4-2004

# Nuclear Export of the Oncoprotein v-ErbA Is Mediated by Acquisition of a Viral Nuclear Export Sequence

Laura J. DeLong

Ghislain M.C. Bonamy

Erin N. Fink

Lizabeth A. Allison
*William & Mary*, laalli@wm.edu



## Recommended Citation

DeLong, Laura J.; Bonamy, Ghislain M.C.; Fink, Erin N.; and Allison, Lizabeth A., Nuclear Export of the Oncoprotein v-ErbA Is Mediated by Acquisition of a Viral Nuclear Export Sequence (2004). *Journal of Biological Chemistry*, 279(15), 15356-15367.
https://doi.org/10.1074/jbc.M308214200

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 15, Issue of April 9, pp. 15356–15367, 2004
Printed in U.S.A.


# Nuclear Export of the Oncoprotein v-ErbA Is Mediated by Acquisition of a Viral Nuclear Export Sequence*

Received for publication, July 28, 2003, and in revised form, January 15, 2004
Published, JBC Papers in Press, January 16, 2004, DOI 10.1074/jbc.M308214200

**Laura J. DeLong‡§, Ghislain M. C. Bonamy‡§¶‖, Erin N. Fink‡, and Lizabeth A. Allison‡****

*From the ‡Department of Biology, College of William and Mary, Williamsburg, Virginia 23187 and ¶Universite Paris 7-Denis-Diderot, Paris Cedex 05, France 75251*

**v-ErbA, an oncogenic derivative of the thyroid hormone receptor α (TRα) carried by the avian erythroblastosis virus, contains several alterations including fusion of a portion of avian erythroblastosis virus Gag to its N terminus, N- and C-terminal deletions, and 13 amino acid substitutions. Nuclear export of v-ErbA occurs through a CRM1-mediated pathway. In contrast, nuclear export of TRα and another isoform, TRβ, is CRM1-independent. To determine which amino acid changes in v-ErbA confer CRM1-dependent nuclear export, we expressed a panel of green and yellow fluorescent protein-tagged mutant and chimeric proteins in mammalian cells. The sensitivity of subcellular trafficking of these mutants to leptomycin B (LMB), a specific inhibitor of CRM1, was assessed by fluorescence microscopy. Our data showed that a nuclear export sequence resides within a 70-amino acid domain in the C-terminal portion of the p10 region of Gag, and *in vitro* binding assays demonstrated that Gag interacts directly with CRM1. However, a panel of ligand-binding domain mutants of v-ErbA lacking the Gag sequence exhibited greater nuclear localization in the presence of LMB, suggesting that the various amino acid substitutions/deletions may cause a conformation shift, unmasking an additional CRM1-dependent nuclear export sequence. In contrast, the altered DNA-binding domain of the oncoprotein did not contribute to CRM1-dependent nuclear export. Heterokaryon experiments revealed that v-ErbA did not undergo nucleocytoplasmic shuttling when the CRM1 export pathway was blocked by LMB treatment, suggesting that the ability to follow the export pathway used by TRα has been lost by the oncoprotein during its evolution. Our findings thus point to the intriguing possibility that acquisition of altered nuclear export capabilities contributes to the oncogenic properties of v-ErbA.**

The v-*erbA* oncogene is transduced by the avian erythroblastosis virus (AEV)[1] from the cellular c-*erbA* protooncogene encoding the thyroid hormone receptor α (TRα), a member of the nuclear receptor superfamily (1). During the course of oncogene activation, v-*erbA* was fused to an AEV *gag* sequence at its N terminus. In addition, v-*erbA* acquired two small deletions in the N and C termini and 13 amino acid substitutions interspersed throughout the DNA-binding domain, hinge region, and ligand-binding domain (2, 3). Studies involving mutant forms of the oncoprotein v-ErbA (also referred to as $p75^{gag\text{-}v\text{-}erbA}$) have illustrated the importance of these sequence alterations for its oncogenic properties (4–12). Although v-ErbA has retained the capacity to bind corepressors (13, 14), it has lost hormone binding and transactivation activity and has altered DNA binding specificity (3, 15–22).

TRα exhibits a dual role as an activator or repressor of gene transcription in response to thyroid hormone. In mammalian and avian cancer cells v-ErbA acts, in part, as a transcriptional repressor of TRα. However, a number of reports have argued against a simple dominant negative function for the oncoprotein (23, 24). Instead it has been proposed that v-ErbA contributes to tumor formation by interfering with the action of both liganded and unliganded TRα (25). Most likely, v-ErbA interferes with TRα-mediated transcription through multiple pathways, including a direct effect on TRα activity (7, 24, 26, 27), dysregulation of the expression of other target genes by inappropriate activation of the transcription factor AP-1 (28–30), and repression of transcriptional activity of the receptors for estrogen and retinoic acid (7, 20).

We are interested in understanding the molecular basis behind the oncogenic conversion of TRα into v-ErbA and the mode of action of dominant negative transcription factors in general. The exact mechanism for transcriptional repression by v-ErbA has not yet been determined, but evidence points to competition for DNA binding sites and cofactors and formation of inactive heterodimers (19). One relatively unexplored mode of oncogenic action is the effect of altered subcellular localization. Regulated nuclear localization of transcription factors can act as a molecular switch to control transcription (31–33). Thus, knowledge of the mechanisms regulating nuclear transport of v-ErbA, in particular its nuclear export, may provide a deeper understanding of its oncogenic activity.

v-ErbA exhibits differential subcellular localization and nuclear export from its cellular homolog TRα. While TRα shuttles rapidly between the nucleus and cytoplasm but is primarily

* This work was supported in part by National Institutes of Health Grant DK058028-01A1, National Science Foundation Grant MCB0090923, Jeffress Memorial Trust Grant J-477, and the Horsley Cancer Research Fund (to L. A. A.); by a Howard Hughes Medical Institute grant through the Undergraduate Biological Sciences Education Program to the College of William and Mary; and by an Endocrine Society Maurice Raben Summer Research Fellowship (to L. J. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

‖ This work is part of the Ph.D. dissertation research under the supervision of A. Guichon Mantel and L. A. Allison.

** To whom correspondence should be addressed: Dept. of Biology, College of William and Mary, P. O. Box 8795, Millington Hall 116, Williamsburg, VA 23187-8795. Tel.: 757-221-2232; Fax: 757-221-6583; E-mail: laalli@wm.edu.

[1] The abbreviations used are: AEV, avian erythroblastosis virus; aa, amino acid(s); TRα, thyroid hormone receptor α; TRβ, thyroid hormone receptor β; LMB, leptomycin B; NES, nuclear export sequence; RSV, Rous sarcoma virus; GFP, green fluorescent protein; YFP, yellow fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione *S*-transferase; N, nuclear; C, cytoplasmic.

localized to the nucleus, v-ErbA accumulates in both nuclear and cytoplasmic compartments in the cell. Leptomycin B (LMB), a specific inhibitor of the export receptor CRM1 (34), blocks nuclear export of v-ErbA; LMB treatment results in complete nuclear localization of v-ErbA, indicating that v-ErbA follows a CRM1-dependent nuclear export pathway (35). In contrast, nucleocytoplasmic shuttling of TR$\alpha$ is not blocked by LMB, indicating that TR$\alpha$ exits the nucleus by a CRM1-independent export pathway (35).

To determine which amino acid changes in v-ErbA confer CRM1-dependent nuclear export, we tested a panel of v-ErbA mutants and chimeric proteins. Using transient transfection assays, their subcellular trafficking was assessed for sensitivity to LMB. Our data showed that the Gag region of v-ErbA interacts directly with CRM1 and mediates v-ErbA nuclear export. More specifically, we showed that the nuclear export sequence (NES) resides within a 70-amino acid domain in the C-terminal portion of the p10 region of Gag. In contrast, the altered DNA-binding domain of the oncoprotein did not contribute to CRM1-dependent nuclear export, whereas data suggested that the ligand-binding domain of v-ErbA may play some role in mediating CRM1-dependent nuclear export. When the CRM1 export pathway was blocked by LMB treatment, nucleocytoplasmic shuttling of v-ErbA was inhibited, suggesting that the ability to follow the export pathway used by TR$\alpha$ has been lost by the oncoprotein during its evolution. Taken together, our findings point to the intriguing possibility that acquisition of altered nuclear export capabilities contributes to the oncogenic properties of v-ErbA.

## EXPERIMENTAL PROCEDURES

*Plasmids*—T7-V3 contains the coding region for a fusion protein termed Gag-TR$\alpha$ comprised of TR$\alpha$ with the Gag sequence from v-ErbA fused to amino acid 13 of TR$\alpha$, thereby deleting the first 12 amino acids of TR$\alpha$ (22). pGEX-KG-v/c/v encodes v/c/v, a chimeric protein in which the DNA-binding domain of v-ErbA was replaced by the DNA-binding domain of TR$\alpha$ (21). In pRS-$\Delta$Gag-v-ErbA the viral Gag sequence is deleted, but it still possesses the N- and C-terminal deletions and the 13 point mutations that distinguish v-ErbA from TR$\alpha$ (36). The $\Delta$Gag-v-ErbA mutants ($\Delta$H10, $\Delta$H11A, $\Delta$H11B, L353R, $\Delta$9heptad, $\Delta$20aa, L360R, and D232A) have substitutions or deletions in regions thought to be important in dimerization (36). For the construction of expression vectors for the viral Gag domain alone, the *gag* sequence was isolated from the 5′ end of v-*erbA* through PCR amplification of that region from a GFP-v-ErbA expression vector (35). *Gag* and v-*erbA* mutant coding sequences were subcloned into pEGFP-C1 (Clontech). In addition, the *gag* sequence from v-*erbA* was subcloned into pGEM-4Z (Promega, Madison, WI) for *in vitro* translation assays. Expression vectors for p53-GFP and GFP-TR$\alpha$ and for *in vitro* translation of TR$\alpha$ and v-ErbA were as described previously (35, 37). GFP-TR$\beta$ was constructed by subcloning the human TR$\beta$ cDNA from RSh-TR$\beta$ (38) into pEGFP-C1. pET-His-CRM1-H, pGST-Ranwt, and pGST-RanQ69L were used for bacterial overexpression of His-tagged full-length human CRM1 (39), GST-tagged wild-type Ran and mutant RanQ69L (35), respectively.

To construct YFP-tagged Gag-(1–70) and Gag NES, coding sequences for the first 70 amino acids of Gag and the putative CRM1-dependent NES in the C terminus of Gag (aa 174–244), plus the addition of a stop codon, were subcloned into pEYFP-C1 (Clontech) between the HindIII and EcoRI restriction sites by PCR amplification of these regions from GFP-v-ErbA. To construct YFP-RSVp10, the Rous sarcoma virus (RSV) p10 sequence was synthesized by hybridizing two long synthetic oligonucleotides with a 25-bp overlap, the appropriate restriction sites, and a stop codon and then completing the fragment by PCR. The PCR product was subsequently purified from a 12% denaturing polyacrylamide gel, PCR-amplified, and subcloned into pEYFP-C1 between the HindIII and EcoRI restriction sites. YFP-Gag$\Delta$CRM1 and YFP-v-ErbA$\Delta$CRM1 were constructed as follows. The v-ErbA$\Delta$CRM1 fragment was made by ligating PCR fragments coding for aa 1–177 and aa 239–640 obtained from expression vectors for GFP-Gag and GFP-v-ErbA, respectively, joined together at a KpnI restriction site. This ligation product was subsequently PCR-amplified into v-ErbA$\Delta$NES and Gag$\Delta$NES with the appropriate primers and then subcloned into pEYFP-C1 between the HindIII and EcoRI restriction sites. The identity of all constructs was confirmed by sequencing with an ABI 3100 Avant Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA).

*Transfection and LMB Treatment*—Transient transfection assays were performed exactly as described previously (35). NIH/3T3 cells were treated with either 10 ng/ml LMB (either from M. Yoshida, University of Tokyo, Tokyo, Japan or from Sigma) or the same volume of absolute EtOH or MeOH (vehicle control). In some trials, EtOH or MeOH was omitted since we have observed no difference in the distribution of GFP-tagged receptors between EtOH- or MeOH-treated and untreated controls. After a 5-h incubation, cells were fixed as described previously (35) and mounted either in Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA) or in Vectashield after staining the DNA with 1 $\mu$M TO-PRO-3 (Molecular Probes, Eugene, OR). Nucleocytoplasmic distribution was analyzed using an Olympus epifluorescence microscope and a Cooke SensiCam high performance digital black and white camera or a Radiance 2100 laser scanning microscope system (Bio-Rad) mounted on a Nikon Eclipse TE300 inverted microscope. Subsequently digital images were pseudocolored using IPLab scientific imaging software (Scanalytics, Fairfax, VA).

*Analysis of Nucleocytoplasmic Distribution*—Transfection experiments were carried out three times for each construct with ~100 cells analyzed per trial. Scoring of cells was performed blindly without prior knowledge of LMB treatment. Cells were categorized into four groups based on qualitative assessment of subcellular distribution: whole cell or cytoplasmic distribution (N $\leq$ C), weak nuclear localization (N > C), strong nuclear localization (N $\gg$ C), and complete nuclear localization (all N). Log-linear analysis was used to determine the statistical significance of differences in subcellular distribution. Since the cell counts for the deletion/substitution v-ErbA mutants contained multiple zeros in classification categories, these data were compressed to two categories: whole cell distribution and nuclear localization (including weak, strong, and complete nuclear localization). Whenever possible, analysis was carried out using both the four-category and two-category schemes, and the results were compared.

*Heterokaryon Assays*—Heterokaryon assays were performed exactly as described previously (35). NIH/3T3 cells were incubated in 10 ng/ml LMB (or an equivalent volume of EtOH or MeOH as a control) in culture medium containing 100 $\mu$g/ml cycloheximide for 2.5 h before fusion with HeLa cells. Following cell fusion, the cells were incubated in LMB (or an equivalent volume of EtOH or MeOH) for 5–10 h at 37 °C. Fixation and staining for DNA and actin were carried out as described previously (35). For heterokaryon analysis, shuttling was indicated by the presence of GFP-tagged protein in both the transfected NIH/3T3 and untransfected HeLa nuclei.

*Pull-down Assays*—Direct interaction between CRM1 and either the Gag domain from v-ErbA, v-ErbA, or TR$\alpha$ was examined by His pull-down assays. The His-CRM1 fusion protein was expressed in *Escherichia coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene, La Jolla, CA). After induction with 0.5 M isopropyl-1-thio-$\beta$-D-galactopyranoside at 30 °C, bacterial cells were harvested and sonicated in B-PER® bacterial protein extraction reagent (Pierce) supplemented with 500 $\mu$g/ml lysozyme. His-CRM1 was purified using TALON™ metal affinity resin (Clontech) according to the manufacturer's instructions. The presence of His-CRM1 in eluted samples was confirmed by 8% SDS-PAGE and staining with Simply Blue™ SafeStain (Invitrogen). Radiolabeled Gag, v-ErbA, and TR$\alpha$ were translated *in vitro* using the TNT-coupled transcription/translation system (Promega) in the presence of [$^{35}$S]methionine (Amersham Biosciences) and T7, SP6, or T3 RNA polymerase, respectively. Recombinant wild-type Ran and RanQ69L were expressed in *E. coli* BL21 cells as GST fusion proteins. After extraction in B-PER reagent, proteins were purified on glutathione-Sepharose 4B beads (Amersham Biosciences) and charged with 20 $\mu$M GTP as described previously (35). Pull-down assays were carried out in the presence of 20 $\mu$M GTP and 140 ng of Ran or RanQ69L using the Profound™ pull-down polyhistidine assay (Pierce) according to the manufacturer's instructions. Samples were analyzed by 10% SDS-PAGE and fluorography as described previously (35). The presence of Ran in the rabbit reticulocyte lysate from the TNT-coupled transcription/translation system was determined by Western blot analysis and chemiluminescence detection (ECL, Amersham Biosciences) (35). The blot of lysate samples was probed with anti-Ran (goat polyclonal antibody, Santa Cruz Biotechnology, Inc.) at 1:100 and horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Inc.) at 1:10,000.

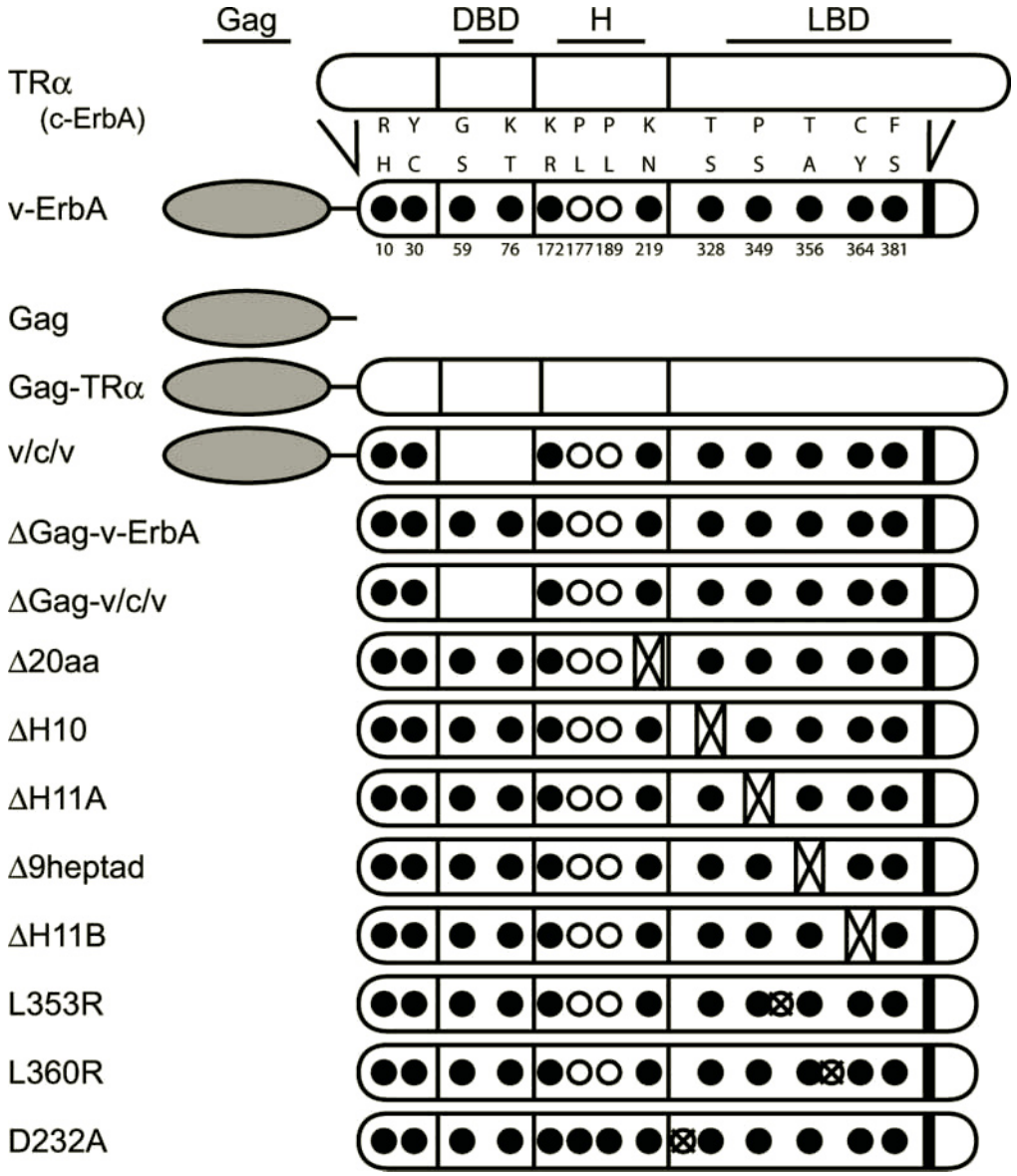


FIG. 1. **Sequence differences between TRα, v-ErbA, and mutants.** The diagram depicts the major domains of TRα and variants consisting of the truncated AEV Gag sequence, the N terminus, DNA-binding domain (*DBD*), hinge region (*H*), ligand-binding domain (*LBD*), and C terminus. Amino acid substitutions in v-ErbA are indicated by *residue number* and standard amino acid abbreviation. Substitution of leucines are indicated by *open circles*, while all other substitutions are indicated by *filled black circles*. For the synthetic v-ErbA mutants, deletions are indicated by *crossed rectangles*, and substitutions are indicated by *crossed circles*. Δ*20aa*, deletion of aa 218–237; Δ*H10*, deletion of helix 10 (aa 322–342); Δ*H11A*, deletion of the region of helix 11 proximal to the ninth heptad (aa 346–352); Δ*9heptad*, deletion of the ninth heptad in helix 11 (aa 353–360); Δ*H11B*, deletion of the region of helix 11 distal to the ninth heptad (aa 361–366); *L353R*, substitution of arginine by leucine at aa 353 in helix 11; *L360R*, substitution of leucine by arginine at aa 360 in helix 11; *D232A*; substitution of aspartic acid by alanine at aa 232.

## RESULTS

*Nuclear Export of the Gag Domain of v-ErbA Is Mediated by CRM1*—During the course of oncoprotein activation, v-ErbA was fused to a portion of AEV Gag, acquired 13 interspersed amino acid substitutions, and obtained small deletions in the N and C termini (2, 3) (Fig. 1). Our prior studies have shown that while TRα exits the nucleus by a CRM1-independent pathway, the nuclear export of v-ErbA is CRM1-dependent (35). Thus, we sought to ascertain which sequence differences between v-ErbA and TRα conferred CRM1-dependent nuclear export.

The short residence time of TRα in the cytoplasm prohibits study of nuclear export by conventional single cell analysis, but in interspecies heterokaryon assays, the human nuclei act as a trap for any TRα that appears, however transiently, in the shared cytoplasm after export from mouse nuclei. Fig. 2*A* illustrates CRM1-independent nuclear export of TRα in a heterokaryon system (*panels a–d*) and extends our earlier findings to another isoform of the thyroid hormone receptor, TRβ (*panels e–h*). Previously we have demonstrated that N-terminal fusion with GFP is a valid probe for studying subcellular trafficking of TRα and variants (35). NIH/3T3 (mouse) cells expressing GFP-tagged TRα or TRβ were fused with nonexpressing HeLa (human) cells both in the presence and absence of LMB. In both cases, TRα and TRβ translocated from the mouse nucleus to the human nucleus, indicating that their nuclear export is CRM1-independent. To ensure that the LMB used in our assays was chemically active, we tested the effect of LMB on the shuttling ability of p53, a transcription factor that follows a CRM1-mediated export pathway (40). As expected, nuclear export of p53 was sensitive to LMB; in the presence of the drug, p53 remained localized to the mouse nuclei (Fig. 2*A*, *panels k* and *l*), whereas in the absence of LMB, p53-GFP accumulated in the human nuclei of the heterokaryons (*panels i* and *j*).

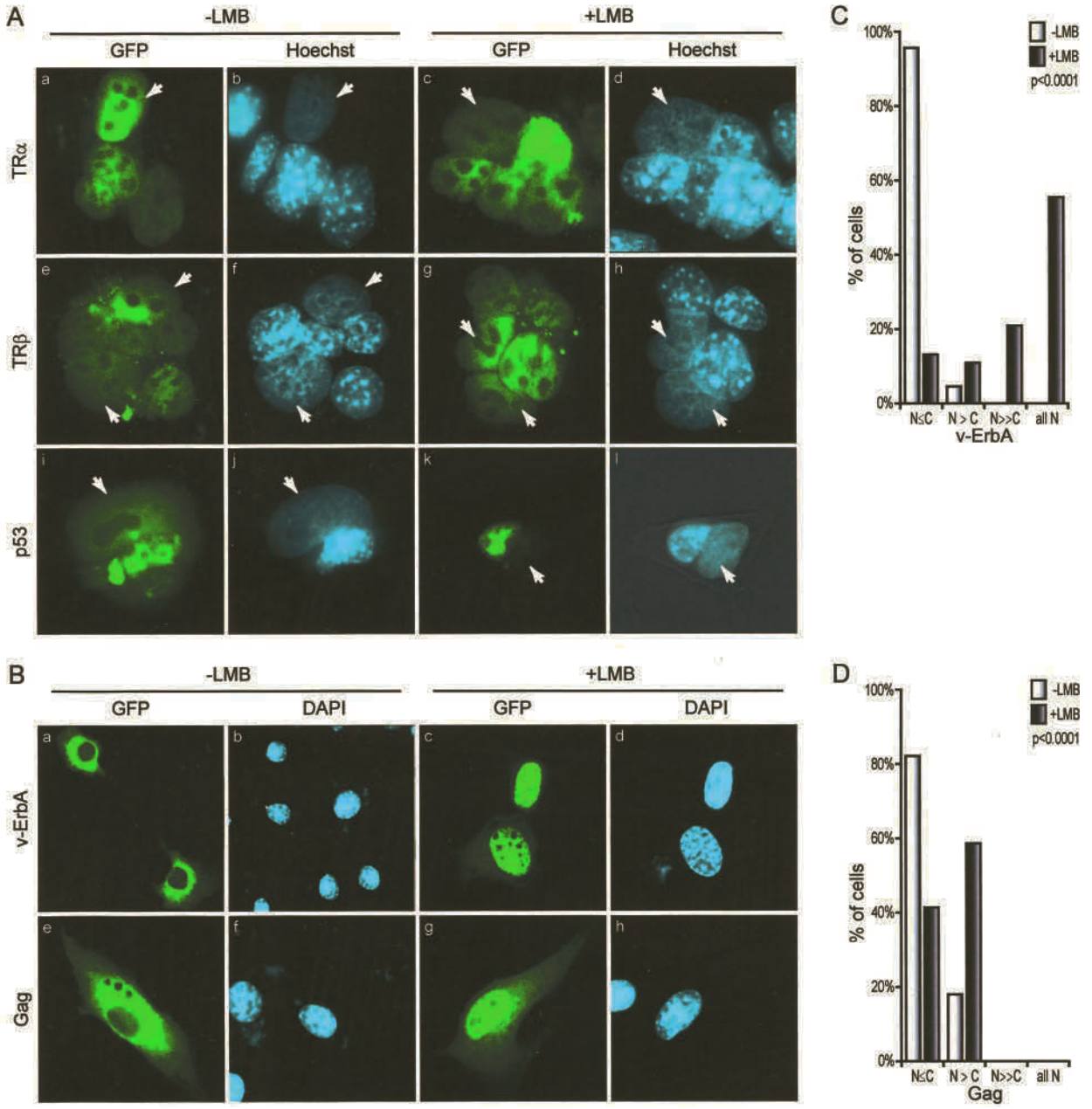


FIG. 2. **Nuclear export of the Gag domain of v-ErbA is mediated by CRM1.** *A*, nucleocytoplasmic shuttling of TR$\alpha$, TR$\beta$, and p53. For the preparation of heterokaryons, NIH/3T3 (mouse) cells were transfected with expression vectors for GFP-tagged TR$\alpha$, TR$\beta$, or p53 and incubated in the presence or absence of LMB as indicated. Subsequently HeLa (human) cells were fused with the mouse cells to form heterokaryons in which human and mouse nuclei share a common cytoplasm. Fused cells were incubated for 5 h in culture medium in the presence or absence of LMB. Shuttling of GFP-tagged proteins was visualized by epifluorescence microscopy (*panels a*, *c*, *e*, *g*, *i*, and *k*). Human nuclei (*white arrows*) are distinguished from mouse nuclei by differential coloration with Hoechst 33258 DNA stain and to some extent by their size (*panels b*, *d*, *f*, *h*, *j*, and *l*). Approximately 10 heterokaryons were analyzed per experiment. Shuttling of TR$\alpha$ and TR$\beta$ was not blocked by LMB. In contrast, p53-GFP shuttling was inhibited. Heterokaryon formation was confirmed by Nomarsky microscopy; the Nomarsky image was merged with the Hoechst-stained image (*panel l*). *B*, subcellular distribution of v-ErbA and Gag. NIH/3T3 cells were transfected with expression vectors for GFP-tagged v-ErbA (*panels a* and *c*) and the Gag domain of v-ErbA on its own (*panels e* and *g*) as indicated. After treatment with LMB, cells were fixed, stained with the DNA stain DAPI to reveal the nucleus (*panels b*, *d*, *f*, and *h*), and visualized by epifluorescence microscopy. *C*, bar graph summarizing the effect of LMB on the subcellular distribution of v-ErbA ($n$ = 300 cells for each treatment). *D*, bar graph summarizing the effect of LMB on the subcellular distribution of Gag. *White bars*, vehicle control (*−LMB*); *black bars*, LMB treatment (*+LMB*) ($n$ = 300 cells for each treatment). $N \leq C$, whole cell or cytoplasmic distribution; $N > C$, weak nuclear localization; $N \gg C$, strong nuclear localization; *all N*, complete nuclear localization.

To ascertain which sequence differences between v-ErbA and TR$\alpha$ confer CRM1-dependent nuclear export, we first tested whether the retroviral Gag domain of v-ErbA exhibits nuclear export activity. In retroviruses, the complete Gag polyprotein directs the assembly and release of virus particles from the plasma membrane (41). Until recently, it was believed that Gag proteins were targeted directly to the plasma membrane after synthesis in the cytoplasm of the host cell; however, it has now been shown that nuclear import and export of the Gag polyprotein are part of the RSV assembly pathway (42). For brevity, in this report we use the term "Gag" to refer to the truncated Gag domain present in v-ErbA as opposed to the complete Gag polyprotein.

To characterize the subcellular localization of the AEV Gag

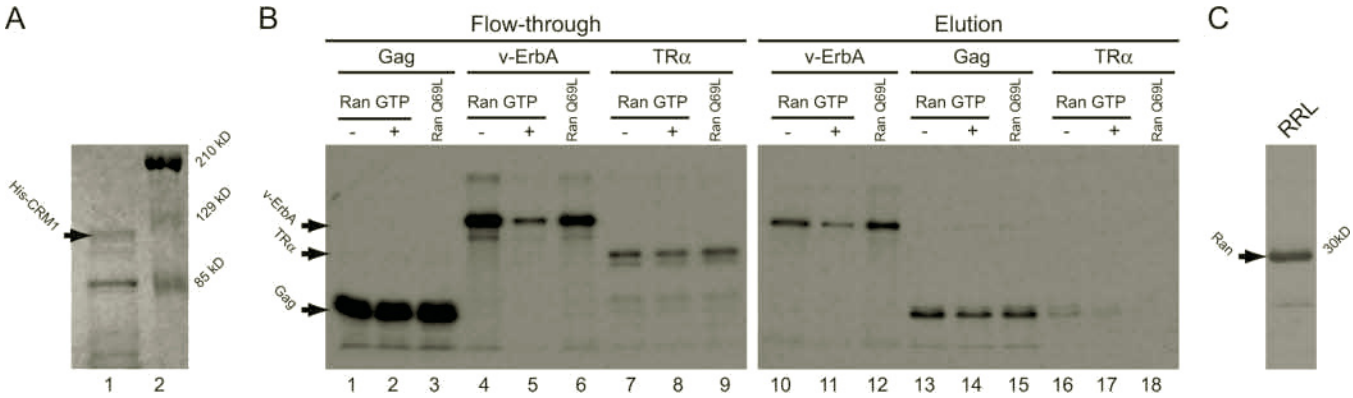


FIG. 3. **The Gag domain of v-ErbA interacts directly with CRM1 in vitro.** *A*, purification of His-tagged CRM1 after overexpression in bacteria. *Lane 1*, His-CRM1; *lane 2*, protein molecular mass standards. *B*, Gag and v-ErbA both interact with CRM1 *in vitro*. His pull-down assays were performed using His-CRM1 and *in vitro* generated $^{35}$S-labeled TR$\alpha$, v-ErbA, or Gag (in rabbit reticulocyte lysate) in the presence or absence of recombinant RanGTP or Ran Q69L as indicated. *Lanes 1–9*, flow-through fractions; *lanes 10–18*, binding fractions (elutions). *C*, Ran is present in rabbit reticulocyte lysate (*RRL*). Western blot analysis was performed on a sample of rabbit reticulocyte lysate (1 $\mu$l). Blots were probed with anti-Ran antibodies and visualized by chemiluminescence detection.

domain of v-ErbA, NIH/3T3 cells were transfected with an expression vector encoding GFP-Gag on its own. In this case, because Gag has a predominantly cytoplasmic localization, it is possible to analyze nuclear export properties by conventional single cell analysis instead of using the heterokaryon system. Cells were scored qualitatively for the nucleocytoplasmic distribution of Gag using four categories: whole cell or cytoplasmic distribution (predominantly cytoplasmic or evenly distributed throughout the cytoplasm and nucleus, N $\leq$ C), weak nuclear localization (whole cell distribution but with a distinct accumulation in the nucleus, N $>$ C), strong nuclear localization (predominantly nuclear with faint cytoplasmic fluorescence, N $\gg$ C), and complete nuclear localization (all N). Interestingly Gag alone exhibited a subcellular distribution almost indistinguishable from that of v-ErbA (Fig. 2, *B*, compare *panels a* and *e*; *C*; and *D*). The majority of cells (82%) exhibited a whole cell or cytoplasmic distribution with a greater accumulation of Gag in the cytoplasm; however, some cells (18%) showed weak nuclear localization of Gag (Fig. 2*D*).

Since some truncated AEV Gag accumulated in the nucleus, this suggested that it is capable of nuclear import either via a signal-mediated pathway or by passive diffusion through the nuclear pore complex. The predominantly cytoplasmic localization, however, also implies the ability to exit the nucleus. To determine whether Gag follows a CRM1-dependent nuclear export pathway, we tested the sensitivity of Gag to LMB. Strikingly Gag showed marked sensitivity to LMB. Treatment for 5 h with LMB resulted in a significant increase ($p < 0.0001$) in nuclear retention of Gag; only 18% of the cells showed weak nuclear localization of Gag in the absence of LMB compared with 59% in the presence of LMB (Fig. 2, *B*, *panel g*, and *D*). However, unlike v-ErbA in which 87% of the cells showed nuclear accumulation in the presence of LMB (Fig. 2, *B*, *panel c*, and *C*), Gag was not completely localized to the nucleus when CRM1 was inhibited (Fig. 2, *B*, *panel g*, and *D*). The continued presence of a cytoplasmic population of Gag (41% of cells) could indicate that nuclear import of the GFP-tagged truncated viral protein alone is less efficient than when it is fused to v-ErbA. However, after 10-h treatment with LMB, there was no further increase in the amount of Gag trapped in the nucleus (data not shown), suggesting that the apparent differential sensitivity to LMB between Gag alone and Gag fused with v-ErbA is not simply a result of altered nuclear import kinetics but rather due to changes in v-ErbA other than the Gag sequence. Alternatively, since GFP-Gag ($\approx$55 kDa) is, in principle, within the size limits (less than 60 kDa) for passive diffusion through the central channel of the nuclear pore complex (35), it is possible that in the presence of LMB Gag passively diffuses in or out of the nucleus to reach a more cytoplasmic distribution than v-ErbA at steady state. Additional possible explanations are that Gag uses the CRM1 pathway with altered affinity when not fused to v-ErbA, or there is a second NES in Gag that is not LMB-sensitive. Experiments are in progress to distinguish between these possibilities. In summary, these data reveal that the truncated Gag domain of v-ErbA can both enter and exit the cell nucleus and follows a CRM1-mediated nuclear export pathway.

*The Gag Domain of v-ErbA Interacts Directly with CRM1*—To demonstrate that the truncated AEV Gag sequence that was fused to v-ErbA during viral transduction interacts directly with CRM1, His pull-down assays were performed. A bacterially expressed His-CRM1 fusion protein (Fig. 3*A*) was incubated with either *in vitro* generated $^{35}$S-labeled truncated Gag, v-ErbA, or TR$\alpha$ (in rabbit reticulocyte lysate), and the flow-through and binding (elution) fractions were analyzed by SDS-PAGE and fluorography (Fig. 3*B*). Gag (*lanes 13–15*) and v-ErbA (*lanes 10–12*) both interacted with CRM1. In contrast, as expected, all input TR$\alpha$ was present in the flow-through fraction (*lanes 7–9*); no TR$\alpha$ bound specifically to CRM1 (*lanes 16–18*). Even when a greater amount of TR$\alpha$ was included in the pull-down assay, there was still no TR$\alpha$ detectable in the bound fraction (data not shown). Rabbit reticulocyte lysate is routinely used as the source of cytosol to support Ran-dependent *in vitro* nuclear transport assays, thus implying the presence of a sufficient supply of Ran in the lysate for specific interaction of CRM1 with cargo. The presence of Ran in the rabbit reticulocyte lysate was confirmed by Western blot analysis (Fig. 3*C*). Not surprisingly, the addition of exogenous wild-type Ran charged with GTP or mutant RanQ69L, which binds GTP but resists hydrolysis, had no effect on the interaction of Gag and v-ErbA with CRM1.

*Fusion of Gag with TR$\alpha$ Confers Partial CRM1-dependent Nuclear Export*—To further characterize the effect of the truncated AEV Gag domain on the subcellular localization and transport of v-ErbA, NIH/3T3 cells were transfected with a GFP-tagged chimeric fusion protein of TR$\alpha$ and the Gag sequence from v-ErbA, termed Gag-TR$\alpha$ (Fig. 1). Cells were scored for nucleocytoplasmic distribution according to the four categories described earlier. Interestingly Gag-TR$\alpha$ exhibited a subcellular localization distinct from that of both TR$\alpha$ (Fig. 4*A*) and v-ErbA (Fig. 2*B*). Unlike TR$\alpha$, Gag-TR$\alpha$ was not completely localized to the nucleus; 49% of cells exhibited a whole cell

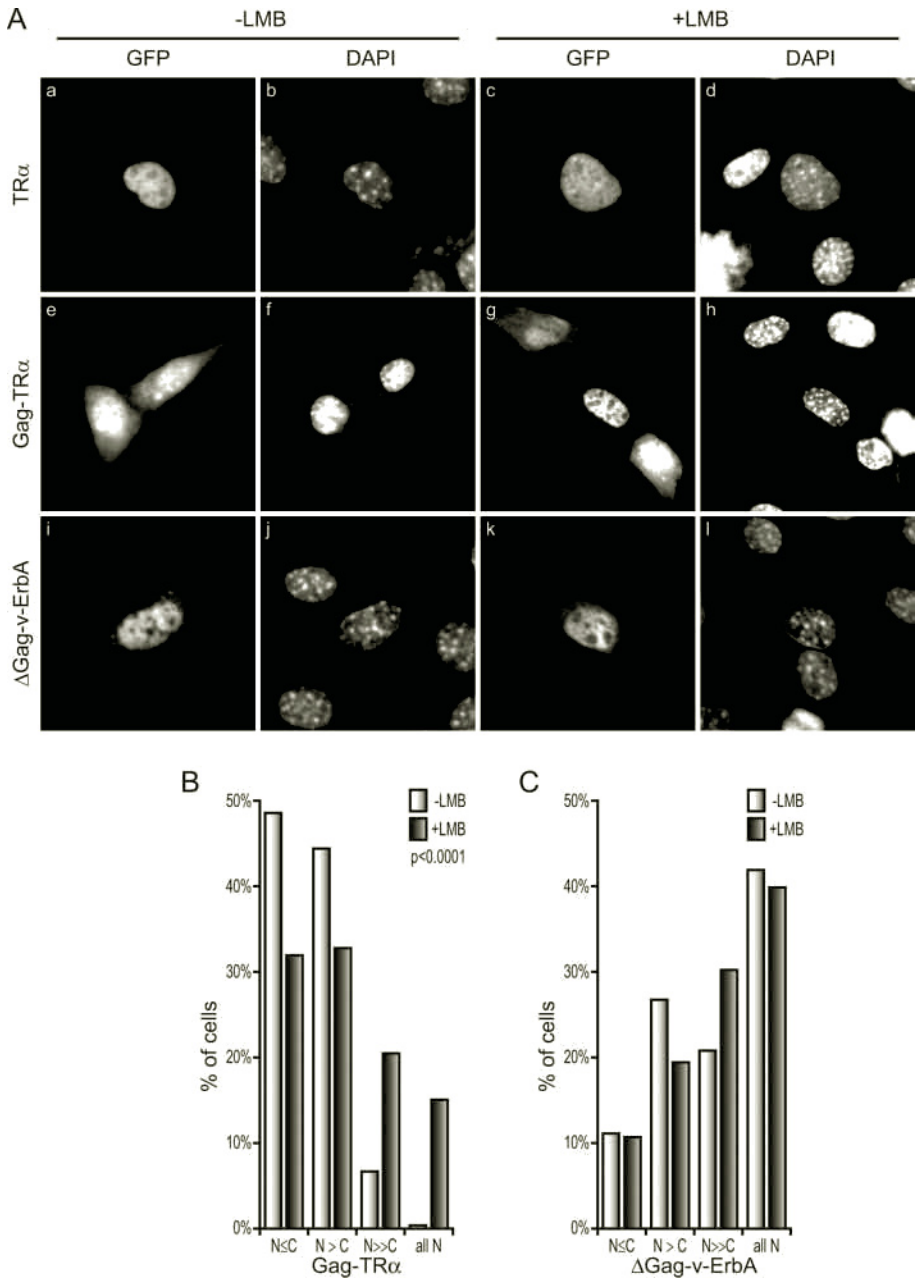


FIG. 4. **Fusion of Gag with TRα confers partial CRM1-dependent nuclear export on the receptor, while the nuclear export of ΔGag-v-ErbA is not sensitive to LMB.** *A*, the subcellular distribution of TRα, Gag-TRα, and ΔGag-v-ErbA. NIH/3T3 cells were transfected with expression vectors for GFP-tagged TRα (*panels a–d*) Gag-TRα (*panels e–h*), or ΔGag-v-ErbA (*panels i* and *j*) as indicated. After treatment with LMB, cells were fixed, stained with DAPI to reveal the nucleus (*panels b*, *d*, *f*, *h*, *j*, and *l*), and visualized by epifluorescence microscopy. *B*, bar graph summarizing the effect of LMB on the subcellular distribution of Gag-TRα. *C*, bar graph summarizing the effect of LMB on the subcellular distribution of ΔGag-v-ErbA. *White bars*, vehicle control (*−LMB*); *black bars*, LMB treatment (*+LMB*) ($n = 300$ cells for each treatment). $N \leq C$, whole cell or cytoplasmic distribution; $N > C$, weak nuclear localization; $N \gg C$, strong nuclear localization; *all N*, complete nuclear localization.

distribution (Fig. 4*A*, compare *panel a* with *panel e*). Further, in contrast to v-ErbA, Gag-TRα did not exhibit a predominantly whole cell or cytoplasmic distribution since 51% of cells expressing Gag-TRα showed weak to complete nuclear localization (compare Fig. 2*B*, *panel a*, with Fig. 4*A*, *panel e*). These hybrid characteristics of Gag-TRα correlate well with previously identified functional properties of the chimeric protein. Liganded Gag-TRα and TRα can both overcome v-ErbA repression of the erythroid carbonic anhydrase II gene in erythroid progenitors and enhance transcription (43), but Gag-TRα subsequently causes aberrant differentiation (4).

To determine whether the truncated Gag sequence alone is sufficient to mediate CRM1-dependent nuclear export of a chimeric protein, we tested the sensitivity of Gag-TRα to LMB. Treatment for 5–10 h with LMB caused a significant difference ($p < 0.0001$) in the subcellular localization of Gag-TRα with a shift toward greater nuclear accumulation; 68% of cells showed weak to complete nuclear localization in the presence of LMB compared with 51% in the absence of LMB (Fig. 4, *A*, *panel g*, and *B*). These data suggest that the AEV Gag sequence alone is sufficient to confer partial CRM1 dependence on the fusion protein Gag-TRα.

To further clarify the influence of the viral Gag domain on the nuclear export of v-ErbA, NIH/3T3 cells were transfected with a v-ErbA mutant with a deletion of the Gag sequence, ΔGag-v-ErbA (Fig. 1), and scored for nucleocytoplasmic distribution. The subcellular localization of ΔGag-v-ErbA was different from that of both TRα and v-ErbA (compare Fig. 4*A*, *panel a*, and Fig. 2*B*, *panel a*, with Fig. 4*A*, *panel i*). ΔGag-v-ErbA was mostly retained in the nucleus with the majority of cells (89%) exhibiting weak to complete nuclear localization (Fig. 4, *A* and *C*).

To determine whether nuclear export of v-ErbA is CRM1-dependent in the absence of Gag, we tested the sensitivity of ΔGag-v-ErbA to LMB. Treatment with LMB had no apparent effect on the subcellular distribution of ΔGag-v-ErbA; in both the presence and absence of LMB, 89% of cells exhibited weak to complete nuclear localization of the mutant protein (Fig. 4, *A* and *C*). These data provide further evidence that the AEV Gag sequence directs nuclear export of v-ErbA.

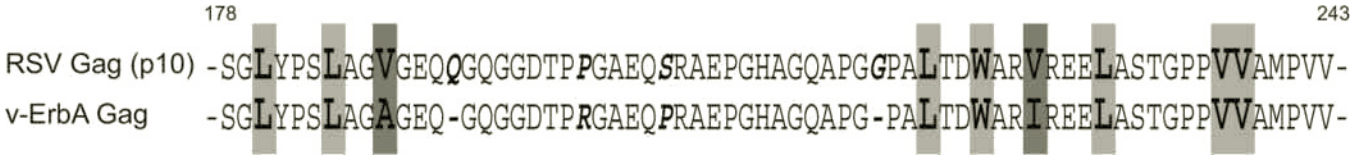


FIG. 5. **The truncated portion of the Gag polypeptide fused with v-ErbA includes the p10 sequence, which has a conserved NES.** A sequence comparison of RSV Gag (p10) and the v-ErbA Gag domain is depicted. *Large*, *bold letters* indicate the RSV region with NES-like properties.

*An NES Resides in the p10 Region of AEV Gag*—When AEV acquired c-*erbA*, the gene sequence was inserted at the viral locus encoding the Gag polyprotein. As a result of this insertion, a portion of the *gag* gene sequence was fused with the c-*erbA* sequence, while additional *gag* coding sequences were displaced downstream (44, 45). Sequence comparison between the portion of the AEV *gag* sequence that is fused with v-*erbA* and the complete coding region for the Gag polyprotein of RSV reveals extensive sequence homology (Fig. 5). After budding, the RSV Gag polyprotein precursor is proteolytically cleaved into the structural proteins MA, p2a, p2b, p10, CA, NC, and PR (42). Of particular interest, the viral sequence fused with v-ErbA comprises the portion of Gag known as the p10 region, which includes a sequence demonstrated to have NES-like properties in RSV (42) (Fig. 5). The CRM1 nuclear export receptor recognizes leucine-rich NESs, originally identified in the human immunodeficiency virus, type 1 Rev protein and the protein kinase A inhibitor (46–48). A cluster of leucine-rich hydrophobic residues in the second half of RSV p10 is thought likely to confer the NES properties (42).

To test whether a CRM1-dependent NES was embedded in the p10 region in AEV Gag, we constructed a series of expression vectors for fusion proteins. We fused to YFP the C-terminal portion of the p10 region from AEV Gag containing the putative NES (YFP-Gag NES). In addition, we fused a highly hydrophobic 70-amino acid region from the N terminus of Gag (YFP-Gag-(1–70)) to YFP to serve as a negative control and the RSV p10 domain (YFP-RSV p10) for a positive control (Fig. 6*A*). Cells were scored for nucleocytoplasmic distribution in the presence or absence of LMB, according to the categories previously described, except for "weak" and "strong nuclear," which were combined into one category (N > C). As predicted, treatment with LMB had no apparent effect on the subcellular distribution of YFP or YFP-Gag-(1–70). In both the absence and presence of LMB, ~68% of cells expressing YFP alone and 47% of cells expressing YFP-Gag-(1–70) showed a weak nuclear localization (Fig. 6, *B*, *panels a–d*, and *C*), suggesting that these small proteins are able to freely diffuse throughout the cell and that their nuclear export is CRM1-independent. Interestingly YFP-Gag-(1–70) did not have exactly the same distribution as YFP; not only did this fusion protein exhibit a greater whole cell distribution, but it often formed foci in the cytoplasm. Although the nature of these foci remains to be determined, it is tempting to speculate that the N-terminal 70 amino acids of AEV Gag may mediate formation of these cytoplasmic foci also characteristic of v-ErbA. In addition, sequestration in the cytoplasm could slow diffusion, thereby causing YFP-Gag-(1–70) to have a greater cytoplasmic distribution compared with untagged YFP at steady state.

In striking contrast to YFP and YFP-Gag-(1–70), in the absence of LMB the putative NES-bearing fusion proteins YFP-RSV p10 and YFP-Gag NES were predominantly localized to the cytoplasm (Fig. 6, *B*, *panels e–h*, and *C*) in ~70% of cells. Addition of the CRM1 inhibitor resulted in a significant increase in their nuclear retention ($p < 0.0001$), suggesting that the viral sequences confer CRM1-dependent nuclear export on YFP. In the presence of LMB, both YFP-RSV p10 and YFP-Gag NES showed a distribution similar to that of untagged YFP with nearly 70% of cells exhibiting a weak or strong nuclear localization (N > C). The continued presence of a cytoplasmic population is most likely due to the small size of these constructs (<40 kDa), which would, in principle, allow their diffusion through the central channel of the nuclear pore complex (Fig. 6, *A*; *B*, *panels f* and *h*; and *C*). Taken together, these data provide strong evidence that the p10 region of AEV Gag contains an NES that is sufficient to mediate CRM1-dependent nuclear export of a heterologous protein.

*The p10 Domain of AEV Gag Mediates CRM1-dependent Export of Gag and v-ErbA*—To confirm that the p10 domain of AEV Gag is necessary for CRM1-dependent nuclear export of Gag and v-ErbA, we generated two deletion constructs, YFP-GagΔNES and YFP-v-ErbAΔNES (Fig. 6*A*), which lack the putative NES-bearing C-terminal portion of the AEV p10 region, and compared their subcellular distribution patterns with those of YFP-Gag, YFP-v-ErbA, and YFP-ΔGag-v-ErbA (see also Fig. 4 for GFP-ΔGag-v-ErbA). In the absence of LMB, YFP-GagΔNES and YFP-v-ErbAΔNES, like YFP-ΔGag-v-ErbA, were significantly ($p < 0.0001$) more nuclear than YFP-Gag and YFP-v-ErbA in their subcellular distribution with only 10–20% of cells showing a cytoplasmic localization (Fig. 6, *B*, *panels i*, *k*, *m*, *o*, and *q*, and *C*). Addition of LMB resulted in a significant increase in the nuclear retention of both YFP-Gag and YFP-v-ErbA ($p < 0.0001$) with a 4–5-fold increase of the number of cells showing strong or complete nuclear localization (Fig. 6, *A*, *panels i* and *j* and *panels m* and *n*, and *C*). In sharp contrast, the subcellular distribution of the corresponding deletion constructs, YFP-GagΔNES, YFP-v-ErbAΔNES, and YFP-ΔGag-v-ErbA, remained unaltered in the presence of LMB (Fig. 6, *B*, *panels k*, *l*, and *o–r*, and *C*), demonstrating that when the C-terminal portion or the entire Gag domain is deleted these constructs are no longer LMB-sensitive. Interestingly YFP-v-ErbAΔNES also showed an altered nuclear distribution, forming nuclear foci similar to those observed in v/c/v (described below) as well as forming cytoplasmic foci similar to those characteristic of v-ErbA (Fig. 6*B*, *panels m–p*). Taken together our data provide strong evidence that the p10 region of AEV Gag (Fig. 5) contains an NES that is necessary and sufficient for CRM1-mediated nuclear export.

*Effect of Amino Acid Substitutions in the Ligand-binding Domain of v-ErbA on CRM1-mediated Nuclear Export*—Since the truncated AEV Gag sequence alone and Gag fused with v-ErbA have differential sensitivity to LMB, we sought to ascertain whether additional sequence changes in v-ErbA contribute to its ability to exit the nucleus by a CRM1-mediated pathway. NIH/3T3 cells were transfected separately with eight different GFP-tagged ΔGag-v-ErbA mutants with deletions or substitutions in the ligand-binding domain (Fig. 1) and scored for nucleocytoplasmic distribution according to the four categories described previously. All of the deletion/substitution mutants of ΔGag-v-ErbA (Δ20aa, ΔH10, ΔH11A, Δ9heptad, ΔH11B, L353R, L360R, and D232A) exhibited on average 89% whole cell or cytoplasmic distribution with the remaining cells showing weak to strong nuclear localization. Data are summarized in Fig. 7.

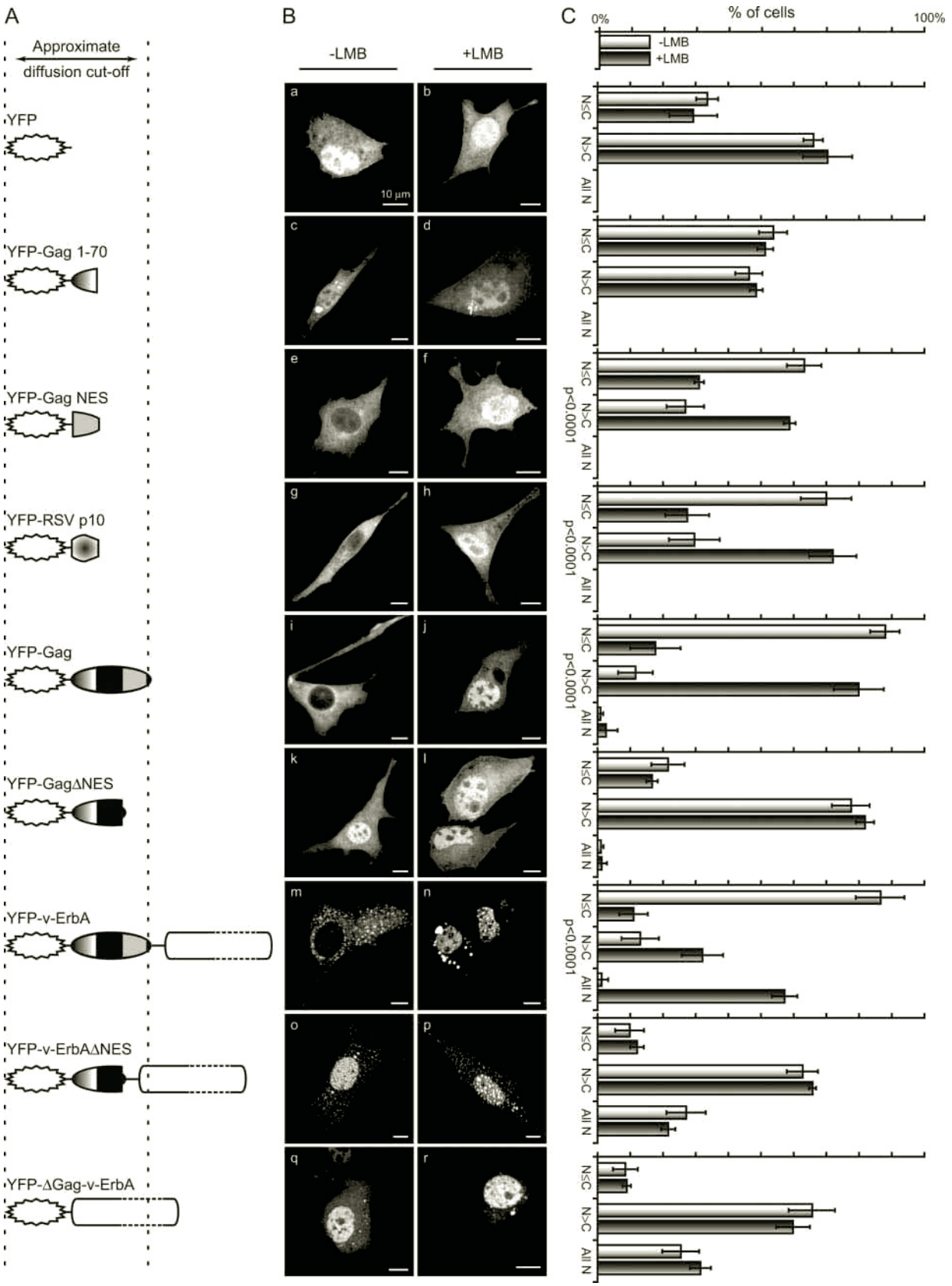


FIG. 6. **The p10 region in AEV Gag mediates CRM1-dependent nuclear export of Gag and v-ErbA.** *A*, the diagram depicts the various YFP constructs used to identify the p10 region of Gag as a CRM1-dependent NES. *Dashed lines* indicate the approximate molecular mass cut-off (60 kDa) for passive diffusion through the nuclear pore complex. *B*, representative examples of the subcellular distribution of the various YFP constructs depicted in *A*. NIH/3T3 cells were transfected with the various constructs and left to shuttle for 6 h in the presence (+*LMB*) or absence of LMB (−*LMB*) as indicated. Cells were fixed, stained with TO-PRO-3, and analyzed by confocal laser scanning microscopy. *C*, graph summarizing the effect of LMB on the various constructs depicted in *A* and imaged in *B*. *White bars*, vehicle control (−*LMB*); *black bars*, LMB treatment (+*LMB*). Three replicate experiments were performed with at least 100 cells scored per replicate. $N \leq C$, whole cell or cytoplasmic distribution; $N > C$, weak or strong nuclear localization; *All N*, complete nuclear localization.

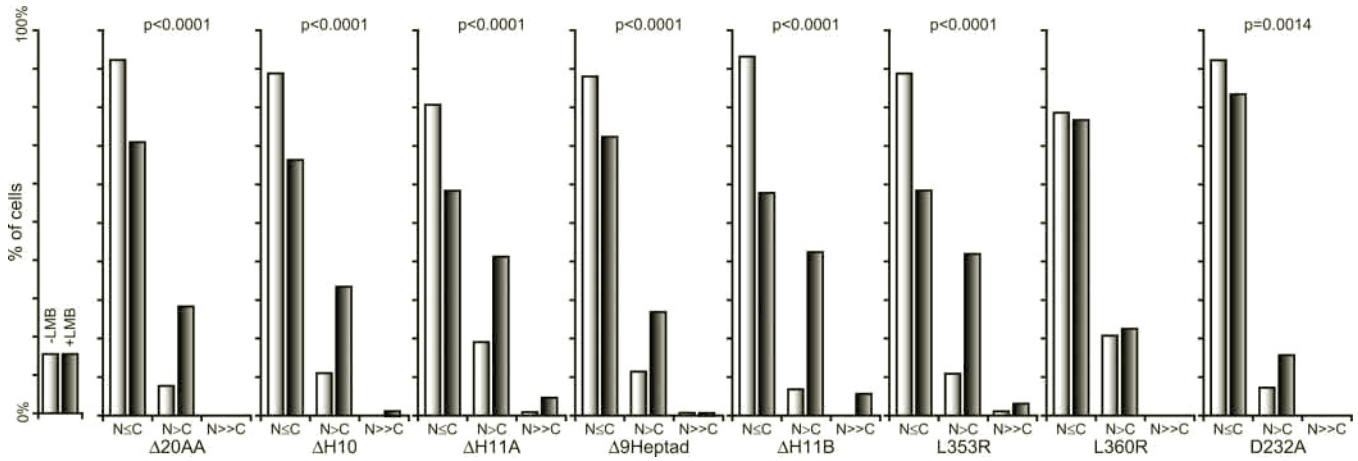


FIG. 7. **The effect of LMB on the subcellular localization of v-ErbA ligand-binding domain mutants.** NIH/3T3 cells were transfected with expression vectors for GFP-tagged ligand-binding domain mutants. The panel of v-ErbA deletion/substitution mutants (lacking the Gag sequence) is depicted in Fig. 1. After treatment with LMB, cells were fixed, stained with DAPI to reveal the nucleus, and visualized by epifluorescence microscopy. For each v-ErbA mutant, a total of 300 cells was scored for nucleocytoplasmic distribution. The bar graphs summarize the effect of LMB on the various constructs. *White bars*, vehicle control (−*LMB*); *black bars*, LMB treatment (+*LMB*). $N \leq C$, whole cell or cytoplasmic distribution; $N > C$, weak nuclear localization; $N \gg C$, strong nuclear localization (there were no cells with complete nuclear localization). Statistically significant results ($p < 0.0001$) are indicated.

Surprisingly treatment with LMB significantly altered the distribution of six of the ΔGag-v-ErbA mutants ($p < 0.0001$ for Δ20aa, ΔH10, ΔH11A, Δ9heptad, ΔH11B, and L353R; Fig. 7); on average, there was a 3-fold shift toward greater nuclear retention, although the majority of cells (67%) still exhibited a cytoplasmic or whole cell distribution. A more modest, 2-fold increase in retention was observed for D232A ($p = 0.0014$; Fig. 7). Only L360R did not exhibit greater nuclear retention in the presence of LMB, 23 and 21% of cells exhibiting a weak nuclear localization in the presence and absence of LMB, respectively. In summary, these data suggest that the ligand-binding domain of v-ErbA may influence interaction of the oncoprotein with CRM1.

*The Altered DNA-binding Domain of v-ErbA Does Not Contribute to CRM1-dependent Nuclear Export*—A recent report suggests that TRα, which lacks a leucine-rich NES for CRM1, may contain an NES in a 15-amino acid sequence located between the two zinc fingers of its DNA-binding domain (49). This NES domain is conserved in the nuclear receptor superfamily and is thought to interact directly with the $Ca^{2+}$-binding protein calreticulin, a recently characterized nuclear export receptor (50). The DNA-binding domain of v-ErbA has sustained two amino acid changes compared with TRα (Fig. 1). To determine whether the altered DNA-binding domain of v-ErbA plays a role in conferring CRM1-dependent nuclear export, v/c/v, a chimeric fusion protein in which the DNA-binding domain of v-ErbA was replaced with the DNA-binding domain of TRα, was analyzed (Fig. 1). NIH/3T3 cells were transfected with GFP-v/c/v and scored for nucleocytoplasmic distribution according to the four categories described previously. v/c/v showed a diversity of subcellular distributions (Fig. 8, *A* and *B*), including entirely cytoplasmic (*panel a*), weak nuclear (*panel b*), strong nuclear (*panel c*), and complete nuclear localization (*panel d*). In cells with complete nuclear localization, v/c/v showed either a diffuse distribution (Fig. 8*A*, *panel d*, nucleus with *no arrow*) or localization to bright foci (*panel d*, nuclei with an *arrow*). Similarly cytoplasmically localized v/c/v either showed a diffuse distribution (not shown) or localization to bright foci (*panels a–c*). The nature of these foci remains to be determined.

To determine whether nuclear export of v-ErbA remains CRM1-dependent in the presence of the TRα NES, we tested the sensitivity of v/c/v to LMB. Treatment with LMB significantly altered the subcellular distribution of v/c/v ($p < 0.0001$). In the presence of LMB, v/c/v localized predominantly to the nucleus (Fig. 8*B*). In contrast, in the absence of LMB, 38% of v/c/v showed a whole cell or cytoplasmic distribution (Fig. 8*B*). These findings suggest that the CRM1-dependent NES within the AEV Gag p10 domain is dominant to the TRα CRM1-independent NES.

When the Gag sequence was deleted, the distribution of ΔGag-v/c/v shifted to a more nuclear distribution with no cells showing a whole cell or cytoplasmic distribution (Fig. 8*C*). Treatment with LMB did not significantly alter the subcellular distribution of ΔGag-v/c/v ($p = 0.0088$). In the presence of LMB, 68% of cells showed strong to complete nuclear localization of ΔGag-v/c/v compared with 57% in the absence of LMB (Fig. 8*C*). These data provide further evidence of the critical importance of the AEV Gag domain in mediating v-ErbA CRM1-dependent nuclear export and cytoplasmic localization.

*v-ErbA Does Not Undergo Nucleocytoplasmic Shuttling When the CRM1 Export Pathway Is Blocked*—In the presence of LMB, v-ErbA appears to be entirely trapped in the nucleus, suggesting that the oncoprotein solely uses the CRM1-mediated nuclear export pathway. However, if v-ErbA was, in fact, undergoing rapid nucleocytoplasmic shuttling, this would not be apparent in a single cell transfection assay. Therefore, to determine whether v-ErbA still undergoes rapid nucleocytoplasmic shuttling in the presence of LMB, heterokaryon assays were performed. In the majority of cells v-ErbA has a predominantly cytoplasmic distribution, thus in the absence of LMB most heterokaryons showed both cytoplasmic and nuclear fluorescence. Accordingly the presence of v-ErbA in the untransfected human nuclei could represent import of a cytoplasmic population rather than shuttling out of the mouse nucleus and into the human nucleus. In those few cells in which GFP-v-ErbA was predominantly nuclear, however, v-ErbA still accumulated in the untransfected human nuclei (Fig. 9, *panel a*). Importantly, in heterokaryons containing LMB-treated, mouse nuclei transfected with GFP-v-ErbA and untransfected human nuclei, only the original transfected mouse nucleus showed an accumulation of v-ErbA (Fig. 9, *panel c*). Moreover v-ErbA did not accumulate in the untransfected human nuclei even when the heterokaryons were incubated for the maximum time possible before cell division (≈10 h), indicating that a lack of shuttling did not simply represent

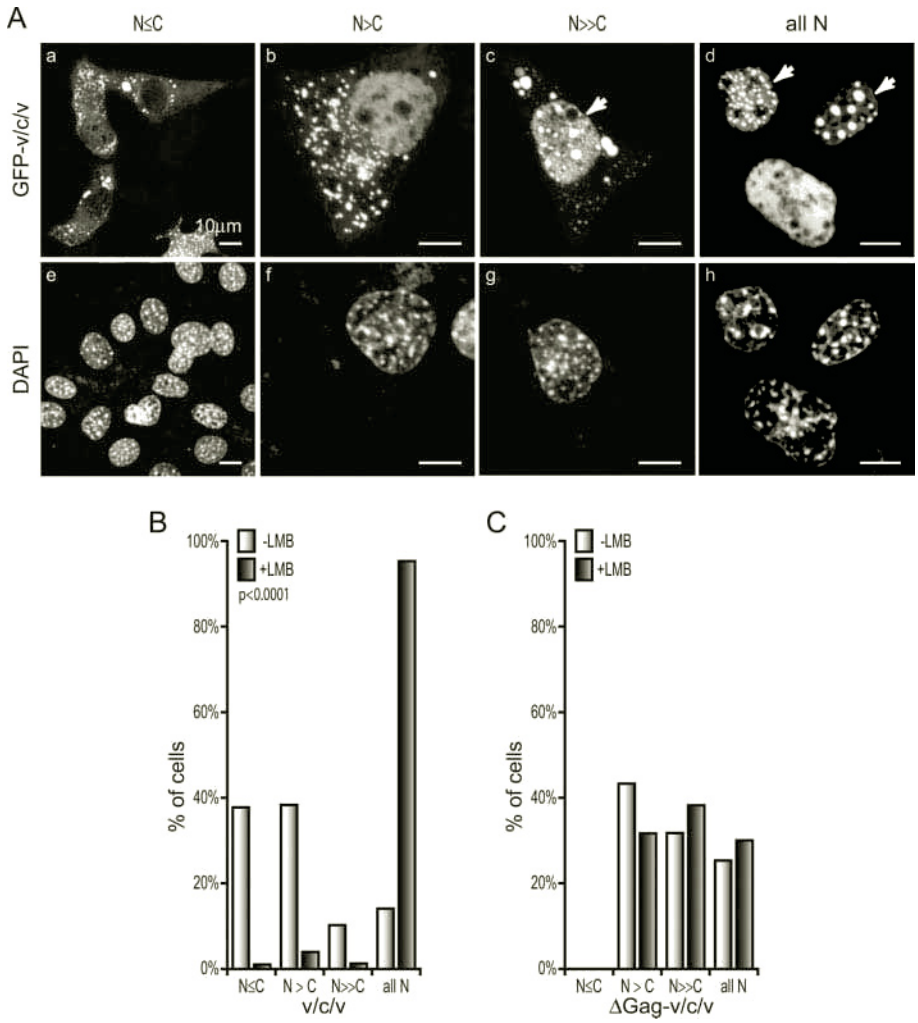


FIG. 8. **The altered DNA-binding domain of v-ErbA does not contribute to CRM1-dependent nuclear export.** *A*, subcellular distribution of v/c/v. NIH/3T3 cells were transfected with an expression vector for GFP-tagged v/c/v (*panels a–d*). Cells were fixed, stained with DAPI to reveal the nucleus (*panels e–h*), and visualized by epifluorescence microscopy. *White arrows* point to nuclei with foci. *B*, bar graph summarizing the effect of LMB on the subcellular distribution of v/c/v ($n$ = 879 cells). *C*, bar graph summarizing the effect of LMB on the subcellular distribution of ΔGag-v/c/v ($n$ = 698 cells). *White bars*, vehicle control (−*LMB*); *black bars*, LMB treatment (+*LMB*). $N \leq C$, whole cell or cytoplasmic distribution; $N > C$, weak nuclear localization; $N \gg C$, strong nuclear localization; *all N*, complete nuclear localization.

slower nucleocytoplasmic transport kinetics. These findings demonstrate that v-ErbA does not shuttle between the nucleus and the cytoplasm when the CRM1 pathway is blocked, suggesting that the ability to follow the export pathway used by TR$\alpha$ has been lost by the oncoprotein v-ErbA during its evolution.

To further test this model for oncogenic loss of the ability to follow the TR$\alpha$ export pathway, we analyzed the nucleocytoplasmic shuttling properties of v/c/v. Heterokaryons were formed by the fusion of LMB-treated or untreated transfected mouse cells with untransfected human cells. In the absence of LMB, v/c/v was found in both mouse and human nuclei (Fig. 9, *panel e*), although as noted above for v-ErbA, this could be due to import of a cytoplasmic population and not "shuttling" *per se*. Interestingly, despite having the DNA-binding domain containing the TR$\alpha$ putative NES, Gag-v/c/v did not shuttle when CRM1 was blocked by treatment with LMB (Fig. 9, *panel g*). These data provide further evidence that the oncoprotein has lost the ability to bind the TR$\alpha$ export receptor due to fusion with Gag and possibly due to other sequence and conformational changes. This further confirms that the altered DNA-binding domain of the oncoprotein does not play an essential role in mediating v-ErbA export.

## DISCUSSION

*Acquisition of a CRM1-dependent NES by v-ErbA*—v-*erbA*, an oncogenic derivative of a TR$\alpha$ gene carried by AEV, influences the transformation capabilities of the virus through interruption of the tightly regulated balance between host cell proliferation and differentiation. The oncogenic effects of v-ErbA result, in part, from direct interference with TR$\alpha$-mediated gene transcription. Here we present findings suggesting that, in addition, the altered subcellular localization of v-ErbA plays a crucial role in its oncogenic properties. While the oncoprotein v-ErbA exits the nucleus by a CRM1-dependent pathway, its cellular homolog TR$\alpha$ follows a CRM1-independent nuclear export pathway (35). In addition, we show here that another isoform of the thyroid hormone receptor, TR$\beta$, also uses a CRM1-independent nuclear export pathway. A recent report states that TR$\beta$ export occurs by a CRM1-mediated pathway (51). Since the data were not shown, we cannot address possible reasons for this apparent discrepancy.

The fusion of a portion of the AEV Gag sequence, including the p10 domain, to the N terminus of v-ErbA allows the oncoprotein to interact directly with CRM1 and confers its CRM1-mediated export. In addition, this finding raises the important point that nuclear import and export of the Gag polypeptide may be of importance for AEV assembly (42). The region that is homologous to the RSV sequence with NES-like properties and causes the CRM1-mediated export of v-ErbA closely resembles the previously identified consensus sequence for the CRM1-dependent NES (48, 52) and therefore should give a better understanding of the sequence requirements for interaction of an NES with CRM1. As a result of these altered export characteristics, the subcellular distribution of v-ErbA has shifted from the predominantly nuclear localization of its cellular homolog

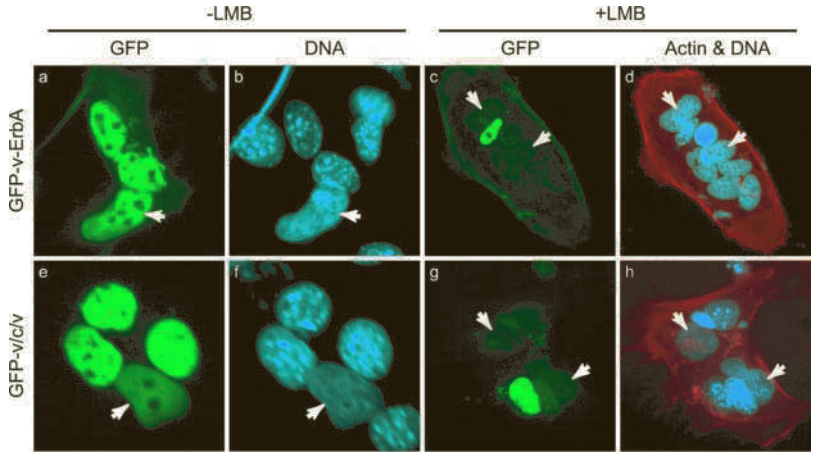


FIG. 9. **Nucleocytoplasmic shuttling of v-ErbA is CRM1-dependent.** v-ErbA does not undergo shuttling when the CRM1 export pathway is blocked. Similarly shuttling of v/c/v, a chimeric protein in which the DNA-binding domain of v-ErbA is replaced by the DNA-binding domain of TR$\alpha$, is also inhibited, indicating that the altered DNA-binding domain of the oncoprotein does not play an essential role in v-ErbA export. Heterokaryon assays were performed as described (Fig. 2) using mouse cells expressing GFP-tagged v-ErbA (*panels a–d*) or v/c/v (*panels e–h*) in the presence or absence of LMB as indicated. *Panels b* and *f*, Hoechst 33258 DNA stain. *Panels d* and *h*, Hoechst 33258 DNA stain overlaid with rhodamine-phalloidin actin stain to visualize the borders of heterokaryons. Approximately 20 heterokaryons were analyzed per experiment with several replicate experiments. *White arrows* indicate human nuclei.

to a more cytoplasmic distribution, thereby limiting access of the oncoprotein to target genes.

Results presented here also suggest that while the truncated Gag sequence, and particularly amino acid residues 178–243 of the p10 domain, is necessary for interaction with CRM1, other sequence differences between v-ErbA and TR$\alpha$ may enhance the ability of the oncoprotein to follow the CRM1-mediated nuclear export pathway. The lack of complete nuclear localization of the Gag sequence alone in the presence of LMB, a specific inhibitor of CRM1, suggests that nuclear import of truncated Gag may be inefficient. This result correlates with the finding that nuclear localization of the RSV complete Gag polypeptide is transient (42). Furthermore the lack of complete nuclear localization of Gag-TR$\alpha$ in cells treated with LMB suggests that the chimeric receptor does not use this CRM1-mediated export pathway exclusively. It is likely that the Gag sequence allows Gag-TR$\alpha$ to follow the CRM1-dependent export pathway in addition to using the export pathway utilized by TR$\alpha$. This interpretation is consistent with the proposed location of the TR$\alpha$ NES in its DNA-binding domain (49), a sequence that is unaltered in the Gag-TR$\alpha$ fusion protein. However, when the DNA-binding domain of v-ErbA was replaced with the DNA-binding domain of TR$\alpha$, this chimeric protein (v/c/v) was not able to follow a CRM1-independent export pathway, suggesting that when fused with v-ErbA other sequence and conformational changes alter the ability to bind to the export receptor and confer dominant properties on the Gag NES. Finally blocking the CRM1 export pathway inhibited nucleocytoplasmic shuttling of v-ErbA, suggesting that the oncoprotein can no longer use the CRM1-independent nuclear export pathway utilized by TR$\alpha$ and that the CRM1-dependent nuclear export pathway is the sole export pathway used by v-ErbA.

*Does the Ligand-binding Domain of v-ErbA Play a Role in CRM1-dependent Nuclear Export?*—The importance of the ligand-binding domain is illustrated by the nuclear export properties of ΔGag v-ErbA mutants, which contain modifications to the ligand-binding domain. The nucleocytoplasmic distribution of these deletion/substitution mutants was at first surprising. Based on the distribution pattern of ΔGag-v-ErbA, we had predicted a shift toward greater nuclear accumulation of these mutants since they also lack the Gag sequence. However, considering the location of the mutations in the ligand-binding domain, their whole cell distribution is consistent with current knowledge of the importance of this region for nuclear import and/or retention (53), dimerization (36), and interference with TR$\alpha$-mediated transcription (4, 5). Further ΔGag-v-ErbA showed no significant difference in subcellular distribution in the presence or absence of LMB. In contrast, seven of the deletion/substitution mutants of ΔGag v-ErbA exhibited greater nuclear localization in the presence of LMB, further suggesting that these mutants, after nuclear entry, follow a CRM1-mediated export pathway. Still, because the majority of cells show at least some cytoplasmic localization, the possibility remains that these mutants may exit the nucleus by another receptor-mediated export pathway when CRM1 is blocked.

How might these v-ErbA ligand-binding domain mutants, which also lack the Gag NES, follow the CRM1-dependent export pathway? One possible explanation is that the various amino acid deletions/substitutions cause a conformation shift, unmasking an additional NES that is not exposed in ΔGag-v-ErbA. This model is supported by the export properties of the mutant D232A; the single amino acid substitution probably causes less of a conformation change than the large sequence deletions in the other mutants, resulting in a weaker interaction with CRM1. Precedence for such a mechanism involving a masked NES is illustrated by a number of proteins that undergo CRM1-dependent nuclear export, including INI1/hSNF5 (a component of the SWI/SNF complex chromatin remodeling factor), the Ret finger protein, the p53 tumor suppressor, the influenza NS1 protein, and adenoviral E4 protein (40, 54–57). Regulation of nuclear export through masking and unmasking of NESs is thought to serve as yet another level of regulation of gene expression.

The ninth heptad in the ligand-binding domain of v-ErbA is leucine-rich, identifying it as a possible location for an additional NES that could enhance the altered nuclear export of v-ErbA. This model is supported by the lack of CRM1 dependence of the substitution mutant L360R, which changes one of these leucines to an arginine. In addition, mutants with modifications near the ninth heptad but not within the leucine-rich region (ΔH11A, ΔH11B, and L353R) exhibit the strongest CRM1 dependence, suggesting that modifications close to the ninth heptad may unmask the NES. However, the ninth heptad clearly does not contain the only additional motif with the

potential to interact with CRM1. The deletion mutant Δ9heptad, which lacks this region, still can follow the CRM1 nuclear export pathway. Moreover Δ20aa and ΔH10, which modify regions further from the ninth heptad, also exhibit CRM1 dependence. In addition to sequence alterations in the ligand-binding domain, it is possible that amino acid substitutions in the hinge region, which include changes to leucines (Fig. 1), act to further enhance interaction of the oncoprotein with CRM1.

In summary, these data suggest two possible mechanisms for CRM1 dependence in addition to the interaction with the truncated AEV Gag sequence. Amino acid substitutions acquired during the evolution of the oncoprotein could by themselves constitute a weak NES through the addition of leucines or other hydrophobic amino acids, or these mutations could cause a conformation shift that indirectly results in stronger interaction of the Gag NES with CRM1.

*Implications for the Oncogenesis and Dominant Negative Activity of v-ErbA*—The shift toward a more cytoplasmic distribution of v-ErbA, which appears to be a direct result of fusion with a viral NES, has implications for the mode of action of v-ErbA. Prior studies provide evidence for a role of competition over DNA binding sites and cofactors and formation of inactive heterodimers in oncoprotein repression of TR$\alpha$-mediated transcription (19). We propose that, in addition, the acquisition of altered nuclear export activity by the oncoprotein v-ErbA may be a factor in its oncogenesis and suggest that altered subcellular localization provides yet another mode of action for dominant negative transcription factors in general.

Evidence from other studies further supports the role of altered nuclear export in oncogenesis. For example, mislocalization of INI1/hSNF5 blocks its normal tumor suppression function (54), p53 is hyperactively exported from the nucleus in some transformed cells (40), and ectopic expression of the hepatitis B virus X protein sequesters CRM1 in the cytoplasm, suggesting that the inactivation of the CRM1-mediated pathway may be an early step during viral hepatitis-mediated liver carcinogenesis (58). The acquired use of CRM1-mediated nuclear export by v-ErbA results in its mislocalization to the cytoplasm, which may affect gene expression directly or indirectly. It is known that overexpression of v-ErbA is required for its oncogenic function (59). Since v-ErbA may need to enter the nucleus to exert its dominant negative activity, if most remains cytoplasmic, then overexpression would be required to increase that amount available to interact or compete with TR$\alpha$.

In summary, our study demonstrates that CRM1-dependent nuclear export of v-ErbA is primarily mediated by an NES within a 70-amino acid region in the C terminus of the AEV Gag p10 sequence and points to the possibility that amino acid changes in other regions of v-ErbA play a cryptic role in CRM1-mediated nuclear export. Further analysis is necessary to more precisely clarify the involvement of these and other domains in CRM1-dependent nuclear export. Taken together our findings suggest that fusion of the viral Gag sequence with v-ErbA was a crucial step in the evolution of the properties of this oncoprotein.

*Acknowledgments*—We thank M. Trosset for assistance with the statistical analysis. We are grateful to A. Muñoz, M. Privalsky, J. Subauste, R. Truant, and J. Kjems for the gift of plasmid expression vectors and M. Yoshida for the gift of LMB.

REFERENCES

1. Zhang, J., and Lazar, M. A. (2000) *Annu. Rev. Physiol.* **62,** 439–466
2. Stunnenberg, H. G., Garcia-Jimenez, C., and Betz, J. L. (1998) *Biochim. Biophys. Acta* **1423,** F15–F33
3. Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986) *Nature* **324,** 635–640
4. Zenke, M., Munoz, A., Sap, J., Vennstrom, B., and Beug, H. (1990) *Cell* **61,** 1035–1049
5. Forrest, D., Munoz, A., Raynoschek, C., Vennstrom, B., and Beug, H. (1990) *Oncogene* **5,** 309–316
6. Selmi, S., and Samuels, H. H. (1991) *J. Biol. Chem.* **266,** 11589–11593
7. Sharif, M., and Privalsky, M. L. (1991) *Cell* **66,** 885–893
8. Damm, K., and Evans, R. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10668–10672
9. Busch, K., Martin, B., Baniahmad, A., and Renkawitz, R. (1997) *Mol. Endocrinol.* **11,** 379–389
10. Wong, C.-W., and Privalsky, M. L. (1995) *Mol. Endocrinol.* **9,** 551–562
11. Boucher, P., and Privalsky, M. L. (1990) *Oncogene* **5,** 1303–1311
12. Saatcioglu, F., Bartunek, P., Deng, T., Zenke, M., and Karin, M. (1993) *Mol. Cell. Biol.* **13,** 3675–3685
13. Andersson, M., and Vennstrom, B. (2000) *Oncogene* **19,** 3563–3569
14. Urnov, F. D., Yee, J., Sachs, L., Collingwood, T. N., Bauer, A., Beug, H., Shi, Y.-B., and Wolffe, A. P. (2000) *EMBO J.* **15,** 4074–4090
15. Subauste, J. S., and Koenig, R. J. (1995) *J. Biol. Chem.* **270,** 7957–7962
16. Subauste, J. S., and Koenig, R. J. (1998) *Mol. Endocrinol.* **12,** 1380–1392
17. Barettino, D., Bugge, T. H., Bartunek, P., Vivanco Ruiz, M. D., Sonntag-Buck, V., Beug, H., Zenke, M., and Stunnenberg, H. G. (1993) *EMBO J.* **12,** 1343–1354
18. Hermann, T., Hoffmann, B., Piedrafita, F. J., Zhang, X., and Pfahl, M. (1993) *Oncogene* **8,** 55–65
19. Yen, P. M., and Chin, W. W. (1994) *Mol. Endocrinol.* **8,** 1450–1454
20. Chen, H., and Privalsky, M. L. (1993) *Mol. Cell. Biol.* **13,** 5970–5980
21. Chen, H., Smit-McBride, Z., Lewis, S., Sharif, M., and Privalsky, M. L. (1993) *Mol. Cell. Biol.* **13,** 2366–2376
22. Munoz, A., Zenke, M., Gehring, U., Sap, J., Beug, H., and Vennstrom, B. (1988) *EMBO J.* **7,** 155–159
23. Damm, K., Thompson, C. C., and Evans, R. M. (1989) *Nature* **339,** 593–597
24. Bauer, A., Ulrich, E., Andersson, M., Beug, H., and von Lindern, M. (1997) *Oncogene* **15,** 701–715
25. Braliou, G. G., Ciana, P., Klaassen, W., Gandrillon, O., and Stunnenberg, H. G. (2001) *Oncogene* **20,** 775–787
26. Mey, A., Gandrillon, O., McNagny, K. M., Clegg, D. O., and Samarut, J. (2002) *Oncogene* **21,** 2864–2872
27. Gandrillon, O., Ferrand, N., Michaille, J. J., Roze, L., Zile, M. H., and Samarut, J. (1994) *Oncogene* **9,** 749–758
28. Trapasso, F., Martelli, M. L., Battaglia, C., Angotti, E., Mele, E., Stella, A., Samarut, J., Avvedimento, V. E., and Fusco, A. (1996) *Oncogene* **12,** 1879–1888
29. Sharif, M., and Privalsky, M. L. (1992) *Oncogene* **7,** 953–960
30. Desbois, C., Aubert, D., Legrand, C., Pain, B., and Samarut, J. (1991) *Cell* **67,** 731–740
31. Kaffman, A., and O'Shea, E. K. (1999) *Annu. Rev. Cell Dev. Biol.* **15,** 291–339
32. Gama-Carvalho, M., and Carmo-Fonseca, M. (2001) *FEBS Lett.* **498,** 157–163
33. Ossareh-Nazari, B., Gwizdek, C., and Dargemont, C. (2001) *Traffic* **2,** 684–689
34. Yoshida, M., and Horinouchi, S. (1999) *Ann. N. Y. Acad. Sci.* **886,** 23–36
35. Bunn, C. F., Neidig, J. A., Freidinger, K. E., Stankiewicz, T. A., Weaver, B. S., McGrew, J., and Allison, L. A. (2001) *Mol. Endocrinol.* **15,** 512–533
36. Shen, Q., and Subauste, J. S. (2000) *J. Biol. Chem.* **275,** 41018–41027
37. Nagl, S. B., Bunn, C. F., and Allison, L. A. (1997) *J. Cell. Biochem.* **67,** 184–200
38. Nagl, S. B., Nelson, C. C., Romaniuk, P. J., and Allison, L. A. (1995) *Mol. Endocrinol.* **9,** 1522–1532
39. Askjaer, P., Jensen, T. H., Nilsson, J., Englmeier, L., and Kjems, J. (1998) *J. Biol. Chem.* **273,** 33414–33422
40. Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999) *EMBO J.* **18,** 1660–1672
41. Wills, J. W., and Craven, R. C. (1991) *AIDS* **5,** 639–654
42. Scheifele, L. Z., Garbitt, R. A., Rhoads, J. D., and Parent, L. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 3944–3949
43. Ciana, P., Braliou, G. G., Demay, F. G., von Lindern, M., Barettino, D., Beug, H., and Stunnenberg, H. G. (1998) *EMBO J.* **17,** 7382–7394
44. Damm, K., Beug, H., Graf, T., and Vennstrom, B. (1987) *EMBO J.* **6,** 375–382
45. Thoraval, P., Savatier, P., Xiao, J. H., Mallet, F., Samarut, J., Verdier, G., and Nigon, V. (1987) *Nucleic Acids Res.* **15,** 9612
46. Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997) *Cell* **90,** 1051–1060
47. Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997) *Cell* **90,** 1041–1050
48. Ossareh-Nazari, B., Bachelerie, F., and Dargemont, C. (1997) *Science* **278,** 141–144
49. Black, B. E., Holaska, J. M., Rastinejad, F., and Paschal, B. M. (2001) *Curr. Biol.* **11,** 1749–1758
50. Holaska, J. M., Black, B. E., Rastinejad, F., and Paschal, B. M. (2002) *Mol. Cell. Biol.* **22,** 6286–6297
51. Maruvada, P., Bauman, C. T., Hager, G. L., and Yen, P. M. (2003) *J. Biol. Chem.* **278,** 12425–12432
52. Bogerd, H. P., Fridell, R. A., Benson, R. E., Hua, J., and Cullen, B. R. (1996) *Mol. Cell. Biol.* **16,** 4207–4214
53. Boucher, P., Koning, A., and Privalsky, M. L. (1988) *J. Virol.* **62,** 534–544
54. Craig, E., Zhang, Z.-K., Davies, K. P., and Kalpana, G. V. (2002) *EMBO J.* **21,** 31–42
55. Harbers, M., Nomura, T., Ohno, S., and Ishii, S. (2001) *J. Biol. Chem.* **276,** 48596–48607
56. Li, Y., Yamakita, Y., and Krug, R. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4864–4869
57. Dobbelstein, M., Roth, J., Kimberly, W. T., Levine, A. J., and Shenk, T. (1997) *EMBO J.* **16,** 4276–4284
58. Forgues, M., Marrogi, A. J., Spillare, E. A., Wu, C.-G., Yang, Q., Yoshida, M., and Wang, X. W. (2001) *J. Biol. Chem.* **276,** 22797–22803
59. Disela, C., Glineur, C., Bugge, T., Sap, J., Stengl, G., Dodgson, J., Stunnenberg, H., Beug, H., and Zenke, M. (1991) *Genes Dev.* **5,** 2033–2047