The GTP-Binding Domain of Class II Transactivator Regulates Its Nuclear Export

Aparna Raval, Jocelyn D. Weissman, T. Kevin Howcroft and Dinah S. Singer

J Immunol 2003; 170:922-930; doi: 10.4049/jimmunol.170.2.922
http://www.jimmunol.org/content/170/2/922

References
This article cites 44 articles, 26 of which you can access for free at:
http://www.jimmunol.org/content/170/2/922.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The GTP-Binding Domain of Class II Transactivator Regulates Its Nuclear Export

Aparna Raval, Jocelyn D. Weissman, T. Kevin Howcroft, and Dinah S. Singer

The transcriptional coactivator class II transactivator (CIITA), although predominantly localized in the nucleus, is also present in the cytoplasm. The subcellular distribution of CIITA is actively regulated by the opposing actions of nuclear export and import. In this study, we show that nuclear export is negatively regulated by the GTP-binding domain (GBD; aa 421–561) of CIITA: mutation or deletion of the GBD markedly increased export of CIITA from the nucleus. Remarkably, a CIITA GBD mutant binds CRM1/exportin significantly better than does wild-type CIITA, leading to the conclusion that GTP is a negative regulator of CIITA nuclear export. We also report that, in addition to the previously characterized N- and C-terminal nuclear localization signal elements, there is an additional N-terminal nuclear localization activity, present between aa 209 and 222, which overlaps the proline/serine/threonine-rich domain of CIITA. Thus, fine-tuning of the nucleocytoplasmic distribution of coactivator proteins involved in transcription is an active and dynamic process that defines a novel mechanism for controlling gene regulation. The Journal of Immunology, 2003, 170: 922–930.

Major histocompatibility complex class I and class II molecules play an important role in immune surveillance. The Ag receptor on T cells (TCR) recognizes antigenic peptides only when the latter are presented in the context of either MHC class I molecules on APCs in the case of cytotoxic T cells or MHC class II molecules in case of Th cells. Regulation of both MHC class I and class II gene expression has been studied extensively and IFN-γ is the primary target of IFN-γ with 18 U.S.C. Section 1734 solely to indicate this fact.

CIITA does not bind to DNA but acts as a classical coactivator (7, 8), interacting with DNA-binding transcription factors such as regulatory factor X (9), regulatory factor X-associated protein (10), basal transcription factors such as human TATA-binding protein–associated factors II 32 and II 70 (TFIIF32, TFIIF70) and transcription factor IIB (TFIIB) (11, 12), and other coactivators including CREB binding protein (CBP) (13, 14) and p300/CBP-associated factor (PCAF) (15). Thus, CIITA plays an active role in regulating transcription by serving as a scaffold for recruitment of a variety of factors that activate transcription. We recently demonstrated that CIITA has intrinsic acetyltransferase (AT) activity which is essential for its function (16). CIITA mutants defective in AT activity are unable to mediate transactivation. Thus, like other coactivators, it may have a role in chromatin remodeling which could explain why the accessibility of MHC class II promoters to DNA binding proteins is enhanced by CIITA in certain cell types (17).

CIITA is a complex molecule of 1130 aa, which contains a number of additional functional domains: an N-terminal activation domain (12), a proline/serine/threonine (PST)-rich domain that overlaps the AT domain, a series of leucine-rich repeats necessary for dimerization and nuclear localization (18–20), N- and C-terminal nuclear localization signals (NLS) (15, 21), and a GTP-binding domain (GBD) (22). Binding of GTP to the GBD increases the AT activity of CIITA (16), suggesting that it plays an important role in regulating transcription.

Endogenous CIITA is primarily located in the nucleus, although a fraction is found in the cytoplasm of normal cells (21). Its subcellular distribution is governed by a complex array of molecular domains. Two NLS have been reported, one at the C-terminal end and the other within the N-terminal domain (18, 19, 21, 23, 24). Binding of the coactivator PCAF to CIITA results in acetylation of lysine residues within the N-terminal NLS of CIITA, leading to its increased nuclear localization (15). The GBD plays a role in the subcellular localization of CIITA (23, 25) and is also necessary for CIITA self-association (20, 26). The leucine-rich repeats also regulate self-association and the rate of nuclear import (18, 19, 27).

Although considerable evidence indicates that the nuclear import of CIITA is regulated, there is as yet little understanding of the role of nuclear export mechanisms in establishing the subcellular distribution of CIITA.

Nucleocytoplasmic shuttling of proteins is a precisely controlled process that occurs through the nuclear pore complex (28). The transport of proteins from the cytoplasm into the nucleus is dependent on the presence of NLS elements, short sequences rich in basic residues (29), which are recognized by the importin family of proteins. The importins act as carriers to transport the substrate protein into the nucleus (30, 31). Nuclear export of many proteins is mediated by the binding of CRM1/exportins to leucine-rich nuclear export sequences (NES) (32). Treatment of cells with leptomycin B (LMB), a specific inhibitor of CRM1-mediated export,
results in nuclear accumulation of such leucine-rich NES-containing proteins (33). Previous studies have demonstrated that CIITA is retained in the nucleus following LMB treatment of cells, suggesting that it has an active CRM1/exportin-dependent NES (15). Although CRM-1 binding sites have been mapped, the NES has not been fully characterized. The relationship between the activities of the NLS and NES is also not understood.

In the present study, we report that the nuclear export of CIITA is negatively regulated by its GBD. CIITA mutants in the GBD bind CRM1/exportin more efficiently than does wild-type (WT) CIITA and are more rapidly exported to the cytoplasm. We also identify an additional sequence that regulates nuclear localization in the N-terminal segment of CIITA. These results collectively provide an insight into the regulatory mechanisms governing the subcellular localization of CIITA. We propose a novel model for the dynamic regulation of CIITA cellular localization mediated by the GBD.

Materials and Methods
Cell line and plasmids
BHK cells and HeLa cells were grown as described previously (34). The MHC class I promoter 313CAT consists of 313 bp of 5' flanking sequences derived from swine class I gene PDI ligated to the chloramphenicol AT (CAT) reporter gene (35). The MHC class II promoter construct pDRA300CAT consists of 300 bp of 5' flanking sequences (22). The mammalian expression vector Flag-CIITA WT, PST deletion mutant and GST-Kpn I reporter gene (35). The MHC class II promoter construct 313CAT consists of 313 bp of 5' flanking sequences (22). The mammalian expression vector Flag-CIITA WT, PST deletion mutant and GST-binding domain mutants CIITA304-135, and CIITA truncation mutants CIITA0-306 have been described previously (22). The CIITA0-306 was generated by cloning the KpnI fragment of Flag-CIITA WT into the KpnI site of pcDNA3. CIITA ATD222 was generated by XcmI/BsaI360 digestion, removing aa 27–222. The CRM1/exportin expression vector pCRM1-1sg143 encodes a green fluorescence protein (GFP)-tagged human CRM1/exportin and was a kind gift of Dr. K. Vousden (National Cancer Institute, National Institutes of Health) (36).

Transient transfections
Transient transfections were done by CaPO4 precipitation method as described previously (37). For cotransfection experiments, HeLa cells were transfected with either 5 μg of 313CAT or 2 μg of pDRA300CAT reporter constructs and the indicated amounts of CIITA WT, mutants, or control plasmid, and the cells were maintained at 37°C for 48 h. CAT activity was normalized to luciferase activity by cotransfecting an internal plasmid control, either pRSVLuc or pSV2Luc. For nuclear localization studies, BHK cells were grown on cover slips in 6-well plates and transfected with 5 μg of Flag-tagged CIITA WT or the mutant constructs, and the cells were maintained at 32°C. After 48 h, cells were assayed for subcellular localization. Where indicated, cells were incubated with 10 ng/ml of LMB (Sigma-Aldrich, St. Louis, MO) for 3 h before fixation.

Subcellular localization of CIITA
After 48 h of transfection, BHK cells were rinsed in 1× HBSS and fixed with 2% paraformaldehyde for 15 min, followed by quenching with 50 mM ammonium chloride made in PBS. Cells were then permeabilized with 1% Nonidet P-40, washed, incubated for 90 min at 37°C with anti-Flag mouse mAb M5 (1/250 dilution), and rinsed with PBS containing 0.1% saponin and 0.01% goat serum. The cells were again incubated for 90 min at 37°C with Alexa 488 goat anti-mouse Ab, at 1/750 dilution (Molecular Probes, Eugene, OR) and Topro at 1/1000 dilution. The cells were rinsed and mounted on slides using ProLong Antifade kit (Molecular Probes). The subcellular localization was studied by Zeiss LSM 410 confocal microscope. For each construct, 100–120 transfected cells were examined.

Table I. Subcellular localization and transactivation of CIITA mutants

<table>
<thead>
<tr>
<th>CIITA</th>
<th>Nuclear/Cytoplasm</th>
<th>Nuclear-Cytoplasm (LMB)</th>
<th>Class I Transactivation (Fold Activation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIITA WT</td>
<td>3.04 ± 0.26</td>
<td>6.4 ± 0.39</td>
<td>2.3</td>
</tr>
<tr>
<td>CIITA0-306</td>
<td>2.3 ± 0.07</td>
<td>2.3 ± 0.009</td>
<td>0.48</td>
</tr>
<tr>
<td>CIITA304-94</td>
<td>3.6 ± 0.04</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>CIITA304-135</td>
<td>2.1 ± 0.18</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>CIITA309-301</td>
<td>2.2 ± 0.27</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>CIITA312-209</td>
<td>1.4 ± 0.17</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIITA0-306</td>
<td>0.86 ± 0.04</td>
<td>3.7 ± 0.3</td>
<td>0.99</td>
</tr>
<tr>
<td>CIITAΔSKAD</td>
<td>0.95 ± 0.04</td>
<td>3.1 ± 0.2</td>
<td>1.24</td>
</tr>
<tr>
<td>CIITAΔGK</td>
<td>1.4 ± 0.2</td>
<td>4.2 ± 0.4</td>
<td>1.28</td>
</tr>
<tr>
<td>CIITA27-222</td>
<td>0.70 ± 0.06</td>
<td></td>
<td>0.98</td>
</tr>
<tr>
<td>CIITA312-301</td>
<td>0.62 ± 0.02</td>
<td>0.93 ± 0.06</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* The subcellular distribution of WT and variant CIITA transfected into BHK cells was determined as described in Materials and Methods.
* Quantitation of the subcellular distribution of CIITA WT and mutants between nucleus and cytoplasm in BHK cells. CIITA mutants with nuclear/cytoplasmic ratios of ≥2 were classified as nuclear.
* Forty-eight hours after transfection, cells were treated for 3 h with 10 ng/ml LMB.

Summary of the ability of each of the CIITA molecules to transactivate the class I promoter in HeLa cells. The activity is expressed relative to CIITA WT.

Immunoprecipitations
HeLa cells were transfected either alone or in combination with 15 μg Flag-CIITA WT, Flag-CIITAΔSKAD, or pCCRM1-sg143 (hCRM-GFP) DNA as described above. Cells were harvested at 48 h, resuspended in lysis buffer (50 mM Tris (pH 8.0), 5 mM MgCl2, 150 mM KCl, 0.1% Nonidet P-40, and 10% glycerol with complete inhibitors (Roche, Basel, Switzerland)), incubated 20 min on ice, and lysed by passage twice through a 23-gauge needle. Lysates were cleared by centrifugation at 100,000 × g for 20 min at 4°C. Cell extract containing transfected hCRM-GFP was incubated with increasing amounts (ratios of 1:2, 1:4, and 1:8) of Flag-CIITA WT or Flag-CIITAΔSKAD containing extracts for 1 h at 4°C. Agarose-conjugated anti-GFP was added, and incubation continued overnight. The immunoprecipitates were pelleted, washed in 1 ml of lysis buffer two times, and resuspended in sample buffer. One half of each sample was resolved on duplicate 6% SDS-PAGE gels under reducing conditions, transferred to membrane, and blotted for hCRM-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or M5 (Flag; Sigma-Aldrich) and developed with HRP (Pierce, Rockford, IL). The resulting signals were measured on Storm Imaging system (Amersham Pharmacia Biotech, Piscataway, NJ). The CIITA results were normalized to the amount of immunoprecipitated CRM1/exportin.

Results
Nuclear export of CIITA is regulated by a C-terminal element
CIITA is primarily nuclear, due to the presence of NLS elements that have been identified in both the C and N termini of the molecule (15, 21). However, a significant fraction of cellular CIITA also is detected in the cytoplasm (Table I (21). This cytoplasmic CIITA could represent recently synthesized molecules en route to
the nucleus. Alternatively, CIITA could be targeted to the cytoplasm from the nucleus by the activity of a previously undescribed nuclear export activity. To examine this latter possibility, the subcellular distribution of a set of truncation and deletion mutants of CIITA was determined by transfection of Flag-tagged CIITA constructs into BHK fibroblast cells. When WT CIITA is introduced into cells, approximately one-quarter of the total CIITA is found in the cytoplasm (Fig. 1A and Table I). In contrast to the largely nuclear localization of the WT CIITA, a CIITA variant molecule spanning aa 1–804 was predominantly cytoplasmic (Fig. 1A and Table I). This finding is consistent with the removal of the previously identified C-terminal NLS. Surprisingly, we found that further C-terminal truncation yielded a CIITA variant (aa 1–306) that was once again predominantly nuclear (Fig. 1A and Table I), raising the possibility that nuclear export is regulated by sequences in the interval between aa 306 and 804.

To further investigate this possibility, the effect of LMB on the subcellular distribution of CIITA and its variants was examined. LMB is a specific inhibitor of CRM1/exportin-mediated nuclear export; CRM1/exportin binds to NES signals within proteins (32, 33). If CIITA contains an NES that depends on CRM1/exportin, LMB treatment would result in the accumulation of CIITA in the nucleus. Indeed, treatment of cells with LMB for 3 h led to a 2-fold enhancement of the nuclear localization of CIITA WT, indicating that nuclear export of CIITA is mediated by CRM1/exportin (Fig. 1B and Table I). The CIITA 1–804 truncation, which is cytoplasmic in the absence of LMB treatment, localizes to the nucleus in the presence of LMB. This finding indicates that 1) NLS sequences reside in this interval and, importantly, 2) sequences necessary for CRM1/exportin-dependent export are present in this variant and mediate its efficient export to the cytoplasm. In contrast, treatment of CIITA 1–306 with LMB did not alter its nuclear localization (Fig. 1B). Thus, regulation of CIITA nuclear export is governed by elements in the interval between aa 306 and 804.

One possibility is that a CRM1/exportin-dependent NES is located in the interval of aa 306–804. Sequence analysis of this region reveals a consensus NES sequence between aa 595 and 605 (LTLLRDPLL). Like known NES elements, it is hydrophobic and leucine rich (Fig. 1A). However, mutation of this sequence did not alter the subcellular distribution relative to WT CIITA (data not shown). Other leucine-rich segments in this region have also been mutated and shown not to mediate nuclear export (38).

The previously characterized GBD is also located within this region, between aa 421 and 561. This GBD has a number of regulatory functions. We have shown that binding of GTP to the GBD regulates the intrinsic AT activity of CIITA (16). Others have shown that the GBD is involved in intermolecular interactions of CIITA (19, 24). Therefore, the finding that the GBD lies within the segment involved in subcellular localization of CIITA raised the possibility that the GBD also plays a role in regulating nuclear export of CIITA.

The GBD regulates CRM1/exportin binding and subcellular distribution of CIITA

We next examined the effect of mutating the GBD on the subcellular localization of CIITA. Two GBD mutants were studied: CIITA ΔSKAD that is unable to bind guanine and CIITA ΔGK that is unable to bind phosphate (23). In transfected BHK cells, these mutants are primarily cytoplasmic (Fig. 2 and Table I), as has been shown in COS7 cells previously (23). This cytoplasmic localization could be due to either decreased nuclear import or increased nuclear export. To distinguish between these possibilities, we examined the effect of LMB treatment on the localization of these

FIGURE 1. CIITA has a NES between aa 306 and 804. A, Schematic representation of full-length and truncated CIITA molecules. The sequence of a consensus NES is also indicated. The positions of previously characterized domains are indicated as follows: acidic domain (A), acetyl transferase (AT), proline (P), serine (S), threonine (T), GBD (GBD), C-terminal nuclear localization sequence (NLS), and LRR (LRR). DNA constructs encoding CIITA WT or C-terminal truncation mutants (5 μg) were transfected into BHK cells and the localization of protein products (shown in green) was examined after 48 h as described in Materials and Methods. B, BHK cells were transfected with each of the constructs; after 45 h, cells were treated with 10 ng/ml LMB for 3 h before harvesting. Nuclei were counterstained with Topro (shown in red). Left, Untreated controls; right, LMB-treated samples.
stained with Topro (shown in red). Left examined as described in LMB for 3 h. Subcellular localization of CIITA (shown in green) was transfected into BHK cells. After 45 h, cells were treated with 10 ng/ml fia possible mechanism to explain the above GBD mutant.Extracts derived from hCRM-GFP transfected cells were combined with increasing amounts of extract derived from cells transfected with either Flag-CIITA WT (●) or Flag-CIITAΔSKAD (■) constructs. (Extract volumes were adjusted to equalize CIITA concentrations, as determined by Western blotting of the whole extract.) The mixes were immunoprecipitated with anti-GFP, resolved on PAGE, and immunoblotted with anti-Flag Ab. The inset shows a representative gel. The amount of CIITA coprecipitated with hCRM-GFP was quantitated by PhosphorImager, normalized to the input CRM1/exportin in each sample, and plotted as a function of input CIITA WT or ΔSKAD.

GTP. If the GBD mutations simply abrogate nuclear localization, LMB should have no effect. However, if the GBD regulates nuclear export, LMB treatment would cause the GBD mutants to be sequestered in the nucleus. As shown in Fig. 2, both CIITA mutants accumulated in the nucleus following LMB treatment (right panel). Thus, the GBD regulates nuclear export by reducing CRM1/exportin-mediated export of CIITA from the nucleus: in the absence of a functional GBD, export is maximally active and CIITA is transported to the cytoplasm.

CRM1/exportin binds to WT CIITA less efficiently than to the GBD mutant

A possible mechanism to explain the above findings is that CRM1/exportin protein binds to the GBD mutant more efficiently than to WT CIITA. To examine this possibility, GFP-tagged CRM1/exportin was combined with increasing amounts of either WT or ΔSKAD mutant Flag-tagged CIITA. Because Ran-GTP is known to be required for the effective interaction of CRM1/exportin with its targets, extracts from transfected HeLa cells rather than purified proteins were used. The complexes were immunoprecipitated with anti-GFP and analyzed by Western blotting to determine the amount of CIITA associated with CRM1/exportin. As shown in Fig. 3, at every concentration of protein, more CIITAΔSKAD than WT CIITA associates with CRM1/exportin. At the highest concentrations of CIITA, the GBD mutant is precipitated by CRM1/exportin approximately six times more efficiently than WT. These findings support the conclusion that the GBD of CIITA is a negative regulator of CRM1/exportin-mediated nuclear export of CIITA. Indeed, we find that the interaction between CIITA and CRM1/exportin is reduced in the presence of 1.25 mM GTP (J. Weissman and D. Singer, data not shown).

N-terminal NLS elements contribute to determination of the subcellular distribution of CIITA

The localization of the 1–306 CIITA truncation largely to the nucleus (Fig. 1A) mapped an NLS to that segment. Consistent with this observation, studies by Spilianakis et al. (15) using CIITA peptides fused to GFP localized an NLS to the segment aa 141–159. However, in those studies mutations within the segment aa 141–159 did not abrogate nuclear localization, but only increased the relative proportion of CIITA in the cytoplasm, leaving the possibility that other NLS elements occur within the segment aa 1–306. To identify any additional NLS elements, deletion mutants of full-length CIITA spanning much of the N terminus were assessed for their subcellular distribution; their distributions are quantitated in Table I.

Whereas WT CIITA is largely nuclear, deletion of a large segment between aa 27 and 222 resulted in the cytoplasmic localization of the mutant CIITA molecule, mapping an NLS to this region (CIITAΔ27–222; Fig. 4A). Two smaller deletions within this same segment, extending from aa 58 to 94 and from 94 to 135, still localized to the nucleus (CIITAΔ58–94 and CIITAΔ94–135; Fig. 4A; Table I), consistent with an NLS residing in the segment aa 135–222. (The possibility that an additional NLS is in the aa 27–58 segment has not been excluded.)

A CIITA variant with a deletion from aa 209 to 301 was predominantly nuclear. Conversely, deletion of a segment extending from aa 132 to 301 was almost completely cytoplasmic (nucleus: cytoplasm of 0.6), mapping an NLS to this interval. Consistent with this conclusion, LMB treatment of cells expressing CIITAΔ132–301 does not alter the subcellular distribution of this variant, indicating that it does not enter the nucleus (Fig. 4C and Table I). These findings are consistent with the presence of the reported NLS between aa 141 and 159.

Surprisingly, a larger fraction of a CIITA variant with a deletion between aa 132 and 209, which spans the reported NLS, partitioned to the nucleus than did CIITAΔ132–301 which has a more
extensive deletion. Thus, the nucleus:cytoplasm ratio of the CI-
ITA/H9004<sub>132</sub>–209 variant is 1.4, while that of CIITA
/H9004<sub>132</sub>–301 is 0.6 (Table I and Fig. 4). This suggests that the segment between 209 and
301 contains a sequence that regulates nuclear localization, poten-
tially an additional NLS. The presence of an additional NLS, albeit
a weak one, in the interval of aa 209–301 is further supported by
the finding that the CIITA/H9004<sub>132</sub>–209, unlike CIITA
/H9004<sub>132</sub>–301, is able to transactivate the MHC class I promoter, indicating that it does
enter the nucleus (Table I).

These data are most simply interpreted as indicating that there
are two N-terminal NLS sequences, one between aa 132 and 209
and another between aa 209 and 222. The NLS between aa 132 and
209 presumably corresponds to the previously identified aa 141–
159 NLS. The second NLS between aa 209 and 222 is novel.
Inspection of the sequence does not reveal homology with known
NLS sequences.

In summary, the localization of CIITA<sub>1–306</sub> in the nucleus and
CIITA<sub>1–804</sub> in the cytoplasm indicates that the N-terminal NLS
elements alone are not sufficient to target CIITA to the nucleus in
the presence of an export signal and require the additional
C-terminal NLS.

Cytoplasmic localization of CIITA mutants correlates with their
failure to transactivate

Functional analyses have mapped a series of regulatory domains
within the CIITA molecule: an N-terminal α helical acidic domain
between aa 58 and 94, an AT domain between aa 94 and 132 that
is required for activation, the PST domain between aa 132 and 301,
the central GBD, and the C-terminal leucine-rich region (LRR)
involved in dimerization. In light of the complex regulatory mech-
anisms governing the subcellular distribution of CIITA, it is im-
portant to determine which apparent activation domains may ac-
tually reflect domains that regulate subcellular localization. To this
end, we next examined the relationship between the subcellular
localization of CIITA and its variants and their ability to transac-
tivate an MHC class I promoter construct (Table I). In contrast to
WT CIITA which is predominantly nuclear and increases promoter
activity 2- to 3-fold above the basal level, the cytoplasmic CIITA
variants—CIITA<sub>1–804</sub>, CIITA<sub>132–301</sub>, and CIITA<sub>27–222</sub>—do not
transactivate. The GBD mutants, CIITA<sub>SKAD</sub> and CIITA<sub>GK</sub>,
which enter the nucleus but are rapidly exported, are weakly
activating.

Whereas all cytoplasmic variants fail to transactivate the class I
promoter, not all nuclear variants are able to transactivate. Thus,
the CIITA<sub>132–301</sub> construct (5 μg) and 45 h later treated with 10 ng/ml LMB for 3 h. Nuclei were counterstained with Topro (shown in red).

FIGURE 4. CIITA has an NLS that lies within the PST domain. A and C, Schematic of CIITA WT and N-terminal deletion mutants used in transfections
below. Domains as in Fig. 1. (B and C) CIITA WT or deletion mutant constructs (5 μg) were transfected into BHK cells, and the localization of protein
products (shown in green) was examined at 45 h as described in Materials and Methods. The corresponding Z sections are shown to the right. D, BHK cells were
transfected with CIITA<sub>132–301</sub> construct (5 μg) and 45 h later treated with 10 ng/ml LMB for 3 h. Nuclei were counterstained with Topro (shown in red).
which activates the class I promoter 4- to 5-fold in HeLa cells, \( \text{IFN-}\gamma \) inhibits basal transcription from the class I promoter in the absence about 2-fold. These findings are consistent with our previous stud-

![FIGURE 5. CIITA\(_{1-306}\) acts as a dominant-negative mutant of promoter activity in both constitutive and CIITA-activated transcription. A, HeLa cells were cotransfected with either 5 \( \mu \)g of the class I promoter construct (313CAT) or 2 \( \mu \)g of the class II promoter construct (pDRA300CAT) and 1.5 \( \mu \)g of control vector (not shown) or with 1.5 \( \mu \)g of CIITA WT with increasing amounts of CIITA\(_{1-306}\). B, HeLa cells were cotransfected with 5 \( \mu \)g of 313CAT and 3 \( \mu \)g of CIITA\(_{1-306}\), WT CIITA, or control vector. In both panels, data are expressed as promoter activity relative to cotransfection with control plasmid. Error bars indicate standard errors.]

Thus, in cotransfections into HeLa cells with full-length CIITA and either class I or class II promoter constructs, increasing amounts of the CIITA\(_{1-306}\) construct increasingly abrogated the CIITA-mediated activation of the promoter (Fig. 5A). The ability of CIITA\(_{1-306}\) to function as a dominant-negative competitor of full-length CIITA suggests that it competes with the full-length CIITA molecule for interactions with other transcription factors.

Of particular interest is the finding that the CIITA\(_{1-306}\), even inhibits basal transcription from the class I promoter in the absence of CIITA (Fig. 5B and Table I). In contrast to full-length CIITA which activates the class I promoter 4- to 5-fold in HeLa cells, CIITA\(_{1-306}\) reduces the basal activity of the class I promoter by about 2-fold. These findings are consistent with our previous studies in which we demonstrated that CIITA has two functional capabilities: mediating activated transcription and supporting basal transcription (16).

**Discussion**

CIITA is a transcriptional coactivator that nucleates the formation of transcription complexes (12, 33) responsible for mediating increased expression of both the MHC class I and class II promoters in response to \text{IFN-}\gamma (1–3). Because of its central role in regulating the activated transcription of these genes, the molecular mechanisms regulating CIITA function have been extensively studied and have revealed a remarkable complexity. CIITA subcellular localization—and thus function—is determined by a variety of factors. CIITA has been mapped to the segment aa 1-141 (24), we deleted these sequences from CIITA did not abrogate nuclear export (19, 24). Surprisingly, some of these mutations actually promote nuclear localization (19, 24). Furthermore, although NES sequences and CRM-1 binding have been mapped to the segment aa 1-114 (24), we find that the CIITA\(_{1-306}\) truncation is primarily nuclear, inconsistent with the entire NES being contained within the segment aa 1-306. Taken together, these studies indicate that the segment aa 1–114 is necessary, but not sufficient, for nuclear export. Thus, nuclear export of CIITA is not localized to a discrete, isolatable element. Rather, we propose that the NES of CIITA spans a large peptide domain similar to the snurportin NES (40). Snurportin, which mediates the nuclear import of SnRNPs, is dependent upon CRM1/exportin for its return to the cytoplasm after its cargo has been delivered. The snurportin NES consists of both a peptide segment in the amino-terminal 64 aa and a peptide segment spanning 74 aa at the carboxyl terminus of the protein. We speculate that the CIITA NES may be similarly discontinuous (40).

Despite the inability to identify a single NES, it is clear that CIITA export is mediated by the CRM-1/exportin pathway, as evidenced by the fact that LMB, which specifically inhibits CRM-1/exportin function, blocks cytoplasmic accumulation of CIITA. CRM-1/exportin binds the CIITA peptide 1–114 both in vitro and in vivo (24) and a segment between aa 408 and 550, which overlaps the GBD (24). However, the mechanisms regulating this export have not been studied previously. In the present study, we have identified a novel regulatory mechanism of CRM-1/exportin-mediated nuclear export. We have demonstrated that export of CIITA is regulated through its GBD, which serves as a negative regulator. Mutations in the GBD of CIITA decreased the efficiency of CRM-1/exportin interaction with CIITA, which results in their activities of the C-terminal NES (aa 955–959) and at least one of the N-terminal NES sequences (aa 141–159 or 209–222). Thus, nuclear localization of CIITA is regulated by a network of NES elements acting in concert to establish its rate of nuclear transfer.

Nuclear localization is further regulated by posttranslational modification of the NLS. Acetylation of Lys\(^{144}\) by either PCAF or CBP is known to enhance nuclear localization (15). Nuclear localization can be further enhanced by dimerization of CIITA which is mediated by both the central GBD and the N-terminal LRR (18, 19, 23). Interestingly, the novel NLS (aa 209–222) maps to the PST-rich domain of CIITA, raising the possibility that phosphorylation may enhance nuclear localization, as has been shown for other proteins (25, 39).

Although a number of regions of CIITA have been identified as nuclear localization sequences, none has been shown to bind importins. It has been reported that the LRR, located between aa 985 and 1096, function as an NLS (23). However, the LRR mediates self-association, a necessary prerequisite for nuclear localization of full-length CIITA (20, 27, 38). Thus, the LRR may not be an NLS, but rather may be required to achieve the proper CIITA conformation that enables nuclear localization. Similarly, the GBD has been thought to be an NLS (23). However, GTP binding to the GBD is required for self-association, which may inhibit the NES activity thereby permitting nuclear accumulation of CIITA.

Considerably less is known about the mechanisms regulating nuclear export than import. Sequences with homology to known NES have been identified, although none has been shown to mediate export of CIITA (19, 24). Inspection of the CIITA sequence reveals the presence of a consensus NES between aa 595 and 605 which is just C-terminal to the GBD, located between aa 420 and 561. However, mutation of this putative element does not alter the distribution of CIITA within the cell. Similarly, whereas various peptide fragments of CIITA have been shown to direct cytoplasmic localization of a fused GFP, deletion of these sequences from CIITA did not abrogate nuclear export (19, 24). Surprisingly, some of these mutations actually promote cytoplasmic accumulation (19, 24). Furthermore, although NES sequences and CRM-1 binding have been mapped to the segment aa 1–114 (24), we find that the CIITA\(_{1-306}\) truncation is primarily nuclear, inconsistent with the entire NES being contained within the segment aa 1–306. Taken together, these studies indicate that the segment aa 1–114 is necessary, but not sufficient, for nuclear export. Thus, nuclear export of CIITA is not localized to a discrete, isolatable element. Rather, we propose that the NES of CIITA spans a large peptide domain similar to the snurportin NES (40). Snurportin, which mediates the nuclear import of SnRNPs, is dependent upon CRM1/exportin for its return to the cytoplasm after its cargo has been delivered. The snurportin NES consists of both a peptide segment in the amino-terminal 64 aa and a peptide segment spanning 74 aa at the carboxyl terminus of the protein. We speculate that the CIITA NES may be similarly discontinuous (40).
activation. Thus, deletion of either the AT domain (CIITA) results in hyperactive nuclear export, do not transactivate. Conversely, not all variants of CIITA that shuttle to the nucleus are able to transactivate. Thus, deletion of either the AT domain (CIITA1-306) or the acidic domain (CIITA135-94) renders the molecule inactive, although still predominantly nuclear. These domains are required for function but do not play a role in subcellular localization. In contrast, the PST domain (aa 132-301) which contains two NLS elements appears to be primarily responsible for subcellular localization. Finally, the CIITA1-306 variant which contains two NLS elements localizes to the nucleus, where it functions as a dominant negative. Interestingly, it inhibits both CIITA-activated transcription and basal transcription of the class I promoter. Although CIITA interacts with CBP (14), squelching by CIITA1-306 is unlikely to account for its dominant negative effect on class I transcription; we have demonstrated previously that CBP is not required for either basal or CIITA-activated class I transcription (16). Rather, this finding suggests that the CIITA peptide interacts with transcription factors that are common to both basal and activated transcription.

We propose the following model for the regulation of CIITA subcellular distribution (Fig. 6). Monomeric, unmodified CIITA has an exposed and active NES that is able to interact with CRM1/exportin and thus accumulates in the cytoplasm. In the presence of GTP, the CIITA dimerizes, thereby masking the NES and activating the NLS. Shuttling of dimeric CIITA to the nucleus can be further enhanced by posttranslational modifications of the NLS, such as phosphorylation or acetylation. Once in the nucleus, CIITA nucleates the formation of an enhanceosome that activates target promoters, such as the MHC class I and class II promoters. We further speculate that chromatin-associated histone deacetylases and GTPases convert the active dimeric form of CIITA to the monomeric form, with an exposed NES. This monomeric form once again localizes to the cytoplasm. In this model, the GBD assumes a central role as an important regulator of CIITA activity, acting as a toggle switch. As we have previously shown, GTP also potentiates the AT activity of CIITA (16). Therefore, GTP enhances CIITA’s transactivation function, both directly and indirectly by reducing nuclear export. Thus, the AT activity and NES of CIITA are coordinately, but inversely, regulated by GTP.

Although nuclear dimeric CIITA may be the transcriptionally active form, this is not a necessary prediction of the model. It is equally plausible that CIITA becomes monomeric in its association with enhanceosome transcription factors that also serve to mask the NES. This model does predict that the structural features of nuclear and cytoplasmic CIITA will be distinct. This prediction is currently being tested.

The existence of a complex regulatory pathway for CIITA cellular localization raises the question of the underlying need for such a system. Transcription factors whose cellular distribution is actively controlled, such as NF-κB, are ubiquitously expressed in all cells. However, CIITA is not normally expressed in cells, with accumulation in the cytoplasm (Figs. 2 and 3). Although the cytoplasmic localization of these mutants was originally interpreted to indicate that the GBD contains an NLS (23), the present studies demonstrate that it results from increased nuclear export. (It is important to note that the present study does not map the CRM1/exportin binding site itself, but rather a regulatory domain that governs CRM1/exportin binding.) Thus, our present data demonstrate that the ability of CIITA to bind to CRM1/exportin and be transported to the cytoplasm is regulated in vivo by GTP. This is the first evidence of regulation of CRM1/exportin-mediated nuclear export by GTP binding to the cargo.

Interestingly, GTP binding to the GBD has also been found to promote CIITA self-association, leading to the suggestion that GTP-mediated dimerization is necessary for nuclear localization. Taken together, these results suggest that GTP regulates export indirectly by catalyzing CIITA dimerization which results in a conformational change that masks the NES and blocks export (Fig. 6). Regulation of target sequence recognition by intermolecular masking has been demonstrated in other systems as well. It governs the cellular localization of the transcription factors NF-κB and NF-AT where binding of IkB and Ca2+, respectively, mask the NLS (41, 42). Similarly, tetramerization of the transcription factor p53 occludes its NES; conversion of p53 into a monomer or dimer exposes the NES that mediates its transfer to the cytoplasm (43). The ability of CIITA to transactivate target promoters is in large part determined by its subcellular localization. CIITA variants that are primarily cytoplasmic, due to either defective nuclear import or hyperactive nuclear export, do not transactivate. Conversely, not all variants of CIITA that shuttle to the nucleus are able to transactivate. Thus, deletion of either the AT domain (CIITA135-94) or the acidic domain (CIITA135-94) renders the molecule inactive, although still predominantly nuclear. These domains are required for function but do not play a role in subcellular localization. In contrast, the PST domain (aa 132–301) which contains two NLS elements appears to be primarily responsible for subcellular localization. Finally, the CIITA1-306 variant which contains two NLS elements localizes to the nucleus, where it functions as a dominant negative. Interestingly, it inhibits both CIITA-activated transcription and basal transcription of the class I promoter. Although CIITA nucleates the formation of an enhanceosome that activates the target promoters. Nuclear histone deacetylases (HDAC) and GTPases convert the active dimeric form of CIITA to the monomeric form, with an exposed NES; binding to CRM1 once again localizes it to the cytoplasm.

FIGURE 6. Model for regulation of subcellular distribution of CIITA. Monomeric, unmodified CIITA has an exposed and active NES determining its accumulation in the cytoplasm. In the presence of GTP, the CIITA dimerizes, which results in the masking of the NES and the dominance of NLS function. Shuttling of dimeric CIITA to the nucleus can be further enhanced by acetylation (AT) of the NLS by either PCAF or CBP (15). Once in the nucleus, CIITA nucleates the formation of an enhanceosome that activates the target promoters. Nuclear histone deacetylases (HDAC) and GTPases convert the active dimeric form of CIITA to the monomeric form, with an exposed NES; binding to CRM1 once again localizes it to the cytoplasm.
the exception of APC. Rather, its expression is induced by de novo transcription of the CIITA gene in response to IFN-γ (4, 6). Why, then, must the localization of CIITA be so tightly controlled? In APC, CIITA is constitutively expressed with a nearly equal distribution of CIITA between the cytoplasm and nucleus. This maintains steady-state levels of MHC class I and II, and also provides a reservoir of CIITA to effect a rapid increase in MHC gene expression in response to changes in the external milieu. Indeed, constitutive MHC class I expression in APCs is twice that of other cells, and its expression is even further activated in response to IFN-γ, presumably due to nuclear localization of the cytoplasmic reservoir. MHC class II expression is similarly enhanced in APCs by IFN-γ. It remains to be determined whether IFN-γ induces nuclear localization of CIITA in APCs.

Similarly, CIITA cellular localization can be used to suppress an immune response. Overexpression of either MHC class I or class II molecules is correlated with autoimmune reactions (29, 44). Thus, once cells have responded to stimulation by IFN-γ with increased expression of the MHC class I and class II, it is important to have a mechanism to rapidly terminate de novo expression. The ability to shuttle CIITA from the nucleus to the cytoplasm is an effective means to shut off transcription. Therefore, both in cells that constitutively express CIITA and in those that are induced to express it, removal of CIITA from the nucleus becomes an important control mechanism. Posttranslational modifications, such as acetylation, phosphorylation, and dissociation of dimers, provide various means to fine-tune CIITA-mediated translocation.

In conclusion, the current studies have confirmed and extended the characterization of regulation of the cellular distribution of CIITA. Importantly, we have identified a novel mechanism to regulate nuclear export of CIITA that is mediated by its GBD. We propose that the overall cellular distribution of CIITA is dynamically determined by its various translocation signals.

Acknowledgments
We thank Drs. Michael Kruhlak, Michael Kuehn, Paul Roche, and Alfred Singer for their critical reading of the manuscript, and Dr. Jenny Ting for helpful discussion. We are grateful to Dr. Tilman Brotz for valuable assistance and training in the confocal microscopy. We also acknowledge Dr. Anne Gogonea and Ms. Heather Lazzusky for helpful discussions.

References
34. Weissman, J. D., T. K. Howcroft, and D. S. Singer. 2000. TAF\textsubscript{II}250-independent transcription can be conferred on a TAF\textsubscript{II}250-dependent basal promoter by upstream activators. \textit{J. Biol. Chem.} 275:10160.


