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A Hierarchy of Nuclear Localization Signals Governs the Import of the Regulatory Factor X Complex Subunits and MHC Class II Expression

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Comprised of RFX5, RFXAP, and RFX-B/ANK, the regulatory factor X (RFX) complex is an obligate transcription factor required for the expression of MHC class II genes. RFX functions by binding to the conserved X1 box sequence located upstream of all MHC class II genes. Using a mutagenesis scheme and a yeast heterologous reporter system, the mechanism by which the RFX complex is transported into the nucleus was examined. The results have identified specific nuclear localization signals (NLS) in both RFX5 and RFXAP that direct the nuclear translocation and expression of MHC class II genes. Additionally, a nuclear export signal was identified in the N terminus of RFXAP. RFX-B was poorly localized to the nucleus, and no specific NLS was identified. Whereas RFX5 could import an RFXAP NLS mutant into the nucleus, it had no effect on the import of RFX-B. The results suggest that although RFX5 and RFXAP could assemble before nuclear import, RFX-B association with the complex does not take place until after the subunits enter the nucleus. The identification of nuclear import and export sites on RFX molecules provides potential targets to modulate MHC class II expression. The Journal of Immunology, 2004, 173: 410–419.

Major histocompatibility complex class II proteins play the important role of presenting peptide Ags to the CD4 Th cells. Recognition of MHC/peptide complexes by CD4 T cells results in T cell proliferation and activation of the regulatory function of the T cell. The importance of MHC class II expression is exemplified in the bare lymphocyte syndrome (BLS), a severe primary immunodeficiency in which patients fail to mount CD4 T cell-mediated immune responses (reviewed in Refs. 1 and 2). BLS patients cannot transcribe MHC class II genes due to mutations in one of four transcription factors required for MHC class II expression. Cell lines derived from BLS patients were used to identify, clone, and characterize the four BLS-specific MHC II transcription factors: CIITA (3), RFX5 (4), RFXAP (5), and RFX-B/ANK (6, 7).

MHC class II genes are coordinately regulated by a set of conserved, upstream elements, termed the X1, X2, and Y boxes (reviewed in Refs. 8 and 9). The three BLS factors, RFX5, RFXAP, and RFX-B, form the regulatory factor X (RFX) complex. RFX binds to the X1 box (reviewed in Refs. 1 and 8) in a cooperative manner with X2BP/CREB and NF-Y, which bind to the downstream X2 and Y boxes, respectively (10–13). The BLS factor CIITA does not bind DNA directly, but interacts with the factors bound to the X-Y box region. CIITA functions to aid in the recruitment/stability of the basal transcription machinery through its potent transcriptional activation domain (14–16). Through the recruitment of histone acetyltransferases, CIITA association with MHC class II promoters results in the opening of the local chromatin structure (17–19).

In recent years associations between the subunits of the RFX complex and CIITA have been described (20–22). RFX5 is the only member of the RFX complex with a distinct DNA binding domain, but it cannot function unless it is associated with RFX-B and RFXAP. Interactions between the subunits of the RFX complex have been mapped to the N terminus of RFX-5, the ankyrin repeats of RFX-B, and the C terminus of RFXAP. Although the interaction domains have been mapped, the question of how this transcription complex is targeted to its site of action within the nucleus has not been addressed.

Although diffusion can play a role in the nuclear localization of small molecules, the import or export of large molecules into and out of the nucleus is a regulated event that requires specific signaling sequences within the protein cargos (23). The most well-characterized nuclear targeting signal is the classical nuclear localization sequence (NLS), which mediates nuclear protein import (24). Classical NLSs can be either monopartite, a single cluster of basic amino acids, or bipartite, two clusters of basic amino acids separated by a nonconserved linker sequence of 6–12 aa (25, 26). Classical NLSs are recognized in the cytoplasm by a heterodimeric receptor composed of importin/karyopherin α and importin/karyopherin β (24). Importin α binds to and recognizes the NLS, and importin β targets the complex to the nuclear pore. Once in the nucleus, the small GTPase, RanGTP, binds to importin β and triggers dissociation of the complex and release of the NLS cargo into the nucleus (24). Export from the nucleus is also a signal-mediated...
GACCG and CTGGTGCCTCCTCCGCGTCATCAGTTGCGTC. The 5′ and 3′ primers used for these PCR were described previously (21). RXFP N-terminal deletions for the nuclear export study were made by PCR using the following forward primers: G-AFp -272; GCCGAAATCCCACCCGGCGGGCTCA; G-AP1 -272; GCCGAAATTCGCCTCTCA; G-APCTCCTGC; G-AP1 -272; GCCGAAATTCGGGCAAGTGGGGCGCAGCAACATTTCGCTTG; and G-APNH2. GAAGGAAAGATGAGAGGGGTAGA. Point mutations at RFX5/H11032 were made by overlap PCR using the primers described below and were cloned by overlap PCR using the primers described below and were cloned into the pEGFP-C2 vector. All point mutations were generated by electroporation as described previously (21). Ten micrograms of the red plasmid pRed2-C1 (BD Biosciences, San Diego, CA) was added to all transfections to gate for transfected red fluorescence microscopy (30). For export studies, COS-7 cells were treated with 20 nM LMB (Sigma-Aldrich, St. Louis, MO) 24 h after transfection, and washed and fixed 3 h later. Cells were examined using a DXM-XR microscope (Zeiss) with a ×40 objective. Image 15 μm captured using the SPOT camera and imaging system. Quantification of images from these experiments was performed using Photoshop software (Adobe Systems, Mountain View, CA) for masking nuclear and cytoplasmic compartments, Slidebook software version 4.0 (Intelligent Imaging Innovations, Baltimore, MD). To determine the percentages of GFP protein in the cytoplasmic and nuclear compartments, Slidebook software version 4.0 (Intelligent Imaging Innovations) was used. Up to 11 cells from multiple fields were masked for their nuclear or cytoplasmic compartments, as delineated by DAPI or phalloidin staining, respectively, and the percentage of GFP in these compartments was determined. Statistical analysis of the data was performed using Student’s t test with the InStat program (GraphPad, San Diego, CA).

For biochemical subcellular localization experiments, the transfection reactions were scaled up. Cells (1 × 10⁶) were plated in a six-well plate. The GFP cDNA constructions were transfected using 1–2 μg of DNA/well as described above.

All B cell lines were transfected with 40 μg of GFP-tagged constructs by electroporation as described previously (21). Ten micrograms of the red fluorescence protein-expressing plasmid pRed2-C1 (BD Biosciences, San Diego, CA) was added to all transfections to gate for transfected red cells during flow cytometry. Cells were stained using unconjugated HLA-DR (L243 monoclonal supernatant), HLA-DQ (clone SK10; BD Biosciences), and HLA-DP (B7/21; BD Biosciences) antibodies. Cells were then stained with allophycocyanin-conjugated anti-mouse Abs (Caltag Laboratories) and analyzed by flow cytometry on an FL4 channel after gating for cells in the FL2 channel (pRed2 positives) using a FACS-Calibur (BD Biosciences).

Subcellular fractionation and Western blot analysis

COS-7 cells were harvested 24 h after transfection with the GFP fusion protein constructions. One quarter of the cells were separated to prepare unfractonated cellular lysates. The remaining cells were processed for nuclear and cytoplasmic fraction separation as described by Cressman et al. (30). The cells were centrifuged and suspended in 200 μl of buffer A (containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 0.5% NP-40) and incu bed before adding 20 μl of 10% Nonidet P-40. The cells were vortexed for 20–30 s and spun for 5 min at 3000 rpm to spin down the nuclei. The cytoplasmic fraction was saved, and the nuclear pellet was washed once with buffer A containing 1% Nonidet P-40. Nuclei were lysed in 50 μl of buffer B (containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). The nuclear lysates were rocked at 4°C for 1 h. The lysate was cleared by centrifugation at 14,000.
rpm for 10 min. The whole cell lysates were prepared using Nonidet P-40 lysis buffer (50 mM Tris (pH 8), 300 mM NaCl, and 1% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSE). The cells lysed with this buffer were sonicated for 30 s and cleared by centrifugation at 14,000 rpm for 10 min. The protein concentration on all extracts was determined using the Bradford dye method (Bio-Rad, Hercules, CA), and 10 μg of lysate was separated using SDS-PAGE (9% acrylamide). SDS-PAGE gels were processed following standard Western blotting procedures as previously described (31).

The immunooblots were stained with anti-GFP Abs and HRP-coupled anti-rabbit secondary Abs (Sigma-Aldrich) and were developed using the ECL system (Amer sham, Arlington Heights, IL.). These experiments were performed three times with similar results.

Raji B cell nuclear and cytoplasmic extracts were prepared as described previously (6). Fifty micrograms of nuclear and cytoplasmic extracts were formed three times with similar results.

Following standard Western blotting procedures as previously described (31). The yeast system was assayed for nuclear import.

The yeast system for assaying nuclear import was performed as previously described with slight modifications (33). The yeast pNIA vector was used as the base vector for testing the NLSs. All NLS test constructions were made by annealing oligonucleotides that created BamHI and PstI overlaps. After digestion with the above enzymes, the fragments were cloned directly in-frame with the Gal4 activation domain within pNIA. The RFXAP NLS, RFX5 NLS1, and RFX5 NLS2 vectors were made using the following oligonucleotide pairs: GATCCAGATCTCAGACCTGGGAAAGAAGATGTACAAGGAGCAAGTCGA and GTCGACTGTTTGGCCACTTTTGTGCTTTCG; and GATCCAGATCTCAGACCTGGGAAAGAAGATGTACAAGGAGCAAGTCGA and GTCGACTGTTTGGCCACTTTTGTGCTTTCG.

The results showed that RFX5 was completely localized to the nucleus, RFXAP was evenly distributed between the nucleus and the cytoplasm, and RFX-B was mostly cytoplasmic in its localization. The GFP control, which was expressed at much higher levels than the others, was found in both the cytoplasm and nuclear compartments, although the level in the cytoplasm was slightly greater than that in the nucleus. A similar analysis was performed on B cell extracts to determine whether the localization was due to over-expression of each of the RFX proteins or the presence of the GFP tags. Nuclear and cytoplasmic extracts were prepared from Raji B cells. Western blot analysis was performed using Abs generated to GFP signal was quantitated, and the percentage of nuclear GFP was determined (Fig. 2A). The results showed that RFX5 was >85% nuclear, and that the distributions of RFXAP and RFX-B were statistically similar to that of GFP itself (38–43% nuclear).

To analyze the subcellular distribution of the RFX proteins, nuclear and cytoplasmic extracts were prepared from the transfected cells. Immunooblots were performed using anti-GFP Abs (Fig. 2B).

To determine whether the presence of G-RFXAP and G-RFX-B in the cytoplasm was due to inefficient import into the nucleus or to active export from the nucleus, cells were treated with LMB, an inhibitor of the CRM-1-dependent nuclear export system (28, 35). Treatment with LMB altered the intracellular distribution of G-RFXAP, with the majority of the G-RFXAP being nuclear (Fig. 1). These experiments suggest that RFX5 and RFXAP both contain nuclear localization signals. In addition, RFXAP contains a CRM-1-dependent export signal. The observed distribution of G-RFXAP therefore reflects an equilibrium between import and export of the protein into and out of the nucleus. RFX-B’s distribution was unaffected by LMB, suggesting that its localization is not influenced by the CRM-1/exportin-1 system.

**Results**

**RFX5 and RFXAP have NLS signals, and RFXAP has an export signal**

To initially identify NLS or NES within the RFX subunits, visual inspection of the primary sequences of the RFX subunits and a computer search program (http://psort.nibb.ac.jp) were used. The analysis predicted two classical NLSs for RFX5, one beginning at aa 144 and the other at aa 469. A single bipartite NLS was predicted for RFXAP beginning at aa 163, but no classical NLS was predicted for RFX-B. No obvious classical NESs were found in any of the RFX proteins.

To assess the roles of these predicted classical NLSs and to examine the localization of the RFX complex in vivo, RFX5, RFXAP, and RFX-B GFP fusion protein expression constructions were generated, with GFP fused to the 5′ end of each of the coding sequences. This created the three expression vectors: G-RFX5, G-RFXAP, and G-RFX-B. To examine the cellular localization of each individual fusion protein, each of the vectors was transfected into COS-7 cells. COS-7 cells were chosen over B cells because of their relatively large and distinct cytoplasmic compartment and the fact that they have very low levels of RFX subunits, allowing the targeting of each subunit to be examined independently of the other subunits. Twenty-four hours after transfection, the cells were examined by fluorescence microscopy (Fig. 1A, top row). Of the three proteins, only G-RFX5 was localized completely to the nucleus. G-RFXAP appeared to be mostly nuclear, but there was significant fluorescence in the cytoplasm. In contrast, G-RFX-B was not clearly targeted, as its distribution in the cytoplasm and nucleus was the same as that of the control GFP protein.

To determine the percentage of GFP visible in cytoplasmic and nuclear compartments for the experiments performed in Fig. 1A, additional slides were prepared with DAPI (nuclear) and phalloidin staining (cytoplasmic filamentous actin). The RFXAP experiment (with or without LMB) is shown as an example in Fig. 1B. Using these stains to highlight nuclear and cytoplasmic compartments, the GFP signal was quantitated, and the percentage of nuclear GFP was determined (Fig. 2A). The results showed that RFX5 was >85% nuclear, and that the distributions of RFXAP and RFX-B were statistically similar to that of GFP itself (38–43% nuclear).

**RFX5 has a distinct NLS at its C terminus**

To map the exact location of the NLS within RFX5, N- and C-terminal deletions of RFX5 were created in the GFP expression vector (Fig. 3A). All the deletion constructs expressed proteins of the expected size (Fig. 3B). Upon transfection into COS-7 cells, the C-terminal deletion, G-RFX5Δ600, was completely excluded from the nucleus, displaying a cytoplasmic and perinuclear distribution, suggesting that the RFX5 NLS is located C-terminal to aa 409 (Fig. 3C). This implies that the predicted NLS at aa 144 is not a functional NLS.
Like the full-length G-RFX5 construct, a GFP fusion containing aa 410–616 of RFX5 (G-RFX5 410–616), was sufficient to confer complete nuclear localization of the chimeric protein (Fig. 3C). However, a slightly smaller construction, G-RFX5 453–616, was no longer efficiently targeted to the nucleus, suggesting that additional signals are likely to reside between 410 and 452.

RFX5 has two functional NLSs

To map the RFX5 NLS site precisely, a full-length G-RFX5 construct containing a complete deletion of the computer predicted...
469 NLS (G-RFX5Δ414–476) was generated and analyzed (Fig. 3A). Surprisingly, this deletion had no effect on the nuclear localization of RFX5 (Fig. 3C). Thus, NLS-469, although capable of targeting some GFP into the nucleus, is not required for nuclear import of RFX5. This result suggested that a second nuclear targeting signal must be located within aa 410–460.

To further map the exact region of RFX5 involved in nuclear import, a series of deletions lacking the sequences between aa 410 and 487 was constructed and analyzed (Fig. 3A). All the expression vectors produced proteins of the expected size (Fig. 3B). The smallest construction, G-RFX51–427, displayed a perinuclear distribution. In contrast, G-RFX51–453, which lacks the predicted NLS beginning at aa 469, was primarily localized to the nucleus. As expected, G-RFX51–487 was completely nuclear. The sequence between aa 427 and 455, which resembles a long bipartite NLS, contains two basic sequence motifs separated by 16 aa (Fig. 3A). To determine whether these sequences functioned as an NLS, each of the basic regions was mutated individually to create G-RFX5NL51 and G-RFX5NL52 (Fig. 3A). Both mutants displayed reduced import into the nucleus as compared with wild-type RFX5 (Fig. 3C). The distribution of G-RFX5NL51 appeared to be equal between the cytoplasm and the nucleus, with no cell displaying complete nuclear localization. In ~10% of the cells containing G-RFX5NL51, the distribution was mostly cytoplasmic. In contrast, the distribution of the G-RFX5NL52 mutant was mostly nuclear, with some cytoplasmic signal. These experiments therefore suggest that the major NLS for RFX5 is located in the region defined by aa 428–455, and that the N-terminal basic amino acid cluster (aa 428–432) within this bipartite NLS plays the major role. The computer predicted NLS at aa 469 could play an additive/supplemental role, because it was sufficient to confer nuclear import of GFP in the chimeric G-RFX5453–616 protein.

**Complementation of MHC class II function by RFX5 deletions and RFX5 point mutations**

An important index of NLS activity is whether the NLS contributes to the function of the protein. RFX5 function and the role of its NLS can be assayed by rescue of MHC class II expression, the cells were stained for all three MHC class II isotypes (HLA-DR, -DQ, and -DP) and analyzed by flow cytometry (Fig. 4). G-RFX5 is fully functional and can rescue all HLA isotypes. The C-terminal deletions G-RFX51–409 and G-RFX51–427 displayed lower activity for HLA-DR, but were unable to fully rescue HLA-DQ and -DP expression. This reduction of activity is not due to a failure to interact with the other RFX subunits, because previous work demonstrated that the N-terminal 409 aa were sufficient for interactions with RFX-B and RFXAP (21). The activity of the RFX5 chimeras increased as the NLSs were included in the constructs (aa 1–455 and 1–487). The influence of the RFX5 NLS mutations, as illustrated by G-RFX5NL51, G-RFX5NL52, and G-RFX5Δ461–476, was most evident when assaying the expression of HLA-DQ and -DP. This is probably due to the fact that these promoters have a lower affinity for binding RFX than HLA-DR (11, 36). It should be noted that none of the RFX5 NLS mutations completely eliminated HLA-DR expression. This suggests that interactions between RFX5 and the other RFX subunits in SJO cells may aid in the nuclear import of RFX5. There was the concern that the overexpression of the GFP fusion proteins overloaded this system. To ascertain whether this was the case, 20-fold less G-RFX5 DNA was used in the above assays. Although fewer MHC class II-positive cells were observed under these conditions, the patterns of MHC class II expression were similar to the amounts used above (data not shown), suggesting that the expression system used did not cause the results observed.

**RFXAP has a functional bipartite NLS**

To analyze the NLS signal within RFXAP, both basic amino acid clusters within the bipartite NLS identified by computer analysis were used to determine the functional importance of RFXAP's NLS. To this end, the G-RFX5 deletion mutants were cotransfected with pDsRed2 plasmid, a red-shifted fluorescent protein that allows for gating of all transfected cells during flow cytometric analysis. The GFP tag on the RFX5 proteins was not used for gating, because the different constructs showed variable green intensities. To measure MHC class II expression, the cells were stained for all three MHC class II isotypes (HLA-DR, -DQ, and -DP) and analyzed by flow cytometry (Fig. 4). G-RFX5 is fully functional and can rescue all HLA isotypes. The C-terminal deletions G-RFX51–409 and G-RFX51–427 displayed lower activity for HLA-DR, but were unable to fully rescue HLA-DQ and -DP expression. This reduction of activity is not due to a failure to interact with the other RFX subunits, because previous work demonstrated that the N-terminal 409 aa were sufficient for interactions with RFX-B and RFXAP (21). The activity of the RFX5 chimeras increased as the NLSs were included in the constructs (aa 1–455 and 1–487). The influence of the RFX5 NLS mutations, as illustrated by G-RFX5NL51, G-RFX5NL52, and G-RFX5Δ461–476, was most evident when assaying the expression of HLA-DQ and -DP. This is probably due to the fact that these promoters have a lower affinity for binding RFX than HLA-DR (11, 36). It should be noted that none of the RFX5 NLS mutations completely eliminated HLA-DR expression. This suggests that interactions between RFX5 and the other RFX subunits in SJO cells may aid in the nuclear import of RFX5. There was the concern that the overexpression of the GFP fusion proteins overloaded this system. To ascertain whether this was the case, 20-fold less G-RFX5 DNA was used in the above assays. Although fewer MHC class II-positive cells were observed under these conditions, the patterns of MHC class II expression were similar to the amounts used above (data not shown), suggesting that the expression system used did not cause the results observed.

**FIGURE 4.** Mutation of the RFX5 NLS region reduces MHC class II expression. The indicated GFP-RFX5 fusion expression vectors described in Fig. 3 were cotransfected into the RFX5-deficient cell line SJO with the constitutive expression vector pDsRed2. The pDsRed2-expressing cells, which represent the transfected pool, were analyzed by flow cytometry. The profiles of the cells stained with Abs against HLA-DR, -DQ, or -DP are indicated by solid histograms. Open histograms represent the control transfection, in which the empty vector (pEGFP-C2) was used. The control profiles were reproduced in each horizontal set of histograms for comparison. The number in the upper left corner indicates the mean fluorescence intensity of control, transfected cells. The numbers in the upper right of each histogram indicate the mean fluorescence intensity for cells transfected with GFP-RFX5 constructs. This experiment was performed twice with similar results.
the bipartite NLS (G-APNLS2) had a more drastic effect on nuclear localization of RFXAP. Mutation of the basic residues in the C-terminal end of G-APNLS2 (Fig. 5A) caused no significant defect in nuclear localization of RFXAP. Mutation of the basic residues in the C-terminal end of the bipartite NLS (G-APNLS2) had a more drastic effect on nuclear localization of RFXAP. Quantitative analysis of the data showed a decrease from 42 to 33% in nuclear content of the G-APNLS2 mutant compared with the wild type (p = 0.0119; Fig. 5D). The difference between G-APNLS2 and wild type was only marginally significant (p = 0.0509). Importantly, LMB had no effect on the distribution of G-APNLS2 (Fig. 5D), implying that G-APNLS2 is poorly imported due to the lack of an NLS rather than to active export from the nucleus. In contrast, LMB increased the nuclear pool of G-APNLS1 from 35 to 52% (p = 0.001). Given these results, it is likely that the second set of lysine residues in the cluster is the functional import sequence for RFXAP.

**RFXAP has an export signal in its N terminus**

The experimental evidence presented in Fig. 1 suggested that RFXAP has a CRM-1-dependent nuclear export signal. To determine the location of the export signal, N- and C-terminal deletions of RFXAP were generated and cloned into the GFP expression vector. The constructs were transfected into COS-7 cells. Localization of the various fusion proteins was then examined in the absence or the presence of the export inhibitor, LMB. Even in the absence of LMB, the fusion protein lacking the first 121 aa of RFXAP, G-AP122-272, was completely nuclear, indicating that the export signal resides in the N terminus of RFXAP (Fig. 6, A and B). The deletion G-AP179-272, which lacks the NLS identified above, also appears to lack the export signal. Hence, it was not targeted specifically to the nucleus (as in the case of G-APNLS2) and was not responsive to LMB. Its even distribution could be due to diffusion, because the chimeric molecule is small. G-AP1-245 lacks the interaction domain with RFX5 and RFX-B (21), but behaves like wild-type RFXAP with regard to nuclear localization and LMB sensitivity. Taken together, these data suggest that RFXAP contains a nuclear export signal upstream of aa 122.

The N-terminal sequence of RFXAP was examined visually for the presence of classical nuclear export signals such as LXXXLXXL (27, 37), but no such sequences were found. To map the export signal further, a high resolution deletion series of the N-terminal sequences between aa 1 and 85 of RFXAP was created (Fig. 6, C and D). The micrographs suggest that the export signal is located between aa 41 and 85, as nuclear import does not increase with further deletion of the N-terminal sequences. LMB treatment of G-AP41-272, G-AP85-272, or G-AP122-272 transfectants caused no further change in nuclear localization (data not shown), indicating that the nuclear export signal was not present in these constructions. The N-terminal sequence within this region of RFXAP is highly hydrophobic, as are conventional NESs, and may form a hydrophobic pocket that is recognized by the export receptor.

**RFXAP NLS mutants affect MHC class II expression**

To test the functionality of the RFXAP NLS mutants, GFP-tagged RFXAP constructs were transfected into the RFXAP-deficient

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**FIGURE 5.** The C-terminal portion of the RFXAP bipartite NLS is required for nuclear localization. A. The sequence of the predicted NLS for RFXAP along with the locations of two series of alanine substitutions are shown. Each of the alanine substitution mutants was created in the context of the full-length RFXAP cDNA. B. Fluorescent microscopy of the wild-type and mutant GFP-RFXAP chimeric vectors. C. Lysates from the transfected COS-7 cells were examined for expression of the GFP-RFXAP constructs from this figure and Fig. 6. D. Transfections of the above constructs in cells untreated or treated with LMB were counterstained, quantitated, and plotted as described in Fig. 1B. #, p < 0.0001 for comparisons between the indicated sample and its no LMB control (by Student’s t test). *, p < 0.0001, comparison between the G-AP and G-APNLS2 samples, both treated with LMB (by Student’s t test).

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**FIGURE 6.** RFXAP contains a nuclear export signal. A and C, GFP-RFXAP deletions are schematically shown. B, GFP-RFXAP deletion constructions shown in A were transfected into COS-7 cells and cultured in the absence or the presence of LMB. The cells were examined by fluorescent microscopy. D, Fluorescent microscopy of COS-7 cells transfected with the deletion series shown in C.
FIGURE 7. NLS mutations in RFXAP show differential MHC class II expression profiles. The 6.1.6 B lymphoblastoid cell line, which is deficient for RFXAP (29), was cotransfected with the indicated GFP-RFXAP constructions (Figs. 5 and 6) and pDsRed2. Cells were stained with anti-HLA-DR, -DQ, or -DP Abs and analyzed as described in Fig. 4.

To examine the function of the RFX5 and RFXAP NLSs, the short NLSs were cloned in-frame with the modified LexA DNA binding domain and Gal4 activation domain in the yeast pNIA shuttle vector and transformed into yeast cells. The ability of the transformed yeast cells to grow on plates lacking histidine (His-) and to turn blue on X-gal plates was determined. Growth on His- plates indicates that the HIS3 reporter gene is expressed, and accumulation of the blue pigment on X-gal plates indicates that the lacZ reporter gene is expressed. Cells transformed with each of the test plasmids as well as the control plasmids were grown to saturation in liquid culture, serially diluted, and spotted onto control (Trp +), His + 3AT + Trp+, and X-gal Trp- plates. The control plate shows an equal number of cells spotted for each sample (Fig. 8B). On the His- plate, cells expressing a fusion protein that contains the wild-type or any of the test NLSs can grow. No growth was observed for the negative control plasmid (pNIA alone), which lacks an NLS. The same NLS-containing constructs turned blue on the X-gal plate. Thus, RFX5-NLS1, RFX5-NLS2, and the combined RFX5-NLS2, which includes both NLS1 and NLS2, were sufficient to transport LexA-Gal4 into nucleus and activate expression of the reporter genes. The NLS of RFXAP can also target the LexA-Gal4 fusion to the nucleus. These results suggest that each of the NLSs identified within RFX5 and RFXAP can function independently.

RFX5 and RFXAP NLSs are sufficient for nuclear transport in a heterologous system

The data indicate that the NLSs identified are necessary for nuclear import of RFX5 and RFXAP. To address whether these sequences are also sufficient to target a heterologous protein into the nucleus, the yeast NLS reporter system developed by Rhee et al. (33) was used. In this assay the reporter genes HIS3 and lacZ are integrated into the yeast genome under the control of a LexA operator (Fig. 8A). This reporter strain (L40) is then transformed with a plasmid (pNIA) that encodes the LexA DNA binding domain fused to the Gal4p activation domain. For this fusion protein to activate transcription of the reporter genes, it must be targeted to the nucleus. However, the fusion protein does not contain a nuclear targeting signal. To assess the function of a putative NLS, that sequence is cloned in-frame with the LexA/Gal4p fusion protein. If the NLS is functional in vivo, the fusion protein will be targeted to the nucleus, and the reporter genes, HIS3 and lacZ, will be expressed. In contrast, when non-NLS control sequences were used, HIS3 and lacZ were not expressed (33). Controls used for this experiment include the canonical SV40 NLS, which serves as a positive control for import into the nucleus, and the fusion protein alone lacking an NLS, which serves as a negative control.

RFX5 is the major importer of the complex, whereas RFXAP plays a secondary role

The fact that RFX5, RFXAP, and RFX-B must associate to perform their functions raises the issue of where they associate (before or after nuclear import) and how RFX-B, which does not contain a classical NLS, is targeted to the nucleus. To address these issues, the ability of an RFX subunit to transport a GFP-tagged, NLS-deficient, RFX subunit was analyzed after cotransfection into COS-7 cells. In the first series the ability of RFXAP to be brought into the nucleus by the other factors was tested (Fig. 9A). In this study G-AP(NLS2), which is deficient for nuclear targeting, was cotransfected with wild-type, His-tagged RFX5; His-RFX-B; or both expression vectors. In the presence of wild-type RFX5, most of the G-AP(NLS2) mutant protein was targeted to the nucleus (Fig. 9A). Visually, RFX-B had a minimal effect on the transport of G-AP(NLS2), and RFX5 and RFX-B together had the same effect as RFX5 itself. Quantitation of the images showed that indeed RFX5 expression resulted in increased nuclear localization, whereas RFX-B had no effect (Fig. 9C). Because it was possible that the ability to measure the nuclear accumulation of G-AP(NLS2) by RFX5 or RFX-B was inhibited by the RFXAP export signal, the
affect of LMB on these experiments was tested. However, no discernable difference was observed (data not shown), suggesting that export did not play a dominant role in these experiments. As a negative control, two previously described deletions of RFX5 were also cotransfected with G-APNL2. The RFX5 deletion His-RFX5261–616 (previously referred to as RFX5Δ2) contains the RFX5 NLS, but lacks sequences necessary for interactions with RFXAP and RFX-B, whereas His-RFX51–409 (previously referred to as RFX5Δ6) can interact with RFXAP and RFX-B (21), but is missing the NLS. Both mutant-RFX5 constructs failed to transport G-APNL2 into the nucleus.

Biochemical analysis of the subcellular localization of RFXAPNL2 after cotransfection with RFX5, RFX-B, or both was also performed as described above (Fig. 9B). The results showed that the transfection of RFX5 led to a significant increase in the amount of the RFXAP NLS mutant found in the nucleus. RFX-B by itself had a very minor effect on RFXAPNL2 import. Together, these results suggest that RFX5 can mediate the nuclear accumulation of RFXAP. Moreover, the results suggest that RFX5 and RFXAP can interact in the cytoplasm and be imported as a single complex.

To determine whether RFXAP can transport RFX5 into the nucleus, the G-RFX51–427 mutant, which lacks the NLS, was used in a similar set of experiments. Cotransfections were performed using His-RFXAP, His-RFX-B, or both. Only minor differences between the control and the cotransfections containing RFXAP were observed (Fig. 9D). The addition of RFX-B did not substantially improve RFXAP’s ability to transport an NLS-defective RFX5 into the nucleus, indicating the requirement for a functional NLS within RFX5.

To determine whether RFX5 or RFXAP mediates nuclear import of RFX-B, GFP-RFX-B was cotransfected with His-RFX5, His-RFXAP, or both. The results of this experiment showed that RFX-B was not efficiently transported into the nucleus by either subunit (Fig. 9E). Because RFX-B can interact with CIITA (20), and CIITA is in itself transported into the nucleus, the possibility that CIITA may transport RFX-B into the nucleus was tested. However, no combination of CIITA with RFX-B and the other RFX subunits resulted in the accumulation of RFX-B in the nucleus over the control (data not shown), suggesting an independent mode of transport.

Discussion
The RFX complex, comprised of RFX5, RFXAP, and RFX-B/ANK, binds the MHC class II promoter and plays an essential role in MHC class II expression. The absence of any one of the three subunits completely abrogates class II expression (reviewed in Refs. 8 and 9). In the present study the link between nuclear localization and assembly of the RFX complex was explored. Using a deletion and mutagenesis scheme, nuclear localization signals for RFX5 and RFXAP were identified and mapped. In contrast, a specific NLS in RFX-B was not identified. Whereas RFX5 was found to be a nuclear protein that was imported efficiently into the nucleus, RFXAP had the characteristics of a nuclear/cytoplasmic shuttling protein, with an LMB-sensitive nuclear export signal. The identified nuclear localization signals for both proteins can

FIGURE 8. The NLSs identified within RFX5 and RFXAP function in a yeast heterologous system. A, The pNIA NLS test vector and yeast reporter genes are schematically shown (33). Test NLSs were cloned into the multiple cloning site (MCS) indicated in the vector. The SV40 NLS used as a positive control was cloned into the site indicated by the arrowhead. R, L40 yeast cells were transformed with the indicated test constructs. Transformants were serially diluted (1/9) and spotted onto control plates lacking Trp (Trp−), Trp− and His− plates containing 3-aminotriazole (Trp− His− 3AT−), or Trp− plates containing X-gal (Trp− X-gal−).

FIGURE 9. RFX5 can import an NLS-defective RFXAP into the nucleus. Cotransfections of the indicated plasmids were performed in COS-7 cells, followed by fluorescent microscopy. A, All cotransfectants contained the G-APNL2 mutant, which does not localize to the nucleus on its own. RFX5261–616 cannot interact with RFXAP, and RFX51–409 lacks the RFX5 NLS. B, COS-7 cells cotransfected with G-APNL2 and empty vector, RFX5, RFX-B, or both were analyzed for the subcellular distribution of the GFP-RFXAP protein by immunoblot after fractionation of cellular lysates into cytoplasmic (C) and nuclear (N) fractions. C, Experiments presented in A were quantitated for the amount of GFP in the nucleus as described above. *, p < 0.011 vs control (by Student’s t test). D, All cotransfectants contain G-RFX51–427, which does not enter the nucleus. Transfection with wild-type RFXAP, but not RFX-B, displays weak nuclear localization. E, Cotransfectants with GFP-RFX-B included empty vector, RFX5, or RFXAP as indicated.
function independently, because they were sufficient to function in a heterologous yeast system. Importantly, the data showed that nuclear localization is a critical event for MHC class II expression, as mutagenesis of the nuclear localization signals in either RFX5 or RFXAP altered their trans-activation function. The results also suggest that RFX5 and RFXAP can associate before nuclear localization.

**NLS signals within RFX5 and RFXAP**

The major RFX5 NLS was found to be an atypical bipartite NLS (aa 427–455), in which the N-terminal lysines were more critical for nuclear import. Bipartite NLSs typically have a 6- to 12-residue spacer. Thus, with 16 residues separating the charged residues, the RFX5 NLS is atypical. Another NLS was found starting at aa 469 that is very similar to those observed in several other proteins. This sequence functions as an excellent NLS in the heterologous yeast system (Fig. 8) and can direct GFP protein into the nucleus. However, complete deletion of this sequence in full-length RFX5 did not affect nuclear localization. This suggests that this signal plays only a minor or an additive role, if any, in RFX5 import. It is possible that in the context of the full-length RFX5 subunit, this region is not on an exposed surface that can be recognized by the importin α NLS receptor. Another member of the RFX family, RFX-1, also contains a strong NLS in its extreme C terminus and a weak NLS in its DNA binding domain, which may play an additive role (38). However, the extreme C terminus of RFX-1 has no homology to other RFX proteins, including RFX5.

RFXAP contains a single NLS, which was sufficient for its transport into the nucleus. Previously, Peretti et al. (36) had suggested that mutations of the lysines (to asparagines) within this NLS did not affect RFXAP activity, leading to the conclusion that this region was not important for RFXAP function. It is likely that the mutations used in that study were less severe than those used in this study. In this study alanine substitution of three of the four lysines in the C-terminal portion of the bipartite NLS resulted in reduced RFXAP nuclear localization. Additionally, the activity of the mutant (G-APNLS2) was significantly reduced when analyzed for HLA-DP and -DQ complementation in 6.1.6 cells.

**RFXAP has an export signal**

Although the relative levels of the RFX proteins may vary between cell types, they are thought to be constitutively transcribed (32). However, their regulation at the post-translational level or through changes in intracellular localization has not been studied. The presence of an LMB-sensitive export signal on the N terminus of RFXAP suggests one possible means of RFX regulation or modulation. The export signal of RFXAP does not conform to other export signals described to date, with no obvious homology to motifs such as LXXXLXXLXX (27). RFXAP’s export signal is, however, located in a very hydrophobic region of the protein. It is possible that the RFXAP export signal is novel and represents a new class of nuclear export signals. Alternatively, this region may bind to another protein with a CRM-1-dependent export signal. The N terminus of RFXAP did not function effectively as an export signal in the heterologous yeast system or when fused to the RFX5 import signal (data not shown). Therefore, the export signal in RFXAP is either weak or may function only in the context of the entire RFXAP protein.

**Consequence of nuclear localization on RFX function**

Many of the studies describing nuclear localization signals of proteins are in COS-7 cells, in which the protein can be overexpressed and studied independently from other factors. However, it was important to determine the roles of the identified NLS in B cells where these proteins normally function. The BLS-derived cell line SJO (RFX5 deficient) and the in vitro BLS-like mutant cell line 6.1.6 (RFXAP deficient) provided the opportunity to study the roles of the RFX5 and RFXAP NLSs in B lymphocytes, respectively. Complete deletion of the entire NLS in RFX5 significantly reduced HLA-DQ and HLA-DP expression, whereas the effect on HLA-DR expression was less pronounced. None of the NLS mutants completely abolished HLA-DR expression. One explanation for this is that all the RFX5 NLS mutants contained domains for interaction with RFXAP, RFX-B, and CIITA. Such interactions could allow nuclear transport of small amounts of RFX5 into the nucleus. Because the MHC class II promoters display differential binding of the RFX complex (11, 36) it is likely that sufficient RFX was present to activate the DR promoters, but not those for the DQ or DP genes.

A significant difference in activity between G-RFXS1–499 and G-RFXS1–427 was observed, although both lack a functional NLS. This could be due to weaker association with CIITA in the G-RFXS1–499 deletion as the interacting region of RFX5 with CIITA is just proximal to this deletion (21). All RFX5 NLS point mutants showed reduced HLA-DQ and HLA-DP expression, suggesting the additive roles of the various NLSs, including the NLS beginning at aa 469. Point mutations within the RFXAP NLS also showed a greater effect on HLA-DQ and HLA-DP expression than they did on HLA-DR expression. As with RFX5 NLS mutations, wild-type RFX5 present in the RFXAP-deficient cell lines could transport some of the RFXAP-NLS mutant protein into the nucleus. Limiting amounts of this factor would be predicted to function at the stronger HLA-DR promoters over the weaker HLA-DQ or -DP gene promoters.

**RFX5 and RFXAP form a complex before translocation**

An interesting aspect of how multicomponent transcription factors and transcription factor complexes function is whether they assemble before or after nuclear import. In the RFX system examined in this study, a hierarchy in nuclear transport efficiency was observed among the subunits (Fig. 10). RFX5 had a strong NLS, as it transported and retained an RFXAP-NLS mutant protein in the nucleus. Similarly, RFXAP played a secondary role in the transport, as it was able to transport a minor portion of an RFX5 NLS mutant into the nucleus. Thus, at least these two subunits of the RFX complex can interact before nuclear import. However, RFX-B transport appears to be separated from these events, as...
neither RFX5 nor RFXAP affected its nuclear import. Although the possibility existed that the GFP-RFX-B fusion protein was not functional, this was ruled out by the ability of the fusion protein to complement an RFX-B-deficient B cell line (data not shown).

How, then, does RFX-B enter the nucleus? One possibility was through binding to CIITA. Experiments to prove this point failed, suggesting that although RFX-B and CIITA associate (20), this association does not take place in the cytoplasm. RFX-B contains several interacting surfaces, including four ankyrin repeats, any of which may function to piggyback RFX-B onto another molecule. Thus, it is possible that RFX-B enters the nucleus in association with another protein that contains an NLS. Likewise, the localization of RFX-B may consist of an export process as well; however, this process would be independent of CRM-1, because RFX-B’s localization was not affected by LMB in our study. Further studies are required to characterize RFX-B translocation across the nuclear membrane.

The presence of import and export signals in RFXAP suggests an interesting possibility for regulation of the RFX complex. As RFXAP and RFX5 form a complex, these signals provide a mechanism for differential regulation of HLA isotypes. Microorganisms such as Chlamydia have been recently shown to target RFX5 for degradation, suggesting an important mode of down-regulating MHC class II expression (39, 40). Likewise, cytokines such as TGF-β and IL-10 that down-regulate MHC class II expression (9) may use the RFX complex as a target. Modulation of the nuclear-cytoplasmic shuttling of these factors provides an instant mechanism for effectors to down-regulate class II expression.

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