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Interaction of the Influenza Virus Nucleoprotein with the Cellular CRM1-Mediated Nuclear Export Pathway

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Influenza virus transcription occurs in the nuclei of infected cells, where the viral genomic RNAs are complexed with a nucleoprotein (NP) to form ribonucleoprotein (RNP) structures. Prior to assembly into progeny virions, these RNPs exit the nucleus and accumulate in the cytoplasm. The mechanisms responsible for RNP export are only partially understood but have been proposed to involve the viral M1 and NS2 polypeptides. We found that the drug leptomycin B (LMB), which specifically inactivates the cellular CRM1 polypeptide, caused nuclear retention of NP in virus-infected cells, indicating a role for the CRM1 nuclear export pathway in RNP egress. However, no alteration was seen in the cellular distribution of M1 or NS2, even in the case of a mutant virus which synthesizes greatly reduced amounts of NS2. Furthermore, NP was distributed throughout the nuclei of infected cells at early times postinfection but, when retained in the nucleus at late times by LMB treatment, was redistributed to the periphery of the nucleoplasm. No such change was seen in the nuclear distribution of M1 or NS2 after drug treatment. Similar to the behavior of NP, M1 and NS2 in infected cells, LMB treatment of cells expressing each polypeptide in isolation caused nuclear retention of NP but not M1 or NS2. Conversely, overexpression of CRM1 caused increased cytoplasmic accumulation of NP but had little effect on M1 or NS2 distribution. Consistent with this, NP bound CRM1 in vitro. Overall, these data raise the possibility that RNP export is mediated by a direct interaction between NP and the cellular CRM1 export pathway.

The influenza virus genome consists of eight segments of single-stranded RNA that encode a total of 10 identified polypeptides. The genomic RNA segments are of negative sense and are always found in association with viral polypeptides: the three subunits of an RNA-dependent RNA polymerase (PB1, PB2, and PA) and, in stoichiometric quantities, a single-strand RNA-binding nucleoprotein (NP) (28). In virions, these ribonucleoprotein (RNP) structures are packaged within a shell of the viral M1 polypeptide underlying the lipid bilayer, along with the hemagglutinin (HA) and neuraminidase integral membrane glycoproteins. Minor virion components include M2, a small transmembrane ion channel, and the NS2 polypeptide (28). Influenza virus particles enter the cell by receptor-mediated endocytosis. Following acidification of the endosome, the M1 polypeptide dissociates from the RNP segments and virion RNPs (vRNPs) are released into the cytoplasm (30, 31). Unusually for a virus with no DNA coding stage, influenza virus transcription occurs in the nucleus (20, 22). Accordingly, after release of the RNPs into the cytoplasm, they migrate into the nucleus, in an active process that is thought to be mediated by the cellular importin α/β pathway (39). Once in the nucleus, vRNPs act as the template for synthesis of mRNAs, which are exported into the cytoplasm for translation. The vRNPs also act as the template for synthesis of full-length cRNA copies of the genome, which are encapsidated by NP and act as replicative intermediates for the synthesis of progeny genomic RNA (28). Transcription and replication of the viral genome require the three components of the RNA-dependent RNA polymerase in addition to NP (21). These proteins, together with newly synthesized virion RNA, are assembled into RNPs in the nucleus. However, since progeny virion formation occurs at the plasma membrane, this necessitates nuclear export of the new RNPs. This occurs by a process that is still only partially understood. Current evidence implicates three virus polypeptides: M1, NS2, and NP itself. RNP export fails in the absence of M1, either in the case of defective viruses (29) or in the absence of late gene expression (4, 29, 51), while microinjection of antibodies to M1 effectively blocks the process of RNP export (29). However, the temperature-sensitive (*ts*) virus *ts*51 accumulates M1 in the nucleus at the nonpermissive temperature but is still able to export RNPs, suggesting that it is not necessary for M1 to be transported across the nuclear envelope in stoichiometric quantities (41, 52). It has recently been proposed that NS2 plays a major role in the transport of vRNPs out of the nucleus (38). Although NS2 does not interact directly with RNPs, it binds to M1 associated with vRNPs (54). Furthermore, NS2 contains a functional nuclear export signal (NES) and interacts with components of the nuclear pore complex (NPC) in a yeast two-hybrid system (38). Accordingly, it has been suggested that NS2 acts as an adapter molecule that links M1-RNP complexes with the NPC, thus mediating their export across the nuclear envelope (38). However, an influenza virus mutant that synthesizes greatly reduced amounts of NS2 replicates normally (44, 53). Similarly, in infected cells with a block to late gene expression, RNP export could be restored by the addition of exogenous M1 in the absence of detectable NS2 (4). Therefore, the virus may have evolved more than one mechanism to transport RNPs across the nuclear envelope. Supporting this possibility, evidence suggests that NP contains

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an intrinsic NES: although it contains two or more nuclear localization signals (5, 35, 49, 50), exogenously expressed NP shuttles between the cytoplasm and nucleus (51) and does not necessarily accumulate in the nucleus. Factors promoting cytoplasmic accumulation of NP include cellular hyperphosphorylation, increased time or levels of NP expression, and the ability of the protein to bind F-actin (9, 35).

Thus, although a plausible and attractive hypothesis has been suggested to explain nuclear export of RNPs late in infection (38), several experimental observations suggest that the process is more complex. To date, there has been no investigation of the possible role of the cellular nuclear export pathway mediated by CRM1 in the export of influenza virus RNPs. CRM1, or exportin 1, is a member of the importin β family, members of which are important in the transport of a variety of proteins in both directions across the nuclear envelope (32). In addition to associating with components of the NPC (16), CRM1 acts as a soluble adapter molecule that binds to leucine-rich NESs in the nucleoplasm and, together with RanGTP, forms a trimeric complex that mediates export of the substrate (15, 46). Many cellular and viral proteins that contain nuclear export signals have been shown to interact with the CRM1 pathway, which therefore appears to act as a general export receptor for proteins and RNA complexes (8, 11).

As a specific inhibitor of CRM1 function, the cytotoxin leptomycin B (LMB) provides a useful reagent for studying nuclear export in cells. LMB inhibits CRM1-mediated nuclear export of a range of proteins and RNAs (2, 11, 15, 45) by covalently modifying a specific cysteine residue in CRM1, which is thought to interfere with the formation of a stable complex with the export substrate (24, 25). Here, we show that LMB treatment retained influenza virus RNPs in the nuclei of infected cells, implying a role for the CRM1 pathway in their export. However, the drug did not alter the cellular distribution of either M1 or NS2, even in the context of a virus mutant that expresses around 5% of the normal amount of NS2. Moreover, LMB treatment caused the nuclear retention of transfected NP while, conversely, overexpression of CRM1 caused increased cytoplasmic accumulation. Neither LMB treatment nor CRM1 overexpression significantly affected the localization of exogenously expressed M1 or NS2. Thus, NP interacts with the CRM1 pathway in the absence of other influenza virus polypeptides and, consistent with this, we show that NP and CRM1 interact in vitro. This raises the possibility that nuclear export of influenza virus RNPs involves a direct interaction between NP and the CRM1 export pathway.

MATERIALS AND METHODS

Viruses, plasmids, and antibodies. Influenza virus strains A/PR/8/34 (PR8) and A/FPV/Rostock/34 (FPV) and the temperature-sensitive FPV mutant mN3 (53) were propagated in 10-day-old embryonated eggs for 2 days at either 37°C (PR8) or 34 (FPVs). The virus mN3 contains a temperature-sensitive lesion in segment 8 which blocks replication at elevated temperatures and an incompletely mapped lesion, probably also in segment 8, which leads to decreased segment 8 splicing at the permissive temperature (44, 53). Recombinant vaccinia virus expressing T7 polymerase, vTF7, has been described previously (17). Plasmids expressing wild-type (WT) or mutant NP genes under control of the T7 promoter (pKT5 series) or as glutathione *S*-transferase (GST) and maltose-binding protein (MBP) fusions have also been described previously (9, 13). pCDNA-CRM1 (2) was kindly provided by S. Swaminathan and G. Grosveld, pT7-hSRP1 was from H. Kent and M. Stewart (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom), pGEM-NS2 (encoding NS2 from influenza virus strain A/Victoria/3/75) was from A. Portela (34), and pT7-703, containing the cDNA of segment 7 from A/PR/8/34 subcloned from pAPR701 (55) under control of a T7 RNA polymerase promoter, was from S. Inglis (Cantab Pharmaceuticals Ltd., Cambridge, United Kingdom). LMB was the generous gift of M. Yoshida and was dissolved in ethanol and stored under argon at −20°C (24, 36). LMB was added to culture medium to a final concentration of 11 nM; this value was chosen after initial titration experiments indicated that concentrations of ≥5 nM were required for nuclear retention of NP and transportin, a marker cellular polypeptide (data not shown). Polyclonal rabbit serum raised against PR8 NS2 has been described previously (10); rabbit anti-RNP and anti-PR8 sera were generously provided by S. Inglis. For double staining of NP and NS2, anti-RNP and anti-NS2 immunoglobulin G sera were purified on protein A-Sepharose and then conjugated to either fluorescein isothiocyanate (Sigma) or Cy3 (Amersham) fluorophore using the manufacturer's recommended protocol. A monoclonal antibody to influenza virus M1 was kindly provided by B. A. Askonas (19), and anti-nucleoporin p62 (Nup62) monoclonal antibody was obtained from Transduction Laboratories.

Cell fractionation. Cells were fractionated essentially as described by Briedis et al. (3). Briefly, BHK cells in 60-mm-diameter dishes were harvested at various times postinfection by scraping into 1 ml of ice-cold phosphate-buffered saline (PBS) and pelleted by centrifugation at 12,000 × *g* for 1 min. Cell pellets were resuspended in 100 μl of ice-cold TMN buffer (10-mM Tris-HCl [pH 7.2], 1.5 mM $MgCl_2$, 140 mM NaCl) containing 0.5% NP-40 and 0.5% Triton X-100, vortexed, and then incubated on ice for 30 min. Following centrifugation at 600 × *g* for 5 min at 4°C, the supernatants were transferred to a fresh tube and pellets (nuclei) were resuspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Equivalent proportions of the two fractions were analyzed by SDS-PAGE and Western blotting. Fractionation efficiency was confirmed by Western blotting for influenza virus HA and β-actin as well as the difference in NP distribution between early and late times postinfection (data not shown).

Labeling of cells with [^{35}S]methionine. Chicken embryo fibroblast (CEF) cells were seeded on 24-well plates and grown on confluency in M199 medium containing 10% fetal calf serum. Cells were infected with influenza virus (FPV or mN3) in allantoic fluid diluted in medium at a multiplicity of infection (MOI) of 10 PFU/cell for 60 min at room temperature (RT) and then incubated at 34°C in M199 medium containing 2% fetal calf serum with or without 11 nM LMB. Cells were transferred to methionine-free M199 medium for 30 min prior to labeling and then labeled for 30 min in 160 μl of medium containing 100 μCi of [^{35}S]methionine (Amersham) per ml with or without LMB. After labeling, cells were harvested in 200 μl of SDS-PAGE sample buffer.

Transfections and immunofluorescence. BHK cells seeded on glass coverslips were infected with vTF7 at an MOI of 5 PFU/cell and transfected 2 h later with 0.01 to 1.0 μg of plasmid DNA using Lipofectin (GIBCO-BRL) according to the manufacturer's directions. LMB was added to desired wells at a final concentration of 11 nM at 3 h posttransfection. Cells were harvested 2 h later and fixed for 20 min in 4% formaldehyde in PBS. Cells infected with influenza virus at an MOI of 10 PFU/cell were fixed in the same way. After permeabilization with 0.2% Triton X-100 in PBS for 5 min at room temperature, cells were incubated for 1 h with rabbit polyclonal anti-RNP serum at 1/250 to stain for NP, anti-PR8 serum at 1/500 to stain for M1, or anti-NS2 serum at 1/200. Cells were then incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit antibodies (Dako) at 1/200 for 30 min at RT. For double staining of PR8-infected cells, directly labeled anti-RNP and anti-NS2 sera were used at a 1/100 dilution for 1 h at RT; an M1 monoclonal antibody was used at 1/150 with anti-mouse Alexa 594 (Molecular Probes) at 1/1,000. Coverslips were mounted in Citifluor and examined using a Leitz Orthoplan microscope or a Leica TCS SP confocal microscope. To generate images of whole cells, serial optical planes of focus (at ~0.5-μm intervals) were taken on the *z* axis, across the depth of the cell, and merged into one image (using extended focus or projection algorithms) by the software package TCS-NT (Leica). Unless otherwise stated, laser power and photomultiplier tube settings were kept identical between matching samples stained with the same antibody. For numerical analysis of influenza virus polypeptide distribution in transfected cells, typically around 100 fluorescent cells per coverslip were examined and scored according to whether the observed fluorescence was predominantly cytoplasmic (C), predominantly nuclear (N), or throughout the cell (N/C). For statistical analysis, numbers of transfected cells falling into the various categories after addition of CRM1 and/or LMB were compared against the distribution obtained in the absence of other agents using the χ^2 test. In certain cases, sums of the N/C and C categories were used to reduce the influence of the relatively low-number C category.

In vitro translation and protein binding assays. Radiolabeled CRM1, hSRP1, NS2, and NP were synthesized using a coupled in vitro transcription-translation

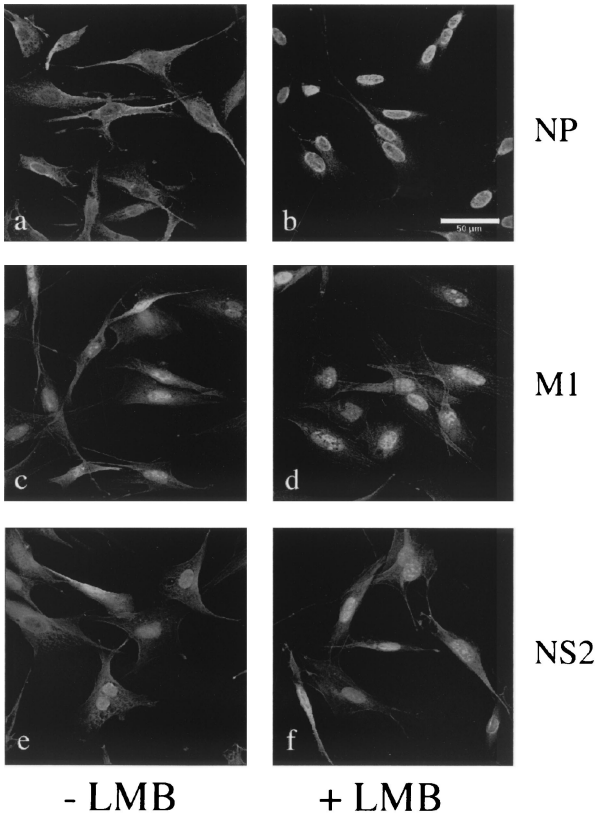

FIG. 1. Effect of LMB treatment on the intracellular localization of influenza virus polypeptides. BHK cells were infected with 10 PFU of influenza A/PR/8/34 virus per cell; incubated from 1 hpi in the absence (a, c, and e) or presence (b, d, and f) of 11 nM LMB, and examined at 12 hpi by confocal microscopy after staining for NP (a and b), M1 (c and d), or NS2 (e and f). Images were generated using an extended-focus algorithm.

system (7) as described previously (12). MBP and GST fusion proteins were affinity purified from lysates of *Escherichia coli* as described previously (9) and left bound to Sepharose beads. Protein binding assays were carried out essentially as described earlier (12). A 50-μl volume of a 50% slurry of glutathione-Sepharose (Pharmacia) or amylose resin (New England Biolabs) containing approximately 1 μg of fusion protein was incubated with 1 μl of in vitro-translated protein in 150 μl of IP buffer (100 mM KCl, 50 mM Tris-Cl [pH 7.6], 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% NP-40) for 1 h at RT. Bound proteins were collected by centrifugation, washed three times with 750 μl of IP buffer, resuspended in 30 μl of SDS-PAGE sample buffer, and analyzed by SDS-PAGE and autoradiography.

RESULTS

LMB inhibits nuclear export of NP in influenza virus-infected cells. Recently, it has been proposed that nuclear export of influenza virus RNPs is mediated by the viral NS2 protein via its NES and ability to interact with cellular nucleoporins (38). In this model, NS2 binds to M1-RNP complexes through interactions with M1 and facilitates nuclear export by direct contact with components of the NPC. However, many proteins that contain NESs interact with the cellular protein CRM1, which appears to act as a general export receptor (8, 11).

To test the involvement of CRM1 in the nuclear export of influenza virus RNPs, BHK cells were infected with virus, treated with 11 nM LMB 1 h postinfection (hpi), and analyzed by indirect immunofluorescence after a further 11 h. In untreated cells, NP localized predominantly to the cytoplasm, with many cells having apparently empty nuclei (Fig. 1a). This is the expected pattern of NP staining late in infection (29), consistent with most of the RNPs having exited the nucleus for assembly into progeny virions. In contrast, cells treated with LMB showed a dramatic change in NP distribution, with the majority of cells showing nuclear accumulation of the polypeptide (Fig. 1b) and very little cytoplasmic staining, indicating a block to the export of RNPs.

To confirm the change in NP distribution induced by LMB, virus-infected cells incubated in the absence or presence of the inhibitor were separated into nuclear and cytoplasmic fractions at different times postinfection and analyzed by Western blotting for NP content. Since immunofluorescence time course experiments indicated that the switch in NP localization from predominantly nuclear to predominantly cytoplasmic occurred at about 5 h in BHK cells and was essentially complete by 9 h postinfection (data not shown), measurements were made at time points from 6 h onward. In cells without drug treatment, the proportion of NP in the cytosolic fraction was greater than that in the nuclear fraction at all of the time points tested (Fig. 2). This is consistent with the immunofluorescence data, which showed that NP was predominantly cytoplasmic at late times postinfection. The overall amount of NP increased with time postinfection, indicating continued synthesis of the polypeptide. In contrast, in the presence of LMB, the majority of the NP was found in the nuclear fractions, particularly at earlier times postinfection (Fig. 2).

Previous studies have shown that use of the protein kinase inhibitor H7 also causes retention of influenza virus RNPs in the nucleus, in part by blocking the synthesis of late viral proteins, including M1 and NS2 (4, 26, 29, 48). To directly examine the effect of LMB on viral protein synthesis, BHK cells were infected with PR8 in the presence or absence of LMB and harvested at 13 hpi and cell extracts were analyzed by SDS-PAGE and Western blotting. No difference was observed in the level of synthesis of HA_0, NP, or M1 between

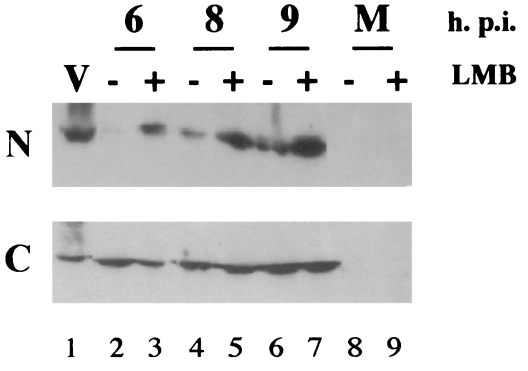

FIG. 2. Effect of LMB on the distribution of viral RNPs between the nuclear and cytoplasmic cell fractions. BHK cells were infected with PR8 at an MOI of 10 PFU/cell, incubated from 1 hpi in the presence or absence of 11 nM LMB, and harvested at 6, 8, and 9 h postinfection. Cells were separated into nuclear (N) and cytosolic (C) fractions, and equivalent amounts were subjected to SDS-PAGE and Western blotting with anti-RNP serum. Samples of purified virus (lane v) were also analyzed in parallel to provide a marker for NP. M, mock infected.

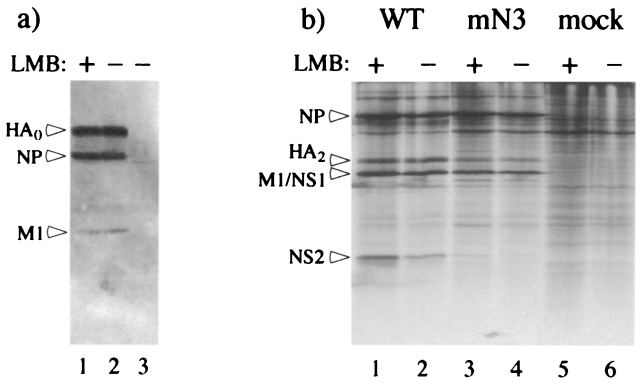

FIG. 3. Effect of LMB on influenza virus polypeptide synthesis. (a) BHK cells were infected with PR8 in the presence (lane 1) or absence (lane 2) of 11 nM LMB (added at 1 hpi), harvested at 13 hpi, and analyzed by SDS-PAGE and Western blotting using anti-PR8 serum. Mock-infected cell lysate is shown in lane 3. The positions of uncleaved HA, NP, and M1 are indicated by arrows. (b) CEF cells were infected at 34°C with WT FPV or the *ts* mutant mN3 in the presence (+) or absence (−) of LMB (added at 1 hpi) and then labeled with [^{35}S]methionine for 30 min before harvesting at 8 hpi. Lysates were analyzed by SDS-PAGE and autoradiography. The positions of NP, HA_2, M1/NS1, and NS2 are indicated.

treated and untreated infected cells (Fig. 3a). Additionally, no decrease in synthesis of the polymerase proteins or NS1 was observed when protein synthesis was examined by metabolic labeling (data not shown, but see later for FPV). Thus, we found no evidence that LMB acts by altering viral protein synthesis.

Since LMB is a specific inhibitor of CRM1-mediated nuclear export, the change in distribution of NP in the presence of LMB strongly suggests that RNPs are exported from the nuclei of virus-infected cells via a pathway involving CRM1. It has been suggested that NS2 is involved in directing egress of the RNPs from the nucleus (38), which could possibly occur through interactions between CRM1 and NS2. Alternatively, since much evidence also indicates the necessity of M1 for RNP export, M1-CRM1 interactions could be involved. We therefore examined the distribution of NS2 and M1 in influenza virus-infected cells in the presence and absence of LMB. In the absence of LMB, NS2 was distributed through both the cytoplasm and nuclei of virus-infected cells but with generally brighter fluorescence in the nuclei (Fig. 1e). In the presence of LMB, this staining pattern was not significantly altered, with the majority of cells still showing a mixture of nuclear and cytoplasmic NS2 (Fig. 1f). In untreated cells, M1 was visible in both the nuclei and cytoplasm of infected cells but at a higher density in the nuclei (Fig. 1c). This pattern did not change in the presence of LMB (Fig. 1d). These results were supported by cell fractionation and Western blot analysis for M1 content, which also showed no significant change in the partitioning of M1 between the nuclear and cytoplasmic fractions of drug-treated cells (data not shown). Thus, although RNP export was inhibited when the CRM1 pathway was blocked, no effect on the distribution of either NS2 or M1 was seen.

Next, we investigated whether the intranuclear distribution of NP retained in the nucleus at late times postinfection by LMB treatment was the same as that seen at early times before the onset of RNP export. Virus-infected cells were harvested at 3.5 hpi in the presence and absence of LMB or at 9 hpi with drug treatment and examined for NP distribution by indirect immunofluorescence assay and confocal microscopy. In addition, to delineate the boundary of the nuclear envelope, cells were double stained for Nup62, a structural component of the NPC (6, 14). At 3.5 hpi in the absence of LMB, NP fluorescence was distributed throughout the nucleus in a stippled pattern, entirely within the distinct from the peripheral ring of Nup62 staining (Fig. 4a to c). This early pattern of intranuclear NP staining was not altered in the presence of LMB (Fig. 4d to f), indicating that inhibition of CRM1 function before the onset of RNP export does not affect NP localization. However, NP retained in the nucleus at 9 hpi by LMB treatment was not distributed evenly throughout the nucleoplasm but, instead, was largely confined to an area just inside the nuclear envelope, producing a ring-like staining pattern (Fig. 4i). Thus, inhibition of the CRM1-mediated export pathway not only prevents nuclear export of NP but also results in specific relocalization of NP to a distinct area within the nucleus.

In the hypothesis invoking NS2 in RNP nuclear egress, complexes of RNP in association with M1 and NS2 are the substrate for nuclear export (38). Therefore, it was possible that inhibition of RNP export by LMB would induce changes in the intranuclear distribution of M1 and/or NS2 similar to those seen with NP and that NP, M1, and NS2 would colocalize. To test this hypothesis, we compared the intranuclear distributions of M1 and NS2 at 9 hpi in untreated and LMB-treated cells and double stained the drug-treated cells for NP. In the absence of LMB, M1 and NS2 were both distributed evenly throughout the nucleus (Fig. 5a and e) in a pattern similar to that of NP early in infection (Fig. 4c). However, the homogeneous distribution of M1 and NS2 throughout the nucleoplasm was not altered by LMB addition (Fig. 5b and f) and only limited colocalization of either polypeptide with the LMB-induced annular NP staining pattern was seen (Fig. 5c, d, g, and h). Thus, the intranuclear distribution of M1 and NS2 is not altered by inhibition of the CRM1 export pathway and both polypeptides localize largely independently of NP.

It was possible that the lack of an observable effect on NS2 localization after LMB treatment could be due to excess NS2 production beyond that required for export of RNPs via the CRM1 pathway. To investigate this possibility, we examined the effect of LMB on RNP export in cells infected with mN3, a mutant avian influenza virus that is defective for segment 8 splicing and consequently expresses greatly reduced levels of NS2 (44, 53). CEF cells were infected with either WT or mN3 influenza A/FPV/Rostock/34 virus, treated with LMB as before at 34°C, harvested at 8 hpi, and examined for NP and NS2 localization. In addition, we also examined the effect of the drug on protein synthesis by metabolic labeling of cells with ^{35}S-methionine at 8 hpi, followed by SDS-PAGE and autoradiography. In the absence of LMB, NP, M1/NS1, HA2, and NS2 could readily be discerned in extracts from FPV-infected cells (Fig. 3b, lane 2). In cells infected with mN3, the pattern of protein synthesis appeared very similar except that significantly reduced amounts of NS2 were observed (Fig. 3b, lane 4), consistent with the known defect in splicing of segment 8 in this virus (44). Comparison of proteins synthesized in the presence of LMB revealed no significant differences for either virus,

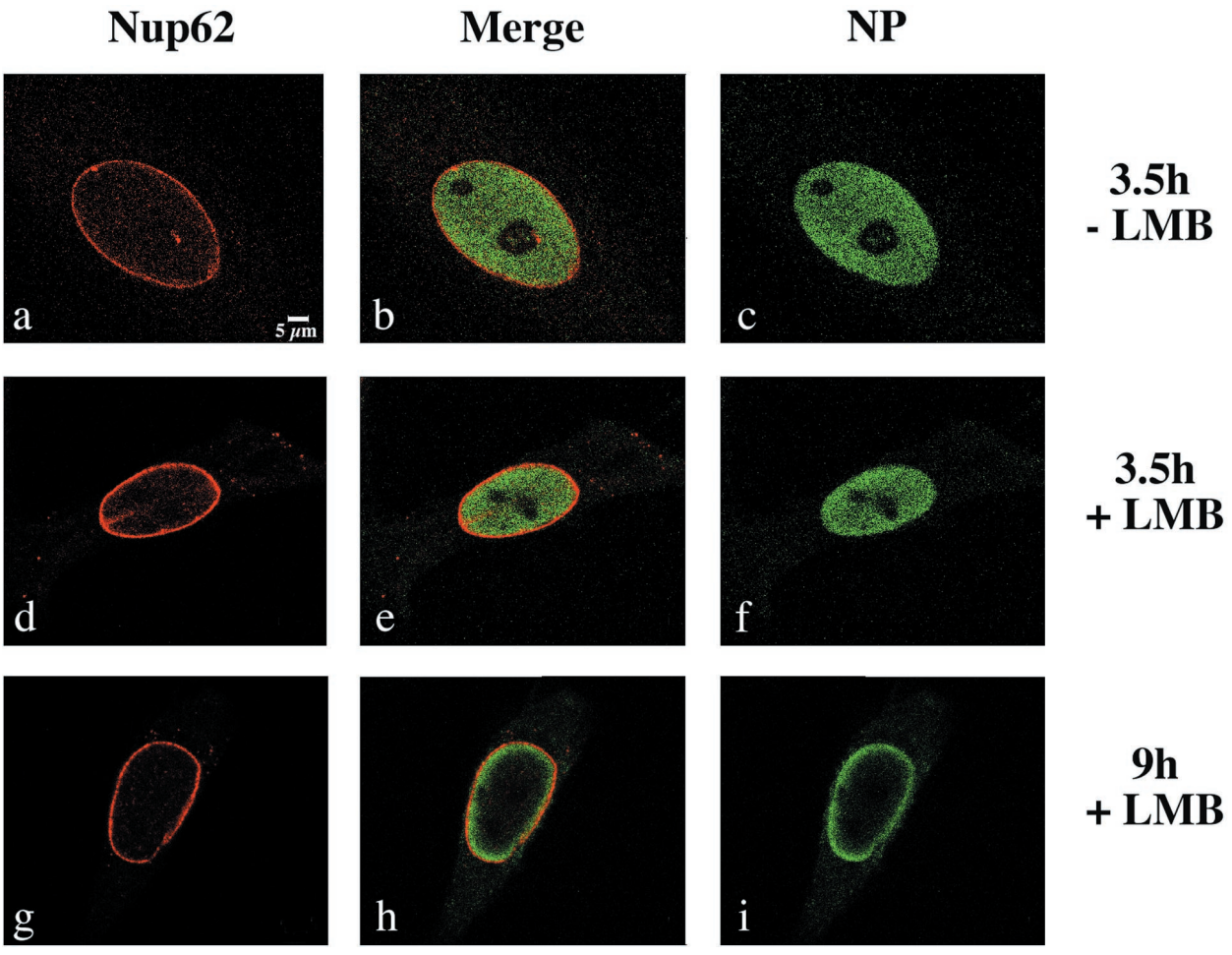

FIG. 4. Effect of LMB on the intranuclear distribution of NP. BHK cells were infected with PR8 in the absence or presence of 11 nM LMB (added at 1 hpi) and harvested at the indicated times postinfection. Cells were stained for NP (green) and Nup62 (red), and single optical planes of focus were examined by confocal microscopy. The green channels in panels h and i were captured at lower sensitivity to keep the maximum fluorescence intensity within the linear range of the detector.

except for a slight increase in the level of NS2 (Fig. 3b). Thus, LMB does not inhibit synthesis of M1 or NS2 in CEF cells, consistent with the lack of an observable effect on viral protein synthesis in BHK cells (Fig. 1 and 3a). Next, we examined the cellular distribution of NP and NS2 in cells infected with WT and mN3 FPV by immunofluorescence assay. In the absence of LMB, NP localized evenly throughout cells infected with WT FPV (Fig. 6a). Thus, as expected, FPV NP is exported into the cytoplasm at late times postinfection in CEF cells, although unlike those of PR8-infected BHK cells, the nuclei do not appear to empty. In the presence of LMB NP was restricted to the nucleus, with only low levels seen in the cytoplasm (Fig. 6b), indicating that the nuclear export of RNPs had been inhibited. These data indicate that inhibition of RNP nuclear export in the presence of LMB is neither cell type nor virus strain dependent. Cells infected with mN3 in the absence of LMB showed the same distribution of NP throughout the cells as those infected with WT virus (Fig. 6c). In the presence of LMB, NP was retained in the nucleus (Fig. 6d), indicating that reduced levels of NS2 had no effect on either the normal export of RNPs or their nuclear retention when CRM1-mediated export was blocked. Consistent with the decreased NS2 synthesis observed by metabolic labeling (Fig. 3b), the level of NS2 fluorescence was lower than that seen with WT FPV (Fig. 6e to h), although still significantly above background levels (Fig. 6i and j). However, the distribution of NS2 in untreated CEF cells infected with either FPV or mN3 was essentially the same for the two viruses in that the protein was distributed throughout the cell and it was not possible to distinguish the nuclei (Fig. 6e and g). In cells treated with LMB, no obvious difference was seen in the distribution of NS2 compared with untreated cells infected with either virus, suggesting that the inhibition of CRM1-mediated nuclear export had no effect on NS2 localization. In addition, while both WT FPV and mN3 NPs displayed the characteristic peripheral nucleoplasmic staining pattern at late times after LMB treatment, the intranuclear distribution of their NS2 polypeptides remained unchanged (data not shown). Thus, even in the presence of minimal amounts of NS2, LMB has no obvious effects on the intracellular distribution of this protein.

Effect of LMB on export of transiently expressed virus proteins. LMB inhibited the nuclear export of NP in virus-infected

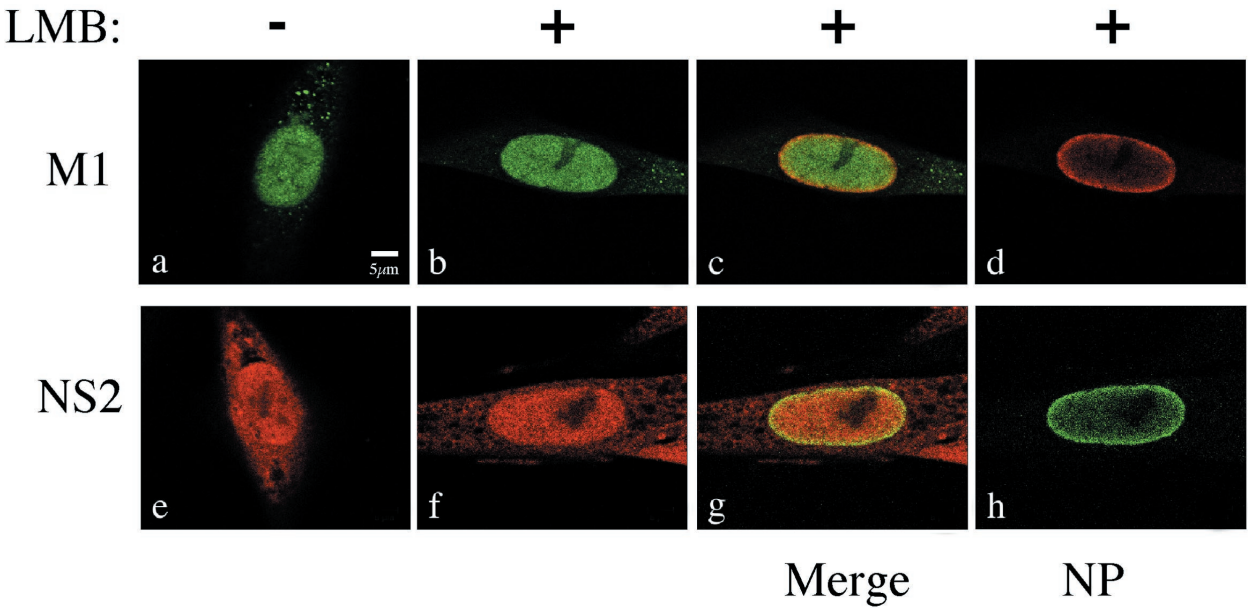

FIG. 5. Effect of LMB on the intranuclear distribution of M1 and NS2. BHK cells were infected with PR8 in the absence (−) or presence (+) of 11 nM LMB and harvested at 9 hpi. Cells were stained for either M1 (green) or NS2 (red) and costained for NP. Single optical planes of focus captured by confocal microscopy are shown. Panels b, c, and d and f, g, and h, respectively, are of the same cells.

cells but had no apparent effect on M1 or NS2 distribution. Since current models for RNP export suggest roles for NS2 and/or M1, it was somewhat surprising that LMB had no effect on either protein. To examine this further, the effect of LMB on the localization of individual virus proteins was tested. BHK cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase and then transfected with plasmids expressing NP, M1, or NS2 under the control of a T7 promoter. We have previously shown that the distribution of NP alters according to the level of expression: cells transfected with a low plasmid dose (0.03 μg of DNA/2 × 10^4 cells) generally show a nuclear staining pattern of NP, but with increasing plasmid doses, the polypeptide tends to accumulate in the cytoplasm (9, 33). We therefore transfected cells with each plasmid at a range of doses, in the case of NP to ensure that cytoplasmic accumulation occurred to some degree, and for M1 and NS2 to test whether a change in the distribution of the protein occurred with various expression levels. In some cases, cell cultures were treated with LMB 3 h after transfection, following which all of the cells were harvested at 5 h posttransfection, the subcellular localization of the influenza virus polypeptides was examined by immunofluorescence assay, and cells were scored N, N/C, or C. The results are expressed graphically as a percentage of the total cells counted, and examples of each staining pattern (obtained in the absence of LMB) for the three polypeptides are shown to the left of the corresponding graphs (Fig. 7). Cells transfected with 0.1 μg of pKT5 displayed the expected NP localization pattern, with the largest proportion (approximately 50%) of the cells containing NP in both the nucleus and cytoplasm, but with substantial populations (about 25% each) showing either predominantly nuclear or cytoplasmic staining (Fig. 7a). At higher plasmid doses, the pattern of NP distribution became more more cytoplasmic, with greater than 60% of the cells scored C and only around 10% scored N (Fig. 7a). This is consistent with our previous observations (9, 33). However, irrespective of the plasmid quantity used, the addition of LMB altered the distribution pattern of NP, decreasing the proportion of cells showing cytoplasmic accumulation and increasing the number with predominantly nuclear protein (Fig. 7a). This effect was most pronounced at a dose of 0.3 μg of pKT5, where the proportion of transfected cells with nuclear NP increased from around 10% in the absence of drug to nearly 50% in its presence. Concomitantly, the number of cells with a cytoplasmic staining pattern decreased from over 70 to 15%. When similar experiments were carried out using a plasmid encoding M1, in the absence of LMB, the majority of the M1-expressing cells (around 70%) showed an even distribution of the polypeptide between the nucleus and the cytoplasm, with only a low percentage of cells showing predominantly nuclear fluorescence (Fig. 7b). In contrast to the behavior of NP, the pattern of M1 localization did not alter significantly with the plasmid dose, even when higher or lower ranges were tried (data not shown). Also in contrast to the behavior of NP, the distribution of M1 did not change significantly in the presence of LMB (Fig. 7b). Cells transfected with NS2 showed a staining pattern similar to that of those expressing M1, with the majority scored N/C, although a slightly higher percentage showed predominantly nuclear protein (Fig. 7c). Similarly, no change in localization pattern was seen with plasmid dose alteration (even when larger amounts of DNA were transfected; data not shown) and no increase in nuclear accumulation was observed in cells expressing NS2 in the presence of LMB (Fig. 7c). We therefore found no evidence that either M1 or NS2 interacts with the CRM1 pathway in the absence of other influenza virus components. In contrast, inhibition of the CRM1-mediated nuclear export pathway results in nuclear retention of NP, indicating

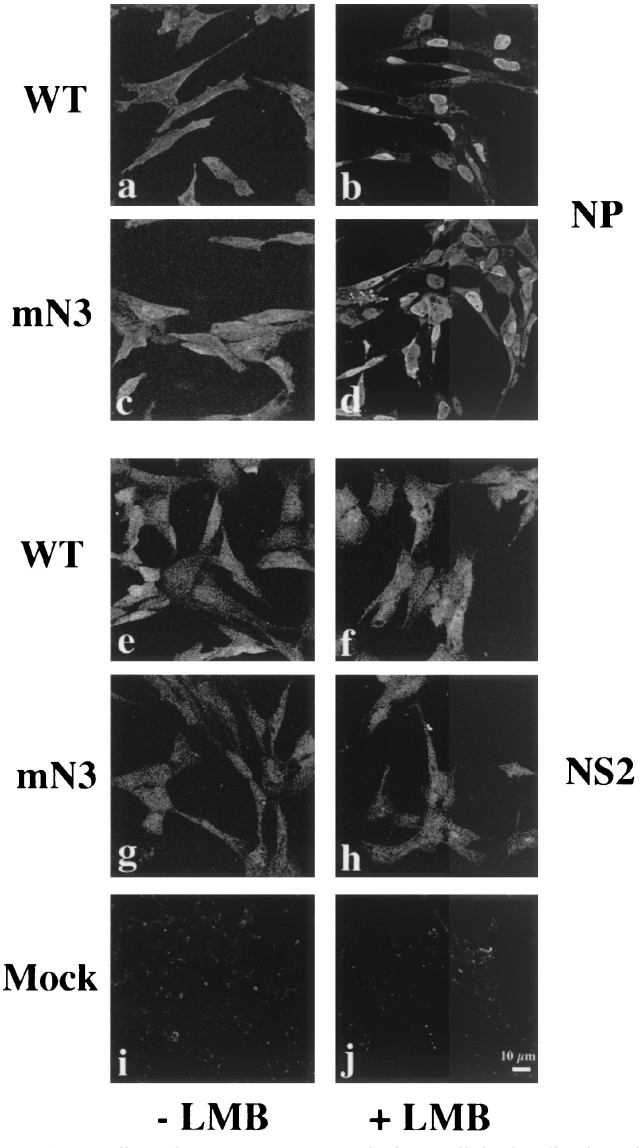

FIG. 6. Effect of LMB treatment on the intracellular localization of NP and NS2 in FPV-infected cells. CEF cells were infected (or mock infected) with either WT or mN3 FPV at 34°C, incubated from 1 hpi in the absence or presence of 11 nM LMB, and harvested at 8 hpi. Cells were stained for NP (a to d) or NS2 (e to j) and examined by confocal microscopy. Images were generated using an extended-focus algorithm.

that NP interacts with this pathway in the absence of other influenza virus proteins.

To further investigate the role of CRM1-mediated export pathway plays in determining intracellular localization of influenza virus polypeptides, we tested the effect of overexpression of CRM1 on the localization of NP, M1, and NS2. Plasmids encoding influenza virus polypeptides were individually cotransfected with 0.2 μg of a construct expressing CRM1 (2) or with pCDNA-3 containing no insert, and the localization of the influenza virus polypeptides was analyzed by indirect immunofluorescence assay as before. In the absence of exogenous CRM1, the percentage of NP-expressing cells showing predominantly cytoplasmic fluorescence increased from 3% at the lowest dose of pKT5 to 15% at the highest (Table 1). When CRM1 was coexpressed with NP, the distribution of NP became more cytoplasmic at each dose of pKT5. This effect was most pronounced at the lowest plasmid dose (0.03 μg) (where NP was predominantly nuclear in the absence of exogenous CRM1), as overexpression of exogenous CRM1 increased the percentage of transfected cells with cytoplasmic NP from 3 to 38% (Table 1). In six independent experiments involving a total of 17 paired samples, cotransfection of CRM1 resulted in a significant increase in cytoplasmic NP in every case at all of the pKT5 doses tested (χ^2 statistic, $P < 0.001$ on 15 occasions and $P < 0.01$ twice). Under transfection conditions which resulted in substantial numbers of cells with cytoplasmic NP in the absence of CRM1, cotransfection of CRM1 resulted in a twofold increase in nuclear exclusion (2.3 ± 0.3 [$n = 5$]). In low-dose pKT5 transfections where the majority of NP-expressing cells showed nuclear staining in the absence of CRM1, the average fold increase in cytoplasmic staining after cotransfection was 23.8 ± 8.5 ($n = 4$) (data not shown). Furthermore, addition of LMB to cells expressing CRM1 and NP prevented the increase in cytoplasmic accumulation of NP at higher doses of pKT5, retaining NP in the nucleus at levels similar to those of cells expressing NP alone without drug (Table 1). At the lowest dose of pKT5, which showed the most dramatic CRM1-dependent increase in NP export, addition of LMB partially reversed the effect, reducing the proportion of cells with predominantly cytoplasmic NP by approximately twofold (Table 1). In contrast, overexpression of CRM1 had little effect on the distribution of M1, causing no more than a 1.5-fold increase in the proportion of cells with cytoplasmic fluorescence (Table 1). In three independent experiments, the greatest increase in cytoplasmic M1 seen was 1.8-fold (average, 1.2 ± 0.4 [$n = 9$]) and overall there was no statistically significant change in the intracellular distribution of the polypeptide (data not shown). LMB treatment of cells cotransfected with M1 and CRM1 also had no major effect on M1 localization (Table 1). When similar CRM1 cotransfection experiments were carried out with NS2, no significant increase in the proportion of cells with predominantly cytoplasmic NS2 was seen (data not shown). However, coexpression of CRM1 consistently caused a slight reduction in the number of transfected cells with predominantly nuclear NS2, which was not reversible by the addition of LMB (Table 1). In three replicate experiments, the maximum decrease in the proportion of cells with predominantly nuclear NS2 was 1.6-fold (average, 1.4 ± 0.2 [$n = 6$]) and the effect was not consistently statistically significant (Table 1 and data not shown). Thus, when expressed in the absence of other influenza virus polypeptides, NP is strongly biased toward cytoplasmic accumulation by the overexpression of CRM1. However, the distribution of M1 is unaffected and that of NS2 is only weakly affected, if at all.

The increased cytoplasmic accumulation of NP after CRM1 overexpression further supports the hypothesis that NP interacts with the CRM1 nuclear export pathway in the absence of other influenza virus proteins. However, as CRM1 is also involved in the export of cellular RNAs (8, 15, 45, 47), it was possible that in the absence of influenza virus RNA, NP was binding nonspecifically to cellular RNA complexes destined for export, rather than interacting directly with CRM1. To investigate this possibility, we tested the effect of overexpression of

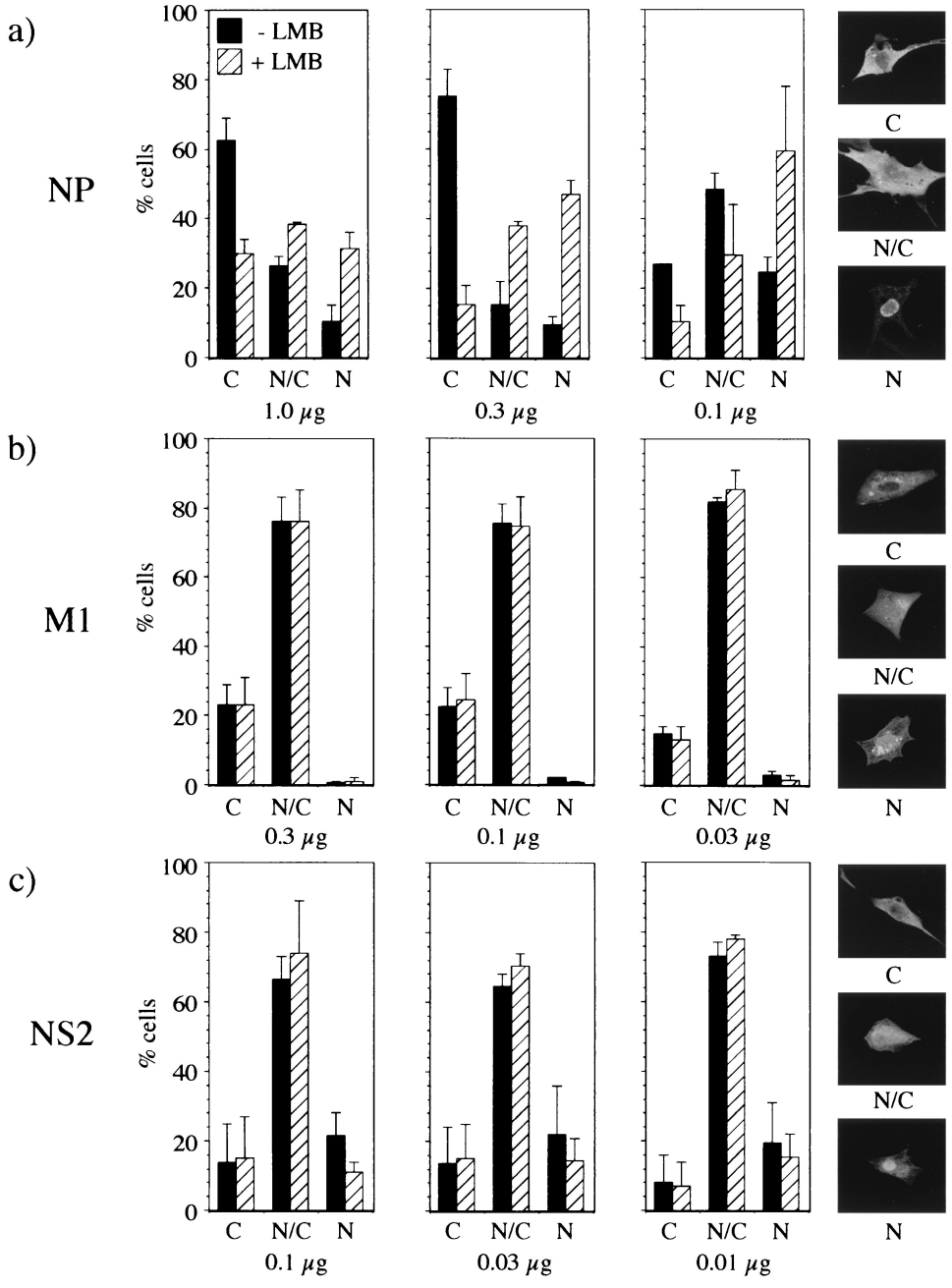

FIG. 7. Effect of LMB on the distribution of NP, NS2, and M1 in transfected cells. BHK cells were infected with vTF7 at an MOI of 10 PFU/cell and then transfected 2 h later with the indicated amounts of plasmid DNA encoding NP, NS2, or M1. At 3 h posttransfection, cells were overlaid with fresh medium containing either 11 nM LMB or no drug and harvested 2 h later. Cells were analyzed by immunofluorescence assay using antibodies for NP, M1, or NS2 and scored as follows for the localization pattern of the influenza virus protein: N, predominantly nuclear; N/C distributed throughout the cell; C, predominantly cytoplasmic. Examples of the staining patterns are shown on the right. The number of cells showing each distribution pattern was expressed as a percentage of the total cell count. The average and range of two independent experiments are shown.

CRM1 on the cellular distribution of an NP mutant that is defective for RNA binding activity (13). NP R267-A was expressed at a range of plasmid doses, as before, and the effects of overexpression of CRM1 in the presence and absence of LMB were examined. In the absence of exogenous CRM1, the mutant NP localized essentially the same way as WT protein (Table 1). However, the mutant polypeptide showed a striking difference in localization when expressed in the presence of exogenous CRM1, with up to 90% of transfected cells showing cytoplasmic NP. The increased cytoplasmic accumulation of R267-A was partially reversible by LMB treatment (Table 1). Similar results were obtained with a second mutant NP, S314-N, which has a *ts* lesion that renders the protein unable to bind RNA at 37°C (data not shown; reference 33). These results

TABLE 1. Effect of CRM1 overexpression on NP, M1, and NS2 localization

Polypeptide and plasmid dose (μg)	Without CRM1[a]	With CRM1[b]	With CRM1 + LMB[b]
NP			
0.3	15	28 (1.9)	14 (0.9)
0.1	11	24 (2.2)	8 (0.7)
0.03	3	38 (13)	17 (5.7)
NP R267-A			
0.3	10	51 (5.1)	47 (4.7)
0.1	5	81 (16.2)	64 (12.8)
0.03	0	88 (>88)	89 (>89)
M1			
0.3	5	5 (1.0)	7 (1.4)
0.1	4	6 (1.5)	5 (1.3)
0.03	1	1 (1.0)	2 (2.0)
NS2			
0.3	63[c]	43 (1.5)	47 (1.3)
0.1	65	54 (1.2)	51 (1.3)
0.03	76	47 (1.6)	57 (1.3)

[a] Percentage of cells scored C.
[b] The fold change in the percentage of cells is in parentheses.
[c] Percentage of cells scored N.

indicate that the CRM1-dependent export of NP operates in the absence of an NP-RNA interaction and is therefore unlikely to be due to fortuitous association with host cell RNAs.

In vitro analysis of interactions among NP, NS2, and components of the cellular nuclear trafficking machinery. The observation that the intracellular localization of NP is sensitive to modulation of the CRM1 export pathway raises the possibility that NP contains an NES and interacts directly with CRM1. To test this hypothesis, we examined the ability of NP fusion proteins to bind CRM1 in vitro. For comparison, we also examined the ability of NP to bind to hSRP-1, a previously characterized cellular NP binding protein from the importin α family (37, 49), to itself, since the polypeptide is known to self-associate (12, 40, 43), and to NS2, as NP is generally held not to interact directly with NS2 (10, 54). Radiolabeled CRM1, hSRP-1, NP, and NS2 were expressed by coupled transcription and translation of the appropriate plasmid templates in rabbit reticulocyte lysate (7), and aliquots of the in vitro-translated material were incubated with either GST, MBP, or NP fused to GST or MBP (GST-NP or MBP-NP, respectively). After subsequent incubation with glutathione-Sepharose or amylose resin, as appropriate, bound material was collected and washed by centrifugation and analyzed by SDS-PAGE and autoradiography. As we have shown previously (12), NP self-associates specifically and efficiently in this assay system, as substantial quantities of the in vitro-translated NP bound to GST-NP but not to GST alone (Fig. 8, lanes 4 to 6). In contrast, no detectable NS2 bound to either GST or GST-NP, (Fig. 8, lanes 10 to 12), consistent with earlier studies that failed to find evidence for a direct interaction between NS2 and NP (10, 54). However, hSRP-1 did bind specifically to GST-NP (Fig. 8, lanes 7 to 9), consistent with the interaction between the polypeptides previously identified by yeast two-hybrid and coimmunoprecipitation analyses (37, 49). Moreover, in vitro-translated CRM1 bound to GST-NP and MBP-NP but not GST or MBP alone (Fig. 8, lanes 1 to 3, 14, and 16), and comparison of the ratios of bound to input polypeptides suggested that the apparent affinity of the interaction between CRM1 and NP was similar to that of the NP-NP or NP–hSRP-1 interaction. However, addition of LMB to the reaction mixtures did not affect the interaction between MBP-NP and CRM1 (lane 15) or that between GST-NP and CRM1 (data not shown). Thus, NP binds CRM1 in vitro, consistent with the effect of up- or down-regulation of the CRM1 nuclear export pathway on the intracellular localization of NP.

DISCUSSION

Influenza virus RNPs display a biphasic pattern of intracellular localization during infection. At early times, they reside in the nucleus, where virus transcription takes place, but at later times in infection, they are exported to the cytoplasm to allow their packaging into progeny virions. The current hypothesis to explain this phenomenon holds that the late viral polypeptides M1 and NS2 enter the nucleus and bind sequentially to the RNPs and that the ability of NS2 to interact with components of the NPC directs export of the complex (38). In light of this model, it has been suggested that NS2 be renamed NEP (nuclear export protein) (38). Here, we show that treatment of cells with the drug LMB blocks nuclear export of RNPs, strongly suggesting that this process requires the cellular export receptor CRM1, rather than a direct interaction of RNPs or RNP complexes with the NPC. Although LMB is generally held to be a specific inhibitor of the CRM1 pathway (8, 18, 24, 25, 36), we cannot formally exclude the possibility that the drug also affects an as yet uncharacterized export function, either directly or indirectly. However, no effect of LMB treatment on the distribution of Ran or Rch-1, a member of the importin α family, was observed (data not shown), suggesting that the importin α- and NTF2-mediated nuclear import pathways and the CAS-mediated export pathway were still functional (27, 42). Similarly, the failure of LMB treatment to inhibit viral protein synthesis argues that the export pathway(s) responsible for viral mRNA egress remains functional (and that this path-

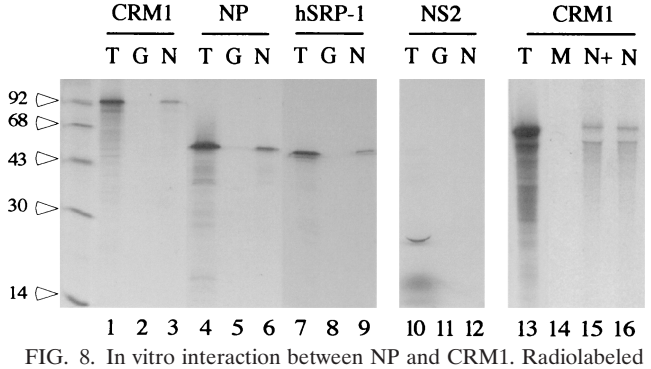

FIG. 8. In vitro interaction between NP and CRM1. Radiolabeled in vitro-translated CRM1, NP, hSRP-1, and NS2 were analyzed by SDS-PAGE and autoradiography before (T) or after binding to GST (G), MBP (M), or GST-NP (N in lanes 3, 6, 9, and 12) or MBP-NP (N in lanes 15 and 16) immobilized on agarose beads. LMB (11 nM) was included in the reaction mixture run in lane 15 (N+). The values on the left are molecular mass markers (kilodaltons).

ways does not involve CRM1). Therefore, LMB treatment does not result in a general inhibition of nucleocytoplasmic transport in the systems examined here. Furthermore, the observation that overexpression of CRM1 biased NP toward cytoplasmic accumulation in an LMB-sensitive manner (Table 1) strongly suggests that the inhibitory target of LMB in this system is CRM1. We therefore conclude that the effects of the drug on influenza virus polypeptide localization are specific. The inhibitory effect of LMB on RNP export was not confined to a particular strain of virus or cell but was observed with the PR8, FPV, and A/Udorn/72 strains in primary CEFs, immortalized fibroblasts such as BHK, CV1, and Cos cells, and polarized epithelial MDCK cells (Fig. 1, 2, 4, and 6 and data not shown). This suggests that use of the CRM1 pathway is a general feature of influenza A viruses.

Part of the experimental support of the NEP hypothesis comes from the identification of a sequence within NS2 that acts as an NES when transplanted onto another polypeptide (38), raising the possibility that it is NS2 that interacts with CRM1. However, the LMB-induced block in RNP export was not accompanied by a major change in the nucleocytoplasmic distribution of NS2 or M1 (Fig. 1) and such a redistribution might be expected if export occurs via an RNP-M1-NS2 complex. Unlike NP in PR8-infected BHK cells, the M1 and NS2 polypeptides are ordinarily resident in substantial quantities within the nucleus late in infection (Fig. 1) and are therefore perhaps less likely to show decreased cytoplasmic and increased nuclear accumulation after LMB treatment. However, a larger proportion of NS2 in FPV-infected CEF cells is cytoplasmic, and although this provides a more sensitive background against which to observe increased nuclear accumulation of the polypeptide after CRM1 inhibition, no change in its distribution was observed (Fig. 6). Moreover, neither NS2 nor M1 showed any apparent intranuclear redistribution after LMB treatment (Fig. 5). This is in contrast to the striking change observed with NP, where at early times postinfection (when the polypeptide is ordinarily resident within the nucleus), the polypeptide displayed diffuse intranuclear staining, but when retained artificially at late times by LMB treatment, it localized to a distinctive peripheral ring within the nucleoplasm (Fig. 4). In the case of M1, our observations are compatible with the observation that although M1 is required for RNP export, it does not necessarily accompany the exported RNPs in detectable amounts (41, 51, 52). However, RNPs have been shown to be tightly associated with the nuclear matrix (4, 22) and it has been suggested that M1 is required for their release (4, 56). As Bui and colleagues pointed out (4), this would effectively make RNP export a two-stage process, which is consistent with the change in intranuclear distribution of NP we observed between early and late times postinfection in the absence of nuclear export (Fig. 4). Experiments to test this hypothesis are currently in progress.

The failure to observe any redistribution of NS2 after LMB treatment is perhaps more surprising, as its putative role as the actual transport factor would predict at least one round of shuttling per molecule of NS2. One could perhaps argue that the unchanged cellular localization of NS2 following LMB treatment arises from an excess of the polypeptide and its nonstoichiometric requirement for RNP export. However, the behavior of the FPV mutant mN3 argues against this hypothesis. This virus is able to replicate to reasonably high titers in tissue culture despite a segment 8 splicing defect which results in the synthesis of 5% or less of the normal amount of NS2 (44, 53). Consistent with the production of a normal infectious titer, the mN3 virus showed no obvious defect in the transport of RNPs from the nucleus to the cytoplasm (Fig. 6). Moreover, in cells infected with mN3 under conditions where the CRM1 pathway was blocked, there was no obvious alteration in the distribution of NS2, either at the level of nucleocytoplasmic distribution (Fig. 6) or at the intranuclear level (data not shown). Thus, if NS2-RNP complexes are the substrate for nuclear export, then only a minor fraction of the total NS2 content is required or the interaction is too transitory to be observed by the techniques used here.

In recent years, much evidence has accumulated to suggest that NP possesses the intrinsic ability to interact with a nuclear export pathway. Despite containing multiple nuclear localization signals (49, 50), exogenously expressed NP is not necessarily resident in the nucleus but can accumulate in the cytoplasm, depending upon the phosphorylation status of the cell, the time and level of NP expression, and its ability to bind F-actin (5, 9, 35). Furthermore, non-RNP-associated NP has been shown to shuttle between the nucleus and the cytoplasm (51). We show here that in the absence of other influenza virus polypeptides, cytoplasmic accumulation of NP was greatly reduced by LMB treatment (Fig. 7), mimicking the effect seen on RNPs in virus-infected cells (Fig. 1, 2, and 6). In contrast, overexpression of CRM1 strongly biased exogenous NP toward a cytoplasmic location (in a manner that was not dependent upon the ability of the polypeptide to bind RNA), but this was reversible by simultaneous LMB treatment (Table 1). Overall, this provides strong evidence that NP interacts with the CRM1 pathway in the absence of other influenza virus polypeptides. In contrast, the distribution of exogenously expressed NS2 and M1 showed very little alteration after up- or down-regulation of CRM1 activity (Fig. 7; Table 1), similar to the lack of effect of LMB on the distribution of each of these proteins in the context of virus infection (Fig. 1, 5, and 6). Consistent with data obtained from infected or transfected cells, we also found evidence for an NP-CRM1 interaction in vitro (Fig. 8). However, the in vitro interaction between NP and CRM1 was not sensitive to LMB (Fig. 8) but it is not clear whether LMB modification of CRM1 prevents its binding to NES sequences. Although the studies of Fornerod et al. (15) and Kudo et al. (24) demonstrated LMB inhibition of the formation of a CRM1-RanGTP-NES peptide complex and a CRM1-NES peptide complex, respectively, the concentrations of both the NES-containing substrate and LMB used were much (~1,000-fold) higher than those used in this study. We also note that the Rev polypeptide of human immunodeficiency virus interacts with CRM1 in vitro in both LMB-sensitive and LMB-insensitive manners, with LMB only inhibiting binding when in the presence of RanGTP (1). Unfortunately, we have not yet been able to obtain an anti-CRM1 serum that is functional in immunofluorescence to perform NP-CRM1 colocalization studies. However, the peripheral nuclear distribution of NP obtained after LMB treatment (Fig. 4) is similar to the nuclear staining pattern shown for CRM1 in non-drug-treated cells (16).

The finding that NP interacts with the CRM1 export path-

way in the absence of other influenza virus components raises the possibility of NS2-independent RNP export. Such a hypothesis is consistent with the lack of NS2 redistribution after CRM1 inhibition (Fig. 1, 5, and 6). We also note the recent experiments of Bui et al. (4) in which RNP export was blocked by treatment with the protein kinase inhibitor H7 but restored by the addition of exogenous M1 in the absence of detectable NS2. In addition, work analyzing the requirements for the formation of influenza virus-like particles has demonstrated that NS2 is dispensable for packaging of a synthetic genome segment (A. Portela, personal communication). However, strong evidence suggesting the requirement of NS2 for RNP export during virus infection has come from recent work showing that NP is retained in the nuclei of cells infected with an NS2 knockout virus generated by reverse genetics (Y. Kawaoka, personal communication). Therefore, it is possible that the direct interaction of RNPs with the CRM1 export pathway represents a redundant alternative to an NS2-mediated pathway, and/or that virus strain and cell type variations are important. In support of a hypothesis of multiple RNP export mechanisms, inhibition of the CRM1 pathway failed to completely block the movement of NP to the cytoplasm, as assessed by cell fractionation studies (Fig. 2). In addition, when the effect of the drug on virus yield was titrated, the maximal decrease in virus titer reached was 90 to 95% (data not shown). While this is a magnitude decrease similar to that caused by H7, another inhibitor of RNP export (26), it implies that sufficient RNPs are still reaching the plasma membrane to form some progeny virus. However, considering the dramatic change in NP localization shown by immunofluorescence assay and cell fractionation and the fact that >90% of the virus titer was lost after LMB treatment, we would argue that, overall, the CRM1 pathway plays a predominant role in influenza virus RNP export.

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