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# The MHC class II transactivator (CIITA) requires conserved leucine charged domains for interactions with the conserved W box promoter element

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## ABSTRACT

**The class II transactivator CIITA is required for transcriptional activation of the major histocompatibility complex (MHC) class II genes. Aside from an N-terminal acidic transcriptional activation domain, little is known about how this factor functions. Extensive mutagenesis of CIITA was undertaken to identify structural motifs required for function. The ability of mutants to activate a reporter gene under the control of MHC class II conserved W-X-Y or X-Y regulatory elements was determined. Two mutants displayed differential activity between the two promoters, activating transcription with the W-X-Y but not the X-Y elements. All mutants were tested for their ability to interfere with wild-type CIITA activity. Five CIITA mutant constructions were able to down-regulate wild-type CIITA activity. Three of these mutants contained targeted disruptions of potential functional motifs: the acidic activation domain, a putative GTP-binding motif and two leucine charged domains (LCD motifs). The other two contained mutations in regions that do not have homology to described proteins. The characterization of CIITA mutants that are able to discriminate between promoters with or without the W box strongly suggests that CIITA requires such interactions for function. The identification of LCD motifs required for CIITA function brings to light a previously undefined role of these motifs in CIITA function.**

## INTRODUCTION

Major histocompatibility complex (MHC) class II genes encode integral components of the immune response. The three human class II isotypes, DR, DQ and DP, each consisting of an α/β heterodimer, present processed extracellular antigenic peptides to helper T cells (1). Class II genes are coordinately expressed in B cells, macrophages, dendritic cells and thymic epithelium. However, their expression can be induced in a number of cell types by treatment with interferon-γ (IFN-γ) (2–5). The temporal and spatial regulation of class II gene expression is vital to the maintenance of a functional immune system. Clues to the mechanism of class II gene regulation have come from analyses of cell lines from patients exhibiting the bare lymphocyte syndrome (BLS), an inherited immunodeficiency (reviewed in 6,7). BLS patients lack all class II expression, resulting in an inability to mount an immune response to soluble antigens (8,9). Four clear BLS complementation groups have been defined, each deficient in a necessary *trans*-acting factor (6,10,11).

The promoters of class II genes contain three conserved regulatory elements, termed the W, X and Y boxes (3,12). The W box is the most upstream of the elements and is required for IFN-γ-induced and maximal B cell expression of class II genes (13–17). While the role of the W box in IFN-γ induction has been well documented (16,17), the mechanism for this requirement has remained elusive, presumably because the factors that bind to the W box have not been well characterized. A recent report suggested that RFX, a factor that binds to the X1 box (see below), binds weakly to the smaller S box region within the W box (18). The X box, both necessary and sufficient for class II expression in B cells (14,15,19), has been biochemically and genetically subdivided into the X1 and X2 boxes (13,14). RFX, a multimeric protein complex consisting of 75, 41 and 36 kDa subunits, binds to the X1 box (20,21). RFX activity is deficient in three of the BLS complementation groups (B, C and D) (13,21–25). The factor X2BP has been shown to bind the X2 box and has been described (26–29). The Y box, an inverted CCAAT motif, has been shown to bind the minor groove DNA-binding protein NF-Y (30). The X and Y box factors form synergistic complexes on all class II promoters (28,31) and therefore appear to coordinate the regulation of all class II genes.

The class II transactivator CIITA appears to function as a master regulatory switch for class II expression. CIITA is mutated in BLS complementation group A (32). Expression of CIITA correlates exactly with class II expression in both antigen-presenting cells and cells induced to express class II genes by IFN-γ (32). Moreover, class II negative cells transfected with CIITA express class II genes (33–37), suggesting that CIITA expression is a limiting component of class II gene regulation. CIITA does not appear to bind DNA. Analysis of the 1130 residue primary amino acid sequence of CIITA (32) revealed an acidic region, a proline/serine/threonine-rich (P/S/T) region, a consensus GTP-binding motif (38), a leucine-rich region and four leucine and charged residue-rich domains (LCD). The acidic domain has

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**Table 1.** Primers used for construction of the CIITA mutants

| CONSTRUCT (bp deleted) | 5' Primer (bp) | OL-PCR Primer* | 3' Primer (bp) |
|---|---|---|---|
| Δ1 (1099-1129) | (799) 5'-GTTTGTCCCCACCATCTCCAC | 5'-GCTGGAGAGGTCTCC/GAGCAGTTCTACCG | (2041) 5'-GACATAGAGTCCCGTGAGCG |
| Δ2 (1217-1246) | (799) | 5'-GGTGGATCTGGTGCA/GCTGGAGCGGGAACTG | (2041) |
| Δ3 (1376-1408) | (799) | 5'-ATTGCTGTGCTGGGC/GCAGTGAGCCGGGCC | (2406) 5'-TGGAAGATCAGCCCAGCCAGAAAGC |
| Δ4 (1436-1465) | (799) | 5'-GCCTGGGCTTGTGGC/GTCCCCTGCCATTGC | (2041) |
| Δ5 (1499-1528) | (799) | 5'-AACCGTCCGGGGGAT/CTGGGCCCACAGCCA | (2406) |
| Δ6 (1862-1894) | (799) | 5'-GTGATGCGCTACTTT/CCCCTGACGCTCCTCCGG | (2406) |
| Δ7 (2165-2194) | (799) | 5'-CAGTTCCCATCCGCA/TTAGTCCAACACCCA | (2406) |
| Δ8 (2486-2530) | (799) | 5'-CAGAAGGTGCTTGCG/CTGCTTGAGCTGCTG | (3118) 5'-GTCCCCGATCTTGTTCTCACTC |
| Δ9 (2771-2806) | (799) | 5'-TGTGTCACCCGTTTC/TCCCTGCGGCAGCAT | (3118) |
| Δ10 (3047-3076) | (2288) 5'-AGTGGCGAAATCAAGGACAAGG | 5'-TTCCCCAAACTGGTG/CATCTGGACCTGGAT | (3566) 5'-CAAGGTCCAGCGTGGTTAGTGTCCTCAG |
| Δ11 (3224-3262) | (2288) | 5'-GCCTACAAACTCGCC/AGCTTGTACAATAAC | (3566) |
| Δ12 (3458-3487) | (2288) | 5'-CCCACCATCCCATTC/TCACGGATCAGCCTG | **(pSVK3') 5'-TACGCCCCGCCCTGCCACTC |
| 161-1130 (a.a. 1-160) | † (599) 5'-GCTCTAGACCATGGAGCCCCCCACTGTG | | (1537) 5' - GGGCCCAGGGAGAAGAGCAGAT |
| mutL1 | (1447) 5' - GCTTGTGGCCGGCTTCCCCAGT | ††5'-GCCTATGGCGCACAGGATGCTGCGTTCTCCCTG | (2406) |
| mutL2 | (1447) | ††5'-CCCTGCTCCGCTCGGGGGGCAGCAGCCGGCCTT | (2406) |
| Δ13 | (104) 5' - GATTCCTACACAATGCGTTGCCTGGCTC | 5'-GCAGAAGTTGGGCAGGCTGAGCCCCCCAGT | (1537) |
| mutCDK | (104) | ††5'-GGCCTCCCAACAGCTGCGGACGCCCCAGGCTCCACC | (1537) |

All primer sequences are given in the 5′→3′ direction with the position of the 5′ base indicated.
*For complementary OL-PCR primers, the sequence is given for the coding strand of CIITA.
**Primer pSVK3′ anneals to the MCS of pSVK-Flag-CIITA, immediately downstream of CIITA.
†The primer adds a *Xba*I site and Koxak consensus translation start site such that the protein begins at wild-type residue 161.
††Sequence changes that lead to alanine substitutions are underlined.

been shown to act as a transcriptional activation domain in both mammalian and yeast systems (39,40). Mahanta *et al.* (41) recently showed that this transcriptional activation domain functions similarly to that of VP16 by interacting with the TATA-binding protein TBP and several TBP-associated factors. No function has been ascribed to the P/S/T region, but it has been shown that it is required for CIITA activity (42).

The mechanism for CIITA action is poorly understood; recent studies have suggested that CIITA functions as a coactivator by interacting with X box DNA-bound transcription factors and activating transcription through its activation domain (39,40). To further investigate the mechanism of CIITA function, a series of internal deletion and substitution mutants was created. The mutants were tested for their ability to activate class II-specific reporter gene constructs and for their ability to interfere with the function of wild-type CIITA. It was found that deletions spanning the C-terminal two thirds of the protein abrogated CIITA function. Two mutants were found to discriminate between a W-X-Y-driven reporter and an X-Y reporter, suggesting direct interaction with the class II-specific transcription factor(s) bound at the W box; both of these mutants contained disruptions of conserved LCD motifs, highlighting the potential involvement of these motifs in CIITA function. Numerous mutations resulted in proteins that were able to interfere with the function of wild-type CIITA, indicating that in addition to the activation domain, CIITA requires additional regions for activity. Thus, the analysis of these mutants brings to light additional functions of CIITA, including the potential for interactions with the W box promoter element and a functional requirement for LCD motifs and neighboring residues.

## MATERIALS AND METHODS

### Cell culture

Raji is a B cell line derived from a patient with Burkitt's lymphoma (43). RJ2.2.5 is an MHC class II negative derivative of Raji and was provided by Dr R. Acolla (44). Both cell lines were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY), supplemented with 5% fetal bovine serum (Intergen Inc., Purchase, NY), 5% calf serum (Hyclone Inc., Logan, UT), 2 mM L-glutamine (Gibco BRL), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco BRL).

### CIITA plasmids

Wild-type CIITA was cloned into the eukaryotic expression vector pSVK3 (Invitrogen Corp., Carlsbad, CA), with an N-terminal FLAG epitope tag (IBI, Corning, NY), which was used as the base vector for mutagenesis. Overlap PCR (OL-PCR) mutagenesis was used to design the internal deletion and amino acid substitution mutants. Two complementary overlap primers containing the desired mutation were used separately in PCRs with CIITA-specific primers 5′ or 3′ of the deletion (Table 1). PCR products were mixed and used as template for OL-PCR amplification using the CIITA-specific primers. The OL-PCR product was cloned into a wild-type CIITA background using available restriction sites (all restriction enzymes were from New England Biolabs, Beverly, MA). Incorporation of mutations was monitored by ablation of restriction sites and all PCR-derived sequences were checked by DNA sequencing. *In vitro* transcribed and translated proteins were prepared using the TnT kit (Promega Corp., Madison, WI) as described by the manufacturer.

### Transient transfections and CAT ELISAs

*CAT* reporter constructs pDRW-X-Y and pDRGX1-X2-Y, containing the W-X-Y or X-Y motifs 5′ of the minimal *DRA* promoter, respectively, were used (13,27). Co-transfections were performed by electroporation as previously described (27). For RJ2.2.5 transfections, cells were harvested 70 h post-transfection, washed, and lysed by three rounds of freezing and thawing. CAT expression was measured by an ELISA (Boehringer Mannheim, Indianapolis, IN). For Raji transfections, cells were harvested at 48 h post-transfection, a time point determined by flow cytometry to be optimal for a dominant negative effect (data not shown), washed, lysed, and assayed by ELISA as above.

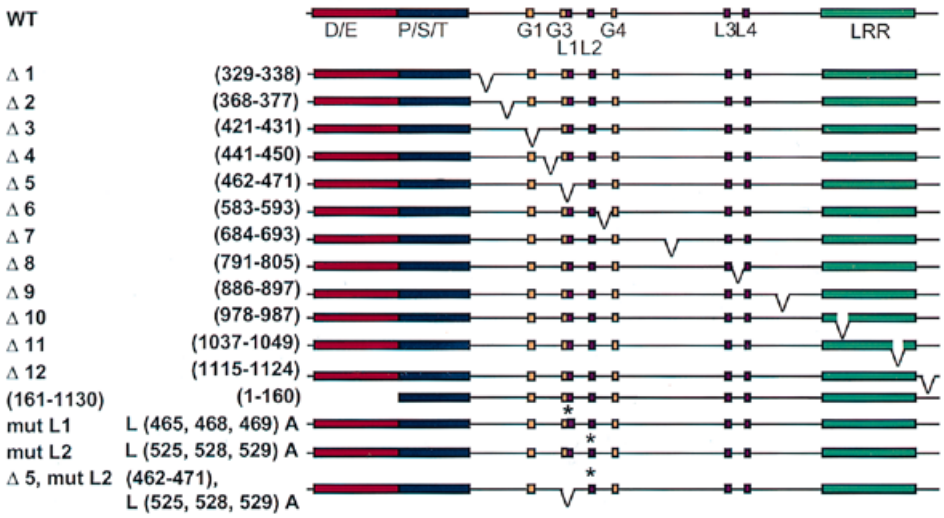


**Figure 1.** CIITA deletion mutants used in this study. (Top) Wild-type CIITA with the acidic activation (D/E), P/S/T, GTP consensus (G1, G3 and G4), LCD (L1–L4) and leucine-rich regions (LRR) indicated. (Below) CIITA mutants created for this study. Amino acid residues deleted or changed are indicated in parentheses, with a schematic approximating the locations of the individual mutations along the length of CIITA. Internal deletion mutants (V) were created using OL-PCR as described in Materials and Methods; the sequences of PCR primers used can be found in Table 1. Asterisks (*) indicate regions containing alanine substitutions.

### Generation of α-CIITA antisera and immunoblot analysis

A bacterial expressed maltose-binding protein–CIITA fusion protein (MBP–CIITA) was used to generate polyclonal antiserum. CIITA-specific IgG antibodies were purified by chromatography over a protein A column as described by the manufacturer (Pharmacia Biotech, Piscataway, NJ). Cell lysates were separated by 7.5% SDS–PAGE and transferred to PVDF membranes as described (45). Western blots were blocked overnight in 10% non-fat dry milk (w/v) in PBS-T (0.1% Tween-20) at room temperature with agitation. Polyclonal α-CIITA antiserum was diluted 1:2000 in block solution; blots were incubated in primary antibody solution for 1 h at room temperature with agitation. Blots were washed in PBS-T and incubated for 1 h in secondary antibody (HRP-conjugated anti-rabbit IgG; Sigma, St Louis, MO) diluted 1:3000 in block solution. Blots were washed as above and developed using enhanced chemiluminescence (ECL system; Amersham Life Science, Arlington Heights, IL).

## RESULTS

### CIITA deletion mutants

To examine the nature of CIITA function, a series of mutations were introduced into the CIITA gene that could be assayed for their ability to distinguish between wild-type and mutant class II promoters. Small in-frame deletions beginning 3′ of the acidic activation domain were created along the length of CIITA (Fig. 1). The mutations were made downstream of the acidic activation domain for three reasons. First, previous work had shown that this domain was required for transcriptional activation (39). Second, the non-functional allele of CIITA in the BLS complementation group A cell line RJ2.2.5 has a fully intact activation domain (46), suggesting that CIITA has properties in addition to transcriptional activation. Third, all complementation group A BLS patient-derived cell lines have mutations downstream of the activation domain (32,33).

The predicted coding region of CIITA (32) was cloned into the pSVK eukaryotic expression vector, which had been modified to contain the FLAG epitope (IBI, Corning, NY) at its N-terminus. The construction containing the wild-type CIITA sequence was termed pFLAG-CIITA. All other constructs were based on this vector. Of the engineered mutations, some were randomly spaced along the length of the CIITA gene, while others targeted specific regions that potentially could affect function. Two mutants, Δ3CIITA and Δ5CIITA, were designed to disrupt the G1 and G3 boxes, respectively, of the putative GTP-binding domain. The Δ5CIITA deletion also eliminated one of the four consensus LCD motifs (termed L1) of CIITA. Δ10CIITA and Δ11CIITA disrupt a leucine-rich region near the C-terminus of the protein. Last, CIITA(161–1130) lacks the activation domain but retains all other sequences of CIITA, including the P/S/T region. Two methods were used to confirm that the mutant constructs encoded the desired mutations. First, all mutations were confirmed by DNA sequencing. Second, each construct was expressed in an *in vitro* transcription/translation reaction to ensure that the proteins produced were of the expected size (Fig. 2A).

Because the FLAG epitope was unable to be detected by western blot when FLAG–CIITA was transfected into RJ2.2.5 or Raji cells, a CIITA-specific antiserum was developed to detect CIITA. Rabbit polyclonal antiserum was generated to an *Escherichia coli*-expressed MBP–CIITA chimera. Use of the MBP–CIITA fusion allowed stable and soluble expression of CIITA in bacteria. The antiserum recognizes CIITA in the wild-type Raji B cell line, but not in the CIITA-deficient cell line RJ2.2.5 (Fig. 2B). To determine if the CIITA mutant series would be expressed and stable in eukaryotic cells, immunoblot analysis was carried out on RJ2.2.5 cells transiently transfected with the mutant CIITA constructs. The results show that the CIITA mutant proteins can be detected at 24 h after transfection, suggesting that the overall levels of CIITA and the stability of the mutant proteins is similar to the wild-type (Fig. 2C). The CIITA(161–1130)

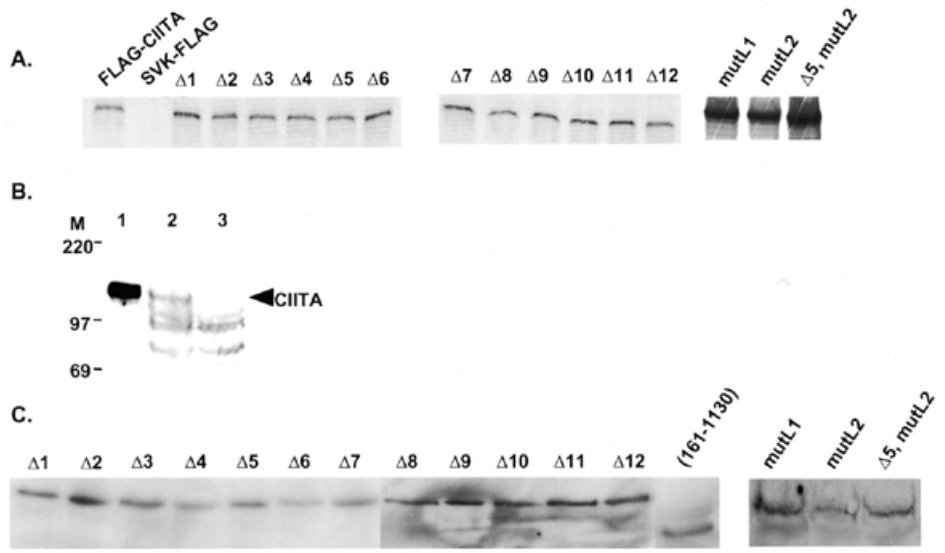


**Figure 2.** Expression of CIITA deletion mutants *in vitro* and *in vivo*. (**A**) Autoradiograph of SDS–PAGE of CIITA deletion mutants. Aliquots of 1 µg of the indicated CIITA deletion constructs were used in individual transcription/translation reactions using the T7 RNA polymerase TnT kit (Promega Corp., Madison, WI) as described by the manufacturer. (**B**) Polyclonal α-CIITA antiserum specifically recognizes recombinant CIITA and native CIITA from Raji but not RJ2.2.5 cell lysates in an immunoblot. Lane 1, recombinant CIITA (2 µg); lane 2, Raji cell lysate ($1.5 \times 10^6$ cells); lane 3, RJ2.2.5 cell lysate ($1.5 \times 10^6$ cells). The arrow indicates the position of the CIITA-specific band. (**C**) CIITA deletion mutants are expressed when transiently transfected into RJ2.2.5 cells. Aliquots of 20 µg CIITA expression constructs were transfected into RJ2.2.5 cells as previously described. Cells were harvested at 24 h post-transfection and lysed by resuspension in SDS–PAGE sample buffer. Lysates were analyzed by immunoblot as above.

mutant is either slightly less stable or not produced in the same quantities as the others, as 1.5-fold more extract was required to generate a similar signal.

## Most mutations in CIITA C-terminal of amino acid 377 result in the loss of CIITA activity

The ability of the CIITA mutants to transactivate the X-Y box-dependent *CAT* reporter plasmid pDRG-X1-X2-Y (39) was determined by co-transfection into RJ2.2.5 cells. The FLAG epitope had no effect on CIITA activity, as it gave comparable transactivation of the reporter as the previously characterized CIITA expression vector pCIITA-8 (39; Fig. 3A). The ability of the CIITA mutants to transactivate pDRG-X1-X2-Y was assayed and normalized to the level of expression produced by the pFLAG-CIITA construct (Fig. 3A). Most CIITA mutations resulted in non-functional alleles. The only exceptions were the most N-terminal mutations, Δ1 and Δ2, which both yielded near wild-type CAT levels as compared with pFLAG-CIITA. Similar results were obtained when using *CAT* reporter constructs driven solely by the X1-X2 sequences (data not shown).

To determine if any of the mutations could function in the presence of the W box, transfections were carried out using the previously described W-X-Y-dependent reporter construct pDRW-X-Y (13). Previous work from our laboratory indicated that the levels of CIITA-dependent transcription increased 2–3-fold when the W box was included in the promoter (27,39). This was also the case for CIITA transactivation in this system, as the reporter levels driven by pDRW-X-Y were 3.2-fold higher than those produced with pDRG-X1-X2-Y. Δ5CIITA showed clear differences in transactivation capabilities between promoters driven by X1-X2-Y and W-X-Y. Where Δ5 was inactive with an X-Y promoter, this mutant activated the W-X-Y reporter to 50.3% of wild-type expression levels. This differential activity for Δ5 suggests that this mutation alters the structure of CIITA in such a manner that it is now able to function only in the presence of the W box, implying that CIITA interacts with the W box proteins. While other mutations also appear to show some W box-dependent activity (e.g. Δ7 and Δ9CIITA), this phenotype is most pronounced for Δ5CIITA.

Primary amino acid homology searches with CIITA highlighted a potential bipartite nuclear localization sequence (NLS) in murine CIITA (47) and a conserved potential CDK phosphorylation site [consensus SPxR (48), CIITA positions Ser280, Pro281, Arg283]. Individual mutations were created that deleted the potential NLS (residues 141–160 of human CIITA) and altered the conserved residues of the CDK phosphorylation site to alanines. Both of these mutants were able to drive transcription to near wild-type levels in all assays tested (data not shown).

## Mutations in the activation domain and central regions of CIITA interfere with the function of wild-type CIITA in Raji cells

Mutations that exhibit dominant negative effects often result from the disruption of a functional domain of the protein without disrupting other portions of the protein. This class of mutants is able to interact with additional factors, but the resulting complexes are biologically inactive or non-functional. To further analyze the mutant CIITA proteins, transient co-transfections of the CIITA mutants were carried out with the pDRW-X-Y reporter in Raji cells to test for dominant negative function of the mutants. Transfection of the wild-type FLAG–CIITA construction in Raji cells resulted in an increase in expression of the reporter gene by a factor of 4.5, indicating that CIITA is limiting even in the high class II-expressing cell line Raji (Fig. 4). In concordance with the

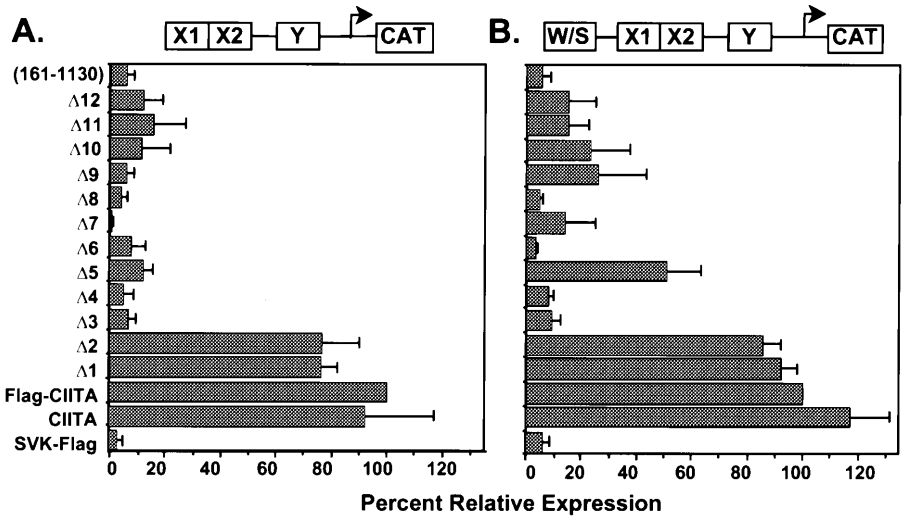


**Figure 3.** Effects of CIITA mutations on class II transactivation. CIITA$^-$ RJ2.2.5 cells were used for transient transfections with 40 µg CIITA expression constructs and 20 µg either pDRG-X1-X2-Y (**A**) or pDRW-X-Y (**B**) reporter constructs. A sample of 1 µg pSV$_2$-Alkphos was included in all transfections to control for transfection efficiency. Cell lysates were analyzed by CAT ELISA as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Results are given as a percentage of the level achieved using FLAG–CIITA with the reporter. The dashed line in (B) (pDR W-X-Y) represents the level of reporter activity corresponding to 100% activity of pDRG-X1-X2-Y in (A). The data represent averages from three independent assays, with SEM indicated.

transfection data in RJ2.2.5 cells shown in Figure 3A, transfection of Δ1 (373.8% of baseline reporter levels), Δ2 (297.3%) and Δ5 (188.9%) resulted in proportional increases in expression of the reporter gene. Transfection of CIITA mutants Δ3 (57.0%), Δ4 (74.5%), Δ7 (71.7%) and Δ8 (70.7%) resulted in significantly reduced levels of the class II reporter gene in Raji cells, indicating that these mutations were in regions that affected some but not all of CIITA function. However, the most significant decrease in reporter levels resulted in transfections receiving CIITA(161–1130) (30.6%), the mutant lacking the acidic activation domain.

The CIITA(161–1130) mutant was expected to function in a dominant negative manner as just the activation domain was deleted. Thus, all other interactions are expected to be functional. The Δ3 mutation specifically deletes the G1 motif of a putative GTP-binding domain, while mutations Δ4, Δ7 and Δ8 are in regions of the molecule that do not show significant homology to described sequences. The dominant negative nature of CIITA(161–1130) is similar to that of other CIITA mutants lacking the activation domain while retaining downstream sequences (49–51). Mutants Δ4, Δ7 and Δ8 are in regions of CIITA that to our knowledge have not been studied; these mutants therefore suggest a functional role for these regions of the molecule.

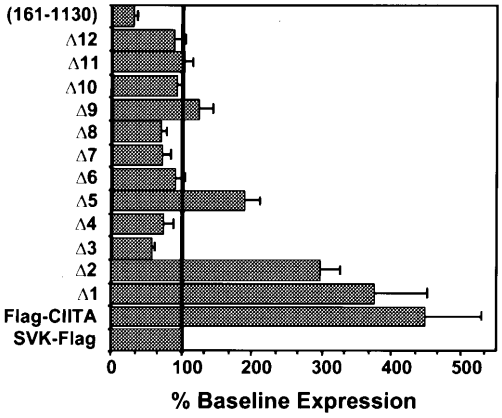


**Figure 4.** Mutations in CIITA function in a dominant negative manner. Transient transfections were performed introducing 20 µg pDRW-X-Y, 40 µg CIITA mutant expression plasmids and 1 µg SV$_2$-Alkphos into CIITA$^+$ Raji cells as described for Figure 3. Cells were harvested 24 h post-transfection and assayed for CAT levels as described above. Reporter activity is expressed as a percentage of each mutant compared with CAT levels in transfections receiving the control expression vector (percent baseline CAT). The data represent averages from three independent assays, with SEM indicated.

## Analysis of the L1 and L2 mutants indicates that these regions are required for CIITA function

LCD motifs have been shown to facilitate interactions between many proteins, including nuclear receptors and their coactivators (52), as well as transcription factors and coactivators (53). These motifs are characterized by the consensus LxxLL, where there is a loose consensus for charged amino acids at both x positions. Analysis of CIITA revealed four such sequences. Of these, three are conserved between human and murine CIITA (47). The Δ5 deletion removed two potential motifs that could be responsible for its dependence on the presence of the W box for function. Both the G3 region of the putative GTP-binding domain and the first LCD motif (L1) were deleted in this mutant. To determine if the W box dependence of Δ5CIITA is attributable to loss of the L1 motif, a mutational analysis of L1 and L2 was undertaken. Previous studies indicate that protein–protein interactions directed by LCD motifs can be mediated by multiple LCDs grouped together in proteins (53). For this reason, mutants were created that disrupted both L1 and L2, which are separated by 56 residues in the primary amino acid sequence. To create more subtle changes, alanine residues were substituted for the conserved leucines in the first two LCD motifs (CIITA-mutL1 and

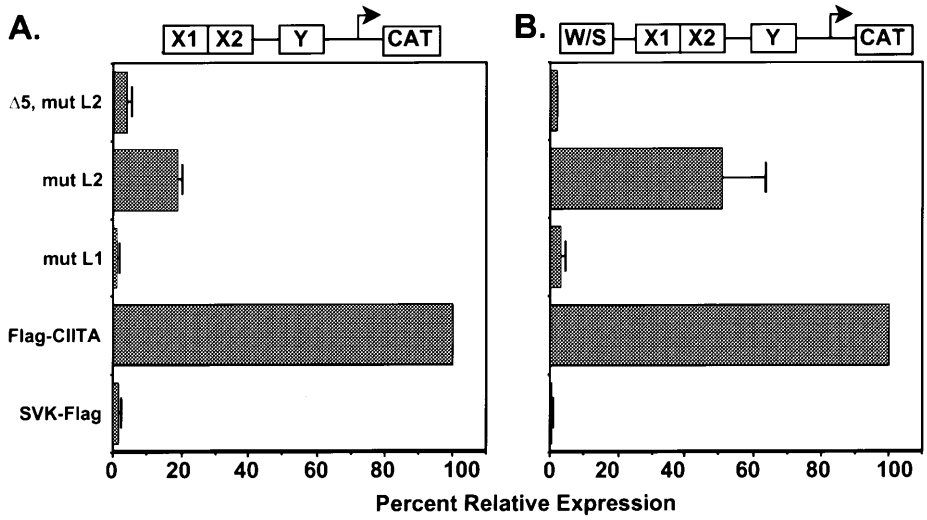


**Figure 5.** LCD motifs L1 and L2 are required for full transactivation by CIITA. Transient transfections were performed in CIITA$^-$ RJ2.2.5 cells as in Figure 3. *CAT* reporter constructs used were either pDRG-X1-X2-Y (**A**) or pDRW-X-Y (**B**). The dashed line in (B) (pDR W-X-Y) represents the level of reporter activity corresponding to 100% activity in (A) (pDRG-X1-X2-Y). The data represent averages from three independent assays, with SEM indicated.

CIITA-mutL2, respectively). A double mutant consisting of the L2 mutation and the Δ5CIITA deletion (Δ5CIITA-mutL2) was also created. These mutants were analyzed by *in vitro* transcription/translation and immunoblot procedures as for the deletion mutants. The expression of each mutant was similar to those described above (Fig. 2C).

When transient transfections into RJ2.2.5 cells were performed in conjunction with both the pDRG-X1-X2-Y (Fig. 5A) and pDRW-X-Y (Fig. 5B) reporter constructs, it was found that neither CIITA-mutL1 nor the double mutant (Δ5CIITA-mutL2) were able to drive transcription from either promoter. CIITA-mutL2 displayed activity similar to Δ5: ~50% activity in the presence of the W box and <20% activity when just the X-Y region was used. When transfected into Raji cells, the double mutant was able to reduce reporter levels to 68.5% of baseline (Fig. 6).

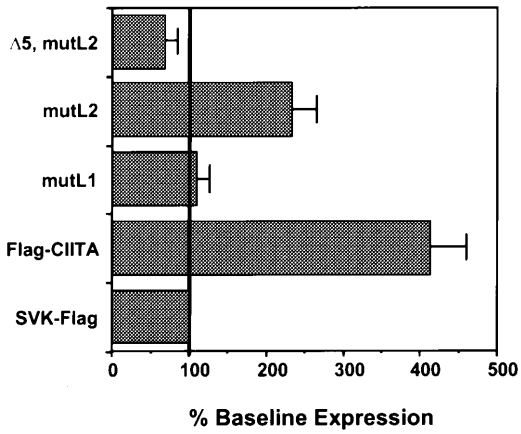


**Figure 6.** Analysis of LCD mutants in CIITA$^+$ Raji cells. Transient transfection and analysis were performed in Raji cells as described for Figure 4, using 40 µg indicated mutant CIITA constructs. The data represent averages from three independent assays, with SEM indicated.

## DISCUSSION

While recent studies using the yeast two-hybrid approach have suggested very weak interactions between CIITA and a subunit of RFX, RFX5 (54), direct physical interactions between CIITA and the class II-specific transcription factors have not been described. Thus, if the activation domain of CIITA is responsible for driving expression, how does CIITA interact with the class II promoter? To elucidate further the mechanism of CIITA action, mutations were created and assayed for their ability to drive transcription or to interfere with the function of wild-type CIITA in transient co-transfection assays. While many of the mutants were inactive, two mutants displayed the ability to distinguish between class II promoter elements. These two mutants required the W box as well as the X box for function. These mutants suggest that in addition to the X box factors, CIITA normally interacts with W box factors. This finding is unique in that these are the first examples of mutations in CIITA that sensitize it to the presence of a promoter element. When considered along with previous work indicating that CIITA acts through the X box (27,39), this result strongly supports a role for CIITA at the promoter as a *trans*-activator.

The W box of class II promoters is the least homologous of the conserved sequences. It was first defined by its requirement for IFN-γ induction of class II expression (4,15) and enhancement of class II expression in B cells (14,16). Subregions within the W box have been identified, termed the S or Z box, that provide some of the effect of the full W box region. Portions of the S region of the *DRA* gene are similar to the X1 box, where RFX normally binds. Recently, Jabrane-Ferrat *et al.* (55) found that the S region could bind RFX *in vitro*, although weakly. This interaction was stabilized by interactions with the Y box factor NF-Y. These results suggested that RFX may bind to both the S and the X1 boxes of class II genes. The isolation of mutant CIITA constructs (Δ5CIITA and CIITA-mutL2) that require the W box in addition to the X-Y boxes for activity suggests that CIITA does

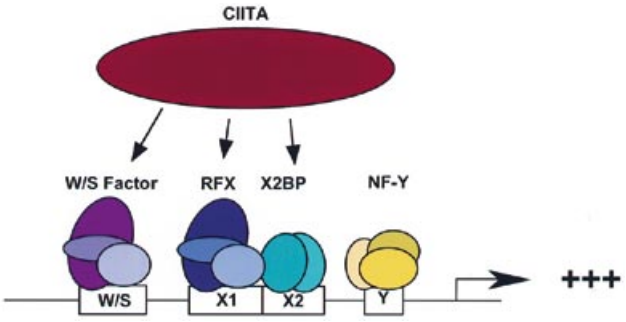


**Figure 7.** Model for transactivation of MHC class II transcription by CIITA. Interactions between CIITA and promoter-bound factors at the W, X1, X2 and Y boxes serve to stabilize the W-X-Y complex and lead to transcriptional transactivation of MHC class II genes. Our data adds to the current model of MHC class II transcriptional regulation by implicating direct interactions between CIITA and the W box, providing one possible explanation for W box involvement in induction of class II transcription by IFN-γ.

indeed interact with this region (Fig. 7). These mutations are not likely to be in the region of CIITA that interacts with W box factors. Alternatively, these mutations define a region of CIITA that interacts with other factors; the loss of these interactions causes the protein to retain *trans*-activation capability only in the presence of 'stabilizing' interactions with factors at the W box.

The above finding suggests that in B cells, the W box may function to stabilize CIITA interactions, allowing optimal transcriptional activation (Fig. 7), thus offering a molecular explanation for the requirement for the W box for class II induction by IFN-γ. In the case of IFN-γ induction in class II negative cells, such as human fibroblasts, a similar situation likely exists with one modification, i.e. the steady-state level of RFX. The level of RFX5, a subunit of RFX, in human fibroblasts is >20-fold lower than that in B cells (20), suggesting that RFX may be limiting in fibroblasts. The W/S box could provide an additional binding site for RFX, allowing for more efficient assembly of factors to class II promoters. Moreover, the induction of CIITA by IFN-γ may further increase the stability of W-X-Y complexes. This model is supported by two lines of evidence. First, while the half-life of an RFX-X2BP-NF-Y–DNA complex is >4 h *in vitro*, these assays were carried out under conditions where the factors were not limiting (56). As RFX concentration is reduced, the ability to form such *in vitro* complexes is also reduced, suggesting that if one of the factors were limiting *in vivo*, then the assembly time of the complex may be increased or the half-life reduced. For example, the half-life of RFX for X1 box DNA and X2BP for X2 box DNA *in vitro* is ~2 and 5 min, respectively (28,29,57). Second, *in vivo* genomic footprinting (IVGF) of the W-X-Y region in fibroblasts detected only partially protected sequences, which became fully protected upon IFN-γ treatment (58–60). Partial occupancy on IVGF indicates that fewer cells in the population have the region occupied at any one time. Thus, it is possible that induction of CIITA by IFN-γ in fibroblasts could stabilize the complex, providing an enhanced footprint.

How much CIITA is required to activate transcription of class II genes? At least nine genes are activated in most cells when CIITA is expressed: *DRA*/*B*, *DQA*/*B*, *DPA*/*B*, *DMA*/*B* and *Ii*. The B cell line Raji expresses high levels of MHC class II and easily detectable levels of CIITA. Yet, even in Raji cells, overexpression of CIITA from a plasmid results in a 4-fold increase in expression of class II promoters, demonstrating that CIITA is limiting. Similar experiments by others have not shown CIITA to be limiting in Raji cells (61). The level of CIITA by immunoblot analysis in IFN-γ treated fibroblasts is barely detectable using our current antiserum and may be 10–20 times lower than in Raji cells (data not shown), suggesting that small amounts of CIITA are sufficient to induce class II expression. The fact that CIITA is limiting may support a model in which CIITA transiently associates with the W-X-Y box factors and highlights the importance of CIITA forming multiple interactions with DNA-bound factors at W-X-Y.

The identification of mutants that functionally interfere with wild-type CIITA molecules highlights regions of importance in the CIITA molecule. While the most straightforward examples of dominant negative mutants have been those that compete with wild-type proteins for homodimer formation (62), there are other explanations for dominant negative mutants that do not require the protein to act as a homodimer, such as deletion of an activation domain from a transactivator. Such a construct would retain all other interactions, yet lose its ability to recruit the general transcriptional machinery to the promoter. Indeed, this type of dominant negative mutant has been described for other coactivators, such as c-Ets2 (63) and NTF-1 (64). CIITA(161–1130) fits this category of dominant negative mutants.

The sequence of CIITA between residues 420 and 561 shows homology to the G1, G3 and G4 domains of a GTP-binding motif (reviewed in 65). The Δ3CIITA allele has a specific deletion in the G1 domain (66), which is involved in phosphate binding, suggesting the intriguing possibility that CIITA requires GTP for function. Chin *et al.* (42) recently showed that mutation of the region corresponding to the G3 motif results in a non-functional, dominant negative CIITA molecule (42). Attempts to show GTP and other nucleotide binding by CIITA were inconclusive (data not shown). It is possible that CIITA binds GTP with low affinity or that other factors are required for this binding that may not be present *in vitro*. Alternatively, the GTP-binding motif homology may be coincidental and the deletion in Δ3CIITA eliminates numerous glycine residues, which may provide needed flexibility at this position.

Comparison of the function of some mutants described underscores the fact that little is known about the three-dimensional structure of CIITA and hence the effect of these mutations on the protein. Δ5 and mutL1 both disrupt the L1 motif, the former with a 10 residue deletion, the latter with alanine substitutions at three conserved leucines. The result that the alanine substitution mutant resulted in a more severe phenotype than the deletion is intriguing and could result from unproductive protein folding in the presence of alanine residues at the position of L1 (amino acid residues 465–469). Because leucine residues have a tendency to be near the N-terminal ends of α-helical structures (67), it is possible that loss of these stabilizing residues results in 'dead end' protein folding and therefore a more severe phenotype of mutL1 as compared with Δ5CIITA.

Most of the recently described CIITA mutations result in non-functional proteins, however, a small number produced a dominant negative effect (42,50). Summarizing the effects of individual CIITA mutations a new understanding emerges (Fig. 8). A clear requirement for the acidic regions, the P/S/T region and the C-terminal regions becomes apparent. However, there appears to be strict boundaries between regions of the molecule

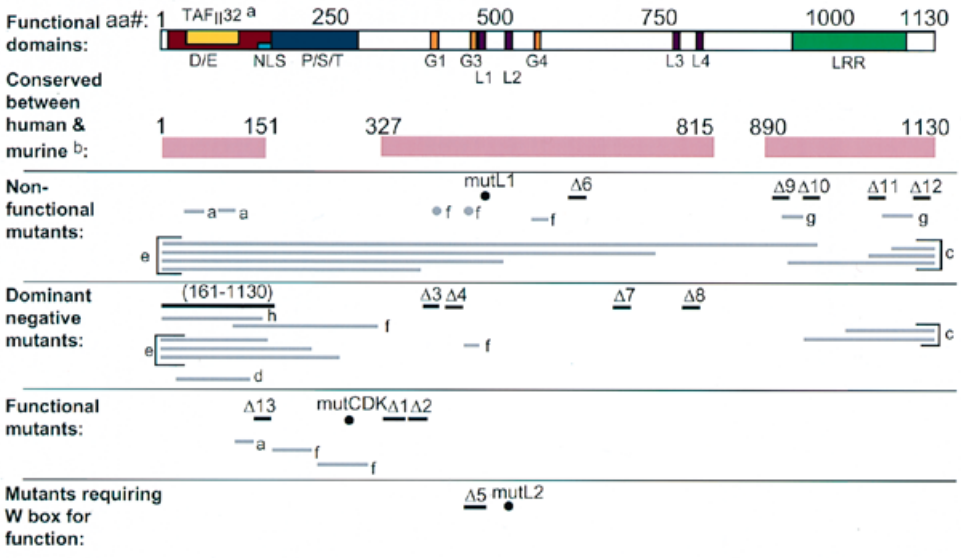


**Figure 8.** Comparison of the described CIITA mutants. Wild-type CIITA with amino acid positions and potential functional domains are indicated. D/E, acidic domain; NLS, nuclear localization signal; P/S/T, Pro/Ser/Thr-rich region; G1–G3, consensus GTP-binding motif; L1–L4, LCD motifs; LRR, leucine-rich region. The regions of homology between human and murine CIITA genes are indicated by shaded boxes (47). Published mutations of CIITA are grouped according to the described functional levels for each mutant. Mutants described herein are labeled. Notation for mutants is as follows: all horizontal bars represent regions deleted from full-length CIITA constructs; •, amino acid substitutions at that position of the protein. Mutants that were created and characterized outside this report are shaded and described: a (68); b (47); c (61); d (49); e (50); f (42); g (69); h (51).

that can be mutated while still retaining enough function to act in a dominant negative manner, both at the N- and C-termini of CIITA. Comparison of mutants surrounding the putative GTP-binding domain is interesting in that mutations within this region are capable of yielding non-functional, dominant negative and functional CIITA molecules. Indeed, similar mutants have been shown to have discordant results in transactivation assays: Δ5CIITA exhibits the ability to transactivate in a W box-dependent manner, while a smaller deletion of solely the G3 residues [CIITA-GTP2 (ΔDAYG); 42] has no activity and has been shown to have a dominant negative effect. A majority of mutants that disrupt regions conserved between human and murine CIITA result in non-functional or dominant negative phenotypes, indicating that the structure of CIITA is highly conserved between species. It is interesting that the P/S/T domain is not conserved, yet this region is required for function of the human gene; an analysis of the role of non-conserved regions of the murine protein may lead to insights into the role of the P/S/T domain in the function of CIITA.

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