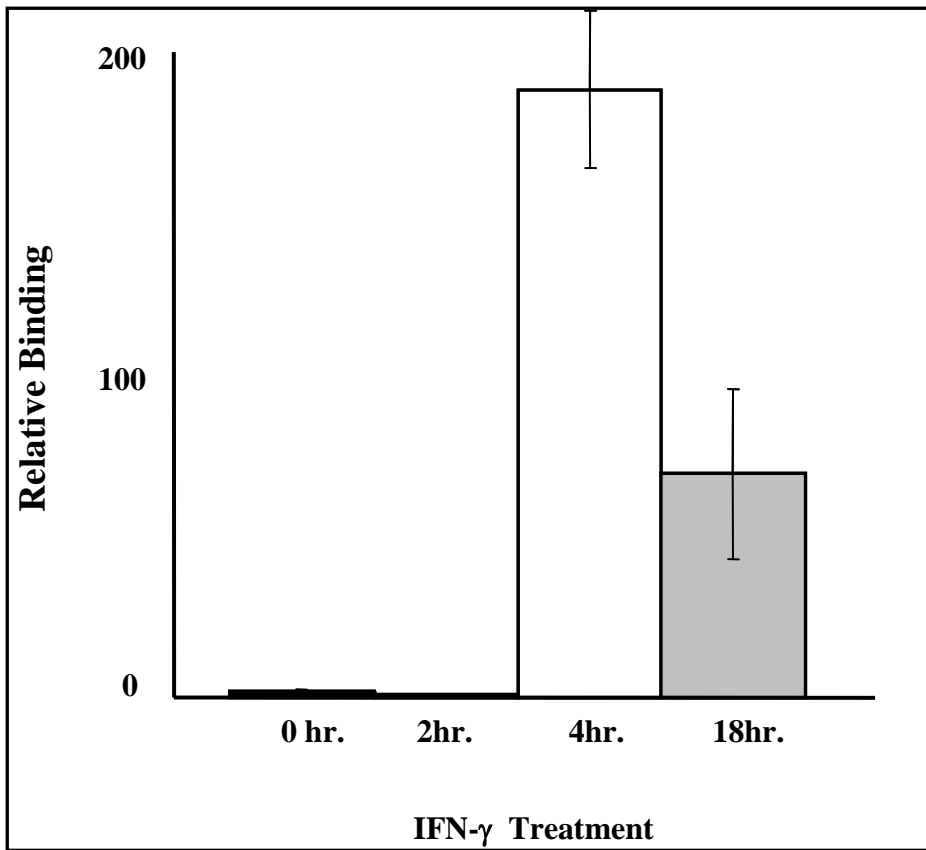
Because the kinetics of any proteasomal ATPases binding to the MHC II promoter were unknown, it was necessary to perform a time course of cytokine stimulation with IFN- $\gamma$ . To determine basal and inducible levels of S7 association at the MHC II promoter, HeLa cells were either untreated (0 hour) or were stimulated with IFN- $\gamma$  for 2, 4, or 18 hours. As a positive control for the ChIP assay, IFN- $\gamma$  induced binding of CIITA to the MHC II promoter was measured (**Figure 6**). Spilianakis et al.,

have shown binding of endogenous CIITA at the MHC II promoter in HeLa cells to peak between 12 and 24 hours following cytokine stimulation (77). Our lab has similarly demonstrated a robust increase of CIITA association at the MHC II promoter to occur 18 hours after IFN- $\gamma$  stimulation (**Figure 6**).

Immunoprecipitation with anti-S7 antibody in unstimulated cells yielded a minimal amount of MHC II promoter DNA, indicating no binding of S7 in the absence of inflammatory cytokine (**Figure 7**). S7 was shown to bind to the MHC II promoter in a cytokine dependent manner as levels of promoter bound S7 increased substantially following 4 hours of cytokine stimulation, with binding decreasing by 18 hours of stimulation (**Figure 7**). Furthermore, the kinetics of S7 binding differed from those of  $\alpha 4$ , a subunit of the 20S proteasome, which showed peak binding at 48 hours (Greer lab unpublished data). This is of consequence as S7 binding at the MHC II promoter at similar time points to other transcriptional activators could be consistent with S7 functioning as a positive regulator of MHC II transcription, while S7 binding at times similar to 20S proteasome components like  $\alpha 4$  would have suggested a proteolytic role.



**Figure 6. CIITA binding at the MHC II promoter dramatically increases with cytokine stimulation.** HeLa cells were stimulated with IFN- $\gamma$  at the indicated time points to induce CIITA transcription. Cells were treated with formaldehyde to crosslink protein and DNA and immunoprecipitated with anti-CIITA antibody. The DNA complexed with the immunoprecipitated CIITA was eluted and subjected to real-time PCR with MHC II promoter specific primers and probe. CIITA showed substantial association at the MHC II promoter following 18 hours of cytokine treatment.



**Figure 7. S7 inducibly binds to the MHC II promoter.** To open MHC II chromatin and induce MHC II transcription, HeLa cells were stimulated with IFN- $\gamma$  at the indicated time points. Cells were treated with formaldehyde to crosslink protein and DNA and immunoprecipitated with anti-S7 antibody. The DNA complexed with the immunoprecipitated S7 was eluted and subjected to real-time PCR with MHC II promoter specific primers and probe. S7 showed substantial association at the MHC II promoter following 4 hours of cytokine treatment. Association was decreased at later time points (18hr.). Graph is representative of 3 separate experiments.

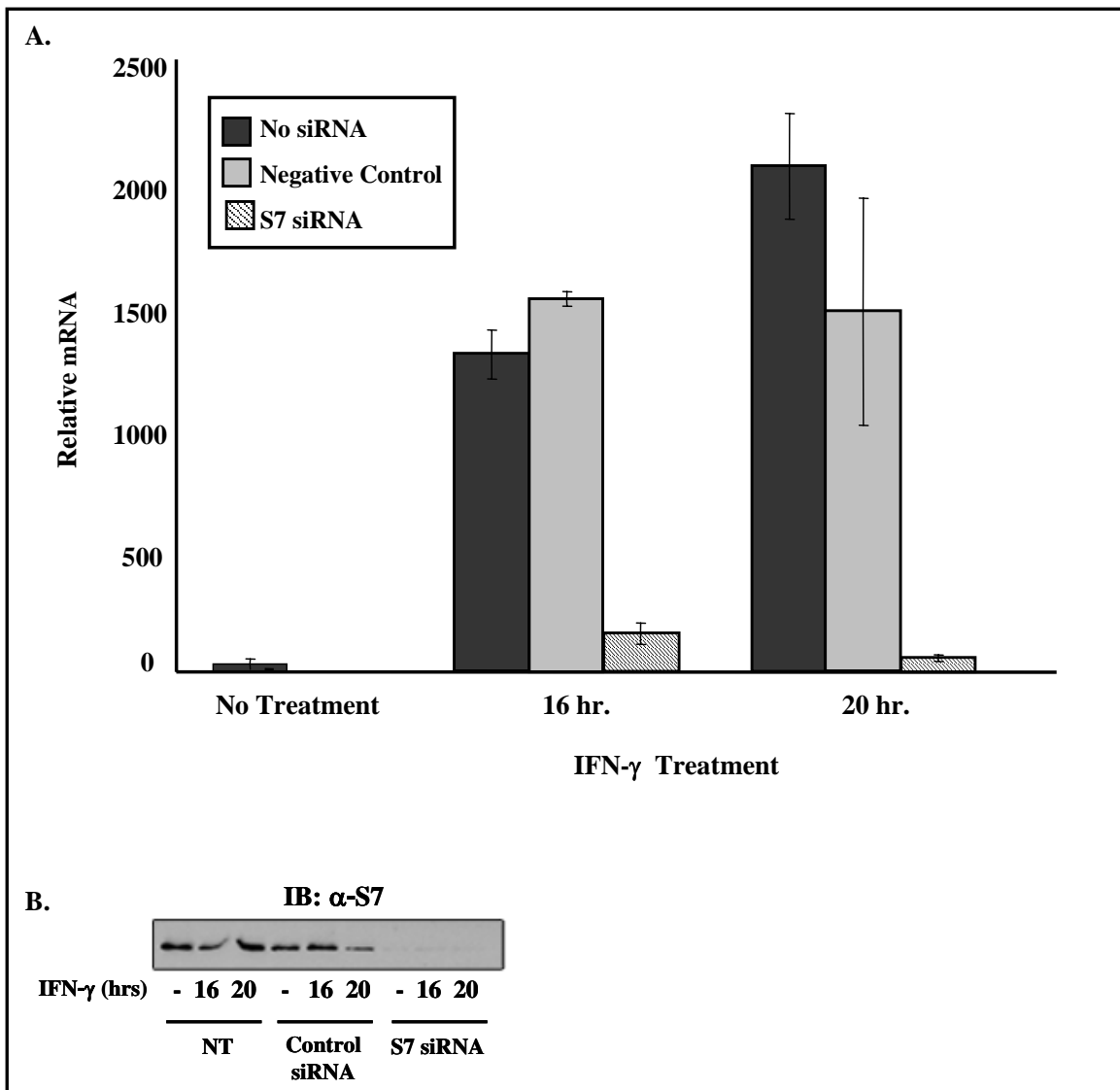
#### **S7 is Required for Optimal Endogenous Transcription of CIITA Promoter IV**

##### **Genes**

Recent studies in yeast have demonstrated that transcription of many genes is regulated by 19S ATPases. The roles of these ATPases in regulating mammalian transcription remain to be determined, but it is likely that the 19S ATPases regulate transcription at many mammalian promoters. Due to fact that IFN- $\gamma$  induced MHC II

transcription is entirely dependent on efficient transcription of CIITA, the effects of altered S7 expression on the level of CIITA transcripts should also be considered to determine if S7 plays a role in regulating transcription at the interferon inducible CIITA PIV. To address the possibility that sub-optimal MHC II transcription after S7 knockdown was due to decreased CIITA transcription, CIITA mRNA from the samples used in the above experiment (**Figure 5.**) was quantified.

In order to determine the effects of decreased S7 expression on interferon gamma induced levels of CIITA, RNA extracted from unstimulated and stimulated cells was reverse transcribed with primers specific to CIITA PIV. Real-time PCR was then performed on the resultant cDNA. We had previously observed that reduced S7 expression decreased CIITA activity (**Figure 4**). It was therefore conceivable that this reduced CIITA activity at the MHC II promoter was the primary cause of decreased MHC II transcription. However, after observing the effect of S7 siRNA on CIITA transcription, it was apparent that silencing S7 expression also caused a decrease in CIITA PIV transcription (**Figure 8**). Similar to levels of MHC II mRNA in unstimulated cells, real-time PCR showed minimal levels of CIITA PIV mRNA in the absence of IFN- $\gamma$  stimulation. Also, like MHC II mRNA, CIITA mRNA exhibited large increases following cytokine stimulation and the increased transcription was abrogated upon silencing of S7 (**Figure 8**). These findings indicate that S7 may regulate MHC II transcription at multiple levels, both during regulatory events at the MHC II promoter and in the transcriptional regulation of CIITA. Of additional importance, this data suggests a non-proteolytic role of S7 in transcriptional regulation, as inhibiting proteolysis should result in an increased number of transcripts.



**Figure 8. S7 is required for endogenous transcription of CIITA PIV Genes.**

To activate CIITA PIV transcription, HeLa cells were stimulated with IFN- $\gamma$  at the indicated time points. Identical samples were used for experiments shown in figures 5 and 6. Relative mRNA was obtained by normalizing the molecules of CIITA PIV mRNA to 18S mRNA. With normal S7 expression, CIITA PIV transcription markedly increased after IFN- $\gamma$  treatment at both 16 and 20 hours (**A. and B. lanes 1-6**). Reduction in S7 expression resulted in a drastic decrease of CIITA PIV transcripts at both 16 and 20 hours (**A. and B. lanes 7-9**). This reduction in CIITA mRNA correlated with MHC II transcripts. Values and western blot are representative of 3 separate experiments.

### **S7 Does not Associate with the CIITA PIV Transcription Factor, IRF-1**

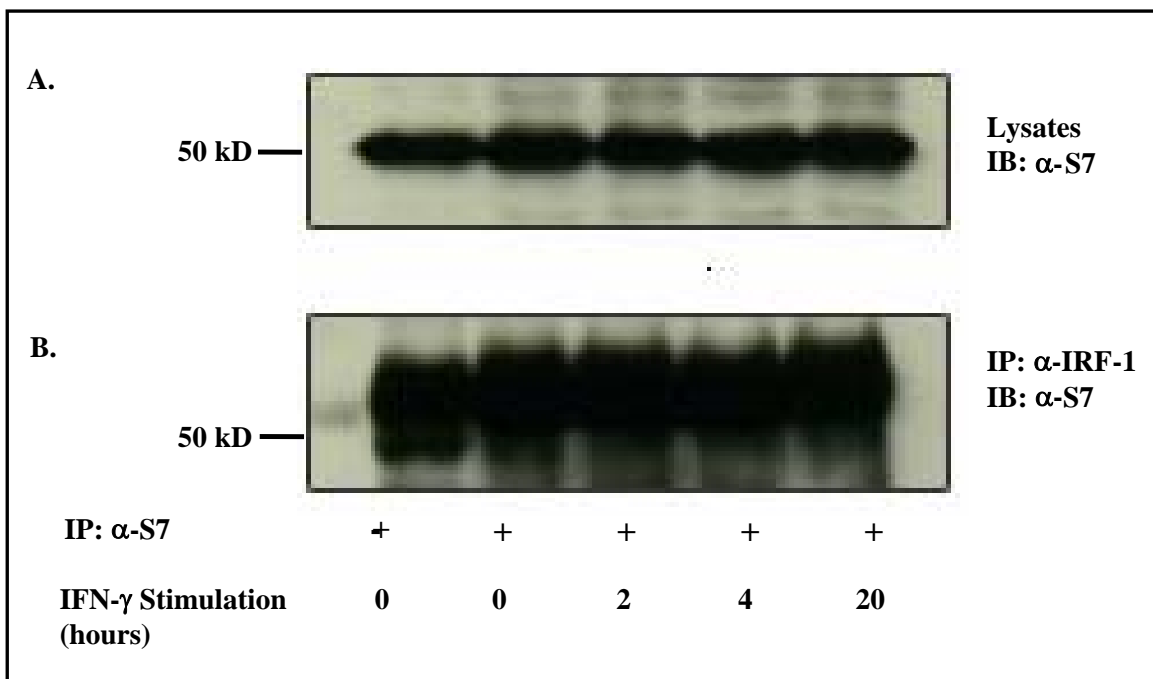
Reduced expression of S7 correlates with decreased endogenous transcription of CIITA PIV transcripts (**Figure 8**). This indicates that, in addition to taking part in the transcriptional regulation of MHC II, S7 may regulate the transcription of inducible CIITA PIV genes (**Figures 5 and 8**). Pertinent to this proposed role of S7 in CIITA PIV transcription is the association of S7 with requisite transcription factors of CIITA PIV.

CIITA PIV is one of four different promoters encoding CIITA genes and is responsible for regulating IFN- $\gamma$  inducible CIITA (28, 36, 38). Following binding of IFN- $\gamma$  to interferon receptors of CIITA inducible cells, a signal transduction cascade promotes localization and binding of STAT1, USF1, and IRF-1 to CIITA PIV (4, 11, 36, 37). Kinetic studies show rapid binding of transcription factors to PIV with STAT1 and USF1 maximally binding half an hour after IFN- $\gamma$  stimulation (28). IRF1 is not present at CIITA PIV until approximately 1 hour after IFN- $\gamma$  stimulation and reaches peak binding at 4 hours (28). Expression of IRF-1 is also increased with IFN- $\gamma$  stimulation in a STAT1 dependent manner (28). Using co-immunoprecipitation experiments, the association of S7 with endogenous IRF-1 was examined.

HeLa cells were plated in 15 cm tissue culture plates at a density of  $3 \times 10^6$  and stimulated with IFN- $\gamma$  at time points ranging from 2 to 20 hours, or not treated. Samples immunoprecipitated with S7 antibody were labeled with a "+", samples not treated with IFN-g were labeled as "0." Cells were lysed with 1% NP40 and 1 % RIPA buffer to lyse the cell nucleus. They were then immunoprecipitated (IP) with a polyclonal antibody against IRF-(SantaCruz, SC-497). Approximately 20  $\mu$ g of the lysate samples were used as loading controls and received no immunoprecipitating antibody. The samples were

then analyzed by immunoblotting (IB) with a primary antibody raised against S7 and an HRP-conjugated secondary antibody.

S7 co-immunoprecipitation results showed no association of S7 with IRF-1 (**Figure 9**). The lack of association between S7 and IRF-1 could argue against a role for S7 in CIITA PIV transcription, but previous experiments illustrated that S7 expression was necessary for transcription of CIITA PIV genes (**Figure 8**). As the co-immunoprecipitation experiment cannot be used to determine direct roles in transcriptional activation, additional experiments must be performed to examine the role of S7 in the transcriptional regulation of CIITA PIV. Specifically, chromatin immunoprecipitation experiments must be performed to find if S7 is bound to active CIITA PIV.



**Figure 9. S7 does not associate with IRF-1.** HeLa cells were assayed for association of endogenous S7 and IRF-1. No immunoprecipitating antibody was added to lysate samples, which were used as loading controls. IRF-1 expression was assayed by immunoblot to verify expression (**A**). S7 expression in loading controls was assayed by immunoblot (**B**). Remaining cell lysates were used for co-immunoprecipitation (**B**).

Samples were immunoprecipitated for IRF-1 and immunoblotted for S7. The negative control (**lane 1**), received control immunoprecipitating antibody. Association between S7 and IRF-1 was seen in unstimulated cells and in cells treated with IFN- $\gamma$  for 2, 4, and 20 hours (**lane 2-5**). Association of IRF-1 was also seen with the control antibody (**lane 1**).

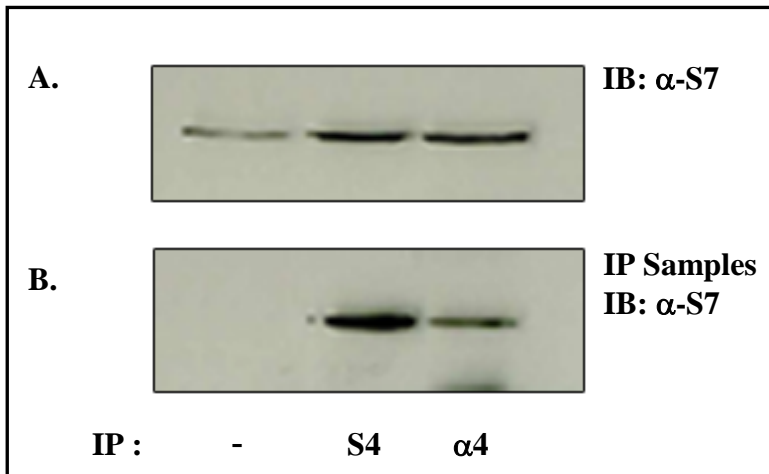
### **S7 Forms a Heterodimer with S4, an ATPase of the 19S Proteasome**

Entry of protein substrates into the 20S core of the 26S proteasome is mediated by Rpt2, the yeast homolog of S4 (78). Along with other members of the 19S proteasome, S4 is believed to participate non-proteolytically in transcription (67). Although extensive studies of the role in S4 transcription have not been undertaken, data has indicated that it associates with TBP and transcriptional activators (67). It is also believed that S4 may associate with transcriptional activators after it forms a heterodimer with S7 (67).

We therefore examined the role of S4 in the transcriptional regulation of MHC II and determined whether formation of the S4/S7 heterodimer augmented the activity of either subunit alone. To explore the possibility that S7 and S4 may cooperatively participate in the transcriptional regulation of MHC II and other mammalian genes, co-immunoprecipitation of endogenous S4 and S7 was undertaken. Previously, *in-vitro* assays were employed to illustrate S4 and S7 association in mammalian cells, but co-immunoprecipitation experiments used in this current study allowed for analysis of associations between endogenous proteins (49).

HeLa cells, plated at a density of  $2 \times 10^6$ , were lysed in 1% NP40 and immunoprecipitated with antibodies (5  $\mu$ g) specific to S4,  $\alpha 4$ , or no antibody bead controls. The non-ATPase subunit of the 20S proteasome,  $\alpha 4$ , was also used as a control immunoprecipitating antibody to determine specificity of S7/S4 association. As a loading control for S7 expression, a portion of the cell lysates received no

immunoprecipitating antibody. The remaining lysate was immunoprecipitated with protein G beads, S4, or  $\alpha 4$ . After immunoprecipitating with the indicated antibodies, samples were immunoblotted with anti-S7 antibody. Immunoblot of protein isolated from immunoprecipitation with anti-S4 antibodies showed that S7 co-immunoprecipitated with S4, while S7 was not isolated from immunoprecipitation with beads only (**Figure 10, lane 1- 2**). S7 also co-immunoprecipitated with  $\alpha 4$ , but the association did not appear to be as strong as the S7-S4 association. These data indicate the possibility that the S7/S4 heterodimer forms at a higher frequency than would be expected if the association occurred only during assembly of the intact 26S proteasome and provide incentive to determine the combined effects of S4 and S7 on MHC II transcription. (**Figure 10, lane 3**).



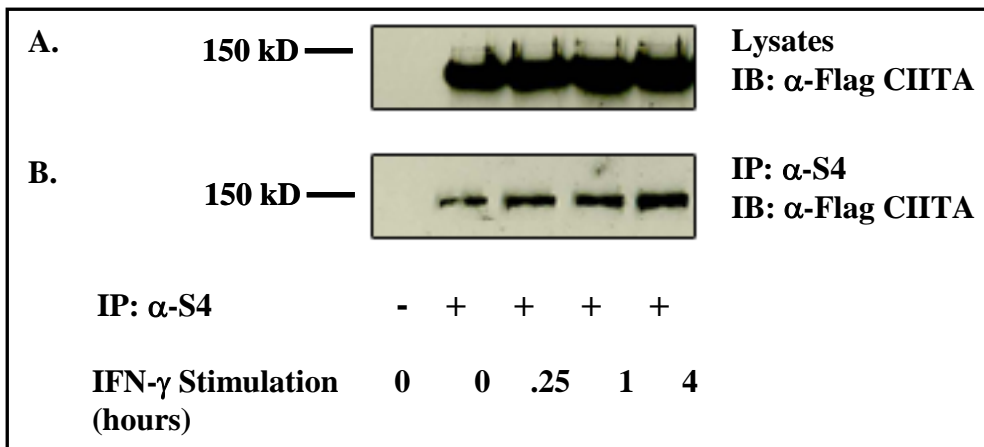
**Figure 10. S7 associates with S4 more so than it associates with  $\alpha 4$ .** No immunoprecipitating antibody was added to lysate samples, which were used as loading controls. S7 expression in loading controls was assayed by immunoblot (A). Remaining cell lysates were used for co-immunoprecipitation (B). Samples were immunoprecipitated with protein G beads only, anti-S4 antibodies, or anti- $\alpha 4$  antibodies. The negative control (**lane 1**) was immunoprecipitated with protein G beads only. Immunoprecipitation of S4 showed association between S7 and S4 (**lane 2**). Immunoprecipitation of  $\alpha 4$  showed association of  $\alpha 4$  and S7, but to a lesser extent than S7/S4 association (**lane 3**).

### **S4 Constitutively Associates with CIITA**

Our previous experiments demonstrate S7 association with CIITA and a requirement for S7 in mediating CIITA activity. These observations raise the possibility that other ATPase subunits of the 19S proteasome may also associate with CIITA and other transcriptional activators where they may play important regulatory roles in transcription. Since S4 is believed to dimerize with S7 in yeast and based on our above observations of associations between S4 and S7 in mammalian cells, examining the interaction of S4 and CIITA was a next logical step. To accomplish this, co-immunoprecipitation experiments with S4 and CIITA were performed.

HeLa cells were transfected with 5  $\mu$ g of Flag-tagged CIITA or with 5  $\mu$ g of the control plasmid, pCDNA3. Following transfection, the cells were either stimulated with IFN- $\gamma$ , at time points ranging from 15 minutes to 4 hours, or not treated at all. Samples were then lysed and immunoprecipitated (IP) with a polyclonal antibody against S4 (Biomol). Approximately 20  $\mu$ g of the lysate samples were used as loading controls and received no immunoprecipitating antibody. Both lysate and immunoprecipitated samples were then analyzed by immunoblotting (IB) for Flag tagged CIITA.

The S4 co-immunoprecipitation results showed association of S4 with CIITA in unstimulated cells and at all indicated time points of IFN- $\gamma$  stimulation (**Figure 11**). This could indicate different regulatory roles of S7 and S4, as S7 associated biphasically with CIITA (**Figure 1**).



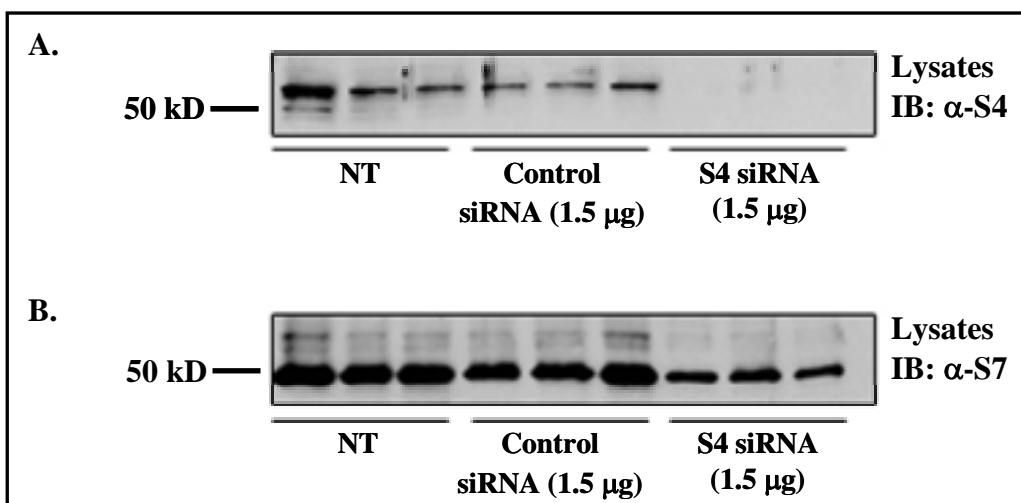
**Figure 11. S4 constitutively associates with CIITA.** HeLa cells were transfected with 5 $\mu$ g Flag-CIITA or PCDNA3. No immunoprecipitating antibody was added to lysate samples, which were used as loading controls. CIITA expression in loading controls was assayed by immunoblot (**A**). Remaining cell lysates were used for co-immunoprecipitation (**B**). Samples were immunoprecipitated for S4 and immunoblotted for Flag-CIITA. The negative control (**lane 1**), transfected with PCDNA3, showed no CIITA expression. Association between S4 and CIITA was seen in unstimulated cells and all indicated time points of IFN- $\gamma$  stimulation (**lane 2-5**), but association disappeared 15 minutes after stimulation with the cytokine, IFN- $\gamma$  (**lane 3**). Western blot is representative of 2 separate experiments.

### The S4 siRNA Construct Specifically Reduces S4 Expression

Since S4 is able to associate with CIITA, the role of S4 in the transcriptional regulation of MHC II molecules was of interest. Paralleling experiments with S7, siRNA was used to knock down S4 expression and observe the effect on MHC II transcription. In order to reduce expression of S4, a siRNA targeting construct with sequence complementarity to S4 mRNA, was chemically synthesized (Quiagen) and transfected into HeLa cells. Similar to the design of the S7 targeting construct, a BLAST search was performed on the sequence of the S4 siRNA construct and no significant sequence homology was found to any mammalian genes other than S4.

Experiments to determine the efficiency of the S4 siRNA construct at various concentrations and time points showed that 1.5 $\mu$ g of S4 siRNA reduced S4 expression in 48 hours (**Figure 12**). Specificity of the siRNA construct for S4 was confirmed by observing protein expression of S4 and expression of S7, after S4 siRNA addition (**Figure 12 B**). As with the S7 siRNA transfection, a non-silencing, negative control siRNA construct was used and was transfected at concentrations identical to S4 siRNA.

As determined by western blot, transfection of 1.5 $\mu$ g of S4 siRNA dramatically reduced expression of S4, while transfection of non-silencing siRNA or treatment with transfection reagent only did not affect S4 expression (**Fig. 12A**). In addition to efficiently reducing S4 expression, the targeting construct did not significantly reduce expression of S7 (**Figure 12B**). Expression of S7 was assayed by western blot, using the same lysates and equal amounts of protein as the S4 western blot.

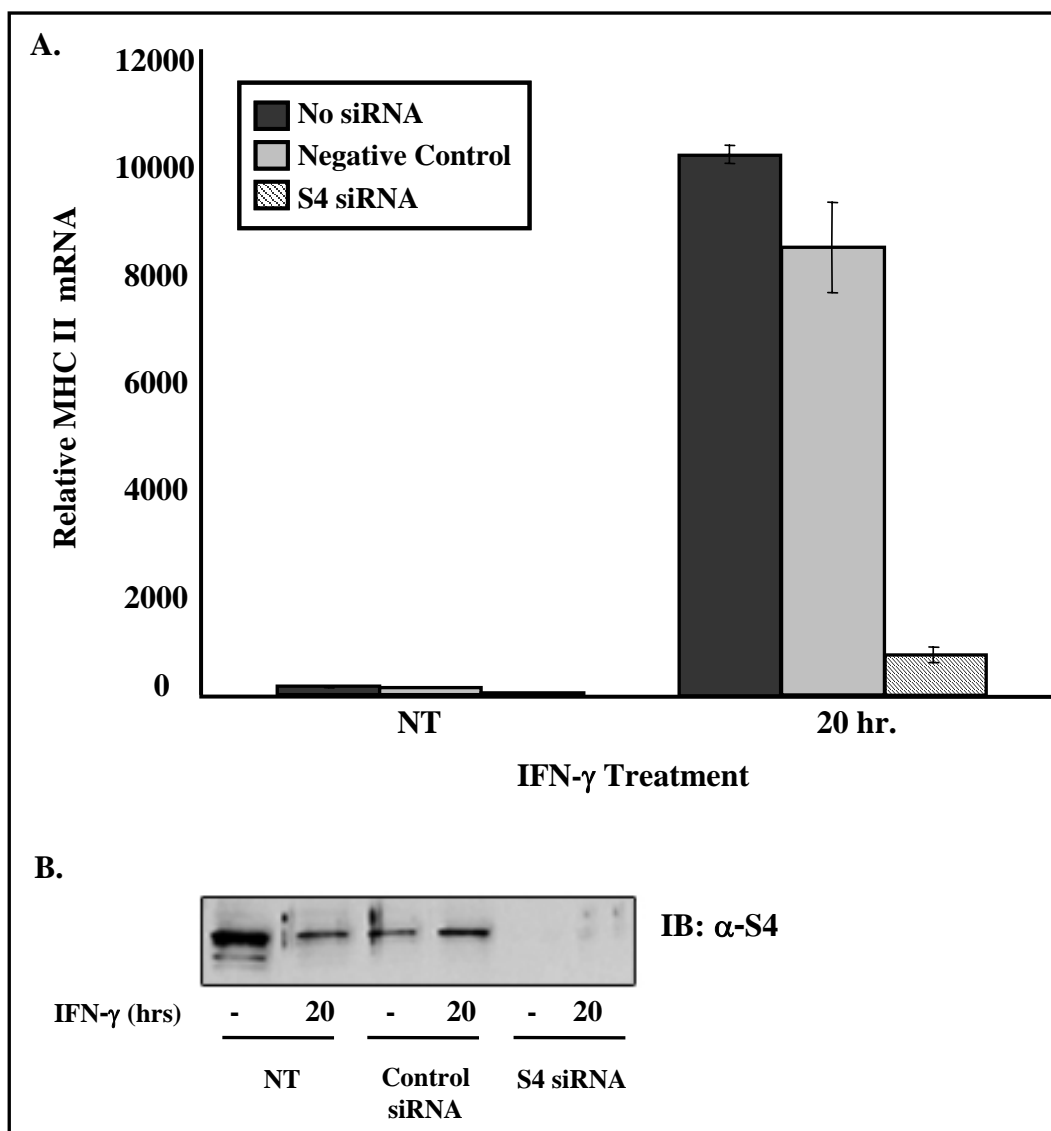


**Figure 12. S4 siRNA targeting construct efficiently reduces S4 expression.** HeLa cells were transfected with reagent only (**lanes 1-3**), 1.5 $\mu$ g of negative control siRNA (**lanes 3-6**), or 1.5  $\mu$ g of the S4 siRNA targeting construct (**lanes 6-9**) and assayed by immunoblot (IB) for expression of S4 (**A**). The S4 siRNA construct did not significantly reduce expression of the control 19S ATPases, S7, as assayed by immunoblot (**B**).

### **S4 is Required for Endogenous MHC II Transcription**

Based on our observations of S4 associations with S7 and CIITA, we next wanted to determine whether S4 plays a role in transcriptional regulation of MHC II. To assay S4 involvement in MHC II transcription, experiments using siRNA were undertaken. To reduce S4 expression in a 48hr. time period, the S4 siRNA construct (1.5 $\mu$ g), non-silencing control (1.5 $\mu$ g), or reagent only was transfected into HeLa cells. Following siRNA transfection, cells were stimulated with IFN- $\gamma$  for 20 hours to induce transcription of CIITA and MHC II (30). Approximately 20% of the cells from each sample were lysed in 1% NP40 and used to determine efficient knockdown of S4 (**Figure 13B**). From the remaining cells, RNA was extracted and quantified. The RNA (1 $\mu$ g) was reverse transcribed using RT-PCR to make MHC II or 18S cDNA. To quantify the molecules of cDNA, real time PCR with primers and probes specific for MHC II HLA-DRA or 18S was performed. The number of MHC II transcripts was then normalized to 18S transcripts.

Samples that did not receive IFN- $\gamma$  treatment had negligible amounts of MHC II mRNA, although the samples not treated with siRNA had a higher basal level of transcripts than those treated with siRNA (**Figure 13A**). As expected, the relative number of transcripts was dramatically increased in samples that were treated with IFN- $\gamma$  for 20 hours. Reduced expression of S4, however, resulted in a significant decrease of MHC II mRNA (**Figure 13A**). Decreased MHC II transcription can be largely attributed to reduced S4 expression as the non-silencing, negative control siRNA did not significantly affect the relative amount of MHC II mRNA. This data indicates that, in addition to S7, S4 may play an important role in the transcriptional regulation of MHC II.



**Figure 13. S4 is required for endogenous transcription of MHC II Genes.** To induce MHC II transcription, HeLa cells were stimulated with IFN- $\gamma$  at the indicated time point. Relative mRNA was obtained by normalizing the molecules of MHC II mRNA to 18S mRNA. With normal S4 expression, MHC II transcription markedly increased after 20 hours of IFN- $\gamma$  treatment (**A. and B. lanes 1-4**). Reduction of S4 expression resulted in a drastic decrease of MHC II transcripts at 20 hours (**A. and B. lanes 5-6**). Values and western blot are representative of 2 separate experiments.

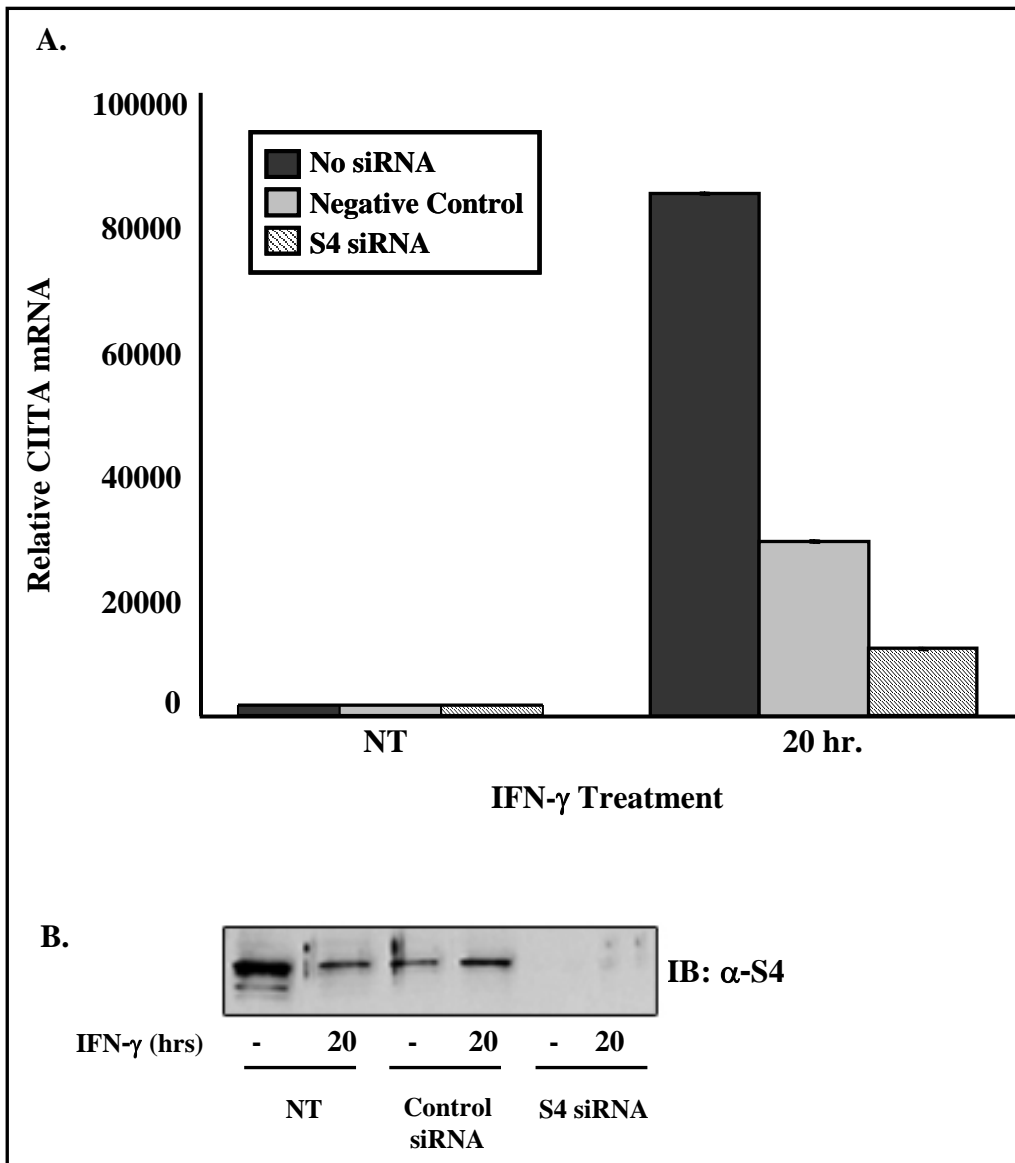
### **S4 is Not Required for Endogenous Transcription of CIITA Promoter IV Genes**

S4 was shown to play a positive role in the transcriptional regulation of MHC II genes, but the potential that S4 exerts its effects on apical transcriptional regulators of MHC II genes also exists (**Figure 13**). In particular, the effect of S4 on the endogenous transcription of CIITA was observed. Experiments were performed exactly as those involving the effect of S7 in endogenous CIITA transcription.

Briefly, HeLa cells were stimulated with IFN- $\gamma$  to induce CIITA transcription and siRNA (1.5  $\mu$ g) was used to knock down S4 expression. A sample that was not treated with siRNA, but that was stimulated with IFN- $\gamma$  (labeled as NT 20hr) served as a positive control for inducible CIITA transcription. Negative controls for IFN- $\gamma$  induced CIITA transcription consisted of samples that were not stimulated with IFN- $\gamma$  (labeled NT -, Control siRNA -, and S4 siRNA -). Negative controls for the effect of siRNA addition were achieved by transfecting cells with non-silencing siRNA (1.5  $\mu$ g) in the presence and absence of IFN- $\gamma$  (labeled Control siRNA - and Control siRNA 20 hr). RNA was extracted from cells and was then reverse transcribed with primers specific to CIITA PIV. Real-time PCR was then performed on the resultant cDNA and all samples were normalized to 18s RNA.

Despite the fact that S4 siRNA addition seems to cause reduced CIITA transcription, the reduction is also observed in the negative control samples (**Figure 14 A. and B. lanes 3-6**). The only reasonable interpretation of this data is that the effect on CIITA transcription is likely due to both endogenous siRNA addition and specific levels of S4 expression. So, dissimilar to S7, knocking down expression of S4 did not seem to have a marked effect on endogenous CIITA transcription (**Figures 8 and 14**). This

indicates that although reduced S4 expression leads to decreased MHC II transcription, this effect is only modestly mediated by reduced CIITA transcription (**Figures 13 and 14**).



**Figure 14. S4 is not required for endogenous transcription of CIITA PIV Genes.** To activate CIITA PIV transcription, HeLa cells were stimulated with IFN- $\gamma$  at the indicated time points. Identical samples were used for experiments shown in figures 13 and 14. Relative mRNA was obtained by normalizing the molecules of CIITA PIV mRNA to 18S mRNA. With normal S4 expression, CIITA PIV transcription markedly increased after IFN- $\gamma$  treatment at 20 hours (**A. and B. lanes 1-4**). Reduction in S4 expression resulted in a decrease of CIITA PIV transcripts at 20 hours (**A. and B. lanes 5-6**), but this reduction

was also observed in the negative control samples (**A. and B lanes 3-4**) Values and western blot are representative of 2 separate experiments.

## **Discussion**

### **Transcriptional Regulation of the Adaptive Immune Response**

The ability of vertebrates to mount an adaptive immune response is dependent on the ability to activate CD4 helper T cells (4, 5, 12). Given the central role of CD4 T cells in adaptive immunity, regulating their activity could open many avenues in the treatment of diseases characterized by excessive or insufficient immune responses (65). As CD4 T cell activation requires interactions of the T cell receptor with both MHC II molecules and antigen fragments, precise regulation of MHC II expression is critical to this process (12, 65). MHC II is regulated entirely at the transcriptional level, so any efforts to modulate the adaptive immune response must first focus on regulating MHC II transcription (4, 12, 65).

Although much is known about MHC II transcription, a complete understanding of events at the MHC II promoter has yet to be attained. MHC II transcription is controlled by binding of the transcription factors CREB, RFX, NFY, CREB to a conserved regulatory region of the MHC II gene (4). Respectively, these transcription factors bind to the S box, X1 box, X2 box, and Y box DNA sequences to form the MHC II enhanceosome (4, 12, 79). Expression of MHC II is unable to commence, however, until CIITA is recruited to the enhanceosome via protein-protein interactions (4, 12). CIITA influences MHC II transcription by recruiting general transcriptional machinery, transcriptional activators, and promoting accessibility of the MHC II promoter (32, 80).

In addition to being controlled by the binding of requisite transcription factors, transcriptional activators, and chromatin remodeling, transcription at the MHC II promoter is mediated by the activity of CIITA (30, 80). CIITA is subjected to a range of post-translational modifications in order to be recruited and stabilized to the MHC II enhanceosome (30, 39). In particular, monoubiquitination of CIITA was shown to enhance its activity and binding to the MHC II promoter (30). This finding, aside from adding to the understanding of CIITA modifications, exposed a potential link between the ubiquitin-proteasome system and the transcriptional regulation of MHC II.

### **Proteasomal Subunits as Non-degradative Transcriptional Regulators**

The participation of the ubiquitin-proteasome system in transcription has been well established, but protein degradation was thought to be the extent of this involvement (43). With recent findings, however, a non-proteolytic function of ATPases of the 19S proteasome in transcription is emerging. Studies by the Germane lab show recruitment of 19S ATPases subunits to activated promoters and association of 19S ATPases with general transcription factors (59-62). Importantly, the 19S ATPases were functioning independently of the 26S proteasome (59-62). This accumulating data makes a strong case for a novel function of 19S ATPases in transcription, but this non-proteolytic mechanism of regulation remains to be elucidated.

An interesting possibility is that, through their ability to recognize ubiquitinated substrates, act as chaperones to unfold proteins, and function as DNA/RNA helicases, the 19S ATPases affect epigenetic modifications and structure of chromatin (51, 52, 63, 64, 81). Epigenetic modifications refer to modifications of the nuclear histone proteins around which DNA is wrapped, with specific histone modifications directly affecting

gene expression (23-25). In support of the 19S ATPases influencing histone modifications, Tansey et. al. have demonstrated that the 19S ATPases S8 and S10b are required for the methylation, in this case a mark of active transcription, of specific histone proteins (64). Evidence that the 19S ATPases are required for the function of the yeast chromatin remodeling enzyme, SAGA, reinforces the concept of proteasomal ATPases acting as transcriptional regulators via interactions with chromatin and chromatin modifiers (63). The possibility, however, that 19S ATPases act to non-proteolytically regulate transcription by recognizing ubiquitinated substrates, such as CIITA, and recruiting them to promoters also exists.

### **S7 Regulates MHC II Transcription Independently of the 26S Proteasome**

Enhanced activity of CIITA by monoubiquitination implied a potential link between the ubiquitin-proteasome system, specifically the 19S ATPase subunits, and MHC II transcription. To explore the viability of this possibility, preliminary experiments were performed to determine how activity of the proteasome affected MHC II transcription. This was accomplished by inhibiting the activity of the 26S proteasome with the general proteasome inhibitor, MG132, and observing the effect on MHC II transcription (Greer lab unpublished data). Interestingly, general inhibition of the proteasome resulted in decreased MHC II transcription, indicating functions of the proteasome in transcriptional regulation extending beyond proteolysis (Greer lab unpublished data).

Based on previously published data, the ATPases of the yeast 19S proteasome were identified as likely mediators of this proteolytically independent transcriptional function (59-62). To extend the understanding of individual mammalian 19S ATPases in

transcriptional regulation, the roles of the individual 19S ATPases, S7 and S4, was examined. S7 was identified as a candidate MHC II transcriptional regulator as it had been tied to transcriptional regulation through activation of HIV genes and associations with general transcription factors (53, 67). S4 and S7 form a heterodimer and share sequence homology (67). The contribution of S4 to MHC II transcription was of interest as it could provide insight to the potentially different transcriptional functions of the individual 19S ATPases.

Preliminary experiments, using reporter assays and co-immunoprecipitations, did in fact illustrate a requirement of S7 expression for efficient CIITA activity and association of S7 with the MHC II enhanceosome components RFX-5, NFY-A, and CIITA (**Figures 1, 2, 4**). This data pointed to a more direct non-proteolytic role in MHC II transcription as S7 expression directly affected the ability of CIITA to induce MHC II expression. Also, this was the opposite effect as would be expected if S7 were only functioning to degrade CIITA. This data, however, did not establish a conclusive role for S7 in MHC II transcription as the experiments employed over expression of enhanceosome components and artificial promoter systems.

A more relevant experiment though, in which endogenous MHC II transcription in both the presences and absence S7 expression was assayed, argued for substantial involvement of S7 in regulation of MHC II (**Figure 5**). Using siRNA and real time PCR, it was found that S7 expression was necessary for endogenous MHC II transcription since reduced S7 expression resulted in a large decrease of INF- $\gamma$  induced MHC transcripts. Further tying S7 to MHC II transcription were chromatin immunoprecipitation assays,

which established localization of S7 at the MHC II promoter after IFN- $\gamma$  stimulation, suggesting a link between S7 function and active MHC II transcription (**Figure 7**).

Although real time PCR experiments evidenced the requirement of S7 for MHC II transcription, it did not delineate the mechanism by which it occurred. Specifically, S7 could be influencing transcriptional events directly at the MHC II promoter or influencing upstream events such as CIITA transcription, both of which are induced in HeLa cells with IFN- $\gamma$  stimulation (28). To address this concern, the IFN- $\gamma$  induced endogenous transcription of CIITA in the presence and absence of S7 expression was assayed. The results showed that, similar to MHC II transcription, the transcription of CIITA PIV genes was positively regulated by S7 (**Figure 8**). This result differed from data obtained for S4, which appeared to be necessary for MHC II transcription, but played a lesser role in CIITA transcription (**Figures 13 and 14**). Importantly, this experiment demonstrated that S7 was required for the transcription of two IFN- $\gamma$  inducible mammalian genes and that the 19S ATPases differentially regulate mammalian genes.

Greater insight into the mechanism of S7 transcriptional regulation was also gained by experiments showing that S7 specifically associated histones that had activating epigenetic modifications (Greer lab unpublished data). This result, in addition to the ability of S7 to regulation transcription of multiple genes, hints that S7 may be non-proteolytically involved in transcription by mediating epigenetic modifications and chromatin structure of target promoters. A common requirement of both MHC II and CIITA PIV inducible transcription is an accessible chromatin structure (28, 36, 80). As with other eukaryotic genes, inducible MHC II and CIITA expression is largely regulated

by the chromatin status and epigenetic modifications to histones (65, 80). An “open” chromatin structure, attained by histone modifications such as acetylation and the actions of chromatin remodeling enzymes, is associated with active transcription (23-25).

In the case of the IFN- $\gamma$  inducible CIITA PIV, an “open” chromatin structure is achieved, in part, by the actions of the ATP dependent chromatin remodeling enzyme BRG-1 (36, 38). Accessible chromatin structure of the MHC II promoter is mediated by the interactions of CIITA with histone modifying enzymes such as HATs and HDACs, and the ATP-dependent chromatin remodeling enzyme BRG-1 (65, 80). Similar to the manner in which BRG-1 mediates chromatin remodeling of multiple IFN- $\gamma$  inducible promoters, S7 may influence the pattern of histone modifications or chromatin structure of both the MHC II promoter and CIITA PIV.

This explanation would fit with the requirement of S7 in the transcriptional regulation of different cytokine inducible genes. It could also explain the different functions of 19S ATPases in transcriptional regulation, as different 19S ATPases could potentially mediate different histone modifications. As studies in yeast cells have clearly indicated a role for the 19S ATPases in influencing histone modifications and recruiting chromatin remodeling enzymes, it seems possible that mammalian 19S ATPases may function in a similar manner (63, 64).

### **Future Directions**

The potential of S7 to mediate chromatin accessibility is an avenue that will most likely be visited in the future, but the in-vivo association of S7 at CIITA PIV must also be determined. Co-immunoprecipitation assays indicate that S7 constitutively associates with IRF-1, a requisite transcription factor for CIITA PIV transcription. To fully

understand the function of S7 at CIITA PIV, chromatin immunoprecipitation assays will be performed to establish the kinetics of S7 binding.

Another set of interesting experiments using siRNA and chromatin immunoprecipitations could be designed to examine if reduced expression of S7 affects binding of transcription factors to the IFN- $\gamma$  inducible PIV. Importantly, if S7 is required for proper assembly of transcription factors at CIITA PIV, the mechanism of S7 regulation at the MHC II promoter would be elucidated. Finally, it would be interesting to determine if S7 and other 19S ATPases regulate transcription at other mammalian promoters that do not exhibit inducible expression.

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