Immunity, Vol. 10, 163–171, February, 1999, Copyright ©1999 by Cell Press

# A Defect in the Nuclear Translocation of CIITA Causes a Form of Type II Bare Lymphocyte Syndrome

**Drew E. Cressman,* Keh-Chuang Chin,*‡**
**Debra J. Taxman,* and Jenny P.-Y. Ting*†**
*Department of Microbiology and Immunology
Lineberger Comprehensive Cancer Center
University of North Carolina
Chapel Hill, North Carolina 27599–7295

## Summary

**The severe immunodeficiency type II bare lymphocyte syndrome (BLS) lacks class II MHC gene transcription. One defect from a complementation group A type II BLS patient is a 24 aa deletion in the MHC class II transactivator (CIITA). We show here that the molecular defect present in this protein is a failure of CIITA to undergo nuclear translocation. This defect was mapped to a position-dependent, novel nuclear localization sequence that cannot be functionally replaced by a classical NLS. Fusion of this 5 aa motif to an unrelated protein leads to nuclear translocation. Furthermore, this motif is not critical for transactivation function. This is a description of a genetic disease resulting from a novel defect in the subcellular localization of a transcriptional coactivator.**

## Introduction

A critical step in initiating an immune response to foreign pathogens is the breakdown and presentation of peptides derived from the invading species. These peptides are presented on the surface of B cells, dendritic cells and macrophages in conjunction with molecules of the class II major histocompatibility complex, thereby allowing recognition of the antigen by T cell receptors. This results in T cell activation and the stimulation of the immune response. MHC class II expression, constitutive in B cells, is induced in a wide variety of cell types by a number of cytokines and other agents (Kara and Glimcher, 1991; Ting and Baldwin, 1993; Mach et al., 1996). These agents stimulate the activity of the class II transactivator CIITA, the master regulator of MHC class II gene expression. CIITA activates the expression of the class II gene products *DR*, *DP*, and *DQ*, as well as the invariant chain and *DM* genes. CIITA is not a DNA-binding protein, but it does mediate class II gene transcription through the specific trimeric regulatory DNA sequences W/X/Y (Abdulkadir and Ono, 1995; Chang and Flavell, 1995; Riley et al., 1995; Boss, 1997; Westerheide et al., 1997). CIITA contains several functional domains typically associated with transcription factors or coactivators, including an amino-terminal acidic domain, a proline-rich domain, and a serine/threonine-rich region. It also contains functionally important sequences that have not been found in other transcriptional factors or coactivators, most notably a functional GTP-binding domain (Chin et al., 1997).

Normal constitutive and inducible CIITA activity is critical for regulating proper expression of MHC class II molecules, whereas aberrant over- or underexpression of class II can result in autoimmune or immunodeficiency diseases, respectively. Loss of CIITA activity leading to the loss of class II expression has been implicated in one such immunodeficiency disease, known as bare lymphocyte syndrome (BLS). While transcription factors play a critical role in modulating temporal and tissue-specific gene expression in response to developmental and physiological stimuli, significantly fewer inherited diseases have been characterized as being caused by a defect in gene regulation as opposed to a defect in a structural gene. The first such group of inherited diseases identified as resulting from mutations in specific transcription factors was type II BLS (Hume et al., 1989; Reith et al., 1995). In humans, the hallmark of this syndrome is the loss of MHC class II expression and severe immunodeficiency. The clinical onset of BLS occurs in the first year of life and is characterized by recurrent gastrointestinal, pulmonary, and respiratory infections (Arens et al., 1987). Lymphocytes are normal in number, but they fail to activate and proliferate in response to stimulation. BLS patients die in early childhood, usually before the age of four.

Individuals afflicted with type II BLS can be divided into one of at least four different complementation groups coinciding with genetic mutations in proteins that are important for the transcription of class II MHC genes, notably RFX5, RFXAP, RFXANK, and CIITA (Hauber et al., 1995; Peijnenburg et al., 1995; Douhan et al., 1996; Masternak et al., 1998; Yang et al., 1988). Patients in complementation group A are defective for the CIITA coactivator. The *CIITA* gene was first cloned by its ability to complement RJ2.2.5, a class II–negative cell line derived from Raji (Accolla, 1983; Steimle et al., 1993). Several different CIITA defects have been identified in complementation group A patients, including nonsense and splicing defect point mutations (Bontron et al., 1997). One specific *CIITA* gene defect that is found in cells from a group A BLS patient (BLS-2) lies outside of all known functional domains defined for the protein. It consists of a 72 bp deletion leading to an in-frame deletion of a 24–amino acid (aa) exon (aa 940–963) (Steimle et al., 1993). The function of this apparently critical exon and the molecular effect of its deletion are completely unknown.

In the current study, we undertook the identification of the specific role of the 24 aa exon deleted in the complementation group A BLS-2 patient. The focus was to determine how loss of this region of the protein leads to a defect in the ability of CIITA to activate the expression of MHC class II genes. The results show that this domain functions as a novel nuclear localization sequence (NLS) critical for targeting the CIITA protein to the nucleus. The NLS in CIITA likely has unique properties because fusion of this immunodeficient form of CIITA with a heterologous SV40 NLS fails to rescue

† To whom correspondence should be addressed (e-mail: panyun@med.unc.edu).
‡ Present address: Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510.

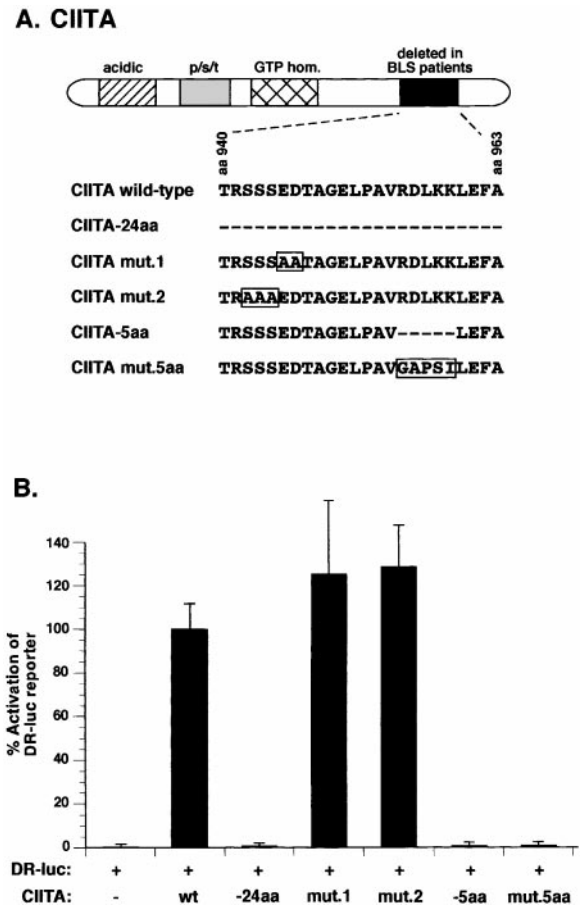


Figure 1. Mutations in the BLS Region Disrupt the Transactivation Ability of CIITA

(A) Schematic of the deletions or mutations of the CIITA 24 aa region that is missing in BLS patients. Acidic, proline/serine/threonine-rich, and GTP-binding homologous domains of CIITA are indicated.

(B) Cos-7 cells were transfected with a luciferase reporter gene under the control of the HLA-DRA promoter (designated DR-luc) and cotransfected with wild-type CIITA (lane 2) or the indicated CIITA mutants (lanes 3–7). Twenty-four hours posttransfection, cells were harvested, extracts prepared and normalized to protein concentration, and luciferase activity of each extract determined. Activation of the DR-luc reporter by all constructs is set relative to 100% activation by the wild-type CIITA (lane 2). Each category within the experiment was performed in triplicate. Standard deviations are shown.

proper CIITA localization and activity. This suggests that this region may mediate interaction of CIITA with other specific proteins of the nuclear import pathway. This paper reports a novel mutation in the NLS of a transcription factor leading to impaired function and resulting in a human disease syndrome.

## Results

### Reproduction of Complementation Group A BLS Mutant CIITA Activity in Cell Culture

To examine the basis for the loss of CIITA activity in cells from the BLS patient, a CIITA expression plasmid that lacks the same 24 aa exon as that deleted in the BLS-2 cells was constructed (Figure 1A, CIITA-24aa). As loss of this region results in the loss of CIITA activity and MHC class II gene expression in the patient, we initially determined whether this same mutation could recapitulate the mutant CIITA inactivity in cell culture lines. Wild-type *CIITA* or *CIITA-24aa* was transfected into Cos-7 cells and examined for its ability to activate a luciferase reporter gene under the control of the MHC class II DR promoter. Whereas transfection of wild-type CIITA strongly activated expression of the reporter gene, CIITA-24aa failed to activate the same reporter (Figure 1B). This confirms the previous findings that the loss of this 24 aa region in a BLS patient correlates with the failure to express MHC class II genes (de Preval et al., 1985; Lisowska-Grospierre et al., 1985; Steimle et al., 1993). To better define the motif and to identify the particular amino acids within the 24 aa exon that specifically caused the abrogation of CIITA activity, a series of mutations or deletions of short amino acid segments was constructed (Figure 1A). The actual mutated residues were selected because of their composition, which may reflect possible functions. Two acidic residues were mutated to alanine (CIITA mut.1), and a short stretch of serine residues was also mutated to alanine (CIITA mut.2). Neither mutation had any impact on the ability of CIITA to stimulate expression from a class II promoter (Figure 1B). In contrast, deletion of a stretch of five predominantly basic amino acids (CIITA-5aa) was sufficient to eliminate the transactivation potential of CIITA. To rule out artifacts that may arise due to deletion mutagenesis, the 5 aa region was also examined by site-specific mutagenesis (CIITA mut.5aa). This CIITA mutant was completely incapable of activating gene expression from a class II MHC promoter. This minimal region contained in aa 955–959 appears to be absolutely essential for the transactivation function of CIITA. Deletion of this region of CIITA in the BLS-2 patient accounts for the failure of the CIITA protein to activate class II gene expression.

### Localization of the BLS Mutant CIITA Protein by Immunofluorescence

The 5 aa region identified as being critical for proper CIITA activity, RDLKK, bears some similarity to NLSs reported for other proteins (Jans and Hubner, 1996). In general, these motifs consist of a series of basic residues concentrated within a short 5–7 aa sequence. The motif present in CIITA conforms to these specifications by containing 3 basic residues, an arginine and two lysines, although it is unusual in that the basic amino acids are separated by an aspartic acid residue. If deletion of the 24 aa exon in the BLS-derived CIITA results in the loss of an NLS, then differences in cellular distribution should be detected between CIITA and its mutants. To address this issue, full-length CIITA and the various mutants were fused to an amino-terminal FLAG epitope and transfected into Cos-7 cells. Gene expression of each of the constructs was under the control of an SV40 promoter. Anti-FLAG antibodies were used to determine the subcellular distribution of the CIITA proteins (Figure 2). Wild-type CIITA (Figure 2A) displayed a dual localization pattern, with high levels of expression in the nucleus

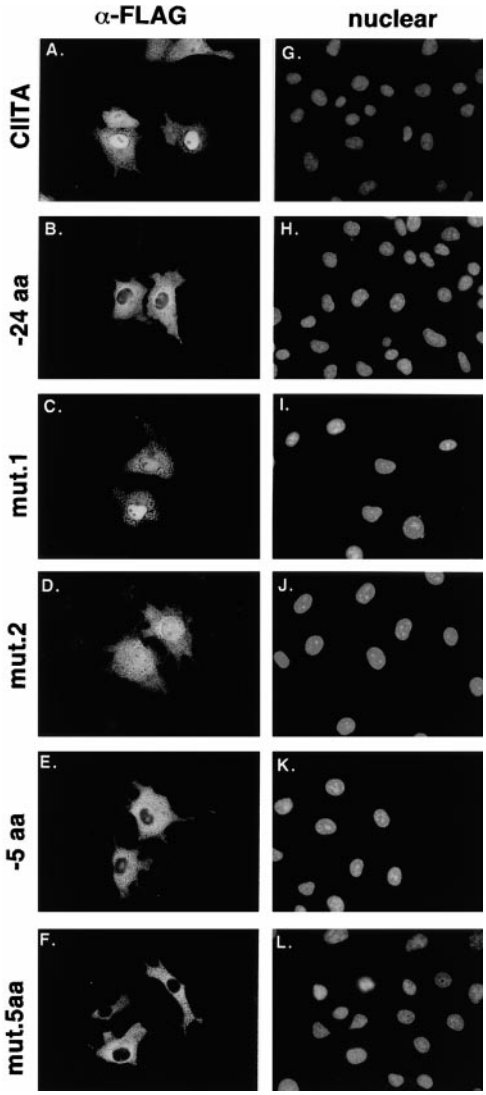


Figure 2. Mutation of the 5 aa Motif in the BLS Region of CIITA Is Sufficient to Eliminate Nuclear Localization of the Protein

FLAG-tagged wild-type CIITA (1.5 μg) (A) or the indicated CIITA mutants (B–F) were transfected into Cos-7 cells growing in 2-well chamber slides and assayed for subcellular localization. The cells were fixed at 24 hr posttransfection and stained with an anti-FLAG antibody/FITC-conjugated secondary antibody to assay for localization of the CIITA proteins in transfected cells (A–F). Hoechst staining indicates the presence and location of nuclei in all cells (G–L). Representative fields are shown. Wild-type CIITA (A) consistently shows cytoplasmic and nuclear localization. Cytoplasmic localization of the BLS mutant forms of CIITA (B, E, and F) is shown by the failure of nuclei to stain with the FLAG-CIITA-specific antibody.

as well as significant expression in the cytoplasm. To determine whether the cytoplasmic expression of CIITA in these studies was simply the result of ongoing mRNA and protein synthesis due to the constitutively active SV40 promoter, cells were also incubated in the presence of actinomycin D to inhibit further transcription. These cells still displayed dual nuclear and cytoplasmic localization of CIITA (data not shown), suggesting a physiological role for the protein in cytoplasm as well as the nucleus. CIITA mut.1 and CIITA mut.2 both displayed

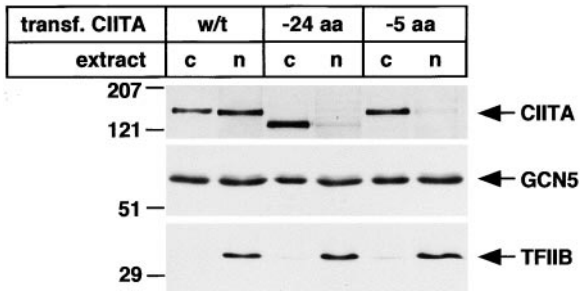


Figure 3. Subcellular Fractionation Demonstrates that Wild-Type CIITA Exhibits Dual Subcellular Localization, while Mutation of the BLS Domain Causes Retention of CIITA Predominantly in the Cytoplasm

Western blot analysis of nuclear and cytoplasmic fractions for the presence of CIITA, GCN5, or TFIIB. Cos cells were cotransfected with tagged CIITA and GCN5. Specific proteins were detected with anti-FLAG (CIITA) or anti-Xpress (GCN5) antibodies. Endogenous TFIIB staining demonstrates integrity of the nuclear extracts despite the apparent absence of the CIITA mutant protein in these subfractions.

localization patterns similar to that seen for the wild-type protein, consistent with the unmitigated ability of these mutants to activate expression from class II MHC promoters. In contrast, deletion of the 72 bp exon (CIITA-24aa) resulted in a strictly cytoplasmic localization of the CIITA protein. Site-specific mutation or deletion of the 5 aa segment of CIITA also resulted in a complete absence of the protein from the nucleus. Cytoplasmic localization of the mutated CIITA molecules correlates with the failure of these same mutants to activate gene expression from the DR promoter.

### Localization of CIITA Protein by Subcellular Fractionation

To confirm the localization patterns of wild-type CIITA and the BLS domain mutants, subcellular fractionation experiments were performed. Cells transfected with wild-type *CIITA*, *CIITA-24aa*, or *CIITA-5aa* were separated into cytoplasmic and nuclear fractions and assayed by Western blot analysis for the presence of the CIITA protein (Figure 3). In accordance with the immunofluorescence studies, wild-type CIITA was present in both cytoplasmic and nuclear fractions, whereas mutant CIITA lacking the 24 aa region was present almost entirely in the cytoplasmic fraction. Likewise, the CIITA-5aa protein was also strictly confined to the cytoplasm. Integrity of the nuclear extracts was confirmed by Western blots using antibodies to the basal transcription factor TFIIB, which is expected to be in the nuclear fraction (Figure 3, lower panel).

In order to demonstrate the capacity of the Cos cells to support nuclear translocation of a transfected gene product, cells were cotransfected with an expression plasmid for *GCN5*, a histone acetyltransferase shown to be active in the nuclear compartment (Brownell et al., 1996). Western blots detect GCN5 in the nuclear fraction, showing that protein from a control plasmid can be translocated to the nucleus. Cytoplasmic GCN5 may be either an inactive form or the result of increased expression in transfected cells. These data support the

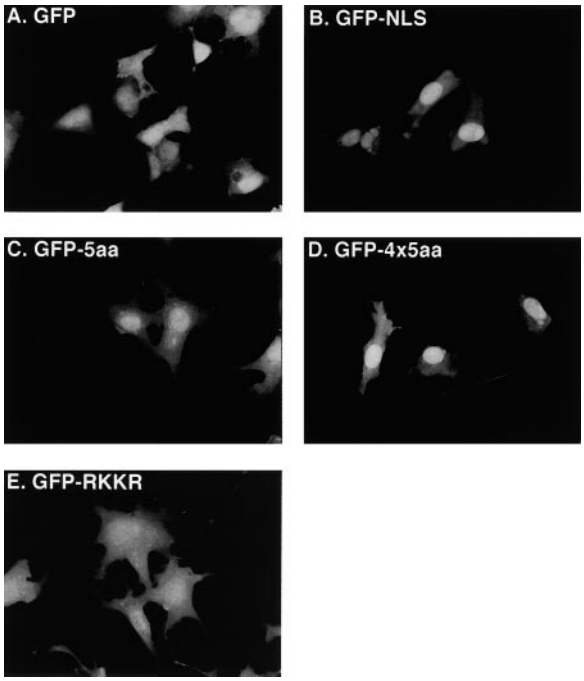


Figure 4. The CIITA 5 aa Motif Drives Nuclear Localization When Fused to Green Fluorescent Protein

Cos-7 cells were transfected with green fluorescent protein alone ([A] GFP), GFP fused to an SV40 NLS (B), GFP fused to one or four copies of the CIITA 5 aa sequence proposed to function as a NLS (C and D), or GFP fused to an unrelated basic sequence located upstream of the BLS deletion site in CIITA (E). Fluorescence of the fusion protein was observed at 24 hr posttransfection. 50× magnification.

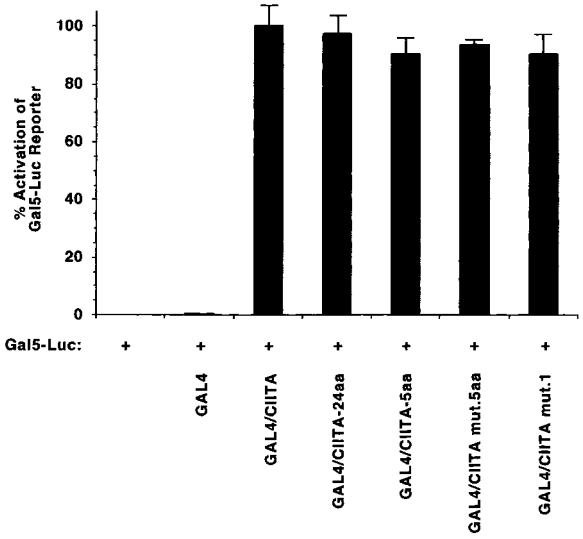


Figure 5. BLS-Derived CIITA Mutants Retain Transactivation Ability When Fused to GAL4

Cos cells were transfected with a Gal5-luciferase reporter plus GAL4 alone (lane 2), GAL4 fused to wild-type CIITA (lane 3), or GAL4 fused to the indicated CIITA mutants (lanes 4–7). Luciferase activity was assayed at 24 hr posttransfection and normalized to protein concentration of the extracts. Results are set relative to 100% activation of the Gal5-luc reporter by GAL4/CIITA (lane 2). Cos cells were transfected with the reporter plasmid alone as a negative control (lane 1).

hypothesis that a 5 aa motif is responsible for the nuclear localization of CIITA and suggest a molecular mechanism for the BLS phenotype resulting in the loss of CIITA activity and MHC class II gene expression.

### Nuclear Translocation of a Heterologous Protein Mediated by the 5 aa Motif

To determine if the 5 aa sequence of CIITA can function independently as a nuclear localization signal, this sequence was fused to green fluorescent protein (GFP). Expression of unmodified GFP transfected into Cos-7 cells resulted in a diffuse fluorescent pattern present throughout the cell (Figure 4A). Fusion of the NLS from the SV40 virus to GFP lead to a strong nuclear localization (Figure 4B). Importantly, GFP fused to one (Figure 4C) or four copies (Figure 4D) of the 5 aa motif of CIITA exhibited nuclear localization. This is a sequence-specific effect, as GFP fused to a sequence of basic amino acids, RKKR, which is found N-terminal to the BLS region (CIITA aa 742–745) and fits the criterion of known nuclear localization domains, displayed a diffuse fluorescent pattern (Figure 4E). In addition, mutations of this RKKR sequence did not affect CIITA transactivation ability, indicating that this region is not functionally critical despite its homology to other NLS (data not shown).

These experiments confirm that the 5 aa sequence of CIITA, RDLKK, is functioning as an NLS.

### The 5 aa Motif Is Not Required for Transactivation Function

Mutation of the BLS domain results in the failure of CIITA to translocate to the nucleus. However, this mutation may also affect the ability of CIITA to transactivate target genes by altering or destroying an activation domain. To distinguish between these two possibilities of a defect in nuclear translocation versus transactivation, a series of fusion proteins was made between the yeast transcription factor GAL4 and the CIITA mutants. The GAL4 protein used in these studies contained its own NLS and DNA-binding domain but lacked the carboxy-terminal activation domain. We assayed the ability of these fusion proteins to activate luciferase gene expression from a promoter containing five GAL4 DNA-binding sites (Gal5-Luc) (Figure 5). GAL4 alone is unable to activate reporter gene expression, while GAL4 fused to full-length, wild-type CIITA induced a 400-fold increase in expression. Fusion of GAL4 to the BLS mutants lacking the 24 aa or 5 aa motif, as well as CIITA mut.1, all resulted in similar degrees of activation. This data indicates that mutations in the BLS region of CIITA do not impair the ability of the protein to mediate activation of target genes and that any activation domains within CIITA remain intact and functional. Therefore, the loss of the 24 aa exon in the group A BLS patient appears to affect only the ability of CIITA to translocate to the nucleus

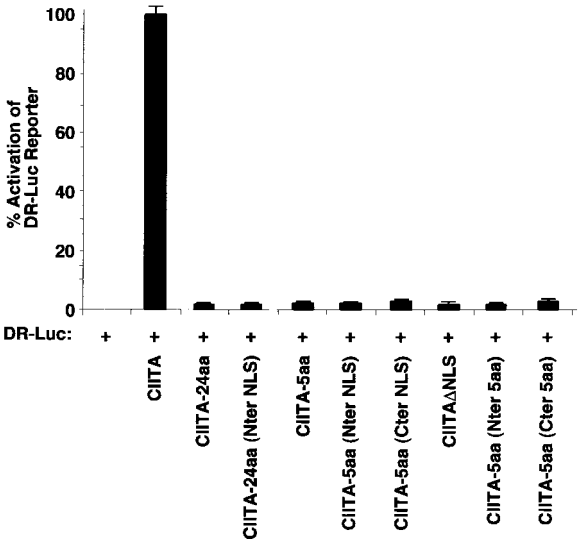


Figure 6. An SV40 NLS or 5 aa Motif Fused to the BLS-Mutated CIITA Fails to Rescue Trascriptional Activation Ability

Cos cells were cotransfected with the DR-luc reporter and the indicated CIITA constructs. For mutant constructs (lanes 4 and 6–11) the SV40 NLS was fused to the amino terminus of CIITA-24aa [CIITA-24aa (Nter NLS)] and CIITA-5aa [CIITA-5aa (Nter NLS)] or to the carboxyl terminus of CIITA-5aa [CIITA-5aa (Cter NLS)]. In another set of constructs, the 5 aa motif was fused to the amino terminus of CIITA-5aa [CIITA-5aa (Nter NLS)] or to the carboxyl terminus of CIITA-5aa [CIITA-5aa (Cter 5aa)], or the BLS deletion sequence was internally replaced with the SV40 NLS (CIITAΔNLS). Luciferase activity was assayed at 24 hr posttransfection and normalized to protein concentration of the extracts. Results are set relative to 100% activation of the DR-luc reporter by cotransfection with wild-type CIITA (lane 2). Presence of the SV40 NLS failed to rescue the activity of the BLS-mutants. CIITA mutants that contained the 5 aa motif at ectopic sites also failed to function.

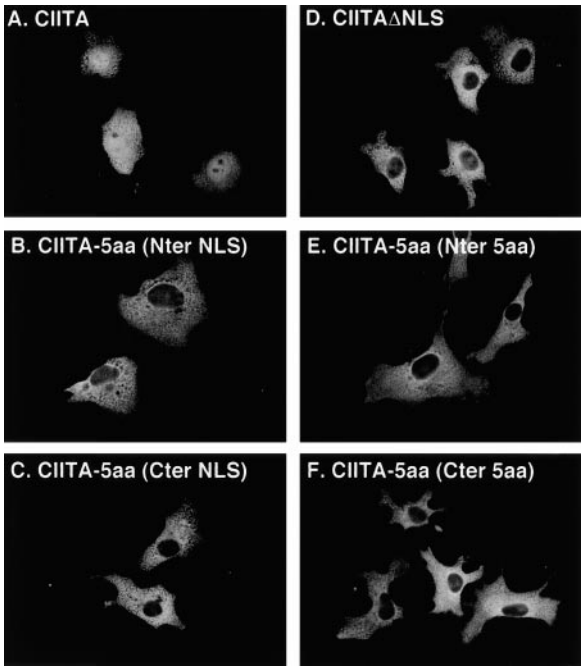


Figure 7. Subcellular Localization of Immunodeficient Forms of CIITA Fused to a Heterologous NLS

Wild-type CIITA (A) or mutant CIITA fused to the SV40 NLS (B–D) or the 5 aa motif (E and F) in various locations were transfected into Cos-7 cells. Mutants are labeled as described in Figure 6. Twenty-four hours posttransfection, cells were fixed and incubated with anti-FLAG antibody. FITC-conjugated secondary antibody was used to visualize localization of the fusion proteins. Wild-type CIITA shows characteristic cytoplasmic and nuclear staining.

without damaging the transactivation capacity of the protein.

### Inability of Heterologous NLSs to Rescue the BLS-Derived Mutant CIITA Activity and Localization

Given the hypothesis that the 5 aa motif present in the exon of CIITA deleted in the BLS patient is functioning as an NLS, we wished to determine whether BLS-derived CIITA activity could be rescued by fusion to a conventional NLS. The classical SV40 NLS was joined to the amino terminus of the CIITA mutants, CIITA-24aa [the plasmid generated is denoted as *CIITA-24aa (Nter NLS)*] and CIITA-5aa [*CIITA-5aa (Nter NLS)*], as well as to the carboxyl terminus of the CIITA-5aa mutant [*CIITA-5aa (Cter NLS)*]. In addition, a mutant protein was constructed in which the SV40 NLS replaced the CIITA 5 aa motif within the same location (*CIITAΔNLS*). We also wished to assay whether the 5 aa motif could function as an independent modular unit to rescue BLS-mutant CIITA activity. Therefore, similar constructs were made using the 5 aa motif and placing it at the amino or carboxyl terminus of CIITA-5aa [denoted *CIITA-5aa (Nter 5aa)* and *CIITA-5aa (Cter 5aa)*, respectively]. Each of these NLS- and 5aa-fused CIITA mutants were then assayed for their ability to activate expression of a luciferase reporter gene under the control of the class II DR promoter (Figure 6). If the NLS of CIITA and SV40 are interchangeable, then it would be expected that CIITA-24aa (Nter NLS), CIITA-5aa (Nter NLS), CIITA-5aa (Cter NLS), and CIITAΔNLS would function properly. Similarly, if the 5 aa motif acts in a position-independent manner, then it would be expected that CIITA-5aa (Nter 5aa) and CIITA-5aa (Cter 5aa) would also function properly. However, none of these mutants exhibited any transactivation function. This demonstrates that the NLSs from SV40 and CIITA defined by the BLS are not interchangeable and indicates that the 5aa motif functions in a position-dependent manner.

Immunofluorescent studies using these same mutants were performed to assess the subcellular localization of CIITA variants fused to the SV40 NLS or 5 aa sequence. The various constructs were transfected into Cos cells and localization of the fusion protein was probed 24 hr later using an antibody to the FLAG epitope. Expression of mutant forms of CIITA remained restricted to the cytoplasm despite fusion of the SV40 NLS to the amino or carboxyl terminus (Figures 7B and 7C). Similarly, substitution of the CIITA 24 aa exon sequence with the SV40 NLS also failed to induce nuclear translocation (Figure 7D). Addition of the 5 aa motif to

the ends of CIITA lacking the internal 5 aa sequence failed to stimulate import of the mutant forms of CIITA (Figures 7E and 7F). Localization of these mutants to the cytoplasm correlates with the failure of the SV40 NLS and 5 aa to rescue the transactivation potential of the BLS-mutant CIITA proteins. These data suggest that while the 5 aa motif is functioning as an NLS in its native position and context, its activity is distinct from that of the classical NLS. Therefore, the proper function of the BLS-mutant form of CIITA cannot be rescued through fusion of the protein with a conventional NLS or the 5 aa motif in another context.

## Discussion

The hallmark of BLS is a failure to express MHC class II antigens on the cell surface. Because of this failure, cells are unable to present antigen peptides in conjunction with the class II complex, resulting in the absence of $CD4^+$ T cell activation and initiation of an immune response to foreign pathogens. This disease was first described nearly 20 years ago (Touraine and Betuel, 1981), ultimately leading to the discovery of the gene responsible for one type of BLS (Steimle et al., 1993). This gene, *CIITA*, is the master regulator of MHC class II expression. Deletion of a 72 bp exon of *CIITA* in a complementation group A patient leads to its loss of function, failure to activate the expression of class II genes, and the establishment of the severe immunodeficiency characteristic of BLS. However, the molecular defect resulting from deletion of this region of *CIITA* has remained unclear. Therefore, this study undertook to examine specifically how loss of this region affects CIITA activity, which should lead to a clearer understanding of the origin and nature of BLS as well as helping to elucidate the mode of action of the CIITA protein and potential control points in modulating its activity.

We demonstrate here that the region missing in CIITA from the BLS-2 patient is critical for the proper nuclear localization of the protein. The CIITA protein normally is present in both the cytoplasm and nucleus. Deletion of the same 72 bps missing in the CIITA gene of the BLS patient causes CIITA to be localized only in the cytoplasm. It would be most desireable to demonstrate localization patterns of endogenous CIITA protein. However, due to extremely low levels of CIITA expression, we were unable to reliably detect native CIITA under a variety of conditions despite all attempts. Therefore, we made use of a cell culture system in which a transfected CIITA gene is tagged with a FLAG epitope. Transfected CIITA has been used by our lab and others (Chang et al., 1994; Zhou and Glimcher, 1995; Chin et al., 1997; Brown et al., 1998) to study CIITA activity and is presumed to function in a manner analogous to endogenous CIITA. Characterization of the BLS-mutant form of CIITA by both immunofluorescence and subcellular fractionation studies confirms its localization to be restricted to the cytoplasm. Mutations within this region identify a critical core domain of 5 aa responsible for nuclear translocation of CIITA. The sequence of this core amino acid motif, RDLKK, contains a concentration of basic residues, indicative of an NLS. While no other nuclear factors have been reported to contain this exact motif, it shares similarity to the carboxy-terminal portion of the bipartite NLSs present in the erb-A, thyroid $\beta$, and estrogen steroid hormone receptors (Jans and Hubner, 1996). Fusion of this domain to a green fluorescent protein is sufficient to cause GFP to accumulate in the nucleus. Furthermore, mutation of this core domain eliminates the ability of CIITA to enter the nucleus to transactivate gene expression from an MHC class II promoter. The BLS-mutant CIITA protein itself remains conformationally intact and fully functional in terms of its ability to transactivate targets, as demonstrated by fusion to a GAL4 factor. This suggests that the 5 aa motif functions as a nuclear localization signal and that the CIITA mutant form present in the BLS-2 patient lacks the exon that contains this NLS. Loss of this NLS results in the failure of CIITA to translocate to the nucleus and activate class II expression, causing the severe immunodeficiency of BLS.

Conventional nuclear localization signals generally act by mediating association of the NLS-containing protein with the karyopherin $\alpha/\beta 1$ heterodimer, resulting in docking to the nuclear pore complex and subsequent translocation into the nucleus (Schlenstedt, 1996). To date, two karyopherin $\alpha$ family member subtypes have been described, karyopherin $\alpha$ and karyopherin $\alpha 2$ (Gorlich and Mattaj, 1996). The SV40 NLS is known to interact with karyopherin $\alpha$ and $\alpha 2$ (Nadler et al., 1997). Complex formation with karyopherin $\beta$ modifies or enhances the ability of the karyopherin $\alpha$ family members to interact with the NLS. However, fusion of the SV40 NLS to the immunodeficient forms of CIITA failed to rescue normal localization and activity of the protein. The fact that the presence of an SV40 NLS is not sufficient to drive nuclear translocation of the BLS-derived mutant form of CIITA suggests that nuclear import of CIITA is not mediated by the same karyopherin $\alpha/\beta$ family members responsive to a classical NLS. The 5 aa NLS, deleted in the BLS form of CIITA, must be present to effect normal nuclear localization of the protein.

The question arises as to how the CIITA NLS specifically mediates translocation to the nucleus. As the 5 aa sequence alone can induce nuclear translocation when fused to a heterologous protein, the most likely explanation is that this motif is functioning as a recognition sequence for binding to other members of the karyopherin family distinct from those utilized by the classical NLS pathway, or it may mediate interaction with an as-yet-unidentified karyopherin family member. The evidence presented here indicates that the 5 aa motif of CIITA is functioning as an NLS. When fused to GFP, one copy of the 5 aa motif is sufficient to cause translocation of GFP. The role of this 5 aa motif in the CIITA protein is position dependent; while this motif can induce nuclear translocation when fused to the carboxyl terminus of a small protein such as GFP (approximately 27 kDa molecular weight), it is not capable of directing the import of CIITA when fused to the amino or carboxyl terminus. The size, three-dimensional structure, and conformational restraints of CIITA may restrict the 5 aa motif, suggesting that in order for the 5 aa motif to function properly as an NLS for CIITA, it must be present in a specific context and position within the protein. Alternatively, the CIITA

5 aa motif may regulate complex formation with another cytosolic protein containing its own NLS, allowing CIITA to cotranslocate into the nucleus at the same time. In either case, mutation or deletion of this region, as observed in one form of BLS, prevents CIITA from properly translocating into the nucleus and results in the cytoplasmic localization of the protein.

Proteins that use the classical NLS-mediated import pathway have interchangeable NLSs. However, not all nuclear localization signals are interchangeable, particularly among proteins that utilize different mechanisms of nuclear import. Analogous to the mode of CIITA import and its failure to be complemented by a SV40 NLS, current evidence indicates that a variety of pathways regulate protein translocation across the nuclear pore complex. Recent studies have demonstrated that these alternate pathways do not involve karyopherin $\alpha$ or $\beta$1 and are mediated by distinct NLSs. The hnRNP A1 protein contains an "M9" NLS, which recognizes karyopherin $\beta$2 (Siomi and Dreyfuss, 1995). While karyopherin $\beta$2 docks the NLS-containing protein to a similar set of nuclear pore proteins (nucleoporins) as that utilized by karyopherin $\beta$1, it recognizes an NLS distinct from the classical NLS (Bonifaci et al., 1997), leading to the suggestion that each of the karyopherin $\beta$ complexes recognizes its own type of NLS. Karyopherin $\beta$2 also appears to mediate the NLS-independent import of $\beta$-catenin (Fagotto et al., 1998). Like the karyopherin $\beta$ family, different subtypes of karyopherin $\alpha$ exist and bind NLSs with differing specificities and in a sequence-specific manner. For example, the high-mobility group transcription factor lymphoid enhancer factor 1 (LEF-1) binds to both karyopherin $\alpha$ and $\alpha$2 through a classical NLS, while a highly related transcription factor T cell factor 1 (TCF-1), which contains an NLS nearly identical to that of LEF-1, does not interact with either karyopherin $\alpha$ or $\alpha$2 (Prieve et al., 1998). An additional novel nuclear import pathway has been demonstrated for the HIV TAT protein. Translocation of TAT is not competed by SV40 NLS peptides and does not utilize the karyopherin $\alpha/\beta$ complex (Efthymiadis et al., 1998). These studies indicate that while the classical NLS pathway of nuclear import is the most well characterized, other related but distinct intracellular pathways also serve to mediate nuclear translocation. Specification of these alternate pathways is likely determined by different types of NLSs.

It is interesting to note that native CIITA has a dual localization in the cytoplasm and nucleus, as shown by both fractionation and immunofluorescence studies. It is possible that this dual localization has functional significance, as many transcription factors including STAT1, NF-AT, and NF-$\kappa$B are controlled at the level of nuclear translocation (Liou and Baltimore, 1993; Rao et al., 1997; Leonard and O'Shea, 1998). The ability of CIITA to properly enter as well as exit the nucleus may be critical in regulating its capacity to transactivate target MHC class II genes. Therefore, restriction of CIITA to the nucleus may interfere with its transactivation potential. It should also be noted that CIITA is itself a GTP-binding protein (J. Harton et al., unpublished data). Since GTP hydrolysis is a central event in the actual translocation though the nuclear pore of the NLS-protein/importin complex, the involvement of this motif with nuclear import and export will be of interest for future investigations.

Several heritable diseases have been described resulting from mutations in transcription factors. Mutations in *Pit-1* lead to combined pituitary hormone deficiency (Pfaffle et al., 1992; Radovick et al., 1992); mutations in *Brn-4* lead to X-linked hearing impairment (de Kok et al., 1995); mutations in *RFXANK*, *RFX5*, and *RFXAP* lead to complementation groups B, C, and D of BLS, respectively (Durand et al., 1997; Masternak et al., 1998; Reith et al., 1988); mutations in *CBFA-1* (Mundlos et al., 1997) lead to cleidocranial dysplasia; and mutations in the Pax family of transcription factors lead to a variety of congenital eye defects, including Waardenburg's syndrome (Tassabehji et al., 1992). While *p53* somatic mutations are recognized as the most frequent genetic abnormality in cancer, rare *p53* germline mutations have also been identified, leading to Li-Fraumeni hereditary cancer syndrome (Malkin et al., 1990). However, each of these transcription factor mutations affect either the DNA-binding domain or the transactivation domain of the protein. In contrast, the mutation in *CIITA* that leads to one form of BLS in the complementation group A patient is the loss of a nuclear localization signal. The molecular defect reported here is a description of a novel mutation affecting the NLS of a transcriptional coactivator, which leads to a hereditary disease.

## Experimental Procedures

### Cells and Plasmids

All mutants were constructed using the pcDNA3.FLAG.CIITA8 parent vector containing an 8 aa FLAG epitope upstream of the first methionine of CIITA (Chin et al., 1997). CIITA-24aa was constructed by overlapping PCR using four primers: 1, (2071-GCCAAAGGCTTAG TCCAACACC-2092); 2, (GCATCGATGATCCTCTGAGTCTGCACAAG CTTTCC-2794); 3, 2(TCATCGATGCTCTGGGCCCTGTCTCAGGCC CCAGG-2914); and 4, (3217-ACACCATGTCCGGAAGCACA-3198). Products from initial PCR reactions using primers 1/2 and 3/4 were combined and reamplified with primers 1/4. The resulting product deleted bp 2818–2890 of CIITA. CIITA mut.1 and mut.2 were constructed by transformer site-directed mutagenesis (Clontech) according to the instructions of the manufacturer using mutagenic primers to alter 945-ED-946 to 945-AA-946 and 942-SSS-944 to 942-AAA-944, respectively. CIITA mut.5aa, deleting bp 2863–2877, was made using the QuikChange site-directed mutagenesis kit (Stratagene) and the primer 2847-CGAGCTCCCTGCTGTTTggggccccatc gataCTGGAGTTTGCGCTGG-2893, according to the instructions of the manufacturer. CIITA-24aa (Nter NLS) and CIITA-5aa (Nter NLS) were made by initially inserting the SV40 NLS sequence (ATGCCGAA GAAAAAGCGAAAGGTA) immediately downstream of the FLAG epitope in the pcDNA3.FLAG.CIITA8 parent vector by overlapping PCR and reamplification. This NLS.CIITA vector was digested with AocI and BsrGI to remove a 2.5 kb fragment internal to the CIITA gene and containing the BLS domain. AocI–BsrGI fragments (2.5 kb) containing the mutated BLS region were isolated from CIITA-24aa or CIITA-5aa and subcloned into the AocI–BsrGI-digested NLS.CIITA vector to generate the NLS.CIITA variants. CIITA-5aa (Nter 5aa) was constructed by mutating the NLS of NLS.CIITA to 5aa with QuikChange, using the primer CAAAGACGATGACGA TAAAATGcgggacctaaagaaaaacgttCGTTGCCTGGCTCCACGCCC TGC, followed by subcloning in the AocI–BsrGI fragment from CIITA-5aa. Carboxy-terminal NLS and 5 aa mutants were constructed by inserting a minipolylinker containing ClaI and BsiWI sites just prior to the stop codon. Double-stranded oligos encoding the SV40 NLS (PKKKRKV) or the 5 aa motif (RDLKK) were ligated into ClaI/BsiWI-digested parental vectors. CIITA$\Delta$NLS was prepared by mutating

aa 955–959 of pCDNA3.FLAG.CIITA8 parental vector to the SV40 NLS by QuikChange, using the primer 2842-GCTGGGGAGCTCCCT GCTGTTccgaagaaaaagagaaaggtcCTGGAGTTTGCGCTGGGCCC-2897. GAL4/CIITA (pSGCIITA) was a generous gift from Jeremy Boss (Riley et al., 1995). GAL4/CIITA mutant derivatives were prepared by subcloning in the AocI–BsrGI fragment from the appropriate CIITA mutant donor vectors. A 1.9 kb BamHI–EcoRI fragment containing the hGCN5 cDNA from pRSET GCN5 was inserted into pCDNA3.1 His (Invitrogen) containing an in-frame Xpress tag sequence. All constructs were sequenced.

**Luciferase Assays**

Cos-7 cells were split to 6-well plates 18 hr prior to transfection. One microgram of each plasmid was transfected into cells using the Qiagen Superfect or Fugene 6 reagent (Boehringer Mannheim) for 3 hr. Cells were then changed to fresh media (Superfect transfections only), incubated, and harvested at 24 hr posttransfection in 200 μl 1× reporter lysis buffer (Promega). Forty microliters were assayed for luciferase activity as previously described (Piskurich et al., 1998). Extracts were normalized to protein concentration. Each transfection was performed in triplicate.

**Immunofluorescence**

FLAG-tagged wild-type CIITA (1.5 μg) or the indicated CIITA mutants were transfected into Cos-7 cells growing in 2-well chamber slides and assayed for subcellular localization. At 24 hr posttransfection, slides were rinsed in PBS, fixed in 3:2 acetone:PBS for 4 min, incubated 1 hr with M5 anti-FLAG mouse monoclonal antibody (Sigma) diluted 1:300, rinsed, incubated 1 hr with FITC-conjugated goat-anti-mouse secondary antibody (1:500 dilution; Pharmingen) supplemented with 0.5 μg/ml Hoechst dye no. 33258 (Sigma), then rinsed and mounted in Vectashield medium (Vector Labs), and observed at 50× magnification. Fluorescence indicates FLAG-CIITA protein localization or Hoechst dye–stained nuclei of the same field.

**Subcellular Fractionations and Western Blots**

Cos-7 cells were transfected in 6-well plates with the indicated plasmids, harvested at 24 hr posttransfection, and fractionated into cytoplasmic and nuclear lysates as previously described (Cressman et al., 1996). Harvested cells were pelleted and resuspended in 400 μl cold buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and incubated on ice for 15 min prior to the addition of 40 μl 10% NP40. Samples were vortexed for 1 min and centrifuged. The cytoplasmic supernatant was reserved, while the pelleted nuclei were resuspended in 50 μl of a high-salt buffer (20 mM HEPES [pH 7.9] 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), rocked vigorously at 4°C for 1 hr, and centrifuged to yield to the nuclear supernatant fraction. Protein concentrations of each fraction were determined by the Bradford method. For Western blot analyses, 10 μg of each fraction were separated on 8% or 12% SDS polyacrylamide gels, transferred to nitrocellulose, blotted with 2 μg α-FLAG or α-TFIIB antibodies (Santa Cruz Biotechnology), and detected by chemiluminescence (Amersham).

**Green Fluorescent Protein Analysis**

Fusion proteins were constructed in which regions of CIITA were subcloned in-frame to the carboxyl terminus of GFP using the pEGFP-C1 parent vector (Clontech) and double-stranded oligonucleotides complementary to CIITA sequences. Regions of CIITA used were: one or four copies of bp 2863–2877 (GFP-5aa, GFP-4x5aa, respectively) and bp 2224–2235 (GFP-RKKR). The SV40 NLS (CCTAAGAAGAAGAGGAAGGTT) was fused to GFP (GFP-NLS) as a positive control. Cos-7 cells ($8 \times 10^4$) were plated into 2-well chamber slides 12 hr prior to transfection. GFP plasmids were transfected into cells, rinsed 24 hr later with PBS, and fixed with 3:2 acetone:PBS. Fluorescence of the fusion protein was observed using an Olympus BX40 microscope at 50× magnification.

**Acknowledgments**

We would like to thank R. Hellendall for assistance with the microscopy and J. Boss for sharing unpublished data. Supported in part by NIH grants RO1-AI29564–07 and AI41751 (J. T.) and by NIH-NRSA grants CA-09156 (D. E. C.) and CA-73063 (D. J. T.). D. J. T. is a Cancer Research Institute Fellow.

Received September 2, 1998; revised December 21, 1998.

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