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Mechanisms of Nuclear Import and Export That Control the Subcellular Localization of Class II Transactivator

Drew E. Cressman, William J. O’Connor, Susanna F. Greer, Xin-Sheng Zhu, and Jenny P.-Y. Ting

The presence of the class II transactivator (CIITA) activates the transcription of all MHC class II genes. Previously, we reported that deletion of a carboxyl-terminal nuclear localization signal (NLS) results in the cytoplasmic localization of CIITA and one form of the type II bare lymphocyte syndrome. However, further sequential carboxyl-terminal deletions of CIITA resulted in mutant forms of the protein that localized predominantly to the nucleus, suggesting the presence of one or more additional NLS in the remaining sequence. We identified a 10-aa motif at residues 405–414 of CIITA that contains strong residue similarity to the classical SV40 NLS. Deletion of this region results in cytoplasmic localization of CIITA and loss of transactivation activity, both of which can be rescued by replacement with the SV40 NLS. Fusion of this sequence to a heterologous protein results in its nuclear translocation, confirming the identification of a NLS. In addition to nuclear localization sequences, CIITA is also controlled by nuclear export. Leptomycin B, an inhibitor of export, blocked the nuclear to cytoplasmic translocation of CIITA; however, leptomycin did not alter the localization of the NLS mutant, indicating that this region mediates only the rate of import and does not affect CIITA export. Several candidate nuclear export sequences were also found in CIITA and one affected the export of a heterologous protein. In summary, we have demonstrated that CIITA localization is balanced between the cytoplasm and nucleus due to the presence of NLS and nuclear export signal sequences in the CIITA protein.


Class II transactivator (CIITA) plays a central role in initiating an immune response by activating the expression of a variety of genes necessary for the presentation of peptides on the cell surface in association with molecules of the class II MHC. CIITA activates the expression of all MHC class II genes as well as the class II-associated molecules DM, DN, and invariant chain (1–4). The activity of CIITA itself and subsequent expression of class II genes is constitutive in B cells and inducible by IFN-γ in most other cell types (5–7). Although it does not directly bind to DNA, CIITA functions as a nuclear coactivator by interacting with other promoter binding factors and components of the basal transcription complex, including RFX5, NF-Y, TATA binding factor-associated proteins, Bob1, CREB, and CBP (8–13). Interaction of CIITA with these factors occurs at the W/X/Y regulatory elements in the promoters of class II target genes (14–17). The CIITA protein contains a number of functional motifs, including an amino-terminal acidic activation domain, a proline-serine-threonine-rich region, and a GTP-binding motif. GTP binding of CIITA has been shown to correlate with nuclear import; mutants defective for GTP binding fail to translocate into the nucleus and fail to activate class II gene expression (18). The GTP-binding motif is not a nuclear localization signal (NLS), but most likely GTP binding causes a conformational change that is compatible with nuclear translocation.

Loss of cell surface MHC class II expression in patients leads to the development of bare lymphocyte syndrome (BLS), a severe immunodeficiency disease characterized by failure of lymphocyte proliferation and activation, multiple recurrent infections, and ultimately death in early childhood (19–21). The genetic defects leading to BLS have been grouped into four complementation groups, with each group corresponding to mutations in different transcription factors necessary for class II gene expression. These factors include RFX5 (complementation group C) (22), RFXAP (group D) (23), RFXANK/RFX-B (group B) (24, 25), as well as CIITA (26). Mutations in CIITA are responsible for complementation group A BLS patients and class II-negative cell lines derived from these patients (27–29). One group A BLS patient was found to contain a 72-bp deletion in the CIITA gene, resulting in the loss of aa 940–963 (28). Recently, we found that the loss of this region results in the loss of a NLS at aa 955–959, abrogating CIITA activity because the protein fails to translocate from the cytoplasm into the nucleus (30). Recently, another NLS has been identified at aa 141–159 of CIITA (31). Interestingly, acetylation of this bipartite sequence increases nuclear accumulation of CIITA.

Translocation of a protein from the cytoplasm into the nucleus is dependent on the presence of a NLS within the protein. Although specific consensus sequences for NLS do not exist, they tend to be short sequences rich in basic residues (32, 33) which mediate binding to members of the importin (karyopherin) α family of proteins in the cytoplasm (34). The NLS-containing protein is translocated through the nuclear pore as part of a trimeric importin α-importin β-NLS protein complex in a GTP-dependent...
manner. Binding of RanGTP to importin β within the nucleus leads to dissociation of the complex and release of the NLS-containing protein (for review, see Ref. 35). Different variations of NLSs have been identified, with the SV40 large T-Ag NLS serving as the prototype of the “classical” NLS (36, 37). Other NLSs that differ from the classical NLS by binding to different members of the importin α family or operating in an importin α-independent manner include the M9 sequence (38), the lymphoid enhancer factor 1 NLS (39), and the NLS of the HIV-1 Tat protein (40).

Similar to nuclear import, export of a protein depends on the presence of a specific nuclear export signal (NES) (reviewed in Ref. 35). These NES are short sequences characterized as being rich in leucine residues and have been identified in a variety of different proteins, the best studied of which being the HIV Rev protein (41). Nuclear export is mediated by CRM1, a importin β-like protein that exports a variety of NES-containing proteins in a RanGTP-dependent manner (42–45). CRM1-mediated nuclear export can be blocked by treatment of cells with the export inhibitor leptomycin B (LMB) (46–48), resulting in the nuclear accumulation of proteins otherwise dependent on the export pathway.

In this paper, we identify an additional NLS present in CIITA. This NLS (NLS2) is located at aa 405–414, amino-terminal to the NLS deleted in the group A BLS patient at aa 955–959 (NLS3). Deletion of NLS2 results in cytoplasmic localization of CIITA and loss of activity, both of which can be rescued in part by replacement with the SV40 NLS. This last property differs from NLS3, which cannot be replaced by the SV40 NLS. This suggests that unlike NLS3, NLS2 can be categorized as a classical NLS. The presence of both NLS2 and NLS3 are required for CIITA to be fully functional in activating class II gene expression. Furthermore, we demonstrate that CIITA localization is sensitive to LMB, leading to increased nuclear concentrations of the protein and suggesting that the presence of CIITA in both the cytoplasm and the nucleus is dependent on NLS and NES sequences in the protein.

Materials and Methods

Plasmids

All mutants were constructed using the pCDNA3.FLAG.CIITA parent vector containing an eight-amino acid FLAG epitope upstream of the first methionine of CIITA (49). Carboxy-terminal CIITA deletion mutants were constructed by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), resulting in a full-length GFP fusion containing the indicated position within the CIITA sequence. Mutations were made at codons for aa 149, 225, 336, 414, 518, 614, 752, 853, and 932. Deletion of aa 405–414 (CIITAΔ405–414) was constructed by overlapping PCR using four primers: primer 1 (657–446), primer 2 (CGGACCGACTCCA CGACAACCGAGGTAGTCTACACTAACGC-1250); primer 3 (GGCTGCC 414–1242 of CIITA were subcloned in-frame to the carboxy-terminal vector using the pEFP-C1 parent vector (CLONTECH Laboratories, Palo Alto, CA). Insertions of one or two copies of aa 405–414 were confirmed by sequence analysis. The SV40 T-Ag NLS (CCTAAG AAGAAGGGGAAGGT) was fused to GFP (GFP-NLS) as a positive control. COS-7 cells (6 × 105) were plated into two-well chamber slides 12 h before transfection. One microgram of GFP plasmids was transfected into cells, rinsed 24 h later with PBS, and fixed for 5 min with 4% paraformaldehyde. Fluorescence of the fusion protein was observed using an Olympus BX40 microscope (Melville, NY) at ×50 magnification.

Results

C-terminal truncations cause nuclear translocation of CIITA and identify a putative NLS

Previously we have shown that CIITA contains a NLS at aa 955–959 (30). Loss of the 24-aa exon that contains this region results in the abrogation of CIITA activity and loss of MHC class II surface expression, leading to the BLS (28). However, we reasoned that because of the size and complexity of CIITA, it might contain additional NLSs, similar to the multiple NLSs seen for other transcription factors such as NF-AT (51), the retinoblastoma protein (52), c-myc (53), and Myo-D (54). Deletion of 200 or 278 residues from the carboxy-terminal terminus, including the aa 955–959 NLS3 (CIITA–931 and CIITA–852, respectively), causes CIITA to be localized to the cytoplasm as demonstrated by subcellular immunofluorescence assays (Fig. 1A). However, CIITA–751 shows a low level of nuclear presence, while shorter forms 1–444, 1–518, and 1–613 (predicted molecular mass of 49, 57, and 67 kDa, respectively) demonstrate nuclear localization stronger than that seen for the wild-type CIITA. Shorter forms of CIITA <366 aa all show protein distribution throughout the cytoplasm and nucleus, likely due to the fact that these are of small enough size (<40 kDa) to diffuse through the nuclear pore complex without the need for a specific NLS. The smallest of the nuclear CIITA deletion mutants, 1–444, has a predicted molecular mass of 49 kDa, well above the maximum size of molecules that can undergo nonselective passive diffusion through the nuclear pore (55). The high concentration of CIITA1–444 in the nucleus compared with the more even distribution of the 1–335 mutant indicates the possible presence of a NLS between aa 336 and 444. In addition, since 1–852 is restricted to the cytoplasm while 1–751 and 1–613 show steadily increasing concentrations of the protein in the nucleus, there may exist a NLS between aa 613 and 852. The presence of any such signal within this region may be sufficient to drive the export of CIITA–852
from the nucleus to the cytoplasm. Therefore, loss of an NES C-terminal to aa 613, combined with the remaining presence of a possible NLS between aa 336 and 444, would account for CIITA1–613, 1–518, and 1–444 to localize more strongly to the nucleus. Despite the presence in the nucleus of these mutant proteins, loss of any portion of the carboxyl terminus eliminates the ability of CIITA to activate reporter gene expression from the class II DR promoter. Luciferase activity of cell extracts was determined at 24 h posttransfection, and values were normalized for protein concentration. Assays were repeated three times and performed in triplicate.

From the above analysis, the presence of a putative NLS between aa 405 and 414 drive the nuclear localization of CIITA. The GTP-binding motif itself has been shown to be necessary for proper CIITA localization and activity, although it likely achieves this through changes in the conformation of CIITA rather than serving as an authentic NLS (18). We have designated this sequence as NLS2 and the NLS we have previously defined in the BLS patient as NLS3.

### Failure of CIITA nuclear import and transactivation by deletion of aa 405–414

Deletion of entire portions of the carboxyl terminus identified the general location of the NLS and sequence analysis pinpointed a specific domain that fit the residue requirements of a NLS motif. To demonstrate that this domain does indeed function as a NLS, it was necessary to specifically determine the effects that deleting aa 405–414 has on protein localization and CIITA activity. Although wild-type CIITA localized to both the cytoplasm and the nucleus, CIITA missing the 10 aa of the putative NLS (CIITA Δ405–414) was only present in the cytoplasm (Fig. 3A). It is important to note that this construct still retains the NLS1 (aa 151–159) and the NLS3 site present in the BLS domain (aa 955–959). Despite the presence of these NLS however, CIITA Δ405–414 did not enter the nucleus, suggesting that NLS2 is critical for normal protein localization. When tested in reporter activation assays using the class II DR promoter fused to luciferase, deletion of aa 405–414 severely inhibited the ability of CIITA to activate gene expression relative to the wild-type form (Fig. 3B). Western blots indicated that expression of the mutant CIITA was equivalent to wild type with no evidence of decreased protein stability (data not shown). Therefore, the failure of CIITA to localize to the nucleus results from a loss of the 10-aa domain, which in turns impairs the ability of CIITA to function as a transcriptional coactivator.

To delineate specific amino acids functioning in the putative NLS, we made arginine to alanine point mutations of the central arginine amino acids at residues 408 and 409 and assayed for localization (Fig. 3C). Compared with wild-type CIITA, R408A and R409A both show decreased presence in the nucleus. Double mutants demonstrated a similar pattern (data not shown). This suggests that mutations of individual amino acids within this region are enough to disrupt the normal localization of CIITA to the nucleus.

### Amino acids 405–414 drive the nuclear localization of a heterologous protein

One characteristic of previously identified NLS is their capacity to mediate the nuclear translocation of a heterologous protein (39, 40, 57). To determine whether the upstream putative NLS of CIITA...
could function in a similar manner, we fused the 10-residue motif of aa 405–414 to the GFP. The 28-kDa GFP by itself localizes to both the cytoplasm and the nucleus (Fig. 4). Fusion of the control SV40 NLS to GFP results in strong nuclear localization of the protein. When fused to one copy of the aa 405–414, GFP shows a moderate increase in nuclear translocation, although not as intense as that seen for GFP-SV40NLS. However, fusion to two copies of aa 405–414 significantly increases the nuclear localization of GFP, such that very little GFP remains in the cytoplasm. This suggests that aa 405–414 can function independently as a NLS, inducing the nuclear translocation of both CIITA and an unrelated protein. Furthermore, the nuclear localization function imparted by the CIITA motif appears to be only slightly weaker than that provided by the well-defined SV40 NLS.

**LMB inhibits the export of CIITA from the nucleus**

The absence of aa 405–414 results in the localization of CIITA to the cytoplasm. However, an alternative explanation for this observation is that the loss of this region allows the mutated CIITA protein to be exported from the nucleus more rapidly than is the wild-type protein. Therefore, blocking nuclear export should reveal whether the cytoplasmic localization of mutant CIITA constructs is due to excessive nuclear export or due to the failure of the protein to enter the nucleus in the first place. Several pieces of evidence indicate that CIITA undergoes not only nuclear import, but also nuclear export. The localization of the wild-type protein in both the cytoplasm and the nucleus indicated by immunofluorescence and immunoblot studies (30) suggests that CIITA resides in both compartments. Additionally, treatment of cells with LMB, an inhibitor of nuclear export, causes the accumulation of nuclear CIITA (Fig. 5, a–d). This nuclear accumulation is first evident 15 min after LMB addition and increases over the course of 1 h. Residual cytoplasmic CIITA is still observed at later time points in these transfected cells. We reasoned that this cytoplasmic CIITA might be due to de novo synthesis from the transfected gene. Actinomycin D is capable of blocking de novo synthesis by inhibiting gene transcription. To determine whether this residual cytoplasmic CIITA is the result of de novo synthesis, we blocked new gene transcription by pretreating cells for 3 h with actinomycin D. This approach allows current mRNA translation to be carried through to completion, resulting in full-length protein, but blocks additional transcription, therefore ensuring that all proteins detected by the Ab are indeed full-length CIITA peptides. Pretreating CIITA-transfected cells with actinomycin D in the presence of LMB shows nearly complete nuclear localization of CIITA protein over time (Fig. 5, e–h). This nuclear localization occurs rapidly, as at 15 min following LMB treatment, there was only a low level of cytoplasmic CIITA present, with the majority localized to the nucleus (Fig. 5j), whereas by 30 min to 1 h following LMB treatment, all CIITA was present in the nucleus (Fig. 5, g–h). This indicates that CIITA in the cytoplasm is primarily the result of nuclear export and that only the residual amount of cytoplasmic CIITA seen in Fig. 5, c and d, is due to de novo synthesis.

**The defect in CIITAΔ405–414 is nuclear import, not export**

Having demonstrated that CIITA protein is susceptible to nuclear export that can be blocked by the addition of LMB, it became possible to further determine whether the cytoplasmic localization of CIITAΔ405–414 is due to the loss of a NLS or to an increase in export from the nucleus. If the latter was true, then any increase in the export of the mutant CIITA should be inhibited by LMB treatment and the resulting pattern of protein localization should be nearly indistinguishable from wild-type CIITA treated with LMB.

**FIGURE 3.** Deletion of aa 405–414 restricts CIITA protein to the cytoplasm and inhibits activation from a class II promoter. A, Localization of wild-type CIITA protein or CIITA with deleted aa 405–414. COS cells were transfected with 1 μg of either plasmid, harvested 24 h later, and stained for protein localization with anti-FLAG primary Ab and FITC-conjugated secondary Ab. Top panels, CIITA or CIITAΔ405–414 protein presence in nuclear or cytoplasmic compartments; bottom panels, all 4',6'-diamidino-2-phenylindole (DAPI)-stained nuclei in the field. B, CIITAΔ405–414 fails to activate expression from the DR-luciferase promoter. COS cells were transfected with 1 μg of CIITA and 1 μg of DR-luciferase and extracts were assayed for luciferase activity 24 h later. Activities are shown relative to CIITA. pCDNA3 plus DR-luciferase was used as a negative control. Extracts were normalized to protein concentration and all assays were repeated three times and performed in triplicate. C, Mutation of arginine residues decreases nuclear localization of CIITA. COS cells were transfected with wild-type CIITA or CIITA containing arginine to alanine single point mutations at amino acid residues 408 or 409. Protein localization was detected as in A.

**FIGURE 4.** The putative NLS motif induces nuclear translocation when fused to a heterologous protein. Oligonucleotides encoding the peptide region for aa 405–414 of CIITA were fused in-frame to GFP in one (GFP-405/414 × 1) or two (GFP-405/414 × 2) copies. All GFP constructs were transfected into COS cells and subcellular localization of GFP proteins was observed 24 h posttransfection. The SV40 NLS fused to GFP served as a positive control for nuclear translocation. Note nuclear and cytoplasmic presence of GFP vs predominantly nuclear staining of GFP-SV40NLS.
However, in cells transfected with CIITA/H9004405–414 and treated with LMB, the mutant CIITA protein remained predominantly in the cytoplasm, in contrast to both wild-type CIITA and CIITA with an N-terminal SV40 NLS (NLSCIITA), which are present primarily in the nucleus (Fig. 6). All cells were pretreated with actinomycin D to inhibit additional gene transcription. This indicates that CIITA/H9004405–414 fails to ever enter the nucleus due to the deletion of the NLS2 motif.

The SV40 NLS rescues CIITA405–414 activity

We wished to determine whether loss of the NLS motif at aa 405–414 can be functionally rescued by the insertion of a classical SV40 NLS (PKKKRKV) into the same region of the protein. Immunofluorescence detection of the NLS-corrected protein shows little increase in nuclear CIITA405–414/NLS, with the majority of the protein restricted to the cytoplasm (Fig. 6). However, following treatment with LMB plus actinomycin D, CIITA405–414/NLS is clearly present in both the nucleus and the cytoplasm. This suggests that insertion of the heterologous NLS into the deleted 405–414 domain is sufficient to partially rescue CIITA localization, most likely by inducing nuclear translocation of the protein at a reduced rate.

To determine whether the inserted heterologous NLS can rescue CIITA405–414 activity as well as localization, cells were cotransfected with the DR promoter-luciferase reporter plus CIITA405–414 or CIITA405–414/NLS. COS cells represent an overexpressed system, while HeLa cells express significantly lower levels of CIITA. Although CIITA405–414 showed minimal ability to activate expression from the DR promoter, cells transfected with CIITA405–414/NLS displayed reporter activity approaching that seen for cells transfected with wild-type CIITA (Fig. 7). DR activation by CIITA405–414/NLS in COS cells was restored to >90% of the level of wild-type CIITA, while HeLa cell activation of DR by CIITA405–414/NLS was 65% that of wild type. This was in contrast to the downstream NLS3 motif present in the BLS domain at aa 955–959, where substitution with the SV40 NLS fails to rescue CIITA activity, thereby confirming previous results (30). These data suggest that the basic residue-rich domain in CIITA from aa 405–414 functions like a classical NLS in directing the CIITA protein to the nucleus where it can then activate target gene expression.
CIITA contains multiple putative NES motifs

LMB inhibits CRM1-mediated nuclear export, and CRM1 specifically binds to leucine-rich NES. Since treatment of CIITA-infected cells with LMB increased the nuclear concentration of CIITA, suggesting that CIITA undergoes CRM1-mediated nuclear export, we analyzed the CIITA sequence for motifs with similarity to a consensus NES (58). CIITA contains 10 motifs that show varying degrees of similarity to the consensus NES (Fig. 8A). To test the function of each of these motifs, they were initially fused to GFP. Fusion of each of these sequences to GFP yielded no changes in GFP subcellular localization, with the exception of the CIITA sequence of residues 1035–1051, which resulted in a cytoplasmic localization of the protein. Note the punctate staining in the cytoplasm of CIITA–1035–1051/GFP as compared with GFP alone evenly distributed throughout the cell.

FIGURE 7. Activation of the DR-luciferase promoter by CIITA deleted for NLS2 or NLS3 and rescue of activity by substitution with the SV40 NLS. COS cells (□) or HeLa cells (□) were transfected with 1 μg each of the indicated CIITA plasmids plus 1 μg of DR-luciferase reporter. Extracts were prepared and assayed for relative luciferase at 24 h posttransfection. △405–414 refers to CIITA deleted for NLS2 at aa 415–414. △955–959 refers to CIITA deleted for NLS3 at aa 955–959, while △955–959/NLS refers to the SV40 NLS inserted into this same deleted motif region, analogous to the CIITA △405–414/NLS. △955–959 is the same as the −5-aa constructs described previously (30). pGL2 Control is used as positive control for transfections. Extracts were normalized to protein concentration and luciferase activities were compared with CIITA activity set to 100% for each cell type. All experiments were repeated three times and performed in triplicate.

Discussion

CIITA has been well characterized as the transcriptional coactivator responsible for initiating the expression of the MHC class II genes as well as genes for DM and invariant chain involved in exogenous Ag processing and presentation (1–3). Although CIITA itself fails to directly bind DNA at the promoters of class II genes, it does interact in the nucleus with other transcription factors at these regions (13, 59). Therefore, for full functionality, CIITA must be able to enter the nucleus to activate target gene expression. Previously we have identified a NLS within CIITA capable of mediating nuclear translocation of the protein (30). This NLS (NLS3) is located toward the carboxyl terminus of CIITA, at aa 955–959, and has been shown to be necessary for CIITA nuclear translocation, as its deletion results in a cytoplasmic-restricted protein and is the molecular basis for one form of complementation group A type II BLS. However, subsequent carboxyl-terminal deletions of CIITA revealed shorter forms of the protein that again displayed the ability to enter the nucleus. The C terminus contains leucine-rich regions that can interact with other regions of the molecule (56), thereby potentially covering up specific motifs within the molecule that are otherwise exposed under particular conditions within the cell. It is possible that deletion of the carboxyl-terminal region of CIITA exposes a previously covered NLS or eliminates an NES, in either case resulting in the increased nuclear

FIGURE 8. Identification of motifs in CIITA with sequence similarity to a consensus NES. A, Putative NES sites were identified by alignment with the consensus NES present in PKI. Location of each sequence within CIITA is identified by amino acid number. Hydrophobic residues are indicated in bold. B, Fusion of residues 1035–1051 to GFP results in cytoplasmic localization of the protein. Note the punctate staining in the cytoplasm of 1035–1051/GFP as compared with GFP alone evenly distributed throughout the cell. C, Leucine to alanine mutation of residue 1049 causes cytoplasmic localization of CIITA. Wild-type CIITA protein and L1049A were FLAG-tagged and detected by immunofluorescence of cells at 24 h posttransfection.

<table>
<thead>
<tr>
<th>Amino Acid #</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>38-47</td>
<td>LNSDADPLCL</td>
</tr>
<tr>
<td>182-190</td>
<td>LPCPLPLPAL</td>
</tr>
<tr>
<td>525-532</td>
<td>LRGLLAGL</td>
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<tr>
<td>633-642</td>
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<tr>
<td>986-994</td>
<td>LQHLDLDAL</td>
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<td>1014-1022</td>
<td>LKSLTTLNL</td>
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<tr>
<td>1035-1051</td>
<td>LAELPSLAASLRLSL</td>
</tr>
<tr>
<td>1068-1076</td>
<td>LPDMSVLRLV</td>
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</table>

Consensus: LxxxxLxxLxL
translocation of the CIITA mutants. These deletion mutant proteins must be specifically directed into the nucleus by a NLS or by association with another NLS-containing protein, as the mutants themselves are too large (>40 kDa) to pass through the nuclear pore complexes by simple diffusion. This observation led to the possibility that CIITA may contain one or more additional NLS amino-terminal to NLS3.

Fine mapping of CIITA using carboxyl-terminal deletion mutants restricted the second CIITA NLS to a smaller region. Deletion mutants 1–444, 1–518, and 1–612 all localize strongly to the nucleus in immunofluorescence studies, whereas 1–335 and smaller CIITA constructs are distributed evenly throughout the cytoplasm and nucleus. Therefore, the 110-aa region between residues 336 and 444 appeared likely to contain a NLS. The equal subcellular distribution of the CIITA constructs shorter than 336 aa in length is likely due to passive diffusion of these proteins through the nuclear membrane, although these observations do completely preclude the possibility of another NLS upstream of aa 335. To be consistent with our observations of equivalent distribution of CIITA1–335 between the cytoplasmic and nuclear compartments, the rate of import due to a NLS in this position would have to be equally balanced by the rate of export or passive diffusion out of the nucleus. Sequence analysis of the region between aa 336 and 444 reveals a single motif that is rich in basic residues, one of the general hallmarks of a NLS (60). This motif at aa 405–414, KEHRRPRETR, is located just upstream from the GTP homology region of CIITA. NLS motifs have traditionally been identified on the basis of three functional characteristics: 1) deletion of the putative NLS results in a protein restricted to cytoplasmic expression and incapable of entering the nucleus; 2) addition of the NLS to a heterologous protein is sufficient to drive the nuclear translocation of that protein; and 3) the NLS is interchangeable with other defined NLS. The CIITA NLS motif at aa 405–414 meets all of these criteria. An internal deletion of aa 405–414 causes the resulting CIITA protein to be expressed only in the cytoplasm. Arginine to alanine mutations at residues 408 and 409 result in decreased nuclear translocation of CIITA as compared with the wild-type protein. Fusion of this 10-aa motif to GFP in one or two copies induces the nuclear localization of GFP. Finally, substitution of the classical SV40 NLS into the region deleted for aa 405–414 in CIITA causes partial restoration of CIITA localization and function. The failure to reach a full 100% restoration of activity by substitution of the SV40 NLS for aa 405–414 may be due to slight differences in charge, size, or sequence between the two NLS, causing a decrease in the rate of nuclear import compared with wild-type CIITA. CIITA that is translocated more slowly into the nucleus could lead to a reduced nuclear accumulation and leave the protein more susceptible to the nuclear export machinery. If the rate of nuclear export of CIITA405–414/NLS is greater than the rate of nuclear import, this would account for the observation of predominantly cytoplasmic CIITA405–414 in untreated cells. This ability of the SV40 NLS to rescue CIITA activity suggests that the functional domain deleted in CIITA405–414 is a NLS and that this region is normally exposed on the surface of the CIITA protein in the proper context such that it is capable of being recognized by the nuclear import machinery of the cell.

The data presented here indicate that CIITA contains three identified NLSs, one at aa 405–414 as well as the previously identified NLS at residues 141–159 (31) and 955–959 (30). We propose that these NLS regions be referred to by the more simplified names of NLS1 (aa 141–159), NLS2 (aa 405–414), and NLS3 (aa 955–959). It is clear that loss of either NLS2 or NLS3 inhibits the ability of CIITA to translocate to the nucleus and activate gene expression. In the absence of a crystallographic structure of CIITA, it is difficult to know how these NLS are aligned relative to one another. It may be that all are part of a pocket domain that stabilizes interaction with importin α or alternatively the NLS may be entirely distinct domains that mediate interactions with separate importin protein family members. Interestingly, the SV40 NLS cannot substitute for NLS3 of CIITA (30). Therefore, while NLS2 is similar to the classical NLS, NLS3 is a distinct form likely to interact with a separate subset of importin family members. Six different isoforms of importin α have been described in humans thus far (61–66) and there is evidence that different isoforms show different affinities for specific NLS-containing substrates (67). Correlating the exact kinetics and binding affinities of different importin α isoforms with different NLS remains to be determined. A number of proteins contain two or more NLS, either sequentially within the protein as part of a bipartite NLS or distributed throughout the protein, including NF-AT, Rh, c-myc, and Myo-D (32).

Bipartite sequences are separated by 10- to 12-aa spacers, although NLS in other proteins may also be separated by >100 residues, as in the case of the influenza NS1 nuclear protein (68). In this case, NS1 is a bipartite NLS, whereas the more carboxy-terminal NLS of CIITA are reminiscent of the latter, with NLS2 and NLS3 likely mediating binding to two different importin molecules, both of which are necessary for nuclear import. Our data suggest that deletion of either NLS alone is sufficient to impair CIITA translocation, resulting in cytoplasmic localization of the protein and loss of gene expression from MHC class II promoters. Because CIITA is a relatively large protein at 140 kDa, multiple NLS may be more efficient at translocating it into the nucleus. The regulation of CIITA must be very tightly controlled to effect MHC class II gene transcription at the proper time in response to cellular stimulation. Control of CIITA translocation mediated by interaction with multiple importin family members may be one of a number of mechanisms by which cells carefully regulate CIITA activity.

Immunofluorescence studies demonstrate that CIITA is present in both the cytoplasm and the nucleus, a finding confirmed by treatment of cells with LMB. As an inhibitor of CRM1-mediated nuclear export (46, 48), LMB increases the nuclear concentration of CIITA, demonstrating that the CIITA protein is normally exported back into the cytoplasm. This accumulation of nuclear CIITA occurs very rapidly following LMB addition, indicating that CRM1-mediated export of CIITA is a highly active process. Despite our initial attempts however, we were unable to definitively identify specific NES motifs in the CIITA protein. Two different possibilities exist that may explain the failure in our initial identification of a CIITA NES. First, the NES exists within another region of CIITA or has an unusual sequence that was not identified by initial sequence homology comparisons to known NES. Second, CIITA may in fact lack a NES but is exported from the nucleus in complex with another NES-containing protein. Because CIITA has been shown to interact with a number of proteins within the nucleus (26), we cannot eliminate this possibility. Several scenarios may exemplify the significance of CIITA being present in both the cytoplasm and the nucleus. It is possible that export is necessary to down-regulate CIITA activity following gene activation; CIITA may be modified in the cytoplasm and reimported into the nucleus; cycling of CIITA between the nucleus and the cytoplasm may allow for the specific import or export of CIITA-associated proteins; or alternatively CIITA may be specifically degraded in the cytoplasm. The identification of the domain
responsible for CIITA export, combined with the present identification of the two NLS, will help clarify the function of this dual subcellular localization of CIITA.

Note added in proof: After this paper was accepted, Kretsovali et al. (69) published data showing that aa 1–114 and 550–850 of CIITA bind CRM1. These regions may correspond to the potential NES sites identified in Fig. 8.

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References


